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Springer Protocols

Peter van Endert *Editor*

Antigen Processing

Methods and Protocols

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Antigen Processing

Methods and Protocols

Edited by

Peter van Endert

*Institut National de la Santé et de la Recherche Médicale, Unité 1013
Université Paris Descartes, Faculté de médecine Paris, France*

Editor

Peter van Endert

Institut National de la Santé et de la Recherche Médicale, Unité 1013
Université Paris Descartes, Faculté de médecine
Paris, France

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Preface

Ever since A. Townsend, A. McMichael, and coworkers reported 26 years ago that T lymphocytes recognize short peptide fragments in association with major histocompatibility complex (MHC) encoded proteins (1), immunologists have been keen to understand how these fragments are produced, how they are transported to MHC molecules and how they assemble with them. It was not surprising that the new field of antigen processing attracted considerable interest, given that peptide presentation by MHC molecules is the key element in immunological self-non self discrimination, pathogen-specific immune responses, autoimmunity, and vaccine development. As a result, many aspects of cellular antigen processing are now understood in great detail, although some other issues (e.g., the nature of endogenous proteins giving rise to MHC class I ligands, or the cell biology of cross-presentation) remain to be clarified.

This volume aims to provide the reader with a comprehensive set of protocols for studying presentation of antigens produced in the standard processing pathways for MHC class I and class II molecules. In both cases, the book attempts to follow the chronology of intracellular events ending with recognition of peptide–MHC complexes at the cell surface by T lymphocytes. Surveying MHC class I antigen processing, we start by examining cytosolic proteases and the kinetics of peptide survival determined by them (Fig. 1). The next steps open to scrutiny are peptide transport into the endoplasmic reticulum and synthesis and loading of MHC class I molecules. Further protocols examine the fate of class I mol-

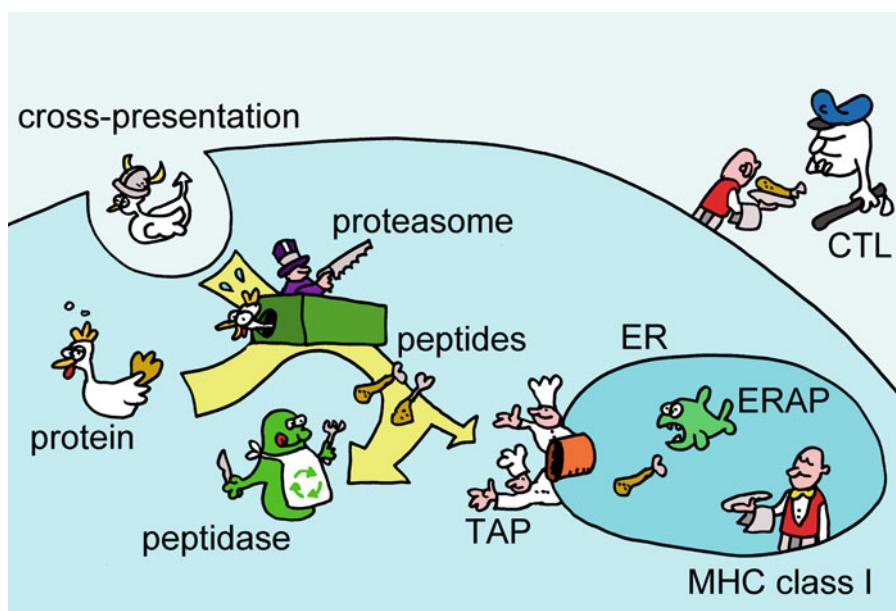


Fig. 1. The principal steps in processing of endogenous antigens for presentation by MHC class I molecules.

ecules beyond the endoplasmic reticulum including disposal upon ubiquitination and viral interference. A number of chapters are devoted to analysis of the final peptide–MHC complexes, using biochemical, immunological (T cell receptor-like antibodies), and immune-informatics approaches. Complementary chapters deal with CD8+ T cell assays and cloning, indispensable tools when studying MHC class I antigen processing.

A second set of chapters (Chapters 24–30) presents methods for studying antigen processing by dendritic cells, a cell population with a critical role in priming and orchestrating antigen-specific cellular immune responses. These chapters will enable the reader to purify and prepare dendritic cells, monitor their activation, transform them with lentiviruses, perform cross-presentation assays, and monitor intracellular routing of antigens in endocytic compartments. Another volume in the series (Dendritic cell protocols, Ed. S.H. Naik) provides a collection of additional protocols that will be of interest to the reader with a special interest in this section.

Section 3 (Chapters 31–40) deals with the principal steps in antigen processing for MHC class II molecules (Fig. 2). These include synthesis, assembly, and peptide loading of class II molecules. Intracellular transport of class II molecules is studied with emphasis on dendritic cells. Additional protocols address the role of macroautophagy, a mechanism with an important role in the loading of class II molecules with endogenous peptides, and endosomal proteases producing class II ligands. The section would not be complete without chapters dealing with class II ubiquitination, which controls MHC class II turnover and disposal, and with production of CD4+ T cell lines and clones. The final two protocols concern presentation of lipid antigens by nonclassical MHC molecules.

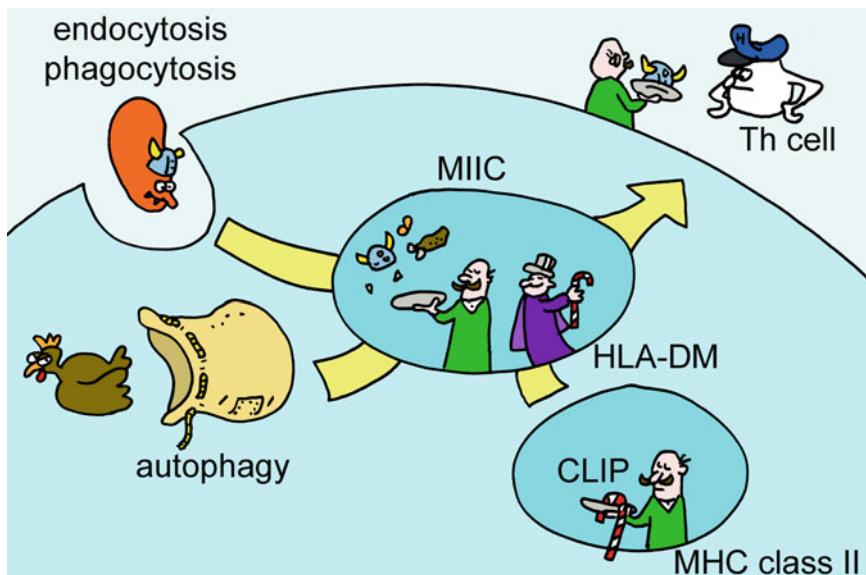


Fig. 2. The principal steps in processing of antigens for presentation by MHC class II molecules

This methods book is designed to be a bench-side companion for beginners and experts interested in studying antigen processing. In keeping with the spirit of the series, the protocols included hopefully will enable readers to confidently venture into the field of antigen processing. The editor wishes to express his gratefulness to all authors for their excellent contributions, to Christophe Marchi for help with editing, and to Eric Reits for his wonderful cartoons.

Paris, France

Peter van Endert

Reference

1. Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ (1986) The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959–968

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Contributors

- CECILE ALANIO • *Immunobiologie des cellules dendritiques, Unité Mixte Pasteur/Inserm U818, Institut Pasteur and Centre d'Immunologie Humaine, Institut Pasteur, Paris, France*
- MATTHEW L. ALBERT • *Immunobiologie des cellules dendritiques, Unité Mixte Pasteur/Inserm U818, Institut Pasteur and Centre d'Immunologie Humaine, Institut Pasteur, Paris, France*
- ANTONY N. ANTONIOU • *Division of Infection and Immunity/Centre of Rheumatology, Department of Immunology and Molecular Pathology, London, UK*
- MICHAEL BASLER • *Biotechnology Institute Thurgau at the University of Constance, Kreuzlingen, Switzerland; Department of Biology, Division of Immunology, University of Konstanz, Konstanz, Germany*
- HENDRIK BERGER • *Institute for Immunology, University of Mainz, Mainz, Germany*
- MARC BEYER • *Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany*
- RICHARD S. BLUMBERG • *Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
- JESSICA M. BONAME • *Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK*
- ISABELLE BOUVIER • *Immunobiologie des cellules dendritiques, Unité Mixte Pasteur/Inserm U818, Institut Pasteur and Centre d'Immunologie Humaine, Institut Pasteur, Paris, France*
- MARLENE BOUVIER • *Department of Microbiology and Immunology, University of Illinois at Chicago, College of Medicine, Chicago, IL, USA*
- SVEN BURGDORF • *Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany*
- MARIAN L. BURR • *Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK*
- ROBERT BUSCH • *Division of Rheumatology, Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK*
- STUART K. CALDERWOOD • *Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
- DAVID H. CANADAY • *Division of Infectious Disease, Case Western Reserve University, Cleveland, OH, USA*
- DOMINIQUE CHARRON • *INSERM UMR-S 940, Institut Universitaire d'Hématologie and Université Paris-Diderot, Paris, France*
- ACHMET IMAM CHASAN • *Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany*
- PETER CRESSWELL • *Department of Immunobiology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, USA*
- ROSA BARREIRA DA SILVA • *Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland*

- FRANCESCA DE ANGELIS RIGOTTI • *Centre d'Immunologie de Marseille-Luminy (CIML), Aix-Marseille University, UM2, Marseille, France; Institut National de la Santé et de la Recherche Médicale (INSERM), U1104, Marseille, France; Centre National de la Recherche Scientifique (CNRS), UMR7280, Marseille, France*
- AUDE DE GASSART • *Centre d'Immunologie de Marseille-Luminy (CIML), Aix-Marseille University, UM2, Marseille, France; Institut National de la Santé et de la Recherche Médicale (INSERM), U1104, Marseille, France; Centre National de la Recherche Scientifique (CNRS), UMR7280, Marseille, France.*
- HENRI DE LA SALLE • *Biologie des cellules dendritiques humaines, U725 Inserm/Université de Strasbourg/Etablissement Français du Sang-Alsace, Strasbourg, France*
- BRIAN P. DOLAN • *Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA*
- LAURENCE C. EISENLOHR • *Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA*
- BEN FANCKE • *Centre for Immunology, Burnet Institute, Melbourne, Australia and Department of Immunology, Monash University, Melbourne, Australia*
- BENOIT FAVIER • *Division of ImmunoVirology, Institute of Emerging Diseases and Innovative Therapies (iMETI), Fontenay-aux-Roses, France and UMR E1, Université Paris-Sud 11, Orsay, France*
- LARS FRANKEN • *Institutes of Molecular Medicine and Experimental Immunology (IMMEI), University Hospital Bonn, Bonn, Germany*
- MONIQUE GANNAGE • *Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland*
- LUIS FERNANDO GARCIA-ALLES • *Institut de Pharmacologie et de Biologie Structurale (IPBS), UMR 5089 CNRS/Université Toulouse III–Paul Sabatier, Toulouse, France*
- EVELINA GATTI • *Centre d'Immunologie de Marseille-Luminy (CIML), Aix-Marseille University, UM2, Marseille, France; Institut National de la Santé et de la Recherche Médicale (INSERM), U1104, Marseille, France; Centre National de la Recherche Scientifique (CNRS), UMR7280, Marseille, France*
- ESTHER GHANEM • *Molecular Life Science, Jacobs University Bremen, Bremen, Germany*
- DENIS GLOTZ • *Service de Néphrologie et Transplantation rénale, Hôpital Saint Louis and Université Paris-Diderot, Paris, France*
- JIANLIN GONG • *Boston University Medical School, Boston, MA, USA*
- MARCUS GROETTRUP • *Biotechnology Institute Thurgau at the University of Konstanz, Kreuzlingen, Switzerland; Division of Immunology, Department of Biology, University of Konstanz, Konstanz, Germany*
- DAVID B. GUILIANO • *School of Health, Sport and Bioscience, University of East London, London, UK*
- ANDREAS V. HADJINICOLAOU • *Division of Transplantation Biology and Immunology, Department of Pediatrics, Stanford University Medical School, Stanford, CA, USA; Division of Rheumatology, Department of Medicine, University of Cambridge, Cambridge, UK*
- TOBIAS HAIN • *Institute for Immunology, University of Mainz, Mainz, Germany*
- ISAMU HARTMAN • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- DANIËLLE HORST • *Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands*

- TIEYING HOU • *Department of Pediatrics, Division of Transplantation Biology and Immunology, Stanford University Medical School, Stanford, CA, USA*
- LAN HUANG • *Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA*
- ISABEL ISHIZUKA • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- MARLIEKE L.M. JONGSMA • *Department of Cell Biology II, The Netherlands Cancer Institute, Amsterdam, The Netherlands*
- HÉLÈNE JUSFORGUES-SAKLANI • *Immunobiologie des cellules dendritiques, Unité Mixte Pasteur/Inserm U818, Institut Pasteur and Centre d'Immunologie Humaine, Institut Pasteur, Paris, France*
- NADINE KÄMPER • *Division of Immunobiology, Institute of Genetics, University of Bonn, Bonn, Germany*
- TAKAYUKI KANASEKI • *Department of Pathology, Sapporo Medical University, Sapporo, Japan*
- EDITA KAROSIENE • *Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark*
- CHRISTIN KELLER • *Institut für Biochemie, Berlin, Germany*
- AERYON KIM • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- PETER-M. KLOETZEL • *Institut für Biochemie, Berlin, Germany*
- NORBERT KOCH • *Division of Immunobiology, Institute of Genetics, University of Bonn, Bonn, Germany*
- DANIEL J. KOWALEWSKI • *Department of Immunology, Interfaculty Institute for Cell Biology, University of Tübingen, Tübingen, Germany*
- ULRIKE KUCKELKORN • *Institut für Biochemie, Berlin, Germany*
- CHRISTIAN KURTS • *Institutes of Molecular Medicine and Experimental Immunology (IMMEI), University Hospital Bonn, Bonn, Germany*
- METTE VOLDBY LARSEN • *Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark*
- PAUL J. LEHNER • *Cambridge Institute for Medical Research, Addenbrooke's Hospital, University of Cambridge, Cambridge, UK*
- FRANÇOIS A. LEMONNIER • *Unité "Immunité Cellulaire Antivirale", Institut Pasteur, Paris, France*
- A.M. LENNON-DUMENIL • *Institut Curie, Inserm U932, Paris, France*
- OLE LUND • *Department of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Kongens Lyngby, Denmark*
- CLAUS LUNDEGAARD • *Department of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Kongens Lyngby, Denmark*
- WENBIN MA • *Ludwig Institute for Cancer Research, Brussels Branch and WELBIO and de Duve Institute, Université catholique de Louvain, Brussels, Belgium*
- NICOLAS MANEL • *Institut Curie, INSERM U932, Paris, France*
- BÉNÉDICTE MANOURY • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- SOPHIA MASCHALIDI • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- JESSICA MATTHIS • *Benaroya Research Institute, Seattle, WA, USA*

- ELIZABETH MELLINS • *Division of Transplantation Biology and Immunology, Department of Pediatrics, Stanford University Medical School, Stanford, CA, USA*
- ESPEN MELUM • *Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
- ALEXANDRE MICHAUX • *Ludwig Institute for Cancer Research, Brussels Branch and WELBIO and de Duve Institute, Université catholique de Louvain, Brussels, Belgium*
- NUALA MOONEY • *INSERM UMR-S 940, Institut Universitaire d'Hématologie and Université Paris-Diderot, Paris, France*
- ARENDE MULDER • *Department of Immunohaematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands*
- CHRISTIAN MÜNZ • *Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland*
- AYESHA MURSHID • *Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
- KEDAR NARAYAN • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- JACQUES NEEFJES • *Department of Cell Biology II, The Netherlands Cancer Institute, Amsterdam, The Netherlands*
- MORTEN NIELSEN • *Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark*
- MEREDITH O'KEEFFE • *Centre for Immunology, Burnet Institute, Melbourne, Australia and Department of Immunology, Monash University, Melbourne, Australia*
- TORSTEN OLSZAK • *Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
- PETRA PAUL • *Department of Cell Biology II, The Netherlands Cancer Institute, Amsterdam, The Netherlands*
- YURI POLUEKTOV • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- HELENA REIJONEN • *Benaroya Research Institute, Seattle, WA, USA*
- ERIC REITS • *Department of Cell Biology & Histology, AMC/University of Amsterdam, Amsterdam, The Netherlands*
- MAAIKE E. RESSING • *Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands*
- CORNELIA H. RINDERKNECHT • *Division of Transplantation Biology and Immunology, Department of Pediatrics, Stanford University Medical School, Stanford, CA, USA*
- SCHEHERAZADE SADEGH-NASSERI • *Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA*
- TAKESHI SATOH • *Institut Curie, INSERM U932, Paris, France*
- LOREDANA SAVEANU • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- HANSJÖRG SCHILD • *Institute for Immunology, University of Mainz, Mainz, Germany*
- ELODIE SEGURA • *Institut Curie, INSERM U932, Paris, France*
- NILABH SHASTRI • *Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA*
- NICHOLAS A. SICILIANO • *Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA*
- SEBASTIAN SPRINGER • *Molecular Life Science, Jacobs University Bremen, Bremen, Germany*

- ANITA STARGARDT • *Department of Cell Biology & Histology, AMC/University of Amsterdam, Amsterdam, The Netherlands*
- STEFAN STEVANOVIC • *Department of Immunology, University of Tübingen, Interfaculty Institute for Cell Biology, Tübingen, Germany*
- CECILE TAFLIN • *INSERM UMR-S 940 Institut Universitaire d'Hématologie and Université Paris-Diderot, Paris, France*
- SEBASTIAN TEMME • *Institute of Molecular Cardiology, University of Düsseldorf, Düsseldorf, Germany; Division of Immunobiology, Institute of Genetics, University of Bonn, Bonn, Germany*
- STEFAN TENZER • *Institute for Immunology, University of Mainz, Mainz, Germany*
- KATHRIN TEXTORIS-TAUBE • *Institut für Biochemie, Berlin, Germany*
- MIRA TOHMÉ • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- NICO TRAUTWEIN • *Department of Immunology, Interfaculty Institute for Cell Biology, University of Tübingen, Tübingen, Germany*
- BENOIT J. VAN DEN EYNDE • *Ludwig Institute for Cancer Research, Brussels Branch and WELBIO and de Duve Institute, Université catholique de Louvain, Brussels, Belgium*
- PETER VAN ENDERT • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- NATHALIE VIGNERON • *Ludwig Institute for Cancer Research, Brussels Branch and WELBIO and de Duve Institute, Université catholique de Louvain, Brussels, Belgium*
- DAVID VREMEC • *The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia*
- PAMELA A. WEARSCH • *Department of Pathology, Case Western Reserve University, Cleveland, OH, USA*
- MIRJANA WEIMERSHAUS • *INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, Faculté de médecine René Descartes, Paris, France*
- EMMANUEL J.H.J. WIERTZ • *Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands*
- M.I. YUSEFF • *Institut Curie, Inserm U932, Paris, France*
- SEBASTIAN ZEISSIG • *Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA; Department of Internal Medicine I, University Medical Center Schleswig-Holstein, Christian-Albrechts-University, Kiel, Germany*

Chapter 1

Purification of Large Cytosolic Proteases for In Vitro Assays: 20S and 26S Proteasomes

Stefan Tenzer, Tobias Hain, Hendrik Berger, and Hansjörg Schild

Abstract

Proteasomes are the main cytosolic proteases responsible for generating peptides for antigen processing and presentation in the MHC (major histocompatibility complex) class-I pathway. Purified 20S and 26S proteasomes have been widely used to study both specificity and efficiency of antigen processing. Here, we describe the purification of active human 20S and 26S proteasomes from human erythrocytes by DEAE-ion exchange chromatography, ammonium sulfate precipitation, glycerol density gradient centrifugation, and Superose-6 size exclusion chromatography and their characterization using fluorogenic substrates and specific inhibitors.

Key words: 20S Proteasome, 26S Proteasome, Antigen processing, Ion exchange chromatography, Ammonium sulfate precipitation, Density gradient centrifugation, Size exclusion chromatography, Peptide digest

1. Introduction

Cytosolic protein degradation depends largely on the ubiquitin-proteasome system (1). Misfolded, aged or no longer needed proteins are tagged for degradation by poly-ubiquitin chains formed by sequential action of E1, E2 and E3 enzymes (1). Ubiquitin-tagged proteins are recognized by the 19S cap of the 26S proteasome, which binds, deubiquitinates, and unfolds substrates for degradation within the core 20S proteasome (2). The 20S-Proteasome is composed of 14 different subunits, which are arranged in four stacked rings with the stoichiometry $\alpha_7\beta_7\beta_7\alpha_7$. The two outer α -rings contain seven related α -subunits ($\alpha_1-\alpha_7$), while the inner β -rings are built from seven different β -subunits ($\beta_1-\beta_7$) (3). The proteolytically active subunits $\beta_1(Y)$, $\beta_2(Z)$, and $\beta_5(MB1)$ are located within the beta rings and can be exchanged to their so-called immuno-counterparts $\beta_1i(LMP2)$, $\beta_2i(MECL1)$,

and β 5i(LMP7) upon stimulation with interferon- γ , which leads to a change in proteasomal specificity (3). Proteasomes produce fragments of 3–20 amino acids in length (4, 5), which are subsequently further trimmed N-terminally by aminopeptidases such as puromycin sensitive aminopeptidase (PSA) (6), bleomycin hydrolase (6, 7), or leucine aminopeptidase (LAP). A fraction of proteasomal products is able to bind to the transporter associated with antigen processing (TAP), which translocates the peptides into the endoplasmatic reticulum (ER), where they can be further trimmed by ER-resident aminopeptidases such as ERAP1/2 before they are loaded onto MHC class I molecules (8). The MHC class I-peptide complexes are subsequently exported to the cell surface for scrutiny by cytotoxic T cells. Numerous studies have used *in vitro* digests of peptides (9) and full-length proteins (4) using purified proteasomes to determine their specificity and antigen processing efficiency (10) for various model substrates and CTL epitope precursor peptides, allowing the development for prediction algorithms for proteasomal cleavages (11) and antigen processing efficiency (12, 13). We here present optimized protocols for the purification of intact, proteolytically active 20S and 26S proteasomes to purities >95%.

2. Materials

All buffers should be prepared using ultrapure water (prepared by purifying deionized water to attain a conductivity of 18 $M\Omega/cm$ at 25°C) and analytical grade reagents. Prepare and store all buffers at 4°C temperature (unless indicated otherwise). Filter all buffers for use in FPLC through a 0.22 μm filter before use. All work should be performed at 4°C to maximize yield of active proteasomes. All waste disposal regulations should be diligently followed when disposing waste materials.

2.1. Common Consumables and Lab Equipment

1. Human erythrocytes.
2. Phosphate Buffer Saline, pH 7.2 (PBS).
3. $(NH_4)_2SO_4$ (solid).
4. Fluorescence Sample Buffer (FSB): 20 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1 mM ATP.
5. Fluorogenic substrates (10 mM in DMSO):
 - (a) suc-LLVY-AMC.
 - (b) Z-ARR-AMC.
 - (c) Z-LLE-AMC.
6. DEAE-52-Cellulose.

7. 1 M NaCl.
8. Wash buffer: 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl.
9. Cold room (4°C).
10. High-Speed Centrifuge with rotor for 6×400 ml flasks, e.g., Sorvall GS-3 or equivalent.
11. Büchner-funnel, suction flask, filter, vacuum pump.
12. Fluorimeter (excitation wavelength between 360 and 380 nm, emission wavelength between 430 and 460 nm).
13. Ultracentrifuge with SW40Ti-Rotor (Beckman) (or equivalent swinging bucket rotor with a capacity of 6×14 ml).
14. Amicon Ultra-15 and Ultra-4 (100 kDa cutoff) concentration units.
15. FPLC-Equipment, e.g., ÄKTA Purifier.
16. Anion-Exchange Chromatography Resin DEAE-Toyopearls 650S.
17. Column HR16/50 (Amersham Pharmacia) or equivalent column for 100 ml gel volume.
18. Superose-6 HR10/30 gel filtration columns.
19. Dialysis tubing (10 kDa cutoff).

2.2. 20S Proteasome Purification

1. Lysis Buffer LB20S: 30 mM Tris-HCl pH 7.6, 2 mM MgCl₂, 0.1 mM EDTA, 1.6 mM DTT.
2. TPDG-Buffers: 20 mM Tris-HCl pH 7.6, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 5% Glycerol. TPDG-Buffers contain variable mM amounts of NaCl indicated by the index, e.g., TPDG₁₀₀ contains 100 mM NaCl.
3. Gradient Buffer 20S-15%: 20 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 15% Glycerol.
4. Gradient Buffer 20S-40%: 20 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 40% Glycerol.
5. Proteasome inhibitors:
 - (a) Lactacystin (10 mM in DMSO).
 - (b) Epoxomicin (10 mM in DMSO).

2.3. 26S Proteasome Purification

1. Lysis Buffer LB26S : 30 mM Tris-HCl pH 7.6, 2 mM MgCl₂, 0.1 mM EDTA, 1.6 mM DTT, 1 mM ATP.
2. TSDGA-Buffers: 20 mM Tris-HCl pH 7.6, 10 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% Glycerol, 1 mM ATP TSDGA-Buffers contain variable mM amounts of KCl indicated by the index, e.g., TSDGA₁₀₀ contains 100 mM KCl.

3. Gradient Buffer 26S-15%: 20 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1 mM ATP, 15% Glycerol.
4. Gradient Buffer 26S-40%: 20 mM Tris-HCl pH 7.6, 100 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1 mM ATP, 40% Glycerol.

2.4. SDS PAGE and Immunoblotting

1. 12% SDS polyacrylamide gels.
2. Coomassie Blue.
3. Nitrocellulose or PVDF membrane.
4. Antibodies against different proteasomal subunits.

3. Methods

3.1. Purification of 20S Proteasomes from Human Erythrocytes

Purification of active 20S proteasomes from human erythrocytes is described in Subheadings 3.1.1–3.1.6. The protocol includes preparation of the erythrocytes from erythrocyte concentrate, batch adsorption to DEAE-52 cellulose, (NH₄)₂SO₄ precipitation, ion-exchange FPLC, glycerol-gradient ultracentrifugation, Superose-6 gel filtration chromatography, and the final concentration by ultrafiltration. The average yield of this protocol is 2 mg of >95% pure 20S proteasomes using two erythrocyte concentrate conserves (2× 330 ml) as starting material.

3.1.1. Preparation of Erythrocytes

1. Empty the two erythrocyte conserves into a large beaker.
2. Add 1,800 ml ice-cold PBS, mix by stirring.
3. Transfer to 400 ml centrifuge flasks.
4. Centrifuge for 20 min, 1,500×*g* at 4°C.
5. After centrifugation carefully remove supernatant and the whitish layer containing leukocytes (see Note 1).
6. Repeat steps 2–5 twice.
7. Add 2.5 volumes of ice-cold Lysis Buffer LB20S (600–800 ml depending on the loss during the washing steps) to the erythrocytes.
8. Shake on an orbital shaker for 30 min at 4°C.
9. Centrifuge at 9,000×*g* for 45 min, 4°C to pellet unlysed erythrocytes and debris (see Note 2).
10. Carefully decant supernatant, ensure that debris pellet is not disturbed as this may lead to very low flow rates in subsequent batch adsorption chromatography.

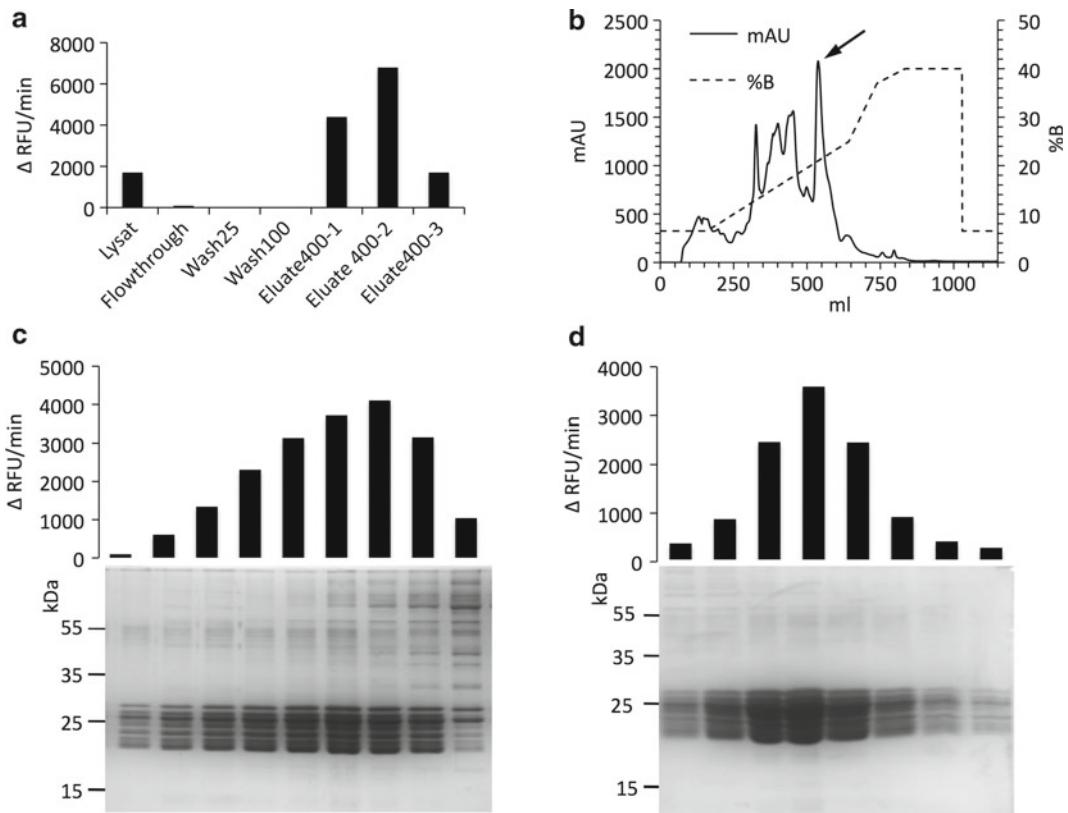


Fig. 1. Purification of 20S proteasomes (a) relative activity of fractions obtained in initial purification steps, (b) elution profile of DEAE-anion-exchange FPLC, the peak containing 20S proteasomes is marked with an arrow (c) relative activity and SDS-PAGE analysis of glycerol gradient fractions (d) relative activity and SDS-PAGE analysis of Superose-6 gel filtration FPLC fractions.

3.1.2. DEAE-Batch Adsorption Chromatography

Batch adsorption chromatography should be performed at 4°C and is required for the removal of hemoglobin, which makes up for >90% of total protein present in the erythrocyte lysate. Relative activities of selected fractions obtained in this are shown in Fig. 1a.

1. Preparation of DEAE-52-Cellulose:

- Add 600 ml dH₂O to 100 g of DEAE-52-Cellulose, allow to settle down for 30 min, and then decant to remove fines. Repeat twice.
 - Resuspend in 300 ml of 1 M NaCl. Transfer material to a Büchner-funnel.
 - Equilibrate with 400 ml Lysis Buffer LB20S.
- Apply erythrocyte lysate to material at a flow rate of approx. 50 ml /min (see Note 3).
 - Wash with a total of 1,000 ml TPDG₂₅ in 200 ml aliquots at a flow rate of 50–100 ml/min.

4. Wash with 500 ml TPDG₁₀₀ in 100 ml aliquots at a flow rate of 50–100 ml/min.
5. Elute with 500 ml TPDG₄₀₀ at a flow rate of 25–50 ml/min; collect 50 ml Fractions.
6. Perform activity assay:
 - (a) Add 20 µl of each fraction to 200 µl FSB containing 100 µM suc-LLVY-AMC.
 - (b) Incubate for 30 min at 37°C.
 - (c) Measure fluorescence intensity (excitation 360 nm, emission: 450 nm) (see Note 4).
7. Pool Fractions containing more than 30% of maximal activity
8. Recycle DEAE-52-cellulose by alternating washes (500 ml each) with dH₂O and wash buffer, until the white color of the resin is restored.

3.1.3. (NH₄)₂SO₄ Precipitation and Dialysis

1. Measure volume of pooled fraction from Subheading 3.1.2, step 7 using a graded cylinder.
2. Slowly add 230 mg/ml (NH₄)₂SO₄ while stirring at 4°C over 30 min.
3. Stir slowly for 1 h at 4°C.
4. Centrifuge at 27,000×*g*, 4°C for 30 min.
5. Transfer supernatant to new beaker, slowly add 242 mg/ml (based on the volume measured in step 1) (NH₄)₂SO₄ while stirring at 4°C.
6. Stir slowly for 1 h at 4°C.
7. Centrifuge at 27,000×*g*, 4°C for 30 min.
8. Discard supernatant.
9. Dissolve pellet in minimal volume (25–50 ml) of TPDG 0.
10. Transfer solution to dialysis tubing (10 kDa cutoff).
11. Dialyze for 6 h to 15 h against 2,000 ml of TPDG₂₅.

3.1.4. DEAE-Ion-Exchange FPLC

For ion exchange FPLC, a HR16/50 column packed with approx. 100 ml TSK-DEAE 650S Toyopearls resin is used. This resin allows high recovery of active 20S proteasomes while offering very high resolution and therefore higher purity of the final preparations. A representative chromatogram is shown in Fig. 1b.

1. Prepare the following buffers:
 - (a) Buffer A: TPDG₀.
 - (b) Buffer B: TPDG₁₀₀₀.
- FPLC should be performed at 4°C using a flow rate of 2 ml/min.
2. If any precipitate is visible in the dialyzed (NH₄)₂SO₄-precipitate (Subheading 3.1.3, step 11), centrifuge for 30 min at 27,000×*g*, 4°C.

3. Filter the dialyzed $(\text{NH}_4)_2\text{SO}_4$ -precipitate through a 0.22 μm filter to remove any precipitates or aggregates, which may clog the column.
4. Equilibrate the column with 8% buffer B for 200 ml.
5. Apply the sample to the column at a flow rate of 1 ml/min.
6. Wash the column with 300 ml of 8% buffer B.
7. Elute bound protein from the column with the following gradient:
 - (a) 8–25% buffer B in 400 ml.
 - (b) 25–36% buffer B in 20 ml.
 - (c) 36% for 150 ml.
 - (d) 40% for 150 ml.
- Collect Fractions with a volume of 4 ml.
8. 20S proteasomes usually elute at a conductivity of 140–160 mM (measured after the column).
9. Perform activity assay of all fractions (see Subheading 3.1.2, step 6).
10. Pool fractions containing more than 50% of maximal activity.
11. Regenerate the column and prepare it for long-term storage: Wash at a flow rate of 1 ml/min with:
 - (a) 500 ml 10 mM Tris–HCl pH 8, 1 M NaCl.
 - (b) 300 ml 10 mM Tris–HCl pH 8.
 - (c) 300 ml dH₂O.
 - (d) 300 ml 20% EtOH.

3.1.5. Glycerol-Gradient Ultracentrifugation

1. Concentrate the pooled fractions from Subheading 3.1.4, step 10 from Ion-Exchange Chromatography to a volume of 0.5–1 ml using an Amicon Ultra-15 concentration cell (100 kDa cutoff) (see Note 5).
2. Prepare linear glycerol density gradients containing 15–40% glycerol using gradient buffers 20S (15%) and 20S (40%) in 14 ml clear ultracentrifugation tubes using a gradient mixer.
3. Centrifuge at 150,000 $\times g$ at 4°C for 18 h (slow acceleration, no brake).
4. Harvest the gradients in 500 μl fractions (see Note 6).
5. Perform Activity assay of all collected fraction: add 10 μl of each fraction to 200 μl Fluorescence Sample Buffer containing 100 μM suc-LLVY-AMC. Incubate for 30 min at 37°C. Measure fluorescence (excitation 360 nm, emission: 450 nm).
6. Pool fractions containing more than 50% of maximal activity. A representative activity assay and SDS-PAGE of obtained fractions is depicted in Fig. 1c.

3.1.6. Superose-6 Size Exclusion Chromatography

1. Concentrate the pooled fractions from Subheading 3.1.5, step 6 to a volume of 0.5 ml using an Amicon Ultra-4 concentration cell (100 kDa cutoff).
2. Equilibrate two Superose-6 HR10/30 columns coupled in series with TSPG₁₀₀ for three column volumes at a flow rate of 0.3 ml/min at 4°C.
3. Apply concentrated sample to column.
4. Elute Column with 1.1 column volumes of TSPG₁₀₀, collect fractions of 0.5 ml.
5. Perform Activity assay of all collected fraction (see Subheading 3.1.5, step 5).
6. Pool fractions containing more than 50% of maximal activity (Fig. 1d).
7. Concentrate the pooled fractions to a protein concentration of approx. 1 mg/ml using an Amicon Ultra-4 concentration cell (100 kDa cutoff).
8. Aliquot the purified proteasomes and freeze at -80°C.

3.2. Purification of 26S Proteasomes from Human Erythrocytes

Purification of active 26S proteasomes from human erythrocytes is described in Subheadings 3.2.1–3.2.7. Analogous to purification of 20S proteasomes, this includes preparation of the erythrocytes from erythrocyte concentrate, batch adsorption to DEAE-52 cellulose, (NH₄)₂SO₄ precipitation, ion-Exchange FPLC, glycerol-gradient ultracentrifugation, and final buffer exchange and concentration. The average yield is 0.8 mg of >95% pure 26S proteasomes from two erythrocyte concentrate conserves (2× 330 ml)

3.2.1. Preparation of Erythrocytes

Purification of erythrocytes for isolation of proteasomes is performed as described in Subheading 3.1.1, steps 1–6.

3.2.2. Hypotonic Lysis of Erythrocytes

1. Add 2.5 volumes of ice-cold Lysis Buffer LB26S (600–800 ml depending on the loss during the washing steps) to the erythrocytes.
2. Shake on an orbital shaker for 30 min at 4°C.
3. Centrifuge at 9,000 × g for 45 min, 4°C to pellet unlysed erythrocytes and debris (see Note 2).
4. Carefully decant supernatant, ensure that debris pellet is not disturbed as this may lead to very low flow rates in subsequent batch adsorption chromatography.

3.2.3. DEAE-Batch Adsorption Chromatography

1. Preparation of DEAE-52-Cellulose:
 - (a) Add 600 ml dH₂O to 100 g of DEAE-52-Cellulose, allow to settle down for 30 min, then decant to remove fines. Repeat twice.

- (b) Resuspend in 300 ml of 1 M NaCl. Transfer material to a Büchner-funnel.
- (c) Equilibrate with 400 ml Lysis Buffer LB26S.
- 2. Apply erythrocyte lysate to material at a flow rate of approx. 50 ml /min (see Note 3).
- 3. Wash with a total of 1,000 ml TSDGA₂₅ in 200 ml aliquots at a flow rate of 50–100 ml/min.
- 4. Wash with 500 ml TSDGA₁₀₀ in 100 ml aliquots at a flow rate of 50–100 ml/min.
- 5. Elute with 500 ml TSDGA₄₀₀ at a flow rate of 25–50 ml/min; collect 50 ml Fractions.
- 6. Perform activity assay (see Subheading 3.1.2, step 6).
- 7. Pool fractions containing more than 30% of maximal activity.
- 8. Recycle DEAE-52-cellulose by alternating washes (500 ml each) with dH₂O and wash buffer, until the white color of the resin is restored.

**3.2.4. (NH₄)₂SO₄
Precipitation and Dialysis**

- 1. Measure volume of pooled fractions from Subheading 3.2.3, step 7 using a graded cylinder.
- 2. Slowly add 242 mg/ml (NH₄)₂SO₄ while stirring at 4°C over 30 min.
- 3. Stir slowly for 1 h at 4°C.
- 4. Centrifuge at 27,000×*g*, 4°C for 30 min.
- 5. Decant and set aside supernatant (this contains 20S proteasomes, which can be further purified as described in Subheading 3.1.4).
- 6. Dissolve Pellet in minimal volume (25–50 ml) of TSDGA₀.
- 7. Transfer solution to dialysis tubing (10 kDa cutoff).
- 8. Dialyze for 6 h to 15 h against 2,000 ml of TSDGA₂₅.

**3.2.5. DEAE-Ion-Exchange
FPLC**

For ion exchange FPLC, a HR16/50 column packed with approx. 100 ml TSK-DEAE 650S Toyopearls resin is used. This resin allows high recovery of active 26S proteasomes while offering very high resolution and therefore higher purity of the final preparates.

- 1. Prepare the following buffers:
 - (a) Buffer A: TSDGA₀.
 - (b) Buffer B: TSDGA₁₀₀₀.
- FPLC should be performed at 4°C using a flow rate of 2 ml/min.
- 2. If any precipitate is visible in the dialyzed (NH₄)₂SO₄-precipitate (Subheading 3.2.4, step 8), centrifuge for 30 min at 27,000×*g*, 4°C.

3. Filter the dialyzed $(\text{NH}_4)_2\text{SO}_4$ -precipitate through 0.22 μm filter to remove any precipitates or aggregates, which may clog the column.
4. Equilibrate the column with 8% buffer B for 200 ml.
5. Apply the sample to the column.
6. Wash the column with 300 ml of 8% buffer B.
7. The protein is eluted from the column with the following gradient:
 - (a) 8–28% buffer B in 400 ml.
 - (b) 28–36% buffer B in 20 ml.
 - (c) 36% for 150 ml.
 - (d) 40% for 150 ml.

Collect Fractions with a volume of 4 ml.
8. 26S proteasomes usually elutes at a conductivity of 180 mM to 210 mM (measured after the column).
9. Perform Activity assay of all fractions (see Subheading 3.1.2, step 6).
10. Pool fractions containing more than 50% of maximal activity.
11. To regenerate the column and prepare it for long-term storage, wash at a flow rate of 1 ml/min with:
 - (a) 500 ml 10 mM Tris–HCl pH 8, 1 M NaCl.
 - (b) 300 ml 10 mM Tris–HCl pH 8.
 - (c) 300 ml dH₂O.
 - (d) 300 ml 20% EtOH.

3.2.6. Glycerol-Gradient Ultracentrifugation

1. Concentrate the pooled fractions from Subheading 3.2.5, step 10 from Ion-Exchange Chromatography to a volume of 0.5–1 ml using an Amicon Ultra-15 concentration cell (100 kDa cutoff) (see Note 5).
2. Prepare linear glycerol density gradients containing 15–40% glycerol using gradient buffers 26S (15%) and 26S (40%) in 14 ml clear ultracentrifugation tubes using a gradient mixer.
3. Centrifuge at 150,000 $\times g$, 4°C for 16 h (slow acceleration, no brake).
4. Harvest the gradients in 500 μl fractions (see Note 6).
5. Perform Activity assay of all collected fraction (see Subheading 3.1.5, step 5).
6. Pool fractions containing more than 50% of maximal activity.

3.2.7. Superose-6 Size Exclusion Chromatography

1. Concentrate the pooled fractions from Subheading 3.2.6, step 6 to a volume of 0.5 ml using an Amicon Ultra-4 concentration cell (100 kDa cutoff).

2. Equilibrate two Superose-6 HR10/30 columns coupled in series with TSDGA₁₀₀ for three column volumes at a flow rate of 0.3 ml/min at 4°C.
3. Apply concentrated sample to column.
4. Elute column with 1.1 column volumes of TSDGA₁₀₀, collect fractions of 0.5 ml.
5. Perform activity assay of all collected fraction (see Subheading 3.1.5, step 5).
6. Pool fractions containing more than 50% of maximal activity.
7. Concentrate the pooled fractions to a protein concentration of 1 mg/ml using an Amicon Ultra-15 concentration cell (100 kDa cutoff).
8. Aliquot the purified proteasomes and freeze at -80°C.

3.3. Characterization of Purified Proteasomes

3.3.1. SDS-PAGE

3.3.2. Western Blot

3.3.3. Fluorogenic Substrates

1. Resolve 10 µg of purified proteasomes on a 12% SDS-PAGE gel.
2. Stain with Coomassie Blue.

20S proteasomal subunits have a size between 20 and 30 kDa, no other bands should be visible in the stained gel (see Fig. 1d).

1. Resolve 0.5 µg of purified 20S or 26S proteasomes on a 12% SDS-PAGE gel
2. Transfer to nitrocellulose or PVDF membrane and analyze by Western blot utilizing antibodies against different proteasomal subunits (see Note 7).

The activity of 20S and 26S proteasomes can be defined by the ability to cleave fluorogenic substrates. For an exact measurement of the activity, a kinetic analysis is preferred over endpoint assays (see Note 8).

1. Prepare standard curve for AMC (0.3, 1, 3, 10, 30 µM) in triplicates.
2. Incubate 0.5 µg of purified proteasomes in 200 µl of FSB containing either 200 µM of either suc-LLVY-AMC, or Z-ARR-AMC, or Z-LLE-AMC. Prepare triplicates for each fluorogenic substrate.
3. Measure fluorescence intensity of each sample every 2 min in a fluorimeter (incubation temperature set to 37°C, excitation at 360 nm, emission at 450 nm) for a period of 100 min.
4. Plot the obtained fluorescence values over time and calculate the fluorescence increase over time for each substrate from the linear part of the curve.
5. Using the AMC-standard curve, calculate the activity of the purified proteasomes in units/µg (1 U is defined as the ability to cleave 1 pmol of substrate in a time period of 1 min).

20S Proteasomes purified by this protocol have activities from 20 to 50 U/ μ g (measured for the substrate suc-LLVY-AMC)

26S Proteasomes purified by this protocol have activities from 100 to 180 U/ μ g (measured for the substrate suc-LLVY-AMC)

3.3.4. Proteasomal Inhibitors

The proteasomal inhibitors lactacystin and epoxomicin can be used to exclude the presence of other proteases. For this assay, purified proteasomes are first preincubated for 30 min before addition of the fluorogenic substrate. Pure 20S and 26S Proteasomes are inhibited more than 98% after preincubation with these inhibitors.

1. Preincubate 1 μ g of purified 20S or 26S proteasomes in 200 μ l of FSB containing 100 μ M lactacystin or 2 μ M epoxomicin for 30 min at 37°C.
2. Add suc-LLVY-AMC to a final concentration of 200 μ M.
3. Measure proteasomal activity as described in Subheading 3.3.3.

4. Notes

1. Depending on purity of erythrocyte preparations, a whitish layer may be present on top of the erythrocyte pellet which contains mostly leukocytes. These contain proteasomes harboring also immuno-subunits. For a pure preparation of constitutive 20S and 26S proteasomes from erythrocytes, a complete removal of this layer is necessary.
2. Erythrocytes are usually completely lysed by this treatment. If incomplete lysis occurs (if a pellet with a volume of more than 10% of the starting erythrocytes remains), the remaining erythrocytes may be lysed by repeating the process, thereby optimizing the yield of the protocol.
3. Due to high viscosity of the erythrocyte lysate, loading by gravity flow is not possible. When applying vacuum, be careful not to let the DEAE material run dry. The capacity of the DEAE-Cellulose is high enough to bind most of the 20S and 26S proteasomes. If the flow through is passed again over the batch adsorption column after regeneration of the material, 20–30% higher yield can be achieved.
4. Standard measuring wavelengths for AMC are excitation at 360 nm and emission at 450 nm, alternative wavelengths are 380 nm for excitation and 430 nm for emission.
5. Alternatively, other centrifugation-based concentration units may be used with a MW cutoff below 300 kDa. In some cases, preblocking the membrane with 200 mM glycine reduces binding of the proteins to the membrane.

6. Harvesting the gradients is achieved either by inserting a capillary tube from the top and emptying the tube using a peristaltic pump or by drilling a small hole in the base of the tube and emptying by gravity flow, which gives slightly better resolution. The fraction size of 500 µl seems to be optimal for the purification of 20S proteasomes, however, slightly higher resolution and therefore higher purity may be achieved by collecting smaller fractions.
7. 20S and 26S proteasomes purified from human erythrocytes are of constitutive phenotype. In Western blot analysis, bands should only be detectable with antibodies against α -subunits and the proteolytically active constitutive β -subunits Y, Z, and δ . When staining with antibodies against the immuno-subunits LMP2, LMP7, and MECL-1, no bands should be detectable.
8. The use of a fluorimeters allowing incubation at 37°C is highly recommended. For kinetic measurements, an interval of 2 min between the measurements is best. For evaluation, a linear part of the curve should be chosen for linear regression, as an initial lag phase due to cold buffers may affect the results, when calculating the activity using an endpoint assay.

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References

1. Ciechanover A (2005) Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat Rev Mol Cell Biol* 6(1):79–87. doi:[nrm1552](#) (pii) [10.1038/nrm1552](#)
2. Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA, Finley D (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* 94(5):615–623. doi:[S0092-8674\(00\)81603-7](#) (pii)
3. Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386(6624):463–471. doi:[10.1038/386463a0](#)
4. Tenzer S, Stoltze L, Schonfisch B, Dengjel J, Muller M, Stevanovic S, Rammensee HG, Schild H (2004) Quantitative analysis of prion-protein degradation by constitutive and immuno-20S proteasomes indicates differences correlated with disease susceptibility. *J Immunol* 172(2):1083–1091
5. Kisseelev AF, Akopian TN, Woo KM, Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 26 and 20S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* 274(6):3363–3371
6. Stoltze L, Schirle M, Schwarz G, Schroter C, Thompson MW, Hersh LB, Kalbacher H, Stevanovic S, Rammensee HG, Schild H (2000) Two new proteases in the MHC class I processing pathway. *Nat Immunol* 1(5):413–418. doi:[10.1038/80852](#)
7. Enomoto Y, Bharti A, Khaleque AA, Song B, Liu C, Apostolopoulos V, Xing PX, Calderwood SK, Gong J (2006) Enhanced immunogenicity

- of heat shock protein 70 peptide complexes from dendritic cell-tumor fusion cells. *J Immunol* 177(9):5946–5955
8. Kloetzel PM, Ossendorp F (2004) Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr Opin Immunol* 16(1):76–81. doi:[S095279150300178X \(pii\)](https://doi.org/10.1016/j.coi.2003.11.003)
9. Asemissen AM, Keilholz U, Tenzer S, Muller M, Walter S, Stevanovic S, Schild H, Letsch A, Thiel E, Rammensee HG, Scheibenbogen C (2006) Identification of a highly immunogenic HLA-A*01-binding T cell epitope of WT1. *Clin Cancer Res* 12(24):7476–7482. doi:[12/24/7476 \(pii\) 10.1158/1078-0432.CCR-06-1337](https://doi.org/10.1158/1078-0432.CCR-06-1337)
10. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, Lamberth K, Chang CH, Harndahl M, Weimershaus M, Gerstoft J, Akkad N, Klenerman P, Fugger L, Jones EY, McMichael AJ, Buus S, Schild H, van Endert P, Iversen AK (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. *Nat Immunol* 10(6):636–646. doi:[ni.1728 \(pii\) 10.1038/ni.1728](https://doi.org/10.1038/ni.1728)
11. Nussbaum AK, Kuttler C, Hadeler KP, Rammensee HG, Schild H (2001) PAProC: a prediction algorithm for proteasomal cleavages available on the WWW. *Immunogenetics* 53(2):87–94
12. Larsen MV, Lundsgaard C, Lamberth K, Buus S, Brunak S, Lund O, Nielsen M (2005) An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur J Immunol* 35(8):2295–2303. doi:[10.1002/eji.200425811](https://doi.org/10.1002/eji.200425811)
13. Tenzer S, Peters B, Bulik S, Schoor O, Lemmel C, Schatz MM, Kloetzel PM, Rammensee HG, Schild H, Holzhutter HG (2005) Modeling the MHC class I pathway by combining predictions of proteasomal cleavage, TAP transport and MHC class I binding. *Cell Mol Life Sci* 62(9):1025–1037. doi:[10.1007/s00018-005-4528-2](https://doi.org/10.1007/s00018-005-4528-2)

Chapter 2

Analysis of Proteasome Generated Antigenic Peptides by Mass Spectrometry

**Kathrin Textoris-Taube, Christin Keller, Ulrike Kuckelkorn,
and Peter-M. Kloetzel**

Abstract

Mass spectrometry (MS) is today one of the most important analytical techniques in biosciences. The development of electro spray ionization (ESI) as a gentle ionization method, in which molecules are not destroyed, has revolutionized the analytic of peptides. MS is an ideal technique for detection and analysis of peptides generated by in vitro experiments using purified 20S proteasomes. It also provides a convenient and sensitive way to monitor the processing activity of enzymes. The combination of high performance liquid chromatography (HPLC) with ESI-MS allows the analysis of complex samples with separation in their specific constituents by LC and their subsequent detection by MS.

Key words: Proteasome, Mass spectrometry, HPLC, Antigen processing, Antigenic peptides, Polypeptides, Substrate degradation, Fragment generation, Epitope

1. Introduction

The protease responsible for the degradation of polyubiquitylated proteins and generation of antigenic peptides is the 26S proteasome, which is composed of the 20S proteasome, representing the catalytic core, and two 19S regulator complexes that confer the binding and unfolding of ubiquitylated substrates (1, 2). The hydrolyzing activities of the 20S core are conferred by three of the seven β subunits located in the two inner heptameric β -rings, whereas the 19S regulator complexes (composed of six ATPase subunits as well as 9–10 non-ATPase subunits) attach to the outer heptameric α rings of the 20S core (3, 4). Major histocompatibility complex (MHC) class I presented peptides are predominantly generated by the proteasome system. Thereby, IFN- γ strongly influences the processing efficiency by inducing immunoproteasome formation and proteasome activator PA28 synthesis. Depending on the protein substrate,

the presence of immunoproteasomes and PA28 (5–10) influence epitope liberation either positively or negatively.

To study proteasomal antigen processing simple in vitro systems have been established that allow mimicking the in vivo situation with surprisingly high fidelity. These assays generally involve the processing of a synthetic polypeptide of 20–30 amino acid residues in length that harbor a known or a potential MHC class I epitope by purified 20S proteasomes, HPLC-separation of the generated peptides followed by MS analysis of the generated peptide products (11) (see Note 1).

2. Materials

2.1. Proteasome Isolation

All solutions are prepared using ultra pure water (MilliQ) or *aqua demineralisata* (A. dem.)

1. Dounce homogenizer.
2. JA20 rotor and 50 ml tubes (Avanti J-E; Beckman & Coulter) and SW40Ti rotor and 14 ml tube (Optima LE-80K; Beckman & Coulter).
3. Beaker.
4. Ponceau red: 1% Ponceau in 5% trifluoroacetic acid (TFA).
5. Ammonium sulfate crystals.
6. 10×TEAbuffer: Dissolve 200 mM 2-amino-2-(hydroxymethyl)-propan-1,3-diol (12.1 g TRIS), 1 mM ethylenediaminetetraacetate (1.86 g Na-EDTA), and 1 mM sodium acid (0.3 g NaN₃) in 450 ml water. Mix and adjust the pH with 25% HCl to 7.2 and add the volume to 500 ml.
7. Stock 0.2 M DTT: Dissolve 308 mg dithiothreitol (DTT = C₄H₁₀O₂S₂) fresh in 10 ml water (see Note 2).
8. Stock solution 2 M NaCl: Dissolve 58.44 g NaCl in 500 ml water.
9. TEAD buffers: Prepare the different TEAD buffers from the 10× TEA stock solution (see Subheading 2.1, item 6), supplement the adequate volume of NaCl from a 2 M stock and 5 ml 0.2 M DTT/l (see below).
 - (a) *TEAD-50*: 10 ml 10× TEAD, 2.5 ml 2 M NaCl, 0.5 ml 0.2 M DTT add to 100 ml.
 - (b) *TEAD-100*: 10 ml 10× TEAD, 25 ml 2 M NaCl, 2.5 ml 0.2 M DTT add to 500 ml.
 - (c) *TEAD-350*: 10 ml 10× TEAD, 17.5 ml 2 M NaCl, 0.5 ml 0.2 M DTT add to 100 ml.
 - (d) *TEAD-1000*: 50 ml 10× TEAD, 250 ml 2 M NaCl, 2.5 ml 0.2 M DTT add to 500 ml.

10. Stock solution 10% NP40: add 9 ml water to 1 g nonyl phenoxypolyethoxylethanol (NP40=tergitol-type NP-40).
11. 40% Sucrose: add 30 ml 1× TEAD to 20 g sucrose.
12. 10% Sucrose: add 45 ml 1× TEAD to 5 g sucrose.
13. Proteasome assay buffer: 50 mM Tris-HCl (pH 7.5), 1 mM DTT in deionized water: dissolve 0.146 g TRIS in 40 ml water, adjust the pH to 7.2 with 25% HCl, pipette 0.25 ml 0.2 M DTT and add the volume to 50 ml (see Note 2).
14. 20 mM Proteasome substrate stock: dissolve 7.64 mg of Suc-LLVY-AMC (Succinyl-leucine-leucine-valine-tyrosine-7-amino-4-methylcoumarin) in 500 µl DMSO (dimethyl sulfoxide).
15. Lysis buffer: supplement 10 ml TEAD-50 with 0.1 ml 0.1% (w/v) NP-40.
16. Diethylaminoethyl-(DEAE) Sephadex®: pre-swollen Suspension in 20% ethanol is washed by water to remove the ethanol. The DEAE-Sephadex has been equilibrated in TEAD-50 over night.
17. Open 50 ml column (BioRad).
18. Anion exchange column: MonoQ 5/50 GL, 1 ml column volume (CV) (GE Healthcare).
19. Fast protein liquid chromatography (FPLC) buffers are filtrated (Millipore filter 0.2 µ) to remove particles and degassed.
 - (a) Buffer A: TEAD-100.
 - (b) Buffer B: TEAD-1000.
20. Black 96-well microtiter plate and plate fluorescence reader (i.e., BioTek Synergy HT).

2.2. Proteasome Digestion Assay Mix

1. Stock solution 10× TEA buffer: see Subheading [2.1](#), item 2.
2. Stock solution 2 mM DTT: dissolve 3.08 mg DTT in 10 ml water (see Note 2).
3. Digestion buffer: 1 ml solution 10× TEA and 1 ml solution 2 mM DTT in 10 ml water.
4. Substrate solution: 1 mg peptide in 1 ml 0.5% trifluoroacetic acid (TFA) in water (see Note 3).
5. Reagent to stop digestion: 3% TFA solution in water (final: 0.3% TFA).

2.3. HPLC Analyses

All reagents in particular the mobile phases used for HPLC and MS analysis should fulfill the following criteria: HPLC or better MS grade.

1. Analytical reversed phase-(RP) chromatography.
Stationary phase:
C18 RP material column, 0.3 cm long with a core of 4.6 mm and a pore size of 1.5 µm.

Mobile phases:

- (a) Solvent A: 0.5% TFA in water, add 5 ml 100 %TFA to 995 ml water.
- (b) Solvent B: 0.45 %TFA in acetonitrile (ACN).

2.4. HPLC-MS Analyses

1. HPLC/ESI-MS:

Stationary phases:

- (a) Analytical column: RP18 ID 1 mm, length 10 cm, particle size 3 µm, pore size 150 Å.
- (b) Precolumn: RP18, ID 1 mm, length 1 cm, 3 µm particle size.

Mobile phases:

- (a) Solvent A: 0.05% TFA in water.
- (b) Solvent B: 0.045 %TFA in 70% ACN and 30% water (see Note 4).

Stock solution of the 9GPS standard:

1 mg YPHFMPTNLGPS (9GPS, MG1359.6) is dissolved in 1 ml 50% methanol (MeOH)-50% water (H_2O), 1% acetic acid (AcOH).

9GPS standard for analysis:

Dissolve 10 µl stock solution of the 9GPS standard in 990 µl 50% MeOH-50% water, 1% (AcOH). Freeze five aliquots a 200 µl (see Note 5).

2. Analysis software.

3. Methods

3.1. Proteasome Preparation (12)

20S proteasomes are purified from cultured cells (see Note 6).

1. The cells are lysed mechanically in 20 ml lysis buffer using a dounce homogenizer (25–30 strokes). Store the cells for 10 min on ice to complete lysis. The cell debris is removed by centrifugation at 17,000 rpm (40,000 $\times g$) for 30 min at 4°C (Avanti J-E centrifuge, JA20 rotor). The cleared supernatant is used for further purification.
2. DEAE-anion-exchange-chromatography
 - (a) First the DEAE Sephadex is equilibrated in TEA buffer (see Note 7) over night (about 20 ml).
 - (b) To bind the protein-fractions the supernatant of the first purification step is incubated with the DEAE sephadex on a bottle roller at 4°C for 1 h.

- (c) The DEAE gel is filled into an open 50 ml column and washed six times with about 10 ml TEAD-50 controlling the protein amount in the flow through (absorption at 280 nm or spotting drops from the eluates onto nitrocellulose and staining with Ponceau red (see Note 8)). The amount of proteins in the flow through should be markedly reduced (see Note 8).
 - (d) In the next step, DEAE bound proteins are eluted with TEAD-350 and collected in about 20 fractions à 2 ml.
 - (e) The proteolytic activity is determined (see Subheading 3.1, step 6).
3. The proteolytically active fractions are combined and subjected to fractional ammonium sulfate precipitation (see Note 9).
- (a) First the volume of the pooled fractions is estimated, and then filled in a beaker, which is placed in an ice bath.
 - (b) Under continuous stirring ammonium sulfate crystals are added very slowly to achieve a 35% saturation (1.94 g ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) per 10 ml solution).
 - (c) The mixture is centrifuged at 12,000 rpm (29,000 $\times g$; JA20 rotor) for 10 min at 4°C.
 - (d) The supernatant is filled in a fresh beaker and 2.91 g of ammonium sulfate crystals per 10 ml are added to 80% saturation.
 - (e) The precipitate is collected by centrifugation at 19,000 rpm (46,000 $\times g$; JA20 rotor) at 4°C for 10 min. The pellet is slowly dissolved in 1 ml TEAD-50 on ice.
4. The completely resolved ammonium sulfate precipitate is overlaid onto a 10–40% sucrose gradient in a 14 ml SW40 tube (see Note 10).
- (a) Centrifugation has to be performed for 16 h at 40,000 rpm (285,000 $\times g$) in a SW40Ti rotor at 4°C (corresponding to $1 \times 10^{12} \omega^2 t$).
 - (b) The gradients are fractionated in 0.6 ml aliquots and the proteolytic activity is determined (see Subheading 3.1, step 6).
 - (c) The proteolytically active fractions are pooled and dialyzed with TEAD-100 over night at 4°C.
5. The dialyzed samples are diluted with 9 ml buffer A. Non-dissolved proteins are removed by a disposable filter holder (0.2 μm).
- (a) The anion exchange column is equilibrated with 5 CV buffer A.
 - (b) The filtrate is applied with a flow of 1 ml/min.
 - (c) After washing the column with buffer A (2CV), proteins are eluted with a linear NaCl-gradient. Within 5 min the

concentration of buffer is raised from 0 to 20%, thereafter the gradient increases from 20 to 40% in 20 min and in 2 min from 40 to 100%. At a concentration of 1 M NaCl (100 %B), the column is washed for 5 min and returned to buffer A within 2 min.

- (d) Fractions are collected in a volume of 1 ml during the elution with 20–40% buffer B (see Note 11).
 - (e) The proteolytic activity is determined (see Subheading 3.1, step 6).
6. To prepare the proteasome activity assay:
- (a) The substrate (Suc-LLVY-AMC; 20 mM stock solution) measuring the chymotrypsin-like activity is added freshly to the proteasome assay buffer (10 µl to 10 ml buffer to a final concentration 20 µM).
 - (b) 10 µl sample or 10 µl water (reference value) are placed into the wells of a black 96-well microtiter plate. 100 µl substrate assay buffer is added. This reaction mixture is incubated for 60 min at 37°C.
 - (c) The released fluorogenic AMC is measured at an emission of 460 nm (excitation at 360 nm) in a plate fluorescence reader.
 - (d) Highly proteolytically active fractions are combined and aliquots are frozen or stored on ice.

3.2. Digestion

1. For time-dependent processing experiments (signal intensity versus time of digestion) 1 mg of the synthetic polypeptide substrate is incubated with 0.1 mg enzyme in an eppendorf tube at 37°C (2).
2. For a five time points kinetic experiment (0, 1, 4, 8, and 24 h to qualify) a master mix of approximately 150 µl is used. The mixture is partitioned in a volume of 29 µl to five labeled tubes and incubated for the indicated time.
3. The digestions are stopped with 3 µl 3% TFA (see Notes 13 and 14) or freezing at -20°C.

3.3. HPLC (See Note 15)

Proteasomal processing products are separated on an analytical reversed phase-(RP) column and generated peptides are monitored by UV at 220 nm.

1. 20 µl of the digestion solution (see Subheading 3.2) is injected into a sample loop of 50 µl (see Note 16) and measured by RP chromatography. The flow through of the mobile phase is 1 ml. Peptide bonds are specifically detected at a wavelength of 220 nm.
2. A short binary gradient (6% in 15 min from 5 to 95% mobile phase B) (see Note 17) is performed to monitor the substrate degradation (Fig. 1).

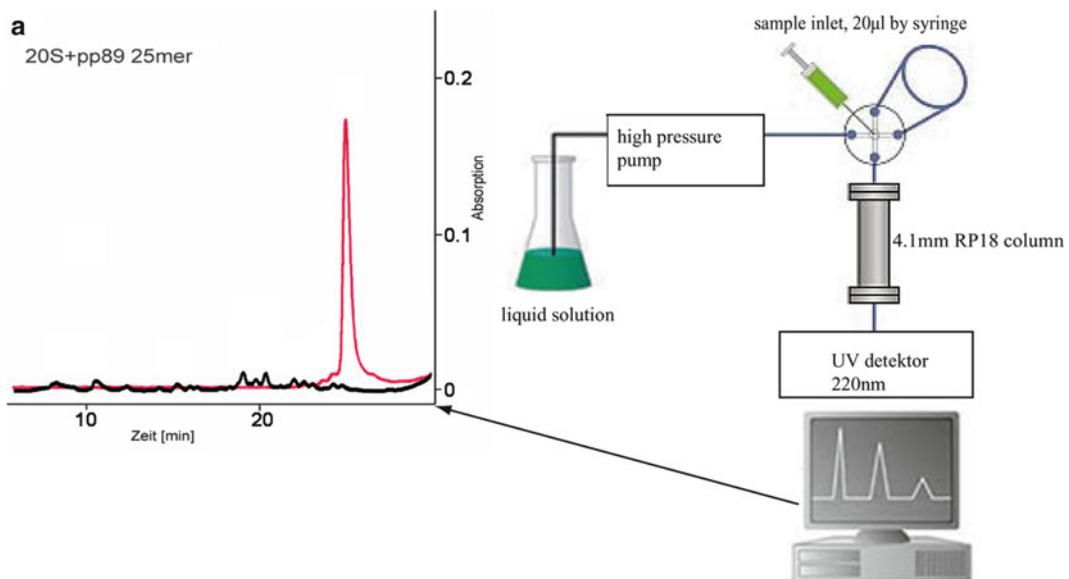


Fig. 1. Schematic configuration of a HPLC instrument with UV detection. The display at the head (a) illustrates two digestion experiments of a peptide with the 20S proteasome. Two chromatograms are displayed; the 0-h incubation period corresponds to the red marked graph, the 20-h digestion to the black one. Non-cleaved pp89 25mer peptide substrate (RLMYDMYDMPHFMPTNLGPSEKRVWMS, (18)) runs under the peak at 22.5 min and it is no longer detectable after digestion by 20S, but on the other hand, many individual peaks of the digestion products can be observed (A. Voigt, unpublished data). Using this experimental set up various options of the digestion approach can be optimized conditions (concentration of substrate and proteasomes, time points of digestion) (see Note 18).

3.4. HPLC/ESI-MS

1. 10 µl of digestion and 1 µl of 9GPS standard for analysis are mixed in a vial. A cooled autosampler (4°C) picks up 10 µl of the sample and loads it onto a 40°C heated (see Note 19) RP18-column with a flow rate of 30 µl/min. The sample is analyzed by a binary gradient starting with 3% eluent B and increasing up to 67% with a slope of 2% for each RP-analysis (see Note 20).
2. The resulting peaks are detected by ESI-MS in a data dependent experiment (see Note 21). Data are used for the identification and quantification of the proteasomal generated polypeptides. The identified fragments are described in a table called cleavage map (Fig. 2).

3.5. Analyze Data

Analysis by mass spectrometry allows identification and quantification of in vitro generated digestion products.

1. Identification (Fig. 4): The processed peptides can be identified in the ion trap by the mass to charge ratio determined by full (Fig. 4b) and zoomscan (Fig. 4c) and subsequent fragmentation (MS/MS, Fig. 4d). An evaluation software (mascot, bioworks (see Note 22)) compares theoretically calculated patterns of

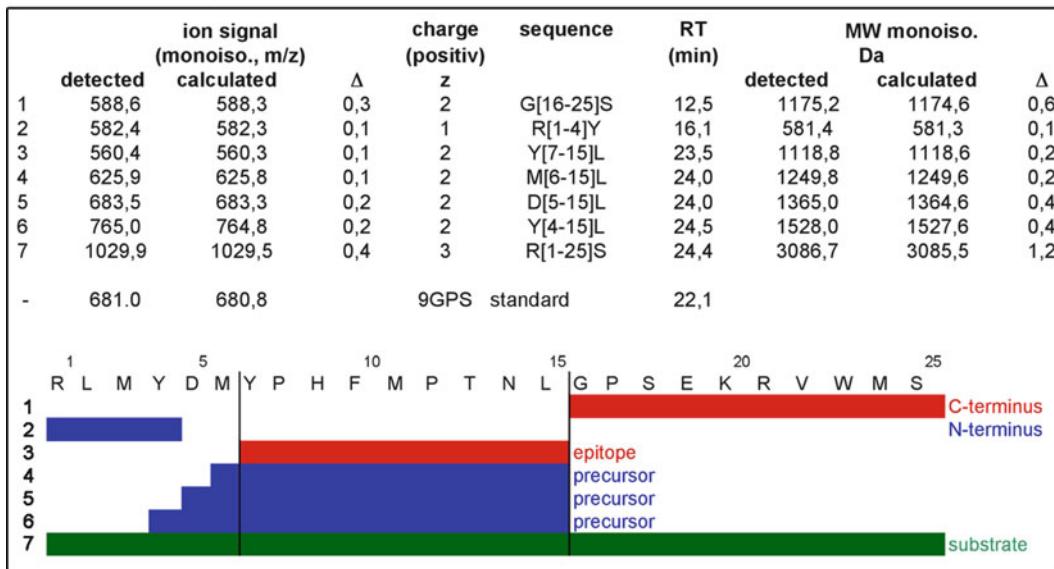
murine cytomegalovirus (MCMV) IE pp89 protein (14)**RLMYDMYPHFMPTNLGPSEKRVWMS-CONH2****mono MG: 3085.4****Proteasome: e.g. T2, T2.27 20S proteasomes****method****gradient: for example 3 - 67 % B (in 30 min), flow 30 µl/min****standard: 9GPS (YPHFMPTNLGPS)**

Fig. 2. This figure shows a so-called cleavage map, an Excel file containing all the important data for identification. The example shows some of the important pp89 (18) antigenic fragments (substrate, epitope, N-terminal elongated precursors of different length, C- and N-terminus).

peptides with the experimentally measured MSMS. Database search is performed using databases of the peptides (in fasta-format: x.fasta) and the following parameters: no enzyme, mass tolerance for fragment ions 1 amu. To get more reliable results the peptide product pattern and retention time should be compared to that of synthetic peptides (13).

- Quantification (Fig. 3): The number of detected ions is proportional to the produced signal. The integration of the signal measured by the profile mode describes the relative rate of product formation (see Notes 22 and 23).

4. Notes

- The mass range of ESI MS (200–2,000 Da) and the column (particle and pore size), which is used for chromatography limit the length of the peptide sequence that can be analyzed. Peptides with a length of up to 35 amino acid residues can be

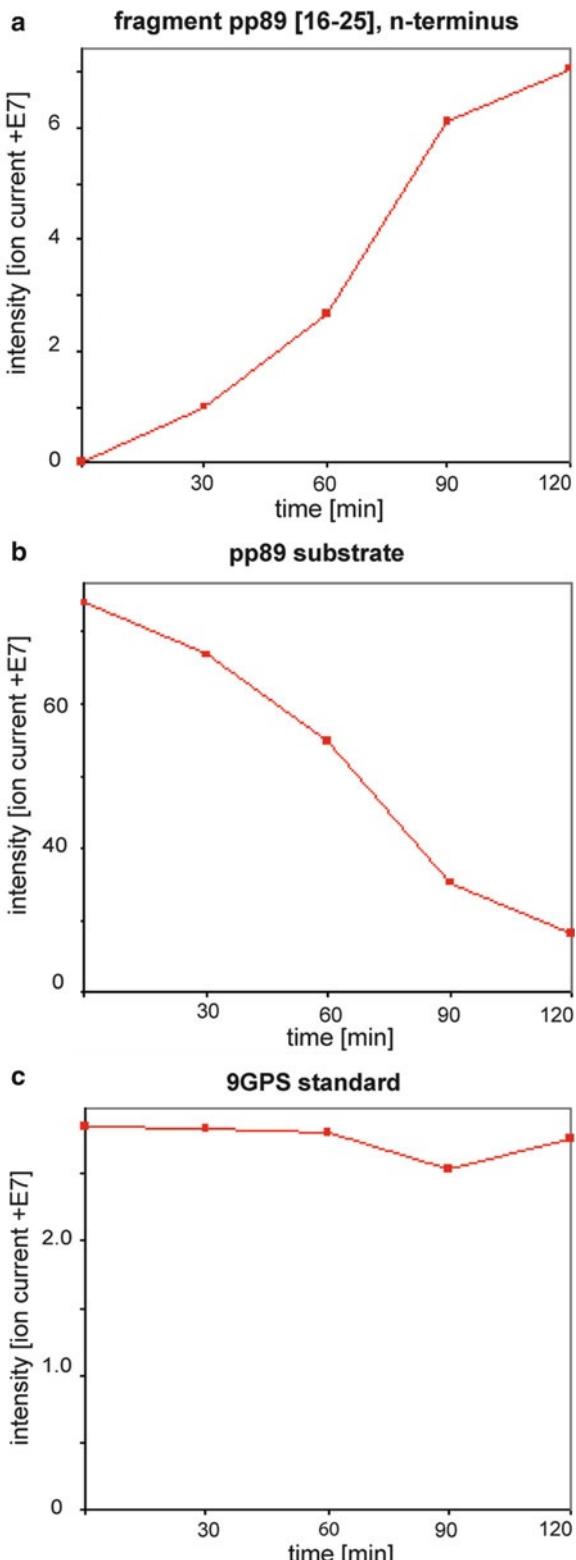


Fig. 3. The graphical plot of the measured intensity versus the incubation time forms so-called digestion kinetics of pp89 (14). The graphs illustrate (a) fragment generation, (b) substrate degradation, and (c) the 9GPS standard as a control for measurement.

measured and analyzed using this ionization method. The peptide sequences derived from proteins of choice (tumor antigen, virus etc.) harboring epitope sequences have a length between 8 and 12 aa residues.

For efficient analysis the amino- (N-) and carboxy- (C-) terminal flanking regions of the epitope sequence should encompass at least 6 residues. Shorter flanking sequences may negatively affect the processing efficiency and analysis by mass spectrometry.

Some of the amino acids tend to be modified: cysteine (C)=dimerization, oxidation, methionine (M)=oxidation.

The analysis of leucine and isoleucine cluster (L, I) is difficult using LC, because of their hydrophobicity. To avoid these methodical problems the number of these amino acid residues may be limited in the flanking regions. In some exceptional cases it is also possible to change an amino acid (for example C to serine (S)).

2. It is useful to prepare 10 ml 0.2 M DTT fresh in deionized water. Store it at -20°C.
3. Some peptides are poorly soluble, on this account we carefully tried to dissolve the solid substance in 100% dimethylformamide (DMF) and dilute it to 20% with mobile phase A (Subheading 2.4). The precipitation of a gelatinous, insoluble pellet should be avoided.

In case a gel-like, insoluble pellet is formed this should be discarded. For physiological reasons the solvent concentration should not be higher than 20% (v/v). To prevent oxidation it is useful to use DMF.

4. We normally prepare a 10% TFA solution. To mix the mobile phase A (Subheading 2.3) 5 ml TFA (10%) is dissolved in 995 ml water.
5. Due to the complexity of the samples it is thus possible to monitor the conditions of measurement via LC and MS. It is not intended to quantify fragments.

9GPS standard derived from pp89 is a peptide, which is not generated in the proteasomal digestion of pp89. This fragment is added prior the MS analysis and serves as a standard. During the whole analysis the peak area of 9GPS in comparable samples should be approximately the same. In addition, the amount of standard must be high enough to generate significant MSMS.

6. For 150–200 µg proteasome 10⁹–10¹⁰ cells should be used.
7. It is important to eliminate the ethanol.
8. A280 control: The absorbance at 280 nm is applicable to calculate the amount of protein.

9. The 20S proteasome is precipitated between 38 and 80% saturation of $(\text{NH}_4)_2\text{SO}_4$.
10. For SW40 tube, prepare the gradient with a gradient mixer: 6.5 ml 10% sucrose in the front chamber and 6.5 ml 40% sucrose in the retral chamber (the higher density solution is underlaided to the lower density solution). The proteins are separated in a SW40Ti rotor (BECKMAN ultra centrifuge Optima LE-80 K at $\omega^2 t = 1.0\text{E}12$, that means 40,000 rpm or $285,000 \times g$ for 16 h).
11. Proteasome is eluted in 28–30% B.
12. In a substrate/enzyme reaction the ratio of mass, not of volume, is critical. In general, an approximate 1,000-fold excess of substrate should be added.
13. The digest is stopped by adding of 0.1 Vol 3% TFA or by freezing. Adding TFA can increase the salt concentration. This may negatively affect the mass spectrometric analysis.
14. The following points should be considered when performing the digest:

Partitioning of the master mix provides reproducible results. The incubation in the whole master mix raises several problems:

 - (a) To achieve a constant temperature during digestion in the whole approach is difficult.
 - (b) The formation of condensation products in the lid of the sample tube can falsify results.

The biological matrix affects the MS signal. The 0-h-value has to include buffer and enzyme. To stop the reaction as quickly as possible, TFA should be placed in the tube for the zero hour time point. Be careful with the TFA concentration, because a high concentration of salt is able to quench the MS signal.
15. The separation of individual substances is based on the differential distribution of samples in two phases. One phase (liquid, mobile) moves to another phase (liquid, stationary) and takes over the constituents with different speeds.
16. For an optimal analysis the sample loop should be half filled.
17. The solvent B increases in 1 min with a slope of 6%, in this example (Subheading 3.3) the eluent B moves from 5 to 95% in 15 min. Eluate A decreases in 1 min 6%, this means it moves from 95 to 5% in the same time.
18. It is important that not more than 50% of the substrate is degraded at the time points that are used for the analyses (better 30–40% degradation time points). This prevents “reentry” of processing products in the 20S proteasome core particle and

reprocessing of the degradation products. To achieve this, we normally increase or decrease substrate or enzyme concentrations accordingly to optimize the substrate turnover. It is also possible to choose different incubation times.

Reliable kinetics should contain at least 5 measured time points (for example, 0, 1, 2, 4, and 8 h). To generate convincing fragmentation pattern (MSMS) the 24 h value is often essential. After 24 h a sufficiently large concentration of fragments has been generated to identify the processed peptides by MS.

19. Hydrophobic or high molecular weight peptides elute in a non-sharp, quenched flat peak. To optimize the resolution, the column temperature can be increased. Higher temperature will lead to a shorter column lifetime and some columns may not be able to tolerate 60°C. But to our experience peaks will be sharper and elute earlier with higher temperature.
20. Usually in the beginning the hydrophilic solvent A rinses (at least 10 min) the polar salts from the column. Salts are able to quench the MS signal.

The hydrophobic eluent B is able to wash the major (non polar) proteins (for example the proteasomes or other enzymes) from the column, because high molecular proteins may block the column. After performing the gradient we generally wash about 10 min with 100% B (if possible longer). Finally, the column is equilibrated for 10 min.

To improve peak separation, the concentration of buffer B should be determined at which the substrate elutes in the chromatogram. At this point in most of the cases the entire elution of the sample is achieved (see Note 17). Ending with this concentration of buffer B makes the gradient shallower and possibly leads to a better separation. The substrate should not elute in the equilibration phase, but also be well separated from processed fragments during the whole gradient.

Chromatography methods often start with a higher non-polar hydrophobic ACN concentration but this can lead to a loss of small polar fragments. To avoid this, these analytical methods (13) often begin with an initially steep gradient, flattening in the course of the chromatogram.

This should be considered depending on substrate and complexity of the sample.

TFA has been reported to suppress MS ionization and often in mass spectrometry a lower percentage of TFA (to 0.02%) is used without significant loss in chromatographic efficiency.

21. To facilitate the identification of many different peptides fragments with little sample consumption a data dependent scan experiment with dynamic exclusion is often used. Data dependent experiments automatically switch between different

scan modes. Without input from the operator the MS System processes the information generated in the experiment to decide about the next step. The mass with the highest intensity is automatically zoomed and fragmented by MS. After several scans (normally 2 or 3) the parent ion (m/z) is set briefly on an exclusion list and the next highest precursor (m/z) can be analyzed. This enables the user to identify as many peptides in a HPLC run as technically possible.

A so-called triple run (Fig. 4) includes a chromatogram (Fig. 4a) and three forms of mass spectra:

- (a) Fullscan (Fig. 4b): all masses in a given range, most of the cases 200–2,000 Da are collected in this kind of scan.
- (b) Zoomscan (Fig. 4c): the growth of the highest mass and its environment to determine the charge state of the parent ion and calculate the molecular weight.
- (c) MSMS-scan (Fig. 4d): the identification of the peptide by fragmentation of the parent ion into product ions.

22. We normally work with Bioworks Rev. 3.3, LCquan 2.5 SUR1, and Xcalibur 2.0 SR2 from Thermo Electron Corporation 1998–2006.
23. To characterize proteasome subtypes and their different cleavage strength and preferences, it is essential to determine the 50% substrate degradation. Whether a proteasome type degrades a substrate only faster or generates more efficiently the fragments in total (epitopes, N-terminal elongated precursors of different length etc. (Fig. 2)) can be seen at this time point. On the other hand, longer incubation periods will result in “reentering” of large cleavage fragments (producing artifacts). The 50% substrate degradation time point is determined from graph analysis displaying 50% substrate concentration compared to time point zero. The example (Fig. 3) shows a 50% substrate degradation in about 70 min.

The generated amount of one fragment cannot be compared with another processed peptide, as intensities in mass spectrometry are directly dependent on the peptide sequence and its individual amino acids. Therefore, a direct quantification is not possible.

To determine the epitope–antitope ratio (14) represents an attempt to quantify antigenic peptides. Another possibility is the usage of a so called “Aqua-peptide” (heavy peptide). This is a peptide with heavy amino acids in the sequence to be analyzed used as an internal standard for quantifying the “natural” peptide (15, 16). To quantify fragments the titration method and QME (17) show success simulating the biological matrix (inactive proteasomes have to be added to the sample).

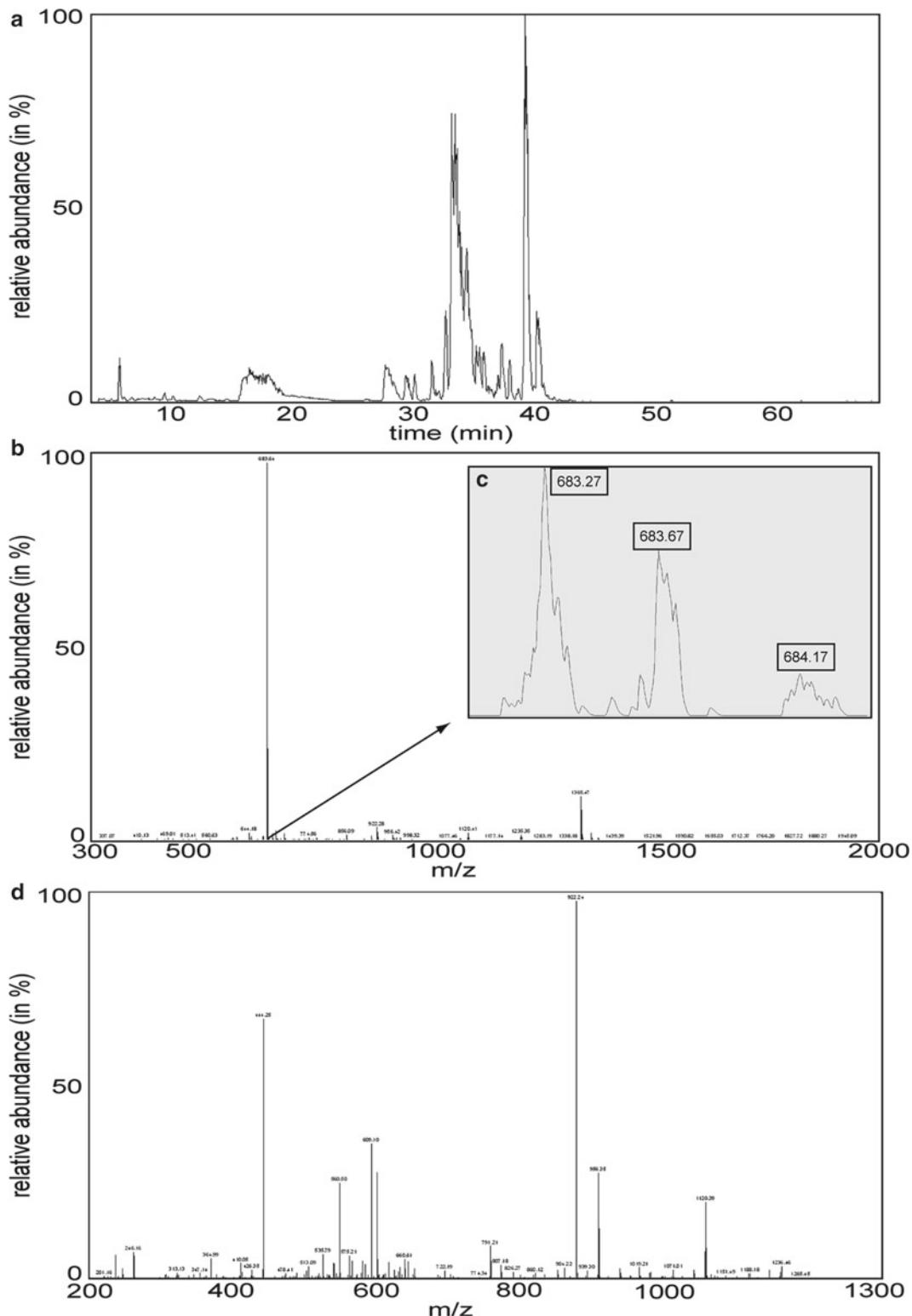


Fig. 4. This figure shows a chromatogram with all generated spectra in a data-dependent experiment. (a) Chromatogram intensity versus time, (b) mass spectra 300–2,000 Da, (c) zoomscan in the spectra (b) concentrated on one (m/z 683.31) mass, charge +2, and (d) MSMS-spectra 150–1,400 Da on this mass (parent ion m/z 683.56).

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References

1. Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA, Finley D (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* 94:615–623
2. Voges D, Zwickl P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 68:1015–1068
3. Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386:463–471
4. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, Finley D (2000) A gated channel into the proteasome core particle. *Nat Struct Biol* 7:1062–1067
5. Sijts A, Sun Y, Janek K, Kral S, Paschen A, Schadendorf D, Kloetzel PM (2002) The role of the proteasome activator PA28 in MHC class I antigen processing. *Mol Immunol* 39: 165–169
6. Textoris-Taube K, Henklein P, Pollmann S, Bergann T, Weisshoff H, Seifert U, Drung I, Mugge C, Sijts A, Kloetzel PM, Kuckelkorn U (2007) The N-terminal flanking region of the TRP2360-368 melanoma antigen determines proteasome activator PA28 requirement for epitope liberation. *J Biol Chem* 282: 12749–12754
7. Sijts AJ, Standera S, Toes RE, Ruppert T, Beekman NJ, van Velen PA, Ossendorp FA, Mielief CJ, Kloetzel PM (2000) MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *J Immunol* 164: 4500–4506
8. Sun Y, Sijts AJ, Song M, Janek K, Nussbaum AK, Kral S, Schirle M, Stevanovic S, Paschen A, Schild H, Kloetzel PM, Schadendorf D (2002) Expression of the proteasome activator PA28 rescues the presentation of a cytotoxic T lymphocyte epitope on melanoma cells. *Cancer Res* 62:2875–2882
9. Schultz ES, Chapiro J, Lurquin C, Claverol S, Burlet-Schiltz O, Warnier G, Russo V, Morel S, Levy F, Boon T, Van den Eynde BJ, van der Bruggen P (2002) The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immuno-proteasome. *J Exp Med* 195:391–399
10. Sewell AK, Price DA, Teisserenc H, Booth BL Jr, Gileadi U, Flavin FM, Trowsdale J, Phillips RE, Cerundolo V (1999) IFN-gamma exposes a cryptic cytotoxic T lymphocyte epitope in HIV-1 reverse transcriptase. *J Immunol* 162: 7075–7079
11. Sijts EJ, Kloetzel PM (2011) The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell Mol Life Sci* 68:1491–1502
12. Kuckelkorn U, Frentzel S, Kraft R, Kostka S, Groettrup M, Kloetzel PM (1995) Incorporation of major histocompatibility complex-encoded subunits LMP2 and LMP7 changes the quality of the 20S proteasome polypeptide processing products independent of interferon-gamma. *Eur J Immunol* 25:2605–2611
13. Liepe J, Mishto M, Textoris-Taube K, Janek K, Keller C, Henklein P, Kloetzel PM, Zaikin A (2010) The 20S proteasome splicing activity discovered by SpliceMet. *PLoS Comput Biol* 6:e1000830
14. Strehl B, Textoris-Taube K, Jakel S, Voigt A, Henklein P, Steinhoff U, Kloetzel PM, Kuckelkorn U (2008) Antitopes define preferential proteasomal cleavage site usage. *J Biol Chem* 283:17891–17897
15. Ong SE, Mann M (2005) Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* 1:252–262
16. Sparbier K, Wenzel T, Dihazi H, Blaschke S, Muller GA, Deelder A, Flad T, Kostrzewa M (2009) Immuno-MALDI-TOF MS: new perspectives for clinical applications of mass spectrometry. *Proteomics* 9:1442–1450
17. Mishto M, Goede A, Textoris-Taube K, Keller C, Janek K, Henklein P, Niewienda A, Kloss A, Gohlke S, Dahlmann B, Enenkel C, Kloetzel PM (2012) Driving forces of proteasome-catalyzed peptide splicing in yeast and humans. *Mol Cell Proteomics* 11:1008–1023
18. Boes B, Hengel H, Ruppert T, Multhaup G, Koszinowski UH, Kloetzel PM (1994) Interferon gamma stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J Exp Med* 179:901–909

Chapter 3

Using Protease Inhibitors in Antigen Presentation Assays

Michael Basler and Marcus Groettrup

Abstract

The major histocompatibility complex (MHC) class I restricted pathway of antigen processing allows the presentation of intracellular antigens to cytotoxic T lymphocytes. The proteasome is the main protease in the cytoplasm and the nucleus, which is responsible for the generation of most peptide ligands of MHC-I molecules. Peptides produced by the proteasome can be further trimmed or destroyed by numerous cytosolic or endoplasmic reticulum (ER) luminal proteases. Small molecule inhibitors are useful tools for probing the role of proteases in MHC class I antigen processing. Here, we describe different methods to test the impact of protease inhibitors in antigen presentation assays.

Key words: Protease, Proteasome, Inhibitor, Antigen presentation, Antigen processing, MHC-I surface staining, lacZ assay, T cell hybridomas, Intracellular cytokine staining, T cell lines

1. Introduction

Antigen recognition by cytotoxic T lymphocytes (CTLs) occurs through the interaction of their T cell receptors with peptide-MHC class I complexes. The main protease involved in this process is the proteasome, which is responsible for the generation of most peptide ligands of MHC-I molecules. Peptides can be further trimmed or destroyed by cytosolic proteases like leucine aminopeptidase (LAP) (1), insulin-degrading enzyme (IDE) (2), bleomycin hydrolase (BH) (3), puromycin-sensitive aminopeptidase (PSA) (3), thimet oligopeptidase (TOP) (4, 5), nardilysin (6), and tripeptidyl aminopeptidase II (TPPII) (7–10). Cytosolic peptides designated for MHC class I presentation are then transported into the lumen of the endoplasmic reticulum (ER) via the transporter associated with antigen processing. In the ER, further N-terminal trimming of peptides can occur by the ER associated aminopeptidases ERAAP or ERAP1/2 (11–13). In case of MHC class I cross-presentation insulin-regulated aminopeptidase (IRAP) mediated

peptide trimming can occur in an endosomal compartment (14). Peptides loaded onto MHC-I molecules are then transported to the cell surface and presented to cytotoxic T cells. Hence, interference with protease inhibitors directly affects the amount of class I molecules on the cell surface. Numerous protease inhibitors affecting different protease activities exist on the market. For example, the use of the LMP7 (a subunit of the immunoproteasome) selective inhibitor ONX 0914 (formerly named PR-957) reduced class I surface expression demonstrating an involvement of LMP7 in the class I processing pathway (15). In this book chapter we describe how to measure the impact of protease inhibition on alteration in class I antigen presentation. Thereby, we introduce how to measure the effect of protease inhibition on total class I surface expression by flow cytometry. The analysis of the impact of protease inhibition on specific cytotoxic T cell epitopes is described by two different methods encompassing antigen presentation by lacZ T cell hybridomas or intracellular cytokine staining of peptide specific T cell lines.

2. Materials

2.1. Common Consumables and Lab Equipment

1. RPMI 10% FCS: RPMI medium containing 10% fetal calf serum.
2. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2, autoclaved.
3. Protease inhibitor.
4. FACS-buffer: 2% FCS (v/v), 2 mM EDTA, 2 mM sodium acid, in PBS.
5. 96-well round bottom tissue culture plates.
6. Fluorescently labeled anti-MHC-I antibody (dilution according to the manufacturer's instruction).
7. Spectra Fluor Plus photometer (TECAN) with Neubauer chamber. Absorbance at 570/620 nm.
8. FACS tubes.
9. Flow cytometer.

2.2. Detection of Class I Peptides by T Cell Hybridomas in lacZ Assays

1. C57BL/6 mice 6–8 weeks of age (see Note 1).
2. Dissecting instruments (scissors and forceps).
3. Grid.
4. 1.66% (w/v) NH₄Cl in ddH₂O, autoclaved.
5. UTY_{246–254} specific T cell hybridoma (15). Maintain cells in RPMI 10% FCS + 0.5 mM β-mercaptoethanol (βME).
6. lacZ-buffer: 9 mM MgCl₂, 0.13% NP40 (v/v) in PBS.

2.3. Detection of Class I Peptides by Specific T Cell Lines

1. IMDM 10% FCS: IMDM medium containing 10% fetal calf serum + 0.5 mM β -mercaptoethanol.
2. Lymphocytic choriomeningitis virus (LCMV) (see Note 2). Propagated on the fibroblast line L929.
3. Brefeldin A (BFA)-solution: 40 μ g/ml in IMDM 10% FCS.
4. Fluorescently labeled anti-CD8 antibody (dilution according to the manufacturer's instruction).
5. 4% Paraformaldehyde (PFA)-solution: 4% PFA (w/v) in PBS, pH 7.4 (see Note 3). Store at -20°C.
6. Permeabilization (Perm)-buffer: 0.1% Saponin (w/v) in FACS-buffer.
7. Fluorescently labeled anti-IFN- γ antibody (dilution according to the manufacturer's instruction).

3. Methods

3.1. MHC-I Surface Staining

In order to analyze the effect of protease inhibitors on the processing and presentation of T cell antigens in the MHC class I pathway, a class I surface staining can be performed. This will provide information regarding the general impact of the inhibited protease on class I presentation. To investigate the effect on specific class-I epitopes, the methods described in Subheadings 3.2 and 3.3 can be used.

1. Choose an appropriate cell line for your experiment (see Note 4).
2. Incubate 5×10^5 cells in 1.5 ml cell culture medium per well (6-well tissue culture plate).
3. Add desired amounts of protease inhibitor in 1.5 ml cell culture medium (see Notes 5–7).
4. Incubate overnight at 37°C (see Note 8).
5. Harvest cells, wash cells once with 15 ml PBS, and count cells.
6. Resuspend at 5×10^6 /ml in FACS-buffer (see Note 9).
7. Add 100 μ l of resuspended cells per FACS tube. For each staining use three tubes.
8. Add 1 μ l (use amount according to manufacturer's instruction) of a fluorescently labeled antibody recognizing MHC-I molecules to one tube (see Note 10). Add 1 μ l of a fluorescence labeled isotype control antibody to the second tube. The third tube is an additional control without antibody (see Note 11).
9. Incubate for 20 min at 4°C in the dark.
10. Wash cells twice with 1 ml FACS-buffer.

11. Resuspend cells in 500 µl FACS-buffer (see Note 12).
12. Measure samples with an appropriate flow cytometer (see Note 13). Comparison of histograms derived from inhibitor treated samples with vehicle treated samples will give you information whether your inhibitor is affecting class-I surface expression.

3.2. Detection of Class I Peptides by T Cell Hybridomas in lacZ Assays

T cell hybridomas are used to monitor the processing and presentation of T cell antigens in the MHC class I pathway (16, 17). Generation of T cell hybridomas is accomplished by the fusion of specific T cells with a T cell receptor-deficient lymphoma line expressing CD8 α and a lacZ reporter construct under the control of the IL-2 promotor/enhancer. Thus, this system permits the monitoring of T cell hybridoma stimulation in chromogenic lacZ assays. In order to investigate the effect of protease specific inhibitors these T cell hybridomas can be used as read-out system to detect altered class I peptide presentation. The UTY₂₄₆₋₂₅₄-specific T cell hybridoma has recently been used to study the effect of an LMP7-selective inhibitor of the immunoproteasome in lacZ assays (15). The following method is described to test the effects of protease inhibitors on the presentation of the male HY-derived CTL-epitope UTY₂₄₆₋₂₅₄. Naturally, this method can also be used to detect the processing of class I epitopes derived from other sources.

1. Remove spleen of one male and one female mouse and take up spleen in 5 ml RPMI 10% FCS (see Note 1).
2. Make a single cell suspension by pressing spleen through a grid.
3. Centrifuge cells for 5 min at 347 $\times g$ and discard supernatant.
4. Lyse the erythrocytes by resuspending cells in 5 ml pre-warmed 1.66% (w/v) NH₄Cl solution (in 15 ml tubes).
5. Incubate for 2 min at room temperature.
6. Fill up to 15 ml with RPMI 10% FCS and centrifuge cells for 5 min at 347 $\times g$ and discard supernatant.
7. Wash cells with 15 ml PBS and centrifuge cells for 5 min at 347 $\times g$ and discard supernatant.
8. Take up cells in 5 ml RPMI 10% FCS and count cells using a Neubauer chamber (see Note 14).
9. Incubate 10⁷ splenocytes in 3 ml RPMI 10% FCS per well (6-well tissue culture plate).
10. Add desired amounts of protease inhibitor (see Note 5). You need one well of male splenocytes without inhibitor for comparison of untreated and treated samples. For female splenocytes you only need one well without inhibitor (see Note 7).
11. Incubate overnight at 37°C (see Note 8).
12. Harvest splenocytes, wash cells twice with 15 ml PBS and count splenocytes.

13. Resuspend cells in RPMI 10% FCS at 10^7 /ml.
14. Use 96-well round bottom tissue culture plate and add 150 μ l per well to wells A1–D1. Make four serial threefold dilutions of splenocytes (100 μ l/per well).
15. Harvest T cell hybridomas, count, and resuspend in RPMI 10% FCS at 10^6 /ml. (We use the UTY_{246–254} specific T cell hybridoma (15).)
16. Add 100 μ l of T cell hybridomas per well (A1–A4; B1–B4). Add to half of your samples (C1–C4; D1–D4) 100 μ l RPMI 10% FCS as background control.
17. Female splenocytes are used as negative control and untreated male splenocytes as positive control for comparison. You can include an additional positive control adding synthetic peptide (we use UTY_{246–254} peptide at a concentration of 10^{-7} M) to female splenocytes (see Note 15).
18. Incubate overnight at 37°C.
19. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
20. Add 100 μ l lacZ-buffer and incubate at 37°C (see Note 16).
21. Measure absorbance at 570/620 nm when color change is visible (approx. after 1–3 h) (see Note 17).
22. The more antigen is presented on class I molecules, the more color will be observed in this assay (see Note 18). Comparison of absorbance values of inhibitor treated samples with vehicle treated samples will give you information whether your inhibitor is affecting UTY_{246–254} processing.

3.3. Detection of Class I Peptides by Specific T Cell Lines

If no T cell hybridomas (Subheading 3.2) are available to study the presentation of certain T cell epitopes, peptide specific T cell lines can alternatively be used. Additionally, T cell lines often have the advantage to be more sensitive compared to T cell hybridomas. The disadvantage of using T cell lines is the time-consuming preparation procedure. Numerous protocols to generate T cell lines can be found in the literature (e.g., (10, 18)). In the following section the use of T cell lines is combined with an intracellular cytokine staining for IFN- γ . Alternatively, secretion of IFN- γ , TNF- α , or IL-2 into the supernatant can also be determined by ELISA. As an example the method is described with a T cell line specific for the lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived GP_{33–41} CD8 T cell epitope. The method can easily be applied to other viral, bacterial, tumor, or endogenous peptides in mouse or human experimental systems.

1. Choose an appropriate cell line. For the detection of LCMV-derived H-2^b epitopes we use the DC2.4 cell line (19). Harvest the stimulator cell line and count cells using a Neubauer chamber.

2. Incubate 5×10^5 cells in 3 ml RPMI 10% FCS per well (6-well tissue culture plate).
3. Add desired amounts of protease inhibitor (see Note 5). You need one well of splenocytes without inhibitor for comparison of untreated and treated samples (see Note 7).
4. Incubate for 1 h at 37°C (see Note 8).
5. Add LCMV (see Note 2) at an m.o.i. of 1 (see Notes 19–21).
6. Incubate overnight at 37°C.
7. Harvest cells, wash cells twice with 15 ml PBS, and count cells.
8. Resuspend cells in IMDM 10% FCS + βME at 4×10^6 /ml.
9. Use 96-well round bottom tissue culture plate and add 150 µl per well to wells A1 and B1. Make four serial threefold dilutions of splenocytes (100 µl/per well).
10. Harvest T cell lines, count, and resuspend in IMDM 10% FCS + βME at 4×10^6 /ml.
11. Add 50 µl of T cell lines per well (A1–A4; B1–B4).
12. Add 50 µl of BFA-solution per well (see Note 22).
13. You can include an additional positive control by adding synthetic peptide (we use the peptide at a concentration of 10^{-7} M) to a separate well containing the T cell line (see Notes 23 and 24).
14. Incubate for 5 h at 37°C.
15. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
16. Add 50 µl FACS-buffer containing fluorescently labeled αCD8 antibody (see Note 25).
17. Incubate for 20 min at 4°C in the dark.
18. Add 100 µl FACS-buffer per well.
19. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
20. Add 150 µl FACS-buffer per well.
21. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
22. Add 70 µl of 4% PFA-solution (see Note 3).
23. Incubate for 5 min at 4°C in the dark.
24. Add 100 µl Perm-buffer per well.
25. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
26. Add 150 µl Perm-buffer per well.
27. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.
28. Add 50 µl Perm-buffer containing fluorescently labeled αIFN-γ antibody (see Note 26).
29. Incubate over night at 4°C in the dark (see Note 27).
30. Add 100 µl Perm-buffer per well.
31. Centrifuge plate at $541 \times g$ for 90 s and discard supernatant.

32. Add 150 µl Perm-buffer per well.
33. Centrifuge plate at 541 ×*g* for 90 s and discard supernatant.
34. Take up cells in 300 µl FACS-buffer and transfer cells to FACS tubes (see Note 28).
35. Measure samples with an appropriate flow cytometer (see Note 13). The more specific antigen is presented on the stimulator cells the more IFN-γ-positive CD8 T cells will be observed. A comparison of inhibitor treated samples with vehicle treated samples will give you information whether your inhibitor is affecting the processing of the investigated T cell epitope.

4. Notes

1. All animals must be maintained in accordance with local and national animal care regulations.
2. Viruses must be maintained in accordance with local and national regulations.
3. Paraformaldehyde-solution is highly toxic and light sensitive.
4. You can also use primary cells (e.g., splenocytes).
5. Be sure that your protease inhibitor is cell permeable and does not affect cell viability.
6. Set up the double concentration of your inhibitor because you will dilute the solution.
7. It is recommended to incubate control cells with the same solvent used for the inhibitor.
8. Unbound protease inhibitor can be removed by washing the cells with PBS. This might prevent undesired off-target effects or toxicity.
9. If you use primary cells or cells expressing Fc-receptors on the cell surface then incubate your cells with Fc-receptor blocker according to the manufacturer's protocol (prior to MHC-I staining).
10. You can use pan class I antibodies or antibodies detecting a certain MHC-I haplotype. Antibodies are available which are labeled with different fluorescent dyes. Choose fluorescent dye according to the flow cytometer available.
11. Light exposure can harm fluorescent dyes.
12. Keep your cells on ice to prevent internalization of antibodies and protect your samples from light exposure.
13. You need an appropriate laser in your flow cytometer to excite your fluorescent dye and also detectors measuring the correct emission wavelength.

14. Do not count residual un-lysed erythrocytes.
15. It is useful to pulse female splenocytes with different concentrations of synthetic peptide (10^{-5} to 10^{-10} M) and use the pulsed cells as stimulators for hybridomas. Comparison of the stimulation of the titrated peptide probes with your samples will provide information regarding the magnitude of observed differences.
16. Seal 96-well plate properly to prevent evaporation.
17. Do not measure after more than 6 h. Color will become unspecific.
18. If your sample is in a saturated range then probably no differences will be visible.
19. This corresponds to 5×10^5 pfu.
20. Be aware that your inhibitor might affect infection efficiency.
21. Vehicle treated and uninfected cells are included as control for unspecific stimulation.
22. Brefeldin A inhibits the secretory pathway and therefore retains IFN- γ inside the stimulated T cells.
23. This will give you information regarding specificity of your T cell line.
24. It is useful to pulse stimulators with different concentrations of synthetic peptide (10^{-5} to 10^{-12} M) and use these cells as stimulators for your T cell line. Comparison of the stimulation of the titrated peptide probes with your samples will give you information regarding the magnitude of observed differences.
25. Antibodies are available in different fluorescent dyes. Choose fluorescent dye according to the flow cytometer available. Use dilution according to the manufacturer's instructions.
26. Antibodies are available in different fluorescent colors. Choose fluorescent dye according to the flow cytometer available. Be aware that the chosen color does not interfere with the anti-CD8 dye. α IFN- γ -FITC and α CD8-APC would be a suitable combination. Use dilution according to the manufacturer's instructions.
27. You can also incubate for a minimum of 90 min.
28. You can use small tubes and transfer your samples using a multichannel pipette.

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References

1. Beninga J, Rock KL, Goldberg AL (1998) Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J Biol Chem* 273:18734–18742
2. Parmentier N, Stroobant V, Colau D et al (2010) Production of an antigenic peptide by insulin-degrading enzyme. *Nat Immunol* 11:449–454
3. Stoltze L, Schirle M, Schwarz G et al (2000) Two new proteases in the MHC class I processing pathway. *Nat Immunol* 1:413–418
4. Saric T, Beninga J, Graef CI et al (2001) Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. *J Biol Chem* 276:36474–36481
5. York IA, Mo AXY, Lemerie K et al (2003) The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation. *Immunity* 18:429–440
6. Kessler JH, Khan S, Seifert U et al (2011) Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* 12:45–53
7. Geier E, Pfeifer G, Wilm M et al (1999) A giant protease with potential to substitute for some functions of the proteasome. *Science* 283: 978–981
8. Seifert U, Maranon C, Shmueli A et al (2003) An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat Immunol* 4:375–379
9. Reits E, Neijssen J, Herberts C et al (2004) A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. *Immunity* 20:495–506
10. Basler M, Groettrup M (2007) No essential role for tripeptidyl peptidase II for the processing of LCMV-derived T cell epitopes. *Eur J Immunol* 37:896–904
11. York IA, Chang SC, Saric T et al (2002) The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues. *Nat Immunol* 3:1177–1184
12. Saric T, Chang SC, Hattori A et al (2002) An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat Immunol* 3: 1169–1176
13. Saveanu L, Carroll O, Lindo V et al (2005) Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* 6: 689–697
14. Saveanu L, Carroll O, Weimershaus M et al (2009) IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325:213–217
15. Muchamuel T, Basler M, Aujay MA et al (2009) A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 15:781–787
16. Schwarz K, van den Broek M, de Giuli R et al (2000) The use of LCMV-specific T cell hybridomas for the quantitative analysis of MHC class I restricted antigen presentation. *J Immunol Methods* 237:199–202
17. Sanderson S, Shastri N (1994) LacZ inducible, antigen/MHC-specific T cell hybrids. *Int Immunol* 6:369–376
18. Mariotti S, Nisini R (2009) Generation of human T cell clones. *Methods Mol Biol* 514: 65–93
19. Basler M, Moebius J, Elenich L et al (2006) An altered T cell repertoire in MECL-1-deficient mice. *J Immunol* 176:6665–6672

Chapter 4

Kinetic Studies of Cytoplasmic Antigen Processing and Production of MHC Class I Ligands

Anita Stargardt and Eric Reits

Abstract

MHC class I molecules present peptides that are derived from intracellular proteins degraded by proteasomes. These peptides often require additional trimming by peptidases to fit into the peptide-binding groove of MHC class I. However, most peptides are rapidly recycled by the large heterogeneous pool of peptidases. Here, we describe a technique to quantify peptide degradation both in living cells and in cell lysates, using quenched peptides that contain a quencher and fluorophore. As degradation results in separation of the quencher and fluorophore, fluorescence will increase. This technique enables the examination of changes in peptide length and amino acid sequence on its half-life, and hence its chances to become presented by MHC class I.

Key words: Peptide, Peptidase, Antigen processing, Proteasome, Quenched peptide, Microinjection

1. Introduction

Inside the cell, protein synthesis and degradation is continuously ongoing. Most proteins fulfill their biological function as a mature protein, but sooner or later they become subjective to proteolysis by the proteasome which leads to the generation of short peptides ranging in size from 3 to 22 amino acids (1). Most peptides are immediately targeted by downstream peptidases that ultimately recycle them into single amino acids. However, some peptides escape complete degradation and are presented as MHC class I antigens to the immune system (2–4). As most MHC class I binding peptides have a length of 8–11 amino acids, additional trimming of the proteasomal products by peptidases is then required (5). Various peptidases have been identified that can trim these peptides before they are translocated by the Transporter associated with Antigen Processing (TAP) into the lumen of the Endoplasmic Reticulum. Here they can be loaded onto MHC class I molecules

and transported to the plasma membrane for presentation to the immune system. The various peptidases show different preferences for specific amino acid sequences and peptide lengths in vitro, which can be examined using purified peptidases together with synthesized peptides. Upon cleavage, the peptide degradation products can be analyzed by mass spectrometry to determine the substrate specificities of each peptidase. This information can subsequently be used to predict its role in the trimming of peptides into antigens (6–9).

Trimming of peptides by isolated peptidases in vitro is obviously very different when compared to the processing by the heterogeneous pool of cytoplasmic peptidases in vivo. To examine peptide processing in the living cell, we previously described the use of a peptide degradation assay to study the effect of peptide sequence or length on antigen processing (4, 10). Not only does this allow to investigate the effect of alterations in peptide sequence on its half-life, but the effect of peptidase inhibitors also can be examined to identify peptidases involved in generation or degradation of the potential epitope. In addition, the effect of N- or C-terminal modifications or posttranslational modifications such as phosphorylation can be investigated (*manuscript in preparation*). Moreover, the incorporation of nondegradable D-amino acids at the termini can be used to examine the involvement of endo-peptidase activities, as exopeptidases are unable to degrade these peptides (10), *manuscript in preparation*). In addition to studying antigen processing, this assay might help to understand and improve the degradation of aggregation prone peptides as observed in neurodegenerative disorders like Alzheimer's disease or Huntington's disease. The technique is based on the principle that the peptide of interest is modified with a quencher and a small fluorophore like fluorescein (see Fig. 1). The quencher is an organic molecule coupled to a lysine that is able to absorb the energy emitted by the fluorophore because its absorption spectrum overlaps with the emission spectrum of the fluorophore. For efficient quenching, they should be into close proximity, e.g., separated by 4–8 amino acids. When a peptidase is cleaving the peptide, quencher and fluorophore are separated and the fluorophore is able to emit its energy as fluorescent light. This allows the determination of the half-life of the peptide of interest under different conditions including the inhibition of particular peptidases. This method can be applied to living cells as well as in vitro using cell lysates or tissue homogenates. It can also be used to study the effect of specific peptidase inhibitors, and examine whether these inhibitors are reversible, irreversible, competitive, or noncompetitive. In this protocol we describe how to prepare the quenched peptides, how to microinject cells or isolate cytoplasmic fractions, and show the possibilities of adding different peptidase inhibitors to investigate the role of peptidases in degrading the peptide of interest.

2. Materials

For preparing the buffers, use ultrapure water, 18 MΩ-cm at 25°C.

2.1. Generation of Quenched Peptides

- To detect peptide degradation using quenched peptides that become fluorescent upon degradation, peptides are synthesized with a fluorescent group (e.g., fluorescein or alexa 488, see Note 1) attached to one amino acid (usually a cysteine) and a quenching group attached to another amino acid (usually a Dabcyl group coupled to a lysine residue). With about five amino acids between the quencher and fluorophore, the Dabcyl group quenches emission of the nearby fluorophore group, and fluorescence will only be detected when the amino acids are separated due to peptide degradation (Fig. 1).
- The synthesis of quenched peptides can be performed by solid phase strategies using an automated multiple peptide synthesizer (Syro II, MultiSyntech) using Fmoc chemistry. Fluorescein can be covalently coupled to the cysteine residue using fluorescein-5-iodoacetamide (Molecular Probes). Various automated peptide synthesizers exist, and synthesized peptides are HPLC purified and validated by mass spectrometry.

2.2. Degradation of Quenched Peptides upon Microinjection in Living Cells

- Adherent cells should be used that are not too easily disturbed by micro-injection, such as HeLa cells (adherent cells such as HEK 293 cells often de-attach upon injection and remain sticking to the needle tip). Ideally, cells are seeded 1–2 days

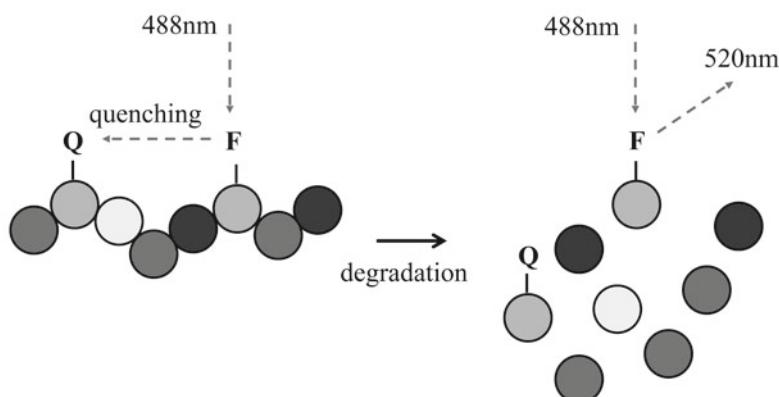


Fig. 1. Schematic representation of a quenched peptide, with a dabcyl group functioning as the quencher (Q) and a fluorescein moiety as a fluorophore (F). As these two groups are fused to two different amino acids, cleavage by peptidase results in the separation of the quencher and fluorophore and subsequent fluorescent light.

before injection on 35 mm glass coverslips in a 6-well plate and kept in regular medium with FCS in a CO₂ incubator.

2. 2× Microinjection buffer: 20 mM Tris-HCl, pH7.4 and 0.2 mM EDTA, and subsequently autoclaved and filter (0.2 µm) sterilized. Store at -20°C.
3. Microinjection mixture: 1 µL of quenched peptide is added to 4 µL microinjection buffer, 2 µL H₂O and 1 µL 2 mM Fura Red (Molecular Probes). Prepare freshly and spin down for 10 min at minimal 20,700×g before use.
4. HBS buffer (HEPES-buffered Saline): 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5.5 mM glucose. Sterilize by filtration (0.2 µm). The buffer can be CO₂ saturated, e.g., by placing the buffer prior to use in a petridish in the CO₂ incubator used for tissue culture. Store at 4°C.
5. Microinjector like the Eppendorf Femtojet coupled to an Injectman NI2 can be used.
6. Sterile microinjection needles like the Eppendorf Femtotips (5242.952.008) can be used, using thin microloader tips (5242.956.003) to load the needles with the microinjection buffer.
7. An inverted fluorescence microscope can be used equipped with a dry 40× or 63× objective and with an additional viewing port connected to a fluorescence detector. Emission of the simultaneously microinjected quenched peptide and Fura Red is measured after splitting the emitted light. Different setups can be used to detect fluorescence; we use a PTI model 612 analog photomultiplier system equipped with a 580 nm dichroic mirror with a 480–530 nm filter for fluorescein emission and a long pass 590 nm filter for Fura Red emission. The two fluorescent signals are simultaneously detected by two PTI model 612 analog photomultipliers (Fig. 2).
8. For data acquisition, software like FELIX (PTI Inc., USA) can be used.

2.3. Degradation of Quenched Peptides in Cytosolic Lysates.

1. 200 µM Digitonin lysis buffer: weigh 1 mg Digitonin (Sigma-Aldrich), add 55 mL H₂O. Heat for 15 min at 98°C in a heating block and cool down at room temperature. Store at 4°C.
2. Bovine Serum Albumin (BSA) solution: dissolve 10 mg BSA in 10 ml water, so that the final concentration is 10 µg/µL. Store at 4°C.
3. Bradford reagent (Sigma-Aldrich).
4. KMH buffer: 110 mM KAc, 2 mM MgAc, 20 mM Hepes-KOH, pH 7.4. In an Erlenmeyer or glass beaker, weigh 260 mg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), add water to a total volume of 50 mL and let dissolve at room temperature with a magnetic stir bar. Adjust pH to 7.4 by adding Potassium hydroxide (KOH). Then add 550 mg potassium

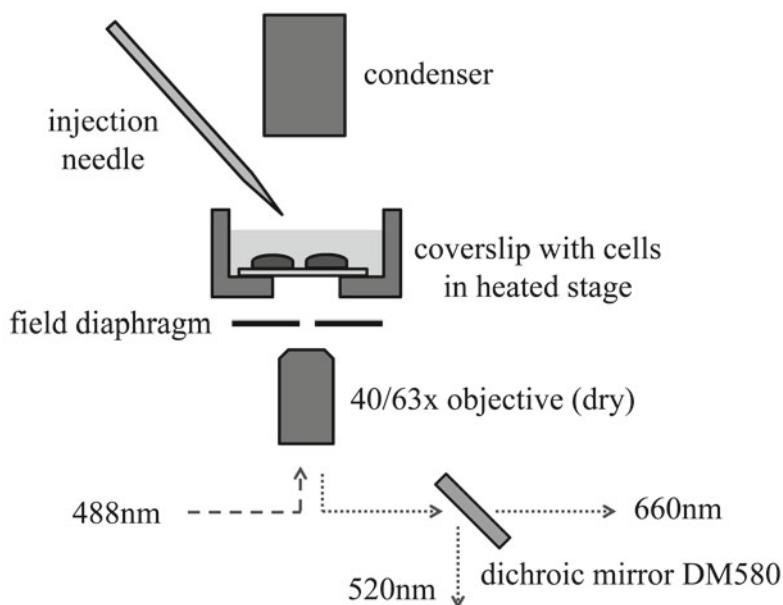


Fig. 2. Microinjection setup to detect quenched peptide degradation in living cells. Cells are grown on thin coverslips and mounted in a heated cell chamber. A high magnification objective (e.g., 40 \times or 63 \times) is used in combination with a small field diaphragm in order to create a small detection window showing only the selected cell that is to be microinjected. When exciting at 488 nm, a di-chroic mirror splitting the emission light at 580 nm can be used to allow simultaneous detection of the peptide fluorophore and the injection marker.

acetate (KAc) and 14.2 mg magnesium acetate (MgAc). The buffer is filter sterilized and can be stored at room temperature.

5. Inhibitors for specific proteases or peptidases. Examples are butabindide for TPPII (Tocris Bioscience, Bristol, UK), bacitracin for IDE (Sigma Chemical Co., St. Louis, MO, USA), amastatin for nardilysin (Enzo Life Sciences, Lörrach, Germany), and MG132 for proteasomes (Sigma-Aldrich, Steinheim, Germany). Bath sonicator, e.g., a Bransonic-221 (Branson Ultrasonics, Danbury, CT, USA).
6. 96-Well Polystyrene Cell Culture Microplates, black. (Greiner Bio-One) (see Note 2).
7. A microplate reader able to detect “time-resolved Fluorescence” like a Polarstar Galaxy microplate reader (BMG Labtech), using filter settings suitable to detect, e.g., fluorescein.

3. Methods

3.1. Measuring Quenched Peptide Degradation in Living Cells

1. Plate cells on glass coverslips and let them grow overnight until a confluence of 50–80% before mounting the coverslip in a heated stage (see Note 3). Replace the medium for CO₂-buffered HBS.

2. Prepare the microinjection mixture containing the quenched peptide and Fura Red. Fura Red is added as a control for microinjection and leakage; as it can be excited at the same wavelength as fluorescein but emits light at a much higher wavelength than fluorescein, the emitted light can be split using a dichroic mirror and separately detected. Since Fura Red is immediately fluorescent, it functions as a control for successful injection, while a decrease in Fura red signal in time indicates leakage from the cell (which will also affect peptide degradation, see Note 4).
 3. About 4–6 µL of the microinjection mixture is loaded in the injection needle, and carefully ticking the needle (kept vertically with the injection tip downwards) will remove any remaining air bubbles that may obstruct the thin end of the needle upon injection (see Note 5).
 4. Since all emitted light from the degraded peptides should ideally go to the detection system (e.g., the PMTs) the microinjection has to be performed “blindly” as no light is going to the oculars. Therefore, a neighboring cell has to be used to set the Z-level limit for the microinjection needle (see Note 6). When the Z-limit is set, the to-be measured cell is centered in the middle of the field. As very little background light should pass through, the field diaphragm of the microscope can be closed until only the single cell is visible by transmitted light. Place the injection needle above the cell. Only now the emitted light can be switched from the oculars to the port connected to the PMTs (and be sure to switch off the transmission light beforehand to prevent over-exposure of the PMTs).
 5. With the detection system switched on and the software running, the experiment can start. When the centered cell is micro-injected, the Fura Red signal should immediately increase and remain on a plateau level (Fig. 3), which indicates a successful injection without leakage. In time, the green fluorescent signal should also increase, indicating degradation of the quenched peptides and no longer quenching of the fluorophore. Also the signal derived from the dequenched peptide should reach a plateau level when all peptides are degraded, and from this curve the half-life ($t_{1/2}$) of the peptide can be calculated, which is the time needed after micro-injection until 50% of the peptides are degraded (Fig. 3, see Note 7).
 6. Upon completion of the experiment, a neighboring cell can be injected after being centered in the middle or a new area in the coverslip can be taken.
1. Collect cells by centrifuging at $180 \times g$ for 3 min, 4°C and wash with PBS. Spin again to obtain the cell pellet and add ice cold Digitonin lysis buffer, 50 µL to 400,000 cells (see Note 8). Vortex and leave on ice for 30 min.

3.2. Measuring Quenched Peptide Degradation in Cytosolic Lysates

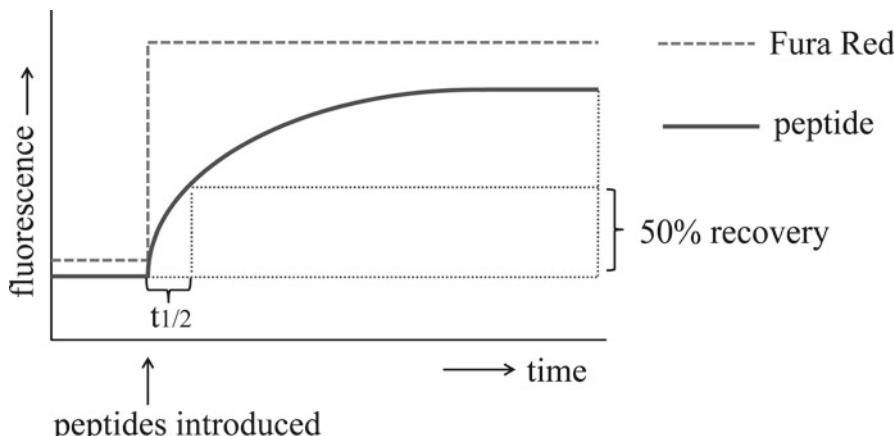


Fig. 3. Model graph of a degraded quenched peptide. Upon microinjection or addition of quenched peptides to cell lysates, the degradation of the peptides will result in fluorescence until all peptides are degraded (leading to a plateau phase). From this graph, the half-life of the peptide can be determined by calculating the time required to reach to 50% of the final plateau phase.

2. Centrifuge lysed cells $16,000 \times g$ for 15 min at 4°C which will spin down membranes but leaves the cytosolic proteins soluble in the supernatant.
3. Move the supernatant to a fresh tube.
4. The protein concentration in the sample lysate can be determined by performing a Bradford assay.
 - (a) Pipet the standard; 0, 0.5, 1, 2, 4, and 8 μL of BSA solution (corresponding to 0, 0.5, 1, 2, 4, and 8 μg BSA) in 6 wells of a clear 96-well plate.
 - (b) Pipet 1 μL of sample lysate in another well.
 - (c) Add 200 μL of Bradford reagent to all conditions and measure the absorbance at 595 nm on the Polarstar Galaxy.
 - (d) Compare value of sample lysate with Standard to determine the protein concentration (see Note 9).

3.3. Preparation of Peptide and Inhibitors.

1. Dissolve quenched peptide in water (see Note 10).
2. Dilute 1 μL quenched peptide in 30 μL KMH buffer (see Note 11).
3. Dilute the inhibitor in KMH buffer to the working dilution. Keep in mind that the 10 μL of the inhibitor working dilution ends up in a total volume of 200 μL . The inhibitor working dilution is thus 20 times more diluted in the final experiment and should be made 20 times more concentrated than the optimal inhibitor concentration (see Note 12).
4. Immediately prior to use, sonicate peptide solution in the ultrasonic water bath for 10 min to prevent aggregation and oligomerization and assure the peptides are soluble monomers (see Note 13).

3.4. Measuring Degradation on the Polarstar Galaxy Microplate Reader

1. In each well of a 96-well Polystyrene Cell Culture Microplate add 20 µg lysate. For the negative control do not add lysate or use lysate pre-incubated with broad spectrum peptidase inhibitors.
2. Add 10 µL inhibitor working dilution and KMH buffer to a total of 190 µL per well. Incubate for 30 min at 4°C to assure the inhibitor is bound to the active site of the proteases of interest when adding the peptide. For the “no inhibitor” control, do not add inhibitor.
3. Add 10 µL of sonicated peptide solution to each well and place the plate in the 37°C pre-warmed Polarstar Galaxy microplate reader.
4. On the Polarstar select the configuration “Time-resolved Fluorescence.” Select the emission and excitation wavelength that correspond to the fluorophore incorporated in the quenched peptide of interest. Set which wells to measure, for how many cycles and over what time span (see Note 14).

4. Notes

1. Various fluorophores can be used to detect degradation of peptides, but ideally a small fluorophore like fluorescein should be used to prevent steric hindrance of peptide degradation. Alternatively, fluorophores like Alexa 488 can be used (which remains fluorescent at low pH, e.g., upon lysosomal degradation). When multiple cysteines are present in the peptide sequence, no specific labeling of one cysteine with a fluorophores is possible. In this case, pre-coupled fluorophores attached to an amino acid can be used, such as Edans (which is coupled to a glutamic acid).
2. Using black plates will eliminate cross talk between wells and reduce back-scattered light, making the measurements more accurate. The Polarstar Galaxy can do both top and bottom readings, but keep in mind to use plates with transparent bottoms in the last case.
3. Different setups can be used to mount living cells prior to micro-injection. We use round 24 mm glass coverslips (Menzel-Glaeser from Thermo scientific) that are mounted in an Attofluor cell chamber (Cat. No. A-7816, Molecular Probes) which is being heated to 37°C.
4. As also shown in Fig. 3, a well-executed microinjection should not result in a perforated cell that leaks all its cytoplasm content in the medium, including the to-be-degraded peptides. Therefore, simultaneous detection of the Fura Red shows

whether cells become leaky or not, and ideally the Fura Red signal should remain similar over time after microinjection.

5. When keeping the needle vertically with the injection tip down, a small air bubble can often be seen below the added peptide solution. Careful ticking the needle with your index finger will make the bubble go upwards. During the actual microinjection, the needle tip may become obstructed by dead cells that adhere to the needle. Briefly pulling the needle out of the medium and putting it back again will often result in the removal of these dead cells.
6. When microinjecting adherent cells, a threshold can be set for the injection level (Z-limit) in order to microinject numerous neighboring cells without having to set the Z-limit for each individual injection.
7. While the height of the plateau phase obviously changes when more or less amount of peptides are introduced, the half-life is much less affected by the amounts of peptides, indicating that the peptidases are not saturated. Therefore it seems not critical to inject the identical amounts of peptides during each microinjection. Still, ideally a similar concentration of (different) peptides should be used to compare the effect of alterations of peptide sequence or upon the addition of peptidase inhibitors.
8. The amount of lysis buffer to add to the cell pellet can be adjusted to obtain the desired protein concentrations in the final cell lysate. Although this may differ for each cell type and experiment, it is important to make sure that enough lysis buffer is added to the pellet that the cells can nicely dissolve and no cell clumps are present.
9. The amount of protein in the cell lysate can be determined by comparing its absorbance value with the values of the protein standard generated with the BSA solution. This can be easily done in a program like Microsoft Office Excel by adding a linear trend line to the graph containing the values obtained for the Standard. The equation corresponding to this trend line can also be added and this is used to calculate the protein concentration in the cell lysate by inserting the measured absorbance value as x -value. The resulting y -value is the amount of protein in the lysate.
10. Peptides are usually supplied as a dry powder. This substance can be dissolved in several solvents. What to use depends on the characteristics of the peptide; non-hydrophobic peptides will most of the time easily dissolve in water, however, peptides that have hydrophobic characteristics may be difficult to dissolve in water and should be dissolved in organic solvent like DMSO. Take into account that DMSO can affect cell culture based assays; therefore make concentrated stock solutions that will be

diluted in the experiment so that no more than 1% of the original solvent is present in the final experimental solution.

11. It is important to make a single diluted peptide solution that will be used for all conditions. Calculate beforehand how much diluted peptide solution is needed for the whole experiment. This will prevent little variations between conditions caused by pipeting differences or differences in preparation of the peptide solutions like sonification or pre-incubation times.
12. The optimal inhibitor concentration is the concentration at which the inhibitor has its maximum effect on peptide degradation. This concentration can be determined by making a dilution series and test what concentration maximally inhibits the degradation of a peptide that is known to be degraded by the protease that is inhibited (a positive control).
13. Peptide degradation can be influenced by peptide aggregation or oligomerization so it is essential to know in what state the peptide is when used in the experiment. Sonification of the peptides can be used to enhance solubility and (re-)dissolve possible aggregated peptides.
14. The amount and length of the measurement cycles depends on the degradation speed of the peptide of interest. When the peptide is quickly degraded (e.g., the degradation curve reaches a plateau within 30 min) then a high amount of short cycles is best (although bleaching due to extensive measurements should be prevented). However, when the peptide is slowly degraded (e.g., the degradation curve reaches a plateau after 10 h) then cycles of a few minutes distributed over 10 h will be best.

Acknowledgment

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References

1. Kisselov AF, Akopian TN, Woo KM, Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 26 and 20S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* 274:3363–3371
2. Yewdell JW, Reits E, Neefjes J (2003) Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat Rev Immunol* 3:952–961
3. Fruci D, Lauvau G, Saveanu L, Amicosante M, Butler RH, Polack A, Ginhoux F, Lemonnier F, Firat H, van Endert PM (2003) Quantifying recruitment of cytosolic peptides for HLA class I presentation: impact of TAP transport. *J Immunol* 170:2977–2984
4. Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, van Veelen P, Janssen H, Calafat J, Drijfhout JW, Neefjes J (2003) Peptide diffusion, protection, and degradation in nuclear

- and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18: 97–108
5. Rammensee HG, Falk K, Rotzschke O (1993) Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 11:213–244
 6. Beninga J, Rock KL, Goldberg AL (1998) Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J Biol Chem* 273:18734–18742
 7. Geier E, Pfeifer G, Wilm M, Lucchiari-Hartz M, Baumeister W, Eichmann K, Niedermann G (1999) A giant protease with potential to substitute for some functions of the proteasome. *Science* 283:978–981
 8. Stoltze L, Schirle M, Schwarz G, Schroter C, Thompson MW, Hersh LB, Kalbacher H, Stevanovic S, Rammensee HG, Schild H (2000) Two new proteases in the MHC class I processing pathway. *Nat Immunol* 1: 413–418
 9. Mo XY, Cascio P, Lemire K, Goldberg AL, Rock K (1999) Distinct proteolytic processes generate the C and N termini of MHC class I-binding peptides. *J Immunol* 163: 5851–5859
 10. Reits E, Neijssen J, Herberts C, Benckhuijsen W, Janssen L, Drijfhout JW, Neefjes J (2004) A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. *Immunity* 20:495–506

Chapter 5

Assaying Peptide Translocation by the Peptide Transporter TAP

Marlieke L.M. Jongsma and Jacques Neefjes

Abstract

MHC class I molecules display peptides at the cell surface that are mostly derived from cytosolic or nuclear proteins. Since peptide loading of MHC class I molecules occurs in the ER lumen, cytosolic peptides have to pass the ER membrane. The peptide transporter TAP translocates peptides over this ER membrane which is critical for successful MHC class I antigen presentation. How peptide translocation by TAP can be assayed and inhibitors of chemical or viral origin can be identified, will be described here.

Key words: MHC class I, TAP, Antigen presentation, Peptide translocation, Assay

1. Introduction

MHC class I molecules present peptides at the cell surface to CD8⁺ T cells for consideration. If these T cells recognize foreign (non-self) peptides in the context of the correct MHC class I molecules, they may respond by eliminating cells displaying these. Most peptides presented by MHC class I molecules are degradation products of cytosolic or nuclear proteins. Since peptides do not diffuse over lipid bilayers, cytosolic peptides have to be actively translocated over membranes to contact MHC class I molecules waiting in the ER lumen (1). A peptide transporter was identified by analyses of mutant cell lines that failed to express MHC class I molecules at the cell surface because of blocked peptide supply in the ER lumen (2–5). As a result, the MHC class I molecules devoid of peptide were not allowed to pass the ER quality control system and were degraded by the ERAD system. The peptide transporter identified was encoded by two multi-pass half-ABC transporter genes located in the MHC locus, close to the two immuno-subunits of the proteasome and was called Transporter associated with Antigen Processing (TAP). The two subunits of TAP, TAP1 and TAP2,

form a heterodimer with two ATP-binding cassettes that can—like all family members of the ABC transporter family—hydrolyze ATP to drive unidirectional transfer of peptide substrates over membranes (6). The multidrug transporter MDRI, that removes a series of different chemical substrates from cells, is a close homologue of TAP.

TAP is located in the ER membrane to translocate peptides from the cytosol into the ER lumen for consideration by MHC class I. The nuclear site of the nuclear envelop does not contain TAP and therefore nuclear peptides have to move into the cytosol for accessing TAP (7). Unlike other ABC transporter family members, TAP acts as the basis of a unique folding platform, dedicated to MHC class I peptide loading. In the ER lumen, a dedicated chaperone called tapasin glues MHC class I molecules devoid of peptide to TAP. This complex is further stabilized by two more common chaperones called calreticulin and ERp57 (8). When peptides bind MHC class I, the chaperones are released and the—now—peptide-containing MHC class I molecules are allowed to leave the ER for presentation at the cell surface.

TAP is a bottleneck in the process of MHC class I antigen presentation but not essential for cell viability. TAP knockout mice are perfectly viable with poor MHC class I surface expression (9). Therefore, it is not surprising that many viruses express inhibitors to block TAP to prevent their own antigen presentation (10). The immune system is blind to such viruses although NK cells may ultimately recognize the cells now lacking MHC class I and destroy these. A series of viral inhibitors for TAP have been defined using experimental protocols for peptide translocation by TAP, as described below.

How can peptide translocation by TAP be detected? The first and most simple assay was described in 1993 (6). It uses a simple cell biological trick of employing defined and localized enzyme activities that add tags to the substrate used in the reaction. When (radioactive or fluorescent) peptides are used including a N-linked glycosylation motif (being amino acid motif NXS/T where X could be any amino acid except P and P should not locate around the glycosylation motif), this peptide will be glycosylated by the N-linked glycosylation machinery present in the ER lumen. The N-glycan (a high-mannose chain) is then used for isolation by a lectin (Con-A) attached to a bead and fluorescence or radioactivity on the peptide is quantified.

In principle, other peptides without N-linked glycosylation motifs can be used as well, but then either binding to or accumulation in ER-derived vesicles is quantified. The complication with this assay is that accumulation is not stable as peptides are removed from the ER after some time (11, 12). In addition, nonspecific binding to membranes is hard to distinguish from actual entry into the ER lumen.

Finally, peptides can be made with a photoaffinity label and added to ER vesicles. After UV-induced cross-linking and protein separation by SDS-PAGE, a (fluorescent or radioactive) band of proper molecular weight can be detected (13, 14). This method is ideal to assay at which point in the TAP peptide-translocation cycle, viral or other inhibitors work but is not the easiest one for quantification neither for large scale analyses of different peptide sets.

Using the assays described above, some rules for the types of substrates binding and peptides translocated by TAP have been defined (see Note 0). These rules still do not allow definition of the efficiency with which a new peptide is translocated by TAP and only experimental testing will define these. The rules do indicate which peptides are unlikely to be successfully entering MHC class I molecules. Direct testing of peptides for TAP translocation can be assessed by measuring direct translocation (when an N-linked glycosylation motif is introduced) or by competition for TAP-dependent translocation of a model peptide with N-linked glycosylation sequences. Various peptides have been reported as model peptide and can be used. When used, the results may allow direct comparison with those from published sources (15, 16).

Below, a detailed protocol is given for the detection of peptide translocation by TAP. We at various points refer to Subheading 4 that includes remarks about alternatives and stress where the protocol does or does not allow flexibility in performance.

2. Materials

Prepare all reagents and solutions under most pure conditions. Store microsomes, ATP solutions, Streptolysin-O, and peptides at -20 or -80°C in aliquoted fractions and do not reuse these after thawing. Store iodinated peptides at 4 or -20°C and use these swift after iodination. Diligently follow all waste disposal regulations when disposing waste materials, especially when using radioactive materials. Sodium azide is not added to any solution.

2.1. Peptide Iodination and Purification

1. Peptide stock: 10–100 µg dissolved in 100 µl PBS or 50 mM Tris-HCl (pH 7.5) (see Note 1). Peptides should contain a tyrosine residue for iodination. Other considerations; (see Note 0).
2. Na¹²⁵I (Perkin Elmer). Order as fresh as possible to obtain highest specific activities (half-life ¹²⁵I is 60 days; specific activity at calibration date 642 GBq/mg).
3. Chloramine T (*p*-toluene sulfonochloramine, Sigma-Aldrich), powder: dissolve in PBS at concentration of 1 mg/ml just before reaction.
4. 50 mM Na-disulfite.

5. 50 mM Ascorbic acid.
6. Phosphate Buffered Saline (PBS): dissolve one tablet in 500 ml of distilled water (GIBCO).
7. DOWEX(OH⁻) beads (AG1-XB resin, BIO-RAD). Store at 4°C.
8. Sep-Pak C18 Cartridge columns (Waters). Store at 4°C.
9. Elution buffer: 60% Acetonitrile, 0.1% TFA.
10. 3 ml Syringe.
11. Dosis calibrator (Capintec) to detect iodinated peptides.
12. Floating Nitrogen gas.

2.2. Fluorescent Peptide Generation, Purification, and Detection

1. Fluorescent peptides: can be ordered from various commercial sources. Any detectable fluorophore can be used (see Note 2). Fluorescent peptides can also be made. Synthesize peptide with cystein group and use Fluorescein-5-Maleimide or 5-IAF to label the sulfur group of cystein.
2. Tris-HCl (pH 7.0) including 5 mM EDTA.
3. 10 mg/ml Fluorescein-5-Maleimide in DMSO.
4. ThermoScientific Dye Removal column (Thermo scientific).
5. Fluorescence plate reader (Cyto fluor; PerSeptive Biosystems).

2.3. Generation of Microsomes

1. Choose appropriate cell line. Cells with high TAP expression work best especially when a TAP-deficient counterpart is available. Such combinations are LCL721-T2, RMA, and RMA-S. EBV transformed B cells but also (insect) cells overexpressing the TAP1 and TAP2 genes work well.
2. PBS.
3. Cell scrapers (Nunc).
4. EMBL Cell cracker with different ball sizes to vary shearing forces (see Note 3).
5. Buffer for microsome production: 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ (see Note 4).
6. 1–5 ml Syringes.

2.4. Cell Permeabilization by Streptolysin-O

1. Choose appropriate cell line. Streptolysin-O is a bacterial toxin that requires cholesterol for membrane binding and not all cells are equally sensitive to permeabilization. In general, large tissue culture cells like Hela and MelJuSo are easily permeabilized. Smaller lymphocyte-derived cells (such as EBV transformed B cells) are more difficult to permeabilize with Streptolysin-O and usually require longer incubations or higher concentrations.
2. PBS.
3. Streptolysin-O (Murex Diagnostics).

4. Streptolysin-O permeabilization/translocation buffer: 130 mM KCl, 10 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 5 mM Hepes (pH 7.3).
5. Tryptan blue (Invitrogen): 0.4 (w/v)% in PBS.

2.5. Peptide Translocation

1. Peptide translocation buffer (see item 4, Subheading [2.4](#)).
2. 10 mM ATP stock (pH 7.5): dissolve 5 mg ATP in 900 µl H₂O and add 100 µl of 1 M Tris (121 g/l in H₂O) for neutralization.
3. TX-100 containing lysis and wash buffer lysis mixture: 0.5 (v/v)% TX-100 (Sigma), 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂.
4. Concanavalin-A (ConA)-Sepharose 4B (GE Healthcare): Wash the beads twice with TX-100 containing lysis and wash buffer before use. Use 25 µl beads for every reaction (see Note 5).
5. Gamma counter.
6. Fluorescence plate reader (Cyto fluor; PerSeptive Biosystems).

2.6. Competition Experiment

1. Radioactive tracer peptide.
2. TAP translocation buffer (see item 4, Subheading [2.4](#)).
3. Other materials are listed under Subheading [2.5](#).

3. Methods

Keep all reagents on ice before the actual peptide translocation assay, which is performed at 37°C.

3.1. Chloramine-T Catalyzed Peptide Iodination

1. Use 10 µl of a 1 mg/ml peptide stock in an Eppendorf tube.
2. Buffer the peptide to pH 7.5 (see Note 6).
3. Add 10 µl Na¹²⁵I from a radioactive iodine stock (see Note 7).
4. Add 10 µl chloroamine-T (see Note 8).
5. Incubate for 5 min at room temperature.
6. Add 10 µl Na-disulfide and 10 µl ascorbic acid to terminate the reaction.
7. Add PBS to a final volume of 500 µl.
8. Separate iodinated peptide from free iodine (see Subheadings [3.2](#) and [3.3](#)).

3.2. Purification of Radioiodinated Peptides by Dowex(OH⁻)

¹²⁵I⁻ will strongly bind to Dowex(OH⁻) beads. If the iodinated peptide does not have affinity to this resin (see Note 9) then radioactive peptides can be easily separated from free radioactive iodine. Avoid acetonitrile as a solvent as this will turn the beads into a solid undissolvable state.

1. Add 50 µl of Dowex(OH⁻) beads to the peptide mixture.
2. Incubate for 30 min on ice while mixing or rotating.
3. Spin down beads for 1 min at 12,000 ×g.
4. Remove supernatant and add to a new tube that now contains the purified peptide.
5. Store radio-iodinated peptide at 4°C or -20°C (see Note 10). Besides Ionidated peptides can be used for a TAP translocation assay (see Subheading 3.7).

3.3. Purification of Radioiodinated Peptides by Sep-Pak C18 Cartridge Columns

1. Wash the Sep-Pak column with 2 ml elution buffer.
2. Wash with 2 ml of H₂O.
3. Put the radio-iodinated peptide on the column.
4. Gently push the radiolabeled peptide through the column (this is fraction 1).
5. Add 1 ml of H₂O to the column, to remove free Iodine.
6. Elute the 1 ml in two fractions of 500 µl.
7. Add 2.5 ml of the acetonitrile containing elution buffer to the column, to elute the Ionidated peptides from the column.
8. Elute the 2.5 ml in five fractions of 500 µl.
9. Measure the radioactivity in the eight fractions and in the column by dosis calibration.
10. Continue with the most radioactive fractions from the peptide elution.
11. Remove acetonitrile and TFA from the elution buffer by a gentle N₂ flow. This usually takes 2 h in the chemical hood.
12. The remaining iodinated peptide in H₂O will be transferred to a new Eppendorf tube, aliquoted and stored at 4 or -20°C until further use. Besides fluorescent peptides can be used for a TAP translocation assay (see Subheading 3.7).

3.4. Synthesis and Generation of Fluorescent Peptides

1. Dissolve peptide in Tris-HCl (pH 7.0) including 5 mM EDTA.
2. Add a 25 times molar excess of 10 mg/ml Fluorescein-5-Maleimide (~3 mg Fluorescein-5-Maleimide to 10 mg of a ten amino acid peptide).
3. Allow the reaction to run for 2 h at room temperature.
4. Remove non-reacted fluorescein by Thermo Scientific Dye Removal Columns according to manufacturers protocol. Alternatively use a G10 Sepharose column. The first fluorescent fraction will contain the fluorescent peptide.
5. Aliquot the fluorescent peptides and store at -20°C.

3.5. Generation of Microsomes

Peptide translocation by TAP can be measured in either microsomes or Streptolysin-O permeabilized (see Subheading 3.6) cells containing TAP. A negative control for peptide translocation is essential to define background of the reaction. A number of cell pairs that do or do not contain TAP have been described. Most common are LCL721 and its mutant T2 for human TAP (17) and RMA and its mutant RMA-S for mouse TAP (18). Alternatively, include a control where ATP to drive TAP is absent as control.

1. Wash 10^7 – 10^9 cells with PBS.
2. Prepare the cells. Adherent cells can be removed by scraping of the plate. Non-adherent cells can be pelleted by centrifugation (1 min at $10,000 \times g$) (see Note 11).
3. Resuspend the cell pellet in PBS and add 1 ml of 50 mM Tris-HCl (pH 7.5) including 5 mM MgCl₂.
4. Prepare the EMBL cell cracker. This device has an 8.020 mm chamber with balls of different sizes (up to 8.010 mm) to vary the shearing forces for cell destruction.
5. Take up the cell suspension in a 1 ml syringe and adjust to the EMBL cell cracker.
6. Adjust another empty 1 ml syringe at the other outlet.
7. Push the cells through the EMBL chamber and push back by pressing the syringes on the other side. Repeat this 10–20 times.
8. Collect fractionated cells via the originally empty syringe and transfer the material to a 1.5 ml Eppendorf tube.
9. Spin down the remaining large cells and nuclei in an Eppendorf centrifuge for 1 min at $500 \times g$, 4°C.
10. Collect the supernatant and transfer to a new 1.5 ml Eppendorf tube.
11. Spin down the microsomes in an Eppendorf centrifuge for 5 min at $12,000 \times g$, 4°C.
12. Remove supernatant and resuspend the pellet in PBS before adding 1 ml of 50 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂.
13. These Microsomes can be used for a peptide translocation assay directly (see Subheading 3.7) or aliquoted and snap frozen (on dry ice) and stored at –80°C for further use.

3.6. Permeabilization of Cells by Streptolysin-O

1. Wash the cells with PBS.
2. Add 2.5 IU/ml Streptolysin O in Streptolysin-O permeabilization buffer (see Note 12).
3. Incubate for 30 min at 37°C (see Note 13).
4. Collect the cells by detaching them from the plate in 1 ml PBS
5. Add 1 ml in an Eppendorf tube and gently spin down cells for 1 min at $12,000 \times g$.

6. Remove supernatant and resuspend the pellet in PBS before adding 400 µl–1 ml of translocation buffer.

7. Take a small volume (25–50 µl) of the permeabilized cells in translocation buffer and check cell permeabilization by adding a 10% volume of trypan blue to cells. Quantify blue permeabilized cells by light microscopy. Typically 60–90% of cells should be permeabilized for the assay.

3.7. Direct Peptide Translocation by TAP

1. Dilute the microsomes or the Streptolysin-O permeabilized cells in 100 µl peptide translocation buffer in a 1.5 ml Eppendorf tube. ~100,000 cell equivalents, microsomes or permeabilized cells per reaction are sufficient.
2. Add (10 mM stock) ATP solution buffered at pH 7.5 till a final concentration of 1 mM.
3. Add 0.1 µg equivalents of iodinated or fluorescent peptides (see Note 14).
4. Mix gently and incubate this cocktail for 10 min in a 37°C water bath (see Note 15).
5. Terminate the reaction by the addition of 1 ml TX-100 lysis mixture of 4°C; mix.
6. Leave the lysates on ice for 30 min.
7. Remove debris by centrifuging 10 min at 10,000×*g* in an Eppendorf centrifuge at 4°C.
8. Transfer lysate to a new Eppendorf tube and add the Con-A beads, to bind N-glycosylated (TAP transported) peptides.
9. Mix and regularly shake the Eppendorf tube to prevent precipitation of the Con-A beads. This step takes 30 min, perform at 4°C.
10. Spin down the Con-A beads for 2 min at 10,000×*g* at 4°C.
11. Remove the supernatant from the beads.
12. Resuspend the Con-A beads in 1 ml TX-100 lysis buffer and spin down the beads as in step 10.
13. Repeat this washing step five times.
14. Take the Eppendorf tubes containing the beads and quantify the radioactive signal with a gamma counter.
15. Fluorescent peptides are quantified by a fluorescent plate reader (e.g., Cyto fluor; PerSeptive Biosystems).

3.8. Competition Experiments for Peptide Translocation by TAP

Often peptides of interest or viral TAP inhibitors do not contain N-glycosylation sites and cannot be monitored directly for translocation by TAP. One method is to follow direct entry in the ER lumen by isolating the microsomes and quantitating the label on the peptides. This method is complicated by the fact that many

peptides (not glycosylated though) are actively removed from the ER lumen in an ATP-dependent manner (11). An effective method is to add peptides or viral proteins of interest along with the radioactive tracer peptide and monitor competition for peptide transfer of the latter. A number of sequences for efficient tracer peptides have been reported (see Note 16).

1. Make a mixture of 2.5 mM ATP (final concentration ATP must be 1 mM) and radioactive tracer peptide in TAP translocation buffer that is used in all samples. Use 50 μ l per sample. If a competition curve is made of eight data points; make at least 400 μ l (preferentially a bit more as stocks easily run out by pipeting errors). Mix carefully.
2. Make a mixture of the highest concentration of the competitor peptide or viral proteins. Make in an Eppendorf tube a 60 μ l volume in TAP translocation buffer containing the highest concentration of competitor peptide (see Note 17). Fill other Eppendorf tubes with 50 μ l TAP translocation buffer.
3. Add 10 μ l of the highest competitor concentration to the next tube; mix carefully and add now 10 μ l of this tube to the next one etc. to make an accurate dilution series. Lowest concentration: only 50 μ l TAP translocation buffer.
4. Add to all tubes 40 μ l of the ATP and radioactive tracer peptide cocktail as generated in step 1.
5. Add 10 μ l microsomes or Streptolysin-O permeabilized cells to the tubes.
6. Mix gently and incubate for 10 min in a 37°C water bath.
7. Lyse all samples in 1 ml TX-100 lysis buffer at 0°C and continue at Subheading 3.7, steps 6–15.
8. The data points are plotted and an IC_{50} value is calculated as the concentration of nonradioactive peptide required for 50% inhibition. Highly accurate numbers are in complicated systems like the ones described here, not easy. When various competitor peptides are used and a reference peptide (for which the IC_{50} related to other peptides is described) is included, relative statements can be made.

4. Notes

(Note 0 can be found at the end of this chapter)

1. Some ideal peptide standards have been described and may be considered. These sequences are sequence TVNKTERAY (15) and RYWANATRSF (19, 20). Such peptides can be ordered at various companies.

2. The fluorescent probe should not be coupled at the N-terminus but as a side chain of for example a cysteine or lysine residue. Successful model peptides include peptide CVNKTERAY, which has fluorescein coupled to the Cysteine residue with Fluorescein-5-maleimide and 5-IAF.
3. Microsomes can also be made by classical douncing—that also applies different modes of exposure of cell membranes to shearing forces. If cells are small and flexible (as most immune cells), it is best to first swell the cells in hypotonic medium (10% normal DMEM diluted in H₂O) for some 20 min at room temperature prior to douncing.
4. MgCl₂ is added to drive ATP hydrolysis but also to maintain the DNA condensed state to prevent sticky DNA in the preparation that would complicate later steps.
5. These beads are supplied with 20(v/v)% ethanol as preservative that should be removed before use by washing.
6. Peptides are often acidic (due to TFA elution from the resin). As peptide iodination is pH sensitive and inefficient at low pH, carefully neutralize the peptide mixture with Tris or NaOH.
7. Higher amounts of Na¹²⁵I will only marginally increase incorporation of radioactivity.
8. Peptides can also be iodinated in a reaction catalyzed by iodo-beads. Our experience is that this is less efficient than the chloro-amine T catalyzed reaction.
9. Some peptides—like model peptide TVNKTERAY—can be easily separated from free ¹²⁵I⁻ by simple exclusion from DOWEX(OH⁻) while free iodine will bind to these beads. If the peptide also binds to DOWEX(OH⁻) then gel filtration (G10 columns or separation on basis of hydrophobicity using Sep-Pak C18 Cartridge columns as described in Subheading 3.8) is an option.
10. Peptides are stable at -20°C but still decay due to radioactivity. Stored peptides can be safely used over a period of 1 month.
11. Lymphoid cell types are usually small and flexible, osmolaric swelling of cells can be used by incubating the cells in 10% DMEM diluted with H₂O for 20 min at 37°C before the cell breakage is starting.
12. Streptolysin O binds to cholesterol and permeabilization can be inhibited by cholesterol. Addition of medium including FCS is not favored. The amount of Streptolysin O required for permeabilization varies dependent on cell line or type. Titration for optimal concentration is a prerequisite. 2.5 IU/ml is usually sufficient for most cells.
13. Streptolysin O can bind to membranes at 4°C. Pore formation occurs only above 20°C.

14. Cytosol and microsomes contain high peptidase activity (15) and peptides should be exposed as short time as possible to these conditions.
15. A water bath allows most efficient warming up of the cocktail in Eppendorf tubes.
16. Peptide #417; sequence TVNKTERAY (also called #417;(15)) and RYWANATRSF (19, 20).
17. A range of competing concentrations of peptides is used usually between 1 μM and 10 mM.

The notes below are related to the different subsections in Subheading 3. We will make cross-references between the various sections when points apply to more than one subsection.

(Note 0) General rules for the substrate specificity of TAP.

These are summarized below and illustrate that TAP specificity has overlap with MHC class I specificity. Since different MHC class I alleles will select different peptides of around nine amino acids and all these MHC class I alleles have to be “fed” by the same TAP, TAP should have a broad specificity. The currently understood rules for TAP substrates are summarized below:

- TAP translocates peptides of minimally 8 to over 40 amino acids in size. Highest efficiency is observed for peptides between 9 and 12 amino acids (15, 21).
- TAP is not very selective for different amino acids at different positions. There are two exceptions: peptides with a P (Proline) residue at position 2 and 3 are very inefficiently handled by TAP (16, 22, 23). Such peptides are however presented by MHC class I molecules and translocated as N-terminally extended peptides that are trimmed to smaller peptides with P at position 2 or 3 in the ER by the ERAAP/ERAP peptidase (24–27).
- MHC class I binds peptides with free N- and C-termini. Also TAP binds peptides with free termini (6).
- Many interactions between the peptide’s peptide bond and MHC class I stabilize the structure. d-amino acids alter the orientation of the peptide bond and introduction of various d-amino acids decreases efficiency of binding. Exchange of peptide bonds for a “double isostere” that cannot make bonds with TAP, in fact improves translocation by TAP. This indicates that peptide bonds in the peptide interact with TAP, similar as observed for peptides in the MHC class I structure (28).
- TAP allows the translocation of very long side chains (including fluorophores). In fact, a side chain of the size of the (9-mer) peptide itself can still be translocated by TAP while longer side chains allow binding but not translocation. In other words, peptides with long extended side chains in effect are competitive inhibitors for TAP (28).

TAP translocation of N-linked glycosylation site containing peptides.

To measure direct translocation, four considerations should be made.

- The substrate peptide should contain an N-linked glycosylation motif and should be labeled by either a radioactive tracer or a fluorophore. Peptides should be larger than eight amino acids with free N- and C-termini.
- The peptides should get access to TAP by using either Streptolysin-A permeabilized cells, microinjection (29) or microsomes.
- TAP can be fuelled by ATP, but also CTP, UTP, and GFP (11). TAP is sensitive to pH and does not work at low pH while the XTPs are highly acidic (15). These should be carefully buffered before use in a TAP peptide translocation assay.
- N-linked glycosylated peptides can be isolated by lectin concanavalin A (ConA)-Sepharose beads and the associated peptides can be quantified by their tracer (usually a fluorophore or radiolabel).

Acknowledgments

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References

1. Neefjes J, Jongsma ML, Paul P, Bakke O (2011) To a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 11(12):823–836
2. Kelly A, Powis SH, Kerr LA, Mockridge I, Elliott T, Bastin J, Uchanska-Ziegler B, Ziegler A, Trowsdale J, Townsend A (1992) Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. *Nature* 355:641–644
3. Spies T, Cerundolo V, Colonna M, Cresswell P, Townsend A, DeMars R (1992) Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature* 355:644–646
4. Powis SJ, Townsend AR, Deverson EV, Bastin J, Butcher GW, Howard JC (1991) Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 354:528–531
5. Attaya M, Jameson S, Martinez CK, Hermel E, Aldrich C, Forman J, Lindahl KF, Bevan MJ, Monaco JJ (1992) Ham-2 corrects the class I antigen-processing defect in RMA-S cells. *Nature* 355:647–649
6. Neefjes JJ, Momburg F, Hammerling GJ (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* 261:769–771
7. Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, van Veelen P, Janssen H, Calafat J, Drijfhout JW, Neefjes J (2003) Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18: 97–108
8. Wearsch PA, Cresswell P (2008) The quality control of MHC class I peptide loading. *Curr Opin Cell Biol* 20:624–631
9. Van Kaer L, Ashton-Rickardt PG, Ploegh HL, Tonegawa S (1992) TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4+ T cells. *Cell* 71: 1205–1214

10. Horst D, Verweij MC, Davison AJ, Ressing ME, Wiertz EJ (2011) Viral evasion of T cell immunity: ancient mechanisms offering new applications. *Curr Opin Immunol* 23:96–103
11. Koopmann JO, Albring J, Huter E, Bulbuc N, Spee P, Neefjes J, Hammerling GJ, Momburg F (2000) Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. *Immunity* 13:117–127
12. Shepherd JC, Schumacher TN, Ashton-Rickardt PG, Imaeda S, Ploegh HL, Janeway CA Jr, Tonegawa S (1993) TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. *Cell* 74:577–584
13. Spee P, Neefjes J (1997) TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. *Eur J Immunol* 27:2441–2449
14. Androlewicz MJ, Ortmann B, van Endert PM, Spies T, Cresswell P (1994) Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc Natl Acad Sci U S A* 91:12716–12720
15. Roelse J, Gromme M, Momburg F, Hammerling G, Neefjes J (1994) Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. *J Exp Med* 180:1591–1597
16. van Endert PM, Riganelli D, Greco G, Fleischhauer K, Sidney J, Sette A, Bach JF (1995) The peptide-binding motif for the human transporter associated with antigen processing. *J Exp Med* 182:1883–1895
17. Ohta N, Bach FH (1986) NO1: an HLA-DQ α 1-associated determinant present on loss mutants not expressing DQ α 1. *Hum Immunol* 16:91–99
18. Townsend A, Ohlen C, Bastin J, Ljunggren HG, Foster L, Karre K (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* 340:443–448
19. Daniel S, Brusic V, Caillat-Zucman S, Petrovsky N, Harrison L, Riganelli D, Sinigaglia F, Gallazzi F, Hammer J, van Endert PM (1998) Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J Immunol* 161:617–624
20. Samino Y, Lopez D, Guil S, Saveanu L, van Endert PM, Del Val M (2006) A long N-terminal-extended nested set of abundant and antigenic major histocompatibility complex class I natural ligands from HIV envelope protein. *J Biol Chem* 281:6358–6365
21. Koopmann JO, Post M, Neefjes JJ, Hammerling GJ, Momburg F (1996) Translocation of long peptides by transporters associated with antigen processing (TAP). *Eur J Immunol* 26:1720–1728
22. Momburg F, Roelse J, Howard JC, Butcher GW, Hammerling GJ, Neefjes JJ (1994) Selectivity of MHC-encoded peptide transporters from human, mouse and rat. *Nature* 367:648–651
23. Neisig A, Melief CJ, Neefjes J (1998) Reduced cell surface expression of HLA-C molecules correlates with restricted peptide binding and stable TAP interaction. *J Immunol* 160:171–179
24. Serwold T, Gonzalez F, Kim J, Jacob R, Shastri N (2002) ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419:480–483
25. York IA, Chang SC, Saric T, Keys JA, Favreau JM, Goldberg AL, Rock KL (2002) The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues. *Nat Immunol* 3:1177–1184
26. Saveanu L, Carroll O, Lindo V, Del Val M, Lopez D, Lepelletier Y, Greer F, Schomburg L, Fruci D, Niedermann G, van Endert PM (2005) Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* 6:689–697
27. Chang SC, Momburg F, Bhutani N, Goldberg AL (2005) The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a “molecular ruler” mechanism. *Proc Natl Acad Sci U S A* 102:17107–17112
28. Gromme M, van der Valk R, Sliedregt K, Vernie L, Liskamp R, Hammerling G, Koopmann JO, Momburg F, Neefjes J (1997) The rational design of TAP inhibitors using peptide substrate modifications and peptidomimetics. *Eur J Immunol* 27:898–904
29. Reits EA, Vos JC, Gromme M, Neefjes J (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature* 404:774–778

Chapter 6

In Vitro Reconstitution of the MHC Class I Peptide-Loading Complex

Pamela A. Wearsch and Peter Cresswell

Abstract

The stability of the MHC (major histocompatibility complex) class I peptide repertoire is optimized during assembly in the endoplasmic reticulum (ER) and depends on the collective function of components of the peptide-loading complex (PLC). The chaperone-like molecule tapasin is the cornerstone of this complex and acts directly on the MHC class I molecule to promote high-affinity peptide loading. Optimal tapasin activity, however, relies on the ability of ERp57 and calreticulin, two proteins involved in general ER glycoprotein folding, to bridge and thereby stabilize its otherwise weak interaction with the MHC class I heavy chain. Here, we describe methods for the recombinant expression of soluble components of the PLC specifically tailored to generate the post-translational modifications required to support subcomplex assembly in vitro. Using recombinant MHC class I molecules bearing monoglycosylated N-linked glycans, calreticulin, and disulfide-linked tapasin/ERp57 heterodimers, this soluble PLC subcomplex can be employed to study the mechanism of peptide loading or the principles governing peptide selection for particular MHC class I alleles.

Key words: MHC class I, Tapasin, Antigen processing, Peptide loading

1. Introduction

The ability of MHC (major histocompatibility complex) class I molecules to present antigens to CD8+ T cells depends on the stability of its associated peptide repertoire. Thus, a highly evolved assembly process takes place in the endoplasmic reticulum (ER) to selectively promote the loading of high-affinity peptides onto MHC class I molecules. Following the proper folding of the class I heavy chain (HC) and assembly with β_2 microglobulin (β_2 m), the empty heterodimer associates with the lectin-like chaperone calreticulin via its monoglycosylated N-linked glycan (1, 2). Calreticulin then recruits the HC/ β_2 m complexes to the tapasin/ERp57 heterodimer by association of the extended P-domain of calreticulin with

the *b* domain of ERp57. These multimeric interactions enhance the intrinsically weak binding affinity of the tapasin N-terminal domain for the $\alpha 2$ domain of the MHC class I binding groove (3). In this complex, known as the PLC (peptide-loading complex), HC/ β_2 m dimers are stabilized and maintained in a conformation that promotes the binding of optimal ligands (4–8). Tapasin also associates with TAP (transporter associated with antigen processing) via its transmembrane domain, to both stabilize the transporter and localize the class I molecule in proximity to the peptide supply (9–12). Following high-affinity peptide loading, a conformational change in the MHC class I binding groove is believed to disrupt the tapasin interaction and promote dissociation of the complex, allowing for class I transport to the cell surface.

The mechanism of PLC activity is poorly defined, largely due to the challenges of studying peptide loading in vitro. Efforts to establish assays for PLC activity have been hampered by the complexity of its components and the intermolecular interactions between them (Fig. 1). Although evidence exists to support TAP regulation of tapasin function, studies using soluble tapasin have demonstrated that high-affinity peptide loading can proceed without TAP incorporation into the PLC (13–15). Thus, biochemical studies may be simplified by excluding TAP, a polytopic membrane protein, and using soluble versions of the remaining components to generate a subcomplex of the PLC. To complicate matters, however, the assembly of this luminal complex (HC/ β_2 m heterodimer, tapasin, ERp57, and calreticulin) involves multiple interactions by each component and protein modifications that cannot be generated using standard recombinant expression approaches (Fig. 1). The considerations are as follows:

1. Calreticulin is an ER chaperone that promotes the folding of newly synthesized glycoproteins. It binds to substrates with a specific N-linked glycan structure ($\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$) that is generated by glucose trimming and is present only transiently during the course of protein folding. The association of calreticulin with monoglycosylated HC glycans regulates MHC class I incorporation into the PLC in cells and in vitro (1, 2, 16, 17).
2. ERp57 is a thiol oxidoreductase that associates with calreticulin and mediates the formation of proper disulfide bonds in newly synthesized glycoproteins. Although ERp57 typically forms transient mixed disulfides with folding substrates, tapasin is a uniquely stable exception. By nature of its interactions with both thioredoxin-like active sites of ERp57 (Fig. 1), a disulfide-linked conjugate is formed between Cys95 of tapasin and Cys57 of the ERp57 *a* domain and cannot be reduced due to interference with the α' active site (3, 18). Tapasin/ERp57 conjugation generates a stable heterodimer and is required for optimal tapasin activity, particularly in vitro, due to stabilizing effects on PLC composition (8, 18, 19).

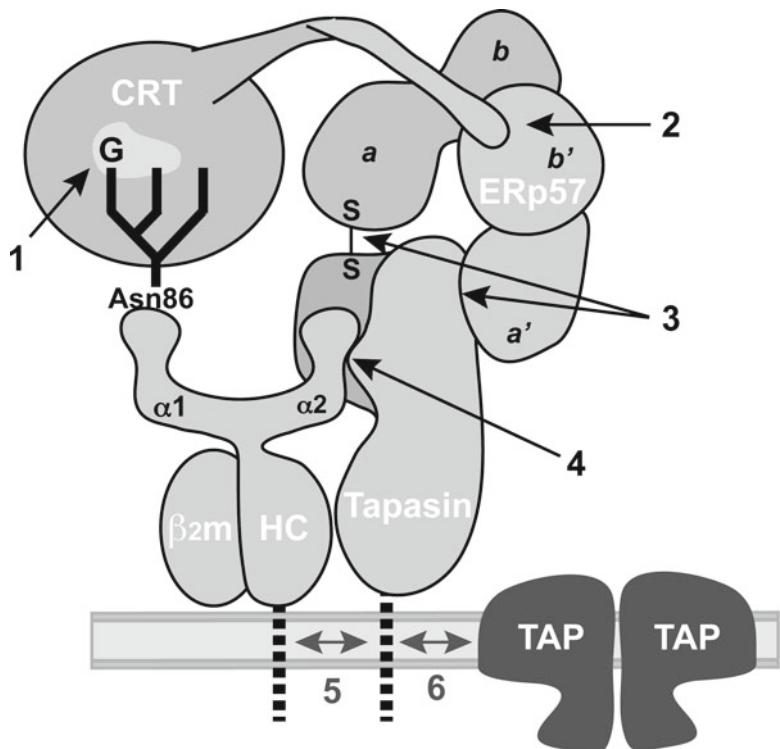


Fig. 1. Model of the MHC class I peptide-loading complex and interactions between its components. Molecular interactions are present between (1) the calreticulin lectin domain and the monoglycosylated N-linked glycan at HC residue Asn86, (2) the calreticulin P-domain and the *b*_c domain of ERp57, (3) the N-terminal domain of tapasin and both (*a* and *a'* domain) active sites of ERp57, (4) the *α2* domain of the class I peptide-binding groove and the N-terminal domain of tapasin, (5) the transmembrane domains of tapasin and the class I HC, and (6) the transmembrane domains of tapasin and TAP. The soluble PLC subcomplex generated for biochemical studies includes the proteins and domains shown in *black outlines*.

3. Only empty MHC class I molecules or those associated with low-affinity ligands are capable of interacting productively with the PLC. Thus, an expression system is required that supports HC folding and $\beta_2\text{m}$ assembly in an environment devoid of tapasin or an adequate peptide supply.

To this end we have customized the recombinant expression of PLC components using the baculovirus-insect cell system (see Note 1). The advantages of this eukaryotic expression system were severalfold. First, constructs (e.g., pFastBac Dual) are available that permit high level expression of two components from the same vector thus enabling the co-expression of tapasin with ERp57 and MHC class I HC with $\beta_2\text{m}$. Second, the ER environment is very similar to mammalian cells. It supports tapasin/ERp57 conjugation and, unlike *E. coli*, the proper folding and heterodimeric assembly of the class I HC and $\beta_2\text{m}$ (bypassing the need for arduous and

inefficient refolding reactions). Finally, N-linked glycosylation in the ER is identical to mammalian cells and, although processing may proceed, the deficiency of tapasin and TAP leads to the retention of a significant percentage (~20%) of class I molecules with Glc₁Man₉GlcNAc₂. This chapter will first present protocols for the recombinant expression of calreticulin, soluble tapasin (sTpsn)/ERp57 heterodimers, and soluble HC/β₂m heterodimers using SF21 insect cells (see Subheadings 3.1 and 3.2). Purifications are performed from cell pellets as a starting material using a sequence of metal-affinity, anion exchange, and gel filtration chromatography (see Subheadings 3.3). Finally, we will describe how the purified recombinant proteins can be assembled into functional PLC subcomplexes and utilized in peptide-binding studies (see Subheadings 3.4 and 3.5).

2. Materials

2.1. Plasmids, Cells, and Media

We have generated the following constructs (available upon request) for the recombinant expression of soluble, human PLC components (see Note 2):

1. pFastBac Dual sTpsn/C60A ERp57.
2. pFastBac Dual soluble HLA-B8/β₂m.
3. pFastBac1 calreticulin.

Specific items required for baculovirus production and insect cell expression include (see Note 3):

1. DH10Bac competent cells (Invitrogen).
2. Cellfectin-II insect cell transfection reagent (Invitrogen).
3. Sf21 insect cells (Invitrogen).
4. Sf900 II SFM and penicillin/streptomycin (Invitrogen).
5. Polycarbonate culture flasks (filter cap, various sizes from 125 mL to 2 L, BD Falcon, San Jose, CA).
6. A 27°C shaking incubator.

2.2. Chromatography Resins and Columns

For protein purification, we employ a sequence of metal-affinity, anion exchange, and gel filtration chromatography using the following matrices. Suitable alternatives may be employed, providing that the sample capacity and resolution range are comparable.

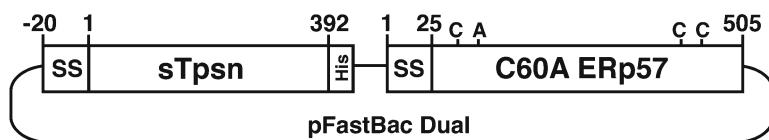
1. Talon metal-affinity resin (Clontech, Mountain View, CA) and 5 mL disposable polypropylene columns (Qiagen, Valencia, CA).
2. MonoQ 5/50GL (GE Healthcare, Piscataway, NJ).
3. HiLoad 16/60 Superdex 200 prep grade (GE Healthcare).

4. AKTA Explorer FPLC and fraction collector (GE Healthcare).
5. Protein G-Sepharose (GE Healthcare).

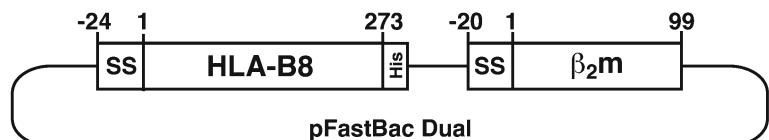
2.3. Buffers and Reagents

1. PBS (phosphate buffered saline).
2. Cell lysis buffer: 1% Triton X-100, 50 mM sodium phosphate, pH 7.0, 250 mM NaCl, 10 mM MMTS (Thermo Scientific), and Complete EDTA-free Protease Inhibitor tablets (Roche, Indianapolis, IN).
3. Suboptimal MHC class I binding peptides or conditional ligands.
4. Talon wash buffer: 250 mM NaCl, 50 mM sodium phosphate, pH 7.0, 5 mM imidazole.
5. Talon elution buffer: 100 mM NaCl, 25 mM Tris-Cl, pH 7.4, 200 mM imidazole.
6. MonoQ buffers: 50–600 mM NaCl, 25 mM Tris-Cl, pH 8.0. (0.2 µM filtered, degassed, and chilled to 4°C. Please refer to Subheading 3 for the specific gradients employed for each recombinant protein.)
7. Gel filtration buffer: 150 mM NaCl, 25 mM Tris-Cl, pH 7.4, 5% glycerol (filtered, degassed, and chilled to 4°C).
8. Amicon Ultra-4 Centrifugal Filter Units (30K and 50K MWCO, Millipore, Billerica, MA).
9. TBS-C: 150 mM NaCl, 25 mM Tris-Cl, pH 7.4, 2 mM CaCl₂.
10. IP (immunoprecipitation) buffer: TBS-C with 0.1% Triton X-100.
11. A spectrophotometer and quartz cuvettes.
12. Reagents for SDS-PAGE and Western blotting. Given the diversity of systems now available, we will not recommend any particular source. These reagents will be necessary, however, to complete the protocols described in this chapter: 10% Laemmli gels, SDS-PAGE reducing and non-reducing sample buffer, SDS-PAGE gel running buffer, Coomassie Blue solution, destaining solution, PVDF membranes, dry milk, PBS with 0.1% Tween-20, secondary HRP-conjugated antibody, and chemiluminescence detection reagent.
13. Antibodies for Western blotting (MHC class I HC, β₂m, calreticulin tapasin, ERp57) and immunoprecipitation (MHC class I, tapasin).
14. ¹²⁵I-labeled high-affinity class I binding peptides and a gamma counter. (Tyrosine-containing peptides are labeled with Na¹²⁵I using the conventional Chloramine T method and separated from unincorporated isotope using Sephadex G10 resin.)

sTpsn/ C60A ERp57 Conjugate



Soluble HLA-B8/ β_2m



Calreticulin

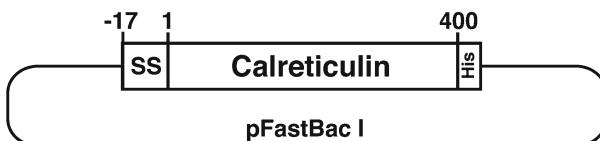


Fig. 2. Constructs employed for the expression of soluble PLC components.

3. Methods

3.1. Insect Cell Culture and Generation of Baculovirus Stocks

Due to space constraints, we shall summarize the methods employed for insect cell culture and baculovirus production.

1. Briefly, expression constructs (Fig. 2) are transformed into DH10Bac competent *E. coli* and positive recombinants are selected.
2. Bacmid DNA is isolated, screened, then transfected into Sf21 cells using the Cellfectin-II reagent.
3. The initial baculovirus stock (~2 mL) is then amplified by two rounds of low MOI (0.01–0.1) infections to obtain 200–500 mL of high titer baculovirus stock.
4. Titration of the stock may be performed using the BacPak Rapid Titre or BacPak qPCR Titration kits (Clontech) or a traditional plaque assay (please refer to the Invitrogen manuals). We have found it most convenient to maintain our cultures or perform baculovirus infections using disposable, baffled flasks and a shaking incubator (see Note 4).

3.2. Infection of Insect Cells for Protein Expression

One benefit of protein purification from Sf21 cell pellets is that baculovirus infections may be performed in several small batches (and cells frozen) rather than performing a single large-scale infection

(see Note 5). Using high titer baculovirus stocks, the expression yields of calreticulin or the sTpsn/ERp57 conjugate can exceed 3 mg/L of culture. We recommend using 1–3 L of culture to obtain 2–5 mg of purified protein. In contrast, the expression yields and stability of the HC/β₂m heterodimers are moderate (typically 250 µg/L). We recommend using 4–8 L of culture to obtain 0.5–1 mg of purified MHC class I complexes.

1. Count the cells and determine the cell viability. Do not continue if the viability is below 90% or if the cell density is below 1.2×10^6 cells/mL.
2. Calculate the amount of baculovirus stock and Sf900 II medium required to obtain an MOI of ~2 and a final cell density of $1.2\text{--}1.5 \times 10^6$ cells/mL.
3. Incubate the cells with shaking at 27°C for 68–72 h.
4. Remove 1 mL of cell culture for screening. Transfer sample to an eppendorf tube, spin in a microfuge for 1 min at $18,000 \times g$, remove the supernatant, then freeze the pellet at –80°C (see Note 6).
5. Harvest the remaining cells by centrifugation at $500 \times g$ for 15 min at 4°C.
6. Resuspend and pool the cells in 50 mL of cold PBS (in conical tubes).
7. Centrifuge at $2,000 \times g$ for 10 min at 4°C.
8. Remove the wash completely and freeze the cell pellets at –80°C.

3.3. Protein Purification

1. Thaw the pellets briefly on ice (10–15 min) then thoroughly resuspend in 75–100 mL of insect cell lysis buffer per liter of initial culture (see Note 7).
2. Incubate on ice for 30 min. If the volume exceeds 100 mL, transfer the mixture to a chilled Erlenmeyer flask with a stir bar and gently mix using a stir plate in the cold room for 30 min.
3. Centrifuge at $12,000 \times g$ for 30 min at 4°C.
4. In the meantime, thoroughly mix the Talon beads and transfer to a 50 mL conical tube. Use 1 mL of 50% bead slurry per mg of anticipated recombinant His-tagged protein. Add deionized water to 50 mL then centrifuge at $500 \times g$ for 5 min at 4°C. Remove the wash then set aside the beads on ice.
5. Carefully collect the cell lysate and transfer to a conical tube or centrifuge bottle. Add the washed Talon beads.
6. Batch bind with rotation for 1 h at 4°C (see Note 8).
7. Centrifuge the sample at $500 \times g$ for 10 min at 4°C. Carefully remove the supernatant without disturbing the beads and set aside on ice (see Note 9).

The following chromatography steps must be performed in a cold room.

8. Transfer the beads to a disposable polypropylene column and collect the remainder of the flow through.
9. Pass 5–10 CV (column volumes) of Talon wash buffer over the resin by gravity flow. Collect the wash and set aside on ice.
10. Recover the recombinant proteins using 10× 1 CV of Talon elution buffer and collecting each fraction in a separate tube (see Note 10).
11. Analyze samples on a 10% non-reducing gel and Coomassie stain. Pool fractions enriched for the protein(s) of interest (see Note 11).
12. Set up the MonoQ column using buffers with the (NaCl) indicated below in 25 mM Tris-Cl, pH 8.0. Inject the sample at a flow rate of 1 mL/ min (see Note 12). Wash with 5 CV of low buffer then elute proteins using a 20 CV linear gradient from 0 to 100% buffer B with 1 mL fraction collection.

	Buffer A (mM)	Buffer B (mM)
sTpsn/ERp57	100	500
Calreticulin	100	600
MHC class I/ β_2 m	50	450

13. Analyze the protein-containing peaks by 10% SDS-PAGE and pool fractions enriched for the protein(s) of interest. If the volume is greater than the loading capacity of the gel filtration column, concentrate the sample using Amicon Ultra-4 Centrifugal Filter Units (see Note 13).
14. Inject the sample onto a Superdex 200 prep column equilibrated with 3 CV of gel filtration buffer. Run a 1 CV program collecting ~40 fractions.
15. Analyze the protein-containing peaks by 10% SDS-PAGE and Coomassie staining. Pool fractions that are enriched for the protein(s) of interest.
16. Estimate yields by measuring the absorbance at 280 nm using a spectrophotometer and a quartz cuvette (use the gel filtration buffer as a blank). Concentrate the sample using Amicon Ultra-4 Centrifugal Filter Units to a final concentration of 0.5–2 mg/mL. Determine the final protein concentration using the Abs 280 nm.
17. Snap freeze aliquots (~50 μ L) in liquid nitrogen and store at –80°C.

3.4. Assembly of the PLC Subcomplex and Co-immunoprecipitation Analysis

We have found that protein concentrations in the 250–500 nM range are sufficient for driving PLC subcomplex assembly. Because both calreticulin and ERp57 are calcium-binding proteins, a buffer containing CaCl₂ is employed.

1. Incubate 500 nM of purified HC/β₂m complexes either alone (as a control) or with 250 nM of purified recombinant calreticulin and sTpsn/ERp57 conjugate (each) in 100 μL TBS-C for 15 min at RT.
2. Chill the tubes on ice then add 400 μL of ice-cold IP buffer.
3. Add 25 μL of protein G-Sepharose and 3 μg of anti-tapasin Ab. Incubate with rotation at 4°C for 1 h.
4. Pellet the beads for 20 s at 3,000 × *g* in a refrigerated microfuge. Remove the supernatant, taking care not to disrupt the beads (see Note 14).
5. Add 1 mL of IP wash buffer and vortex. Pellet the beads for 20 s at 3,000 × *g* and remove the wash using an aspirator. Repeat twice more. After the final spin, remove as much of the wash as possible with a gel loading tip, without disturbing the beads.
6. Add *non-reducing* sample buffer (see Note 15).
7. Analyze the samples by 10% SDS-PAGE and transfer to PVDF membranes. Perform Western blot using standard procedures for class I HC and/or calreticulin.

3.5. Functional Analysis of the PLC Subcomplex

Although removal of the transmembrane domains eliminates the participation of TAP and somewhat reduces the stability of the tapasin/MHC class I interaction, the remaining soluble PLC components are fully capable of mediating peptide loading and exchange reactions *in vitro* (see Note 16).

1. Incubate 1 μM ¹²⁵I-labeled high-affinity peptide and 500 nM of purified HC/β₂m complexes either alone (as a control) or with 250 nM of purified recombinant calreticulin and sTpsn/ERp57 conjugate (each) in a final volume of 100 μL TBS-C. Also set up a peptide-only sample as a control for background correction.
2. Incubate for 15–30 min at room temperature.
3. Add 800 μL of IP buffer, 25 μL of protein G-sepharose, and 3 μg of W6/32 Ab to immunoprecipitate the MHC class I molecules.
4. Incubate with rotation at 4°C for 1 h.
5. Pellet the beads for 20 s at 3,000 × *g* in a refrigerated microfuge. Remove the supernatant and wash the beads with 3 × 1 mL of IP buffer.
6. Determine the amount of bound peptide on the beads with a gamma counter. To calculate specific binding, subtract the cpm from the “peptide-only sample” as background.

4. Notes

1. This system has been optimized for components of the human PLC. Although we expect no expression issues for the murine equivalents of our constructs, there may be additional requirements for in vitro PLC reconstitution. In particular, these considerations are (1) murine class I molecules are modified with multiple glycans and (2) calnexin, another lectin-like chaperone, is incorporated into the murine PLC, likely via interactions with these additional glycans (20, 21).
2. Schematics of these constructs are presented in Fig. 2. We have incorporated His₆-tags into calreticulin, tapasin, and the class I HC to allow for metal-affinity chromatography. *sTpsn/ERp57 conjugate*: WT ERp57 supports stable formation of the disulfide-linked heterodimer, but co-expression of sTpsn with the C60A ERp57 trapping mutant is recommended. Although we initially expressed ERp57 constructs with deleted QEDL (ER retrieval) motifs, we found that the majority of the conjugate remained in the cells, likely the ER. We also observed that the extracellular environment promoted sTpsn/ERp57 conjugate reduction and dissociation of the heterodimer. Our final strategy employed an ERp57 construct that included the QDEL motif to promote cellular accumulation and the C60A trapping mutant to improve stability during biochemical isolation. *MHC class I HC*: We chose to express the HLA-B*0801 allele, but it is certainly possible that other alleles may be employed with the following considerations: (1) expression levels and stability without high-affinity ligands, (2) the tapasin-dependence of peptide loading, and (3) availability of either suboptimal or conditional peptide ligands. Suboptimal peptides are those that can bind to the class I molecule with intermediate affinity, but are displaced by action of the PLC. Conditional ligands are high-affinity peptides containing photocleavable residues (22). They may be used to bind and stabilize the class I molecule during purification, but released by UV irradiation to generate empty heterodimers for functional assays. *Calreticulin*: Although we have previously employed *E. coli* expression, the yields are moderate due to issues with toxicity, codon usage, or cleavage from GST fusions. In contrast, we have recently discovered that calreticulin expression is very robust in Sf21 cells.
3. It is beyond the scope of this chapter to provide a complete description of methods for insect cell culture and baculovirus production. We have employed the following reagents for these techniques from Invitrogen (Carlsbad, CA) and refer the reader to the excellent, detailed manuals provided by the manufacturer

(*Growth and Maintenance of Insect Cell Lines; Guide to Baculovirus Expression Vector Systems and Insect Cell Culture*).

4. The culture volume should be 30–50% of the flask volume. Overfilling the flasks will compromise cell growth and performance. To obtain optimal results, we also recommend maintaining Sf21 cells at a density between 0.6 and 2×10^6 /mL with 5–6 feedings per week. When the viability and/or expression yields drop (after ~2 months in culture), the cells should be discarded and a new vial thawed.
5. Furthermore, recombinant class I molecules *must* be purified from cells. Soluble HLA-B8 is completely retained in the ER of Sf21 cells (1), and, even if other alleles should be secreted, their glycans will be processed to a form that will not bind calreticulin.
6. Prior to large-scale purification, screen these individual pellets by Western blotting to confirm and compare expression levels. Discard pellets from suboptimal baculovirus infections.
7. *It is absolutely essential to work quickly through the MHC class I purification since the complexes are unstable.* The entire procedure should be completed within 2 days. To limit dissociation of HC/ β_2 m dimers, the following tactics may be employed: (1) addition of conditional peptide ligands to the lysis buffer, (2) addition of suboptimal peptides (25–50 μ M) and/or purified β_2 m (2 μ M, commercial sources available) to the lysis and purification buffers.
8. Make sure to select *EDTA-free* protease inhibitors for use with the Talon beads. Also, prolonged incubations in Triton X-100 (i.e., batch binding for longer than 90 min) will affect the resin and reduce yields.
9. Always save the column “flow through” and “wash” analyzed by SDS-PAGE. Do not discard until the presence of recombinant proteins in the eluates has been verified.
10. For consistent volumes (if a fraction collector is not available), we have found it convenient to apply 1 CV of elution buffer carefully to the top of the resin using a pipet for each elution step.
11. When pooling fractions, do not include fractions that contain excessive amounts of contaminants: small increases in yield are not worth significant increases in protein contamination.
12. Prior to injection, dilute the MHC class I samples twofold with water to reduce the ionic strength.
13. Amicon Ultra-4 Centrifugal Filter Units: use 30K MWCO for HC/ β_2 m complexes and 50K MWCO for sTpsn/ERp57 dimers or calreticulin.

14. To preserve weak interactions, make sure to use ice-cold buffers and refrigerated microfuges for the remainder of the protocol.
15. It is important to use non-reducing sample buffer since DTT or β -Me will reduce the antibody and, thus, introduce background in the Western blots.
16. Although we have developed this assay using radiolabeled peptide, it is feasible to adapt this protocol using fluorescent labeling and detection.

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References

1. Wearsch P, Peaper D, Cresswell P (2011) Essential glycan-dependent interactions optimize MHC class I peptide loading. *Proc Natl Acad Sci U S A* 108:4950–4955
2. Del Cid N, Jeffery E, Rizvi S, Stamper E, Peters L, Brown W, Provoda C, Raghavan M (2010) Modes of calreticulin recruitment to the major histocompatibility complex class I assembly pathway. *J Biol Chem* 285:4520–4535
3. Dong G, Wearsch PA, Peaper DR, Cresswell P, Reinisch KR (2009) Insights into MHC class I peptide loading from the structure of the tapasin-ERp57 thiol oxidoreductase heterodimer. *Immunity* 16:21–32
4. Garbi N, Tan P, Diehl AD, Chambers BJ, Ljunggren HG, Momburg F, Hammerling GJ (2000) Impaired immune responses and altered peptide repertoire in tapasin-deficient mice. *Nat Immunol* 1:234–238
5. Grandea AG III, Golovina TN, Hamilton SE, Sriram V, Spies T, Brutkiewicz RR, Harty J, Eisenlohr LC, Van Kaer L (2000) Impaired assembly yet normal trafficking of MHC class I molecules in tapasin mutant mice. *Immunity* 13:213–222
6. Williams AP, Peh CA, Purcell AW, McCluskey J, Elliott T (2002) Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity* 16(4):509–520
7. Chen M, Bouvier M (2007) Analysis of interactions in a tapasin/class I complex provides a mechanism for peptide selection. *EMBO J* 26:1681–1690
8. Wearsch PA, Cresswell P (2007) Selective loading of high-affinity peptides onto major histocompatibility complex class I molecules by the tapasin-ERp57 heterodimer. *Nat Immunol* 8: 873–881
9. Ortmann B, Androlewicz MJ, Cresswell P (1994) MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature* 368(6474): 864–867
10. Suh WK, Cohen-Doyle MF, Fruh K, Wang K, Peterson PA, Williams DB (1994) Interaction of MHC class I molecules with the transporter antigen processing. *Science* 264(5163): 1322–1326
11. Bangia N, Lehner PJ, Hughes EA, Surman M, Cresswell P (1999) The N-terminal region of tapasin is required to stabilize the MHC class I loading complex. *Eur J Immunol* 29:1858–1870
12. Garbi N, Tiwari N, Momburg F, Hammerling GJ (2003) A major role for tapasin as a stabilizer of the TAP peptide transporter and consequences for MHC class I expression. *Eur J Immunol* 33:264–273
13. Lehner PJ, Surman MJ, Cresswell P (1998) Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line 220. *Immunity* 8:221–231
14. Leonhardt RM, Keusekotten K, Bekpen C, Knittler MR (2005) Critical role for the tapasin-docking site of TAP2 in the functional integrity of the MHC class I-peptide-loading complex. *J Immunol* 175:5104–5114

15. Vigneron N, Peaper D, Leonhardt R, Cresswell P (2009) Functional significance of tapasin membrane association and disulfide linkage to ERp57 in MHC class I presentation. *Eur J Immunol* 39:2371–2376
16. Sadasivan B, Lehner PJ, Ortmann B, Spies T, Cresswell P (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* 5:103–114
17. Wearsch PA, Jakob CA, Vallin A, Dwek RA, Rudd PM, Cresswell P (2004) Major histocompatibility complex class I molecules expressed with monoglycosylated N-linked glycans bind calreticulin independently of their assembly status. *J Biol Chem* 279:25112–25121
18. Dick TP, Bangia N, Peaper DR, Cresswell P (2002) Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. *Immunity* 16:87–98
19. Peaper DR, Wearsch PA, Cresswell P (2005) Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex. *EMBO J* 24:3613–3623
20. Suh W, Mitchell E, Yang Y, Peterson P, Waneck G, Williams D (1996) MHC class I molecules form ternary complexes with calnexin and undergo peptide-regulated interaction with TAP via their domains. *J Exp Med* 184(2): 337–348
21. Harris MR, Yu YY, Kindle CS, Hansen TH, Solheim JC (1998) Calreticulin and calnexin interact with different protein and glycan determinants during the assembly of MHC class I. *J Immunol* 160:5404–5409
22. Rodenko B, Toebe M, Hadrup S, van Esch W, Molenaar A, Schumacher T, Ova H (2006) Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* 1:1120–1132

Chapter 7

Studying MHC Class I Peptide Loading and Exchange In vitro

Marlene Bouvier

Abstract

In the endoplasmic reticulum (ER), MHC class I molecules associate with several specialized proteins, forming a large macromolecular complex referred to as the “peptide-loading complex” (PLC). In the PLC, antigenic peptides undergo a stringent selection process for binding onto MHC class I molecules. This ensures that the immune system elicits robust CD8+ T-cell responses to viruses and solid tumors. The ability to reconstitute in vitro MHC class I molecules in association with key proteins of the PLC provides a mean for studying at the molecular level how antigenic peptides are selected for presentation to CD8+ T-cells. Here, we describe practical procedures for generating a cell-free system involving MHC class I molecules and tapasin, a critical protein of the PLC, that can be used as a versatile tool for biochemical and mechanistic studies of peptide loading and exchange.

Key words: MHC class I molecule, Peptide association, Peptide dissociation, Tapasin, Peptide-loading complex, Fluorescence anisotropy, Peptide exchange

1. Introduction

Proteins expressed at high levels in *Escherichia coli* are often found in an insoluble form called inclusion body (IB). IBs require high denaturant concentrations to be solubilized (1). Importantly, precise conditions under which the solubilized protein can be refolded efficiently to its native structure have to be identified. The complexity of this problem is magnified for proteins made up of more than one subunit such as MHC class I molecules, where the correct refolding and assembly of the ER-luminal domain of heavy chain and β_2m subunits is essential to generate a biologically active protein. Here, we describe experimental procedures for reconstituting soluble MHC class I molecules from IBs of the ER-luminal domain of heavy chain and β_2m together with an antigenic peptide (2). We also discuss a strategy based on leucine zippers (3–5) to stabilize interaction between soluble MHC class I molecules and tapasin (6);

the MHC class I/tapasin interaction is intrinsically low affinity and thus normally difficult to study experimentally. The attachment of leucine zippers to the C-terminal ends of class I heavy chain and tapasin greatly facilitates studies of tapasin catalytic effects on peptide association onto and dissociation from MHC class I molecules. Based on the leucine zipper strategy, we describe a fluorescence anisotropy assay for the biophysical characterization of equilibrium and kinetics parameters between MHC class IFos molecules and peptide ligands in the presence and absence of tapasinJun. Overall, this chapter describes the main techniques and strategies for generating a soluble, stable, and functional MHC class IFos/tapasinJun complex that can be used as a cell-free system for biochemical and mechanistic studies of peptide loading and exchange. The functional implication of such studies is significant given that the repertoire of peptides presented by MHC class I molecules to CD8+ T-cells plays a key role in cellular immunity.

2. Materials

2.1. Consumables and Lab Equipment

1. Glass homogenizer.
2. Stainless steel beaker.
3. Water bath.
4. Plastic pail.
5. 6,000–8,000 MWCO membrane dialysis bags.
6. Stirred cell.
7. Centriprep-10.
8. Gel filtration columns; 10 kDa spin columns; and Ni-NTA affinity columns.
9. Gel electrophoresis materials.
10. 1-cm fluorescence cuvettes and a stopped-flow spectrofluorometer.

2.2. Buffers

1. Resuspension buffer: 50 mM Tris-HCl, pH 8.0, 25% sucrose, 1 mM EDTA, 0.1% sodium azide, 10 mM DTT.
2. Lysis buffer: 50 mM Tris-HCl, pH 8.0, 1% triton-X100, 1% sodium deoxycholate, 100 mM NaCl, 0.1% sodium azide, 10 mM DTT.
3. Purification buffer 1: 50 mM Tris-HCl, pH 8.0, 0.5% triton-X100, 100 mM NaCl, 1 mM EDTA, 0.1% sodium azide, 1 mM DTT.
4. Purification buffer 2: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium azide, 1 mM DTT.

5. Solubilization buffer: 25 mM MES, pH 6.0, 8 M urea, 10 mM EDTA, 0.1 mM DTT.
6. Oxidative refolding buffer: 50 mM Tris-HCl, pH 8.0, 400 mM L-arginine, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride.
7. FPLC running buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl.
8. Denaturation buffer: 20 mM Tris-HCl, pH 7.5, 6 M guanidinium hydrochloride, 150 mM NaCl.
9. Dialysis buffer 1: 20 mM Tris-HCl, pH 7.5, 8 M urea, 150 mM NaCl.
10. Dialysis buffer 2: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl.
11. Dialysis buffer 3: 10 mM Tris-HCl, pH 8.0, 200 mM NaCl.
12. Dialysis buffer 4: 10 mM Tris-HCl, pH 8.0, 20 mM imidazole, 200 mM NaCl.
13. Equilibration buffer: 10 mM Tris-HCl, pH 8.0, 20 mM imidazole, 200 mM NaCl.
14. Washing buffer: 10 mM Tris-HCl, pH 8.0, 25 mM imidazole, 500 mM NaCl.
15. Elution buffer: 10 mM Tris-HCl, pH 8.0, 110 mM imidazole, 500 mM NaCl.
16. Incubation buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol.
17. Native gel loading buffer : 50 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 10% glycerol.
18. Running buffer: 25 mM Tris-HCl, pH 8.3, 200 mM glycine.
19. Dissociation and association buffer: 20 mM Hepes, pH 7.5, 150 mM NaCl.

2.3. Common Reagents

1. DNase I: stock solution at 2 mg/mL.
2. Liquid nitrogen.
3. Lysozyme: stock solution at 50 mg/mL.
4. MgCl₂: stock solution at 4 M.
5. EDTA: stock solution at 0.5 M.
6. Glycerol.
7. Synthetic peptide: stock solution at 10 mg/mL in water or DMSO.
8. DTT: stock solution at 1 M.
9. Coomassie Blue.

3. Methods

3.1. Preparation of Purified Inclusion Bodies of Class I Heavy chainFos and β_2m

1. Add resuspension buffer to the bacterial cell pellets of class I heavy chainFos and β_2m (5–10 mL buffer/L cell culture) and transfer the mixture into a glass homogenizer. Resuspend the cell pellets to homogeneity (see Note 1).
2. Transfer the resuspended cell pellets into a stainless steel beaker (see Note 2) and add lysis buffer (2.5 mL/mL resuspension buffer) followed by lysozyme (to 1 mg/mL, see Note 3). Stir the mixture intermittently. The mixture will become gradually viscous as the DNA is released from the lysing cells. After ~25 min, add DNase I (to ~20 μ g/mL) followed by MgCl₂ (to 5 mM). The viscosity of the mixture will decrease gradually under the action of DNase I. After ~20 min, add EDTA (to 5 mM).
3. Freeze the lysed cells by placing the stainless steel beaker in liquid nitrogen. This is followed up by a thawing step in which the beaker is transferred into a warm water bath, swirl the mixture gently as it thaws (see Note 4). When the mixture is thoroughly thawed, add MgCl₂ (to 10 mM) to reactivate the DNase. Stir the mixture gently. After ~15 min, add EDTA again (to 10 mM).
4. Centrifuge the mixture at 9,800 $\times g$ (JA-14 rotor) for 20 min at 4°C. Discard the supernatant and transfer the crude IBs into a glass homogenizer.
5. Resuspend the IBs in ~30 mL of purification buffer 1 and homogenize thoroughly (see Note 1). Centrifuge the mixture at 9,800 $\times g$ (JA-14 rotor) for 20 min at 4°C. Discard the supernatant and repeat the washing step two more times. Resuspend the IBs in ~30 mL of purification buffer 2 and repeat the washing and centrifugation steps three times.
6. Dissolve the purified IBs in solubilization buffer (~2 mL buffer/L cell culture). Centrifuge in a tabletop centrifuge for ~10 min at 4°C (see Note 5). Aliquot the solubilized IBs (see Note 6) and store at -80°C.

3.2. Reconstitution of Peptide-Filled MHC Class IFos Molecules

1. Prepare 1 L of oxidative refolding buffer and cool it down thoroughly in an ice-water bath.
2. Under vigorous stirring, add synthetic peptide (to 10 μ M) (see Note 7) followed by solubilized IBs of β_2m (to 2 μ M) and class I heavy chainFos (to 1 μ M) (see Note 8). Incubate the oxidative refolding buffer at 10°C for 48 h.
3. Dialyze the refolding mixture in a plastic pail filled with FPLC running buffer using a 6,000–8,000 MWCO membrane at 4°C for 12 h.

4. Concentrate the refolding buffer to ~1–2 mL using both a stirred cell and centriprep-10. Purify the crude MHC class IFos molecules on a gel filtration column in the FPLC running buffer. Collect the fractions corresponding to MHC class IFos molecules (see Note 9). Concentrate the combined fractions (see Note 10) and store the purified protein at –80°C.

3.3. Preparation of Peptide-Deficient MHC Class IFos Molecules

Peptide-deficient MHC class IFos molecules are prepared from the denaturation of peptide-filled MHC class IFos molecules after removal of the bound peptide (7).

1. Incubate peptide-filled MHC class IFos molecules in the denaturation buffer for 4 h at room temperature (see Note 11).
2. Transfer the mixture to a 10 kDa spin column and wash extensively with the denaturation buffer to dilute away the peptide. Collect the retentate containing the denatured class I heavy chainFos and β_2m subunits and dilute it with the denaturation buffer to 0.1 mg/mL protein (based on the starting concentration of peptide-filled MHC class IFos molecules).
3. Dialyze the mixture in 500 mL of dialysis buffer 1 using a 6,000–8,000 MWCO membrane at 15°C for 12 h. Continue dialysis for an additional 12 h with fresh 500 mL of dialysis buffer 1.
4. Add 30% molar excess of folded β_2m (relative to the starting concentration of peptide-filled MHC class IFos molecules) (see Note 12) to the dialysis bag. Dialyze the solution in 1 L of dialysis buffer 2 at 4°C for 24 h. Continue dialysis for an additional 18 h with fresh 1 L of dialysis buffer 2.
5. Add glycerol (to 15%) to the dialyzed solution (see Note 13), mix well, and concentrate to ~1 mL.
6. Purify the crude peptide-deficient MHC class IFos molecules on a gel filtration column in the FPLC running buffer. Collect the fractions of peptide-deficient MHC class IFos molecules and add glycerol (to 15%) immediately to each collected fraction. Concentrate the combined fractions (see Note 14) and store the purified protein at –80°C. Determine the activity of peptide-deficient MHC class IFos molecules (see Note 15).

3.4. Expression and Purification of Soluble tapasinJun

TapasinJun is expressed in High Five cells using a recombinant baculovirus system (6, 8).

1. Dialyze the concentrated supernatant of cell culture containing crude tapasinJun in dialysis buffer 3 at 4°C, include two changes of dialysis buffer 3 over 48 h. Change the dialysis buffer 3 to dialysis buffer 4 and continue dialysis for an additional 24 h.
2. At 4°C, apply the dialyzed mixture onto a Ni-NTA affinity column that was previously equilibrated with the equilibration buffer. Wash the column with the washing buffer to remove

nonspecifically bound proteins. Then, elute tapasinJun in the elution buffer. Add DTT (to 1 mM) to the eluate and concentrate it using both a stirred cell and centriprep-10.

3. Purify the eluate containing crude tapasinJun (see Note 16) on a gel filtration column in the FPLC running buffer. Collect the fractions corresponding to tapasinJun and add DTT (to 1 mM) and glycerol (to 10%) immediately to each collected fraction. Concentrate the combined fractions (see Note 17) and store the purified protein at -80°C.

3.5. Formation of MHC Class IFos/tapasinJun Complexes; a Cell-Free System

The Fos/Jun leucine zippers help tethering MHC class I molecules to tapasin generating a biologically active cell-free system. This cell-free system has been very helpful for mechanistic studies of how tapasin acts as a chaperone protein towards peptide-deficient MHC class I molecules and a catalyst of peptide dissociation (Subheading 3.6) and association (Subheading 3.7) (6) (see Note 18). The ability of tapasin to influence the peptide repertoire is rooted in these two critical functions.

1. Incubate peptide-filled or peptide-deficient MHC class IFos molecules (10 µg) and tapasinJun (10 µg) (1:1 molar ratio) on ice in the incubation buffer for 30 min.
2. Add native gel loading buffer (see Note 19) and run the samples on 8% native PAGE gel at 4°C (Fig. 1) with running buffer. Stain the gel with Coomassie Blue.

3.6. Studies of Peptide Dissociation from Peptide-Filled MHC Class IFos Molecules

Fluorescein-labeled peptides are used to monitor the dissociation of ligands from MHC class IFos molecules (6). The fluorescence anisotropy-based assay described here (and in Subheading 3.7) shows good reproducibility and requires small amounts of MHC class IFos molecules. Similar methodologies have been described for determining equilibrium and kinetics parameters for MHC class I/peptide interaction (9–11).

1. To a 1-cm fluorescence cuvette, add under stirring MHC class IFos molecules (40 nM), loaded with a tagged peptide (see Note 20), in the dissociation buffer containing nontagged peptide (40 µM) (to block the rebinding of dissociated tagged peptide) and β_2m (4 µM) (to avoid complications due to dissociation of β_2m from MHC class IFos molecules) in the absence or presence of tapasinJun (400 nM).
2. Monitor peptide dissociation kinetics, at 20°C, by fluorescence anisotropy using a spectrofluorometer. Set the polarization wavelength at 524 nm and excitation wavelength at 495 nm. Repeat the dissociation experiment three to five times using different batches of peptide-filled MHC class IFos molecules and tapasinJun.
3. See Notes 21 and 22 for processing fluorescence anisotropy data.

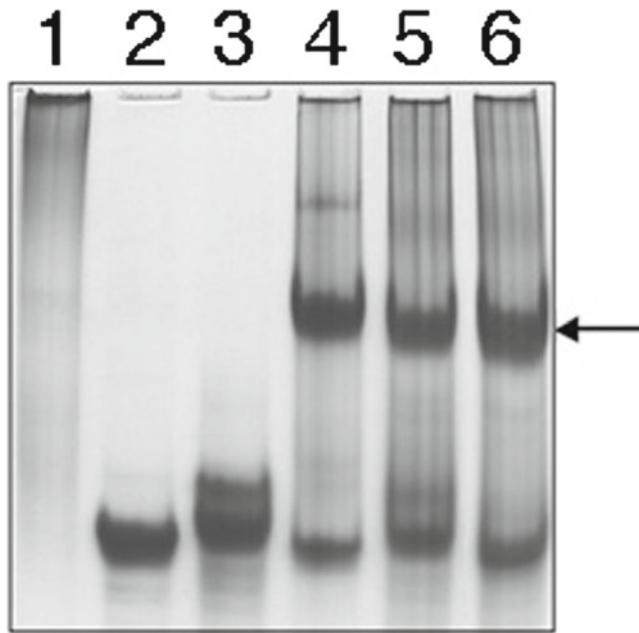


Fig. 1. Formation of MHC class IFos/tapasinJun complexes. HLA-B*0801Fos (10 µg) and tapasinJun (10 µg) (1:1 molar ratio) were incubated on ice in the incubation buffer for 30 min. Samples were analyzed on native gel (8%): *lane 1*, tapasinJun; *lane 2*, peptide-filled HLA-B*0801Fos; *lane 3*, peptide-deficient HLA-B*0801Fos; *lane 4*, mixture of tapasinJun and peptide-filled HLA-B*0801Fos; *lane 5*, mixture of tapasinJun and peptide-deficient HLA-B*0801Fos; and *lane 6*, tapasinJun/peptide-deficient HLA-B*0801Fos complex incubated with an excess of FLRGRAYGL peptide. The gel was run at 4°C in 25 mM Tris–HCl, pH 8.3, 200 mM glycine. The gel was stained with Coomassie Blue. The *solid arrow* indicates the position of MHC class IFos/tapasinJun complexes.

3.7. Studies of Peptide Association onto Peptide-Deficient MHC Class IFos Molecules

1. Using a stopped-flow spectrofluorometer, mix automatically equal volumes (125 µL) from chamber A containing peptide-deficient MHC class IFos molecules (active concentration of 800 nM) and β_2m (80 µM) and chamber B containing a tagged peptide (160 nM) with or without tapasinJun (1,200 nM).
2. Monitor peptide association kinetics, at 20°C, by fluorescence anisotropy. Set the polarization wavelength at 510 nm and excitation wavelength at 495 nm. Average together the fluorescence polarization signals of ~3 independent experiments.
3. See Notes 23 and 24 for processing fluorescence anisotropy data.

3.8. Studies of Tapasin-Mediated Peptide Exchange

The availability of peptide-filled and peptide-deficient MHC class IFos molecules and tapasinJun, and the use of fluorescence anisotropy-based assays for monitoring kinetics of peptide dissociation and association using a MHC IFos/tapasinJun cell-free system, provides a valuable mean for studying tapasin-mediated peptide exchange. Tapasin-mediated effects on peptide exchange critically

shape the repertoire of peptides presented by MHC class I molecules. An example of how to set-up a tapasin-mediated peptide exchange experiment is provided here (See Note 25). Briefly, in these experiments, a tapasin-sensitive peptide competes for binding onto peptide-deficient MHC class IFos molecules with a tapasin-insensitive control peptide in the presence of tapasinJun ([6](#)). Changes in IC₅₀ values, determined as described in Note 25, are used to assess the effects of tapasin on peptide loading.

4. Notes

1. The use of a motor-driven Teflon pestle allows to resuspend and wash thoroughly the cell pellets.
2. A stainless steel beaker is used because of the subsequent freezing/thawing step (see Note 4).
3. Lysozyme is added to the buffer as a clear solution, rather than in a powder form, to prevent the formation of lysozyme clumps.
4. The freezing/thawing step ensures that cells are completely lysed. Care should be taken not to let the IBs mixture warm up in this process.
5. Avoid freezing the urea solution by using short centrifugation times.
6. The concentration of IBs can be determined by the Bradford protein assay.
7. Synthetic peptides do not have to be purified for this experiment. Many peptides restricted to MHC class I molecules are hydrophobic and DMSO can be used for dissolving these peptides. The final concentration of DMSO in the refolding buffer should be less than ~5%. See Note 20 on fluorescein-labeled peptides.
8. The solubilized IBs should be added under vigorous stirring to ensure that they are rapidly and thoroughly dispersed into the buffer. This is important to promote the correct refolding of class I heavy chainFos and β₂m subunits. Use a stock solution of IBs of class I heavy chainFos at less than 0.3 mM.
9. MHC class IFos molecules elute from a calibrated gel filtration column at ~50 kDa.
10. Stock solutions of MHC class IFos molecules should be less than 10 mg/mL to minimize dimer formation through Fos/Fos association. The protein concentration can be determined by the Edelhoch's method ([12](#)).
11. The concentration of MHC class IFos molecules in the denaturation buffer should be ~10–20 mg/mL.

12. A 30% molar excess of folded $\beta_2\text{m}$ (not IBs of $\beta_2\text{m}$) is added to promote the correct pairing of class I heavy chainFos with $\beta_2\text{m}$ subunits.
13. Glycerol helps to stabilize the intrinsically unstable groove of peptide-deficient MHC class IFos molecules.
14. Stock solutions of peptide-deficient MHC class IFos molecules should be less than 10 mg/mL. The protein concentration can be determined by the Edelhoch's method (12).
15. It is recommended that the activity of peptide-deficient MHC class IFos molecules be determined quantitatively as described in ref. 6 to assess the number of peptide-deficient molecules in solution that are capable of effectively binding peptides.
16. This additional purification step removes any remaining protein impurities and aggregates of tapasinJun.
17. Stock solutions of tapasinJun should be less than 10 mg/mL to minimize dimer formation through Jun/Jun association. The protein concentration can be determined by the Edelhoch's method (12).
18. A cell-free system in which peptide-deficient MHC class I molecules, tapasin, calreticulin, and ERp57 are incorporated together has been described in the literature for its effect on influencing peptide selection (13).
19. The total volume of each sample should not exceed 20 μL for loading into the native gel wells.
20. A fluorescein-derivatized lysine residue can be introduced into the peptide amino acid sequence at any positions where the side chain points up (into the solvent), as informed by X-ray crystallography. This way, the bulky fluorescein-labeled side chain will not interfere with reconstitution of peptide-filled MHC class IFos molecules (see Subheading 3.2) (6).
21. For experiments carried out in the absence of tapasinJun, fit the observed averaged intrinsic anisotropy data to an exponential decay function of the form: $y(t)_{\text{int}} = y_{0\text{int}} + \sum i(Ai \cdot \exp(-ki \cdot t))$, where $y_{0\text{int}}$ is the baseline offset, the summation is over the number of phases i , Ai and ki ($ki = 0.693/\tau_i$) are the amplitude and rate constant for phase i , respectively, and t is the time (6).
22. For experiments carried out in the presence of tapasinJun, fit the averaged observed anisotropy data to an exponential decay function described by the sum of tapasinJun-dependent (tpn) and intrinsic dissociation data: $y(t)_{\text{tpn}} = y_{0\text{tpn}} + f_{\text{tpn}} \cdot \sum i(Ai \cdot \exp(-ki \cdot t)) + ((1 - f_{\text{tpn}}) \cdot (y(t)_{\text{int}} - y_{0\text{int}}))$, where f_{tpn} represents the fraction of dissociation reaction under the action of tapasinJun, $y(t)_{\text{int}}$ is the intrinsic decay function of the MHC class I molecule as computed in Note 21, and other parameters are as described in Note 21. The dissociation rate enhancement factor is defined as $\tau(\text{intrinsic; slow phase})/\tau(\text{tapasinJun})$ (6).

23. For experiments carried out in the absence of tapasinJun, the averaged observed intrinsic anisotropy data were fitted to an exponential function of the form: $y(t)_{\text{int}} = y_{0\text{int}} + \sum i(A_i \cdot (1 - \exp(-k_i \cdot t)))$, where parameters are as defined in Notes 21 and 22 (6).
24. For experiments carried out in the presence of tapasinJun, the averaged observed anisotropy data were fitted to an exponential function described by the sum of tapasinJun-dependent (tpn) and intrinsic association data: $y(t)_{\text{tpn}} = y_{0\text{tpn}} + f_{\text{tpn}} \cdot \sum i(A_i \cdot (1 - \exp(-k_i \cdot t))) + ((1 - f_{\text{tpn}}) \cdot (y(t)_{\text{int}} - y_{0\text{int}}))$, where f_{tpn} represents the fraction of dissociation reaction under the action of tapasinJun, and other parameters are as described in Note 23 (6).
25. IC_{50} values are determined as follows. Incubate peptide-deficient MHC class IFos molecules (100 nM in the absence of tapasin-Jun and 30 nM in the presence of tapasinJun due to the effect of tapasin on increasing the concentration of active peptide-deficient molecules in solution (6)) with a tapasin-insensitive control fluorescein-tagged peptide (100 nM) and different doses of a tapasin-sensitive competitor nontagged peptide (0–10 μM) together with an excess of $\beta_2\text{m}$ (1 μM) in the absence or presence of tapasinJun (100 nM). After 20 h incubation, record the fluorescence anisotropy value of each sample. Calculate percentages of inhibition from the observed anisotropy signals in the absence, r_o , and presence, r , of the competitor peptide: % Inhibition = $(r_o - r)/(r_o - r_f) \cdot 100$, where r_f , the minimum anisotropy signal corresponding to free control peptide, was measured in a separate experiment. The concentration of competitor peptide required for 50% inhibition of control peptide represents the IC_{50} value. Changes in IC_{50} values are used as a measure of tapasin function in “editing” peptides in favor of those that form kinetically stable peptide-filled MHC class I molecules.

Acknowledgement

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References

1. Burgess RR (2009) Refolding solubilized inclusion body proteins. *Methods Enzymol* 463:259–282
2. Garboczi DN, Hung DT, Wiley DC (1992) HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc Natl Acad Sci U S A* 89:3429–3433
3. Busch R, Pashine A, Garcia KC, Mellins ED (2002) Stabilization of soluble, low-affinity HLA-DM/HLA-DRI complexes by leucine zippers. *J Immunol Methods* 263:111–121

4. Scott CA, Garcia KC, Carbone FR, Wilson IA, Teyton L (1996) Role of chain pairing for the production of functional soluble IA major histocompatibility complex class II molecules. *J Exp Med* 183:2087–2095
5. Kalandadze A, Gallenno M, Foncarrada L, Strominger JL, Wucherpfennig KW (1996) Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane motif allows the assembly and secretion of soluble DR alpha beta heterodimers. *J Biol Chem* 271:20156–20162
6. Chen M, Bouvier M (2007) Analysis of interactions in a tapasin/class I complex provides a mechanism for peptide selection. *EMBO J* 26: 1681–1690
7. Bouvier M, Wiley DC (1998) Structural characterization of a soluble and partially folded class I major histocompatibility/β₂m heterodimer. *Nat Struct Biol* 5:377–382
8. Chen M, Stafford WF, Diedrich G, Khan A, Bouvier M (2002) A characterization of the luminal region of human tapasin reveals the presence of two structural domains. *Biochemistry* 41:14539–14545
9. Gakamsky DM, Davis DM, Strominger JL, Pecht I (2000) Assembly and dissociation of human leukocyte antigen (HLA)-A2 studied by real-time fluorescence resonance energy transfer. *Biochemistry* 39:11163–11169
10. Binz AK, Rodriguez RC, Biddison WE, Baker BM (2003) Thermodynamic and kinetic analysis of a peptide-class I MHC interaction highlights the noncovalent nature and conformational dynamics of the class I heterotrimer. *Biochemistry* 42: 4954–4961
11. Springer S, Doring K, Skipper JCA, Townsend ARM, Cerundolo V (1998) Fast association rates suggest a conformational change to the MHC class I molecules H-2D^b upon peptide binding. *Biochemistry* 37:3001–3012
12. Edelhoch H (1967) Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* 6:1948–1954
13. Wearsch PA, Cresswell P (2007) Selective loading of high-affinity peptides onto major histocompatibility complex class I molecules by the tapasin-ERp57 heterodimer. *Nat Immunol* 8: 873–881

Chapter 8

Measuring Synthesis and Degradation of MHC Class I Molecules

David B. Giuliano and Antony N. Antoniou

Abstract

Major histocompatibility complex (MHC) class I molecules function to present pathogen-derived peptides to cytotoxic T cells or act as ligands for Natural Killer cells, thus alerting the immune system to the presence of invading pathogens. Furthermore MHC class I molecules can be strongly associated with autoimmune diseases. Therefore understanding not only the biosynthesis and the degradation pathways of MHC class I molecules has become important in determining their role in pathogen and autoimmune-related diseases. Here we describe how using epitope-tagged MHC class I molecules can aid in the analysis of MHC class I molecule biosynthesis and degradation and also complement studies using conventional conformationally specific antibodies. Coupled together with pharmacological manipulation which can target both biosynthetic and degradative pathways, this offers a powerful tool in analyzing MHC class I molecules.

Key words: MHC class I molecules, Degradation, Biosynthesis, Pulse chase, Immunoblotting

1. Introduction

Major histocompatibility complex (MHC) class I molecules are composed of a heavy chain of approximately 45 kDa, which non-covalently associates with the light chain beta-2 microglobulin ($\beta 2m$) of approximate 16 kDa and a peptide of 8–10 amino acids in length. The peptide is bound within the peptide binding groove of the MHC class I heavy chain which is made up of two α -helices and a series of antiparallel β -sheets, which form the walls and floor of the groove, respectively (1, 2). The peptide binding groove is composed of pockets that can determine the amino acid sequence of the peptide at both the amino (N) and carboxy (C) terminus. Therefore, peptides bind according to amino acid sequences that fit these pockets. As MHC class I molecules bind a wide spectrum of peptides, these molecules, especially in human beings, express extensive polymorphisms within the peptide binding domain (3, 4).

MHC class I molecule biosynthesis occurs within the endoplasmic reticulum (ER) and is primarily dependent on peptide availability and the acquisition of optimal peptide. MHC class I biosynthesis can be viewed as a two-stage event. Newly synthesized MHC class I molecules during the initial stages of folding can bind peptides of low affinity until they become part of the peptide-loading complex, whose main function is to optimize the peptide cargo (5, 6). These early and latter stages of MHC class I biosynthesis require transient interactions with ER resident chaperones, which are proteins whose function is to aid folding of newly translated molecules. Newly synthesized MHC class I heavy chains associate with the chaperones, calnexin, and immunoglobulin binding protein (BiP). Following the non-covalent association of β 2m, calnexin is displaced and replaced with calreticulin. These partially folded complexes are then tethered to the peptide transporter heterodimer known as the transporters associated with antigen presentation (TAP) via the specific accessory molecule tapasin. This complex of MHC class I heavy chain- β 2m, calreticulin, TAP, and tapasin, which also includes members of the protein disulfide isomerase family of oxidoreductases PDI and ERp57, forms the peptide-loading complex. Peptides generated in the cytosol by the proteolytic activity of the proteasome are transported via TAP in an ATP-dependent manner and peptide optimization is performed by tapasin (7, 8).

MHC class I heavy chains are glycoproteins, with human class I molecules glycosylated at the sugar acceptor site (asparagine-X-serine, where X is any amino acid) at position 86, whilst mouse and rat MHC class I molecules have additional glycosylation sites (9, 10). These glycosylation units serve different functions depending on the sugar modification and location of the protein within the cell. Within the ER, the sugar unit serves both as a chaperone binding site and a signal of the folding status of the heavy chain. These signals can either allow the MHC class I heavy chain to proceed with productive folding or instruct the cell that the heavy chain is terminally misfolded and should be removed. Detection of these sugar signals can allow us to determine the maturation status and rate of MHC class I heavy chains. During folding and transit through the ER, glycan structures on MHC class I heavy chains remain sensitive to the enzyme Endoglycosidase H (Endo H). Once folding of the MHC class I molecule is completed, it transits from the ER to the Golgi apparatus and subsequently to the surface of the cell. Within the Golgi apparatus, MHC class I heavy chain glycan structures become insensitive to Endo H cleavage as a result of Golgi mannosidase II activity, which yields a mannose core present in complex oligosaccharides and renders the bond between the two N-acetylglucosamines which are located proximal to the asparagine residue resistant to further Endo H digestion. Thus Endo H sensitivity can be used to specifically determine if a class I molecule has matured to the point of exiting the ER.

As well as synthesis, unwanted MHC class I molecules that are misfolded, mistranslated, or fail to acquire appropriate peptides within the ER must be disposed off. MHC class I molecules within the ER are degraded by being flipped out off the ER into the cytosol and tagged with ubiquitin which targets them for degradation by the proteasome. This process is known as ER-associated degradation (ERAD) (11). MHC class I molecules can be targeted for degradation throughout their maturation and their state of folding determines the pathway of degradation (12, 13).

There are a variety of pharmaceutical reagents which can be used to disrupt or perturb points in the biosynthetic pathway of MHC class I molecules. For disruption of biosynthesis, reagents that inhibit protein translation such as cycloheximide are commonly utilized. These are often used in the context of experiments where researchers are interested in measuring the half-life of a molecule but do not or cannot employ radioactive methodologies. MHC class I molecules present some particular challenges in these sort of studies because some alleles have very long half-lives (48–72 h) such as HLA-G (14), making treatments with cytotoxic drugs such as cycloheximide problematic. Also, different cell types display different turn over rates of MHC class I molecules or sensitivities to pharmacological reagents. This requires extensive pre-experimental optimization of treatment regimes for each cell type to be tested. A number of different reagents can be used to disrupt maturation of MHC class I molecules within the ER. These include reagents such as tunicamycin, which inhibits the glycosylation of proteins or kifunensine which inhibits the activity of mannosidase I, an enzyme involved in processing of glycans as proteins mature within the ER (15). Again like cycloheximide because of their broad effects on protein synthesis these drugs can induce a number of cytopathic responses such as the unfolded protein response, which induces a set of transcriptional and translation changes aimed to alleviate and restore ER homeostasis at times of cellular stress (16, 17). Thus the induction of such cytopathic responses can complicate interpretation of any experimental observations (17). A number of reagents are also used to examine the degradative pathways and inhibitors of ERAD such as kifunensine and deoxymanojirimycin can be used to interrogate the roles of different enzymes in the targeting of MHC class I molecules to the ERAD machinery. Downstream, such proteolytic degradation events are often examined using broad spectrum proteasome inhibitors such as MG132. Again the cytotoxic effects of these molecules can often complicate interpretation of experimental results if extended treatments of cells are required to observe an effect.

Biosynthesis and degradation of MHC class I molecules can vary depending on the cell type, stage of differentiation, whether a cell is infected or undergoing cellular stress. Determining these parameters has become increasingly important when trying to

understand the role of certain MHC molecules in disease states such as autoimmunity or during infections. Analysis of these biochemical features of MHC class I molecules has usually employed various monoclonal antibodies. These antibodies can recognize folded MHC class I molecules, though due to the shared structure and sequence these antibodies are very rarely specific to a single MHC class I molecule. Many studies employ antibodies that are conformationally specific such as W6/32, which detects fully folded MHC class I molecules of HLA-A, B, and C type (18). However some antibodies are more restricted in their specificity such as the ME1 antibody (19), which detects various mature HLA-B alleles, whilst the BB7.2 antibody predominantly detects folded HLA-A2 (20). Thus, when measuring biosynthesis using these conformationally specific antibodies, it must be considered that the analysis will be biased towards the conformation of MHC class I molecules recognized by the antibody employed and therefore may not provide a global measure of MHC class I folding. To try and overcome these issues, the levels and folding rates of molecules are sometimes compared to experiments performed in parallel with antibodies specifically detecting partially folded/unfolded molecules such as the HC10 antibody (21). Again, these experiments are limited to the pool of molecules that acquire or possess the epitope for the desired antibody. Using antibodies that can distinguish various but limited numbers of different MHC class I conformational states can also influence what chaperones can be detected. Of course the detection of proteins associating with MHC class I molecules is also dependent on the experimental conditions employed.

To overcome the limitations of analyzing specific antibody reactive pools of MHC class I molecules we have employed the use of small epitope tags located after the cytoplasmic tail at the C-terminal end of the MHC class I heavy chain (see Fig. 1) (22). We have found that inclusion of these tags does not effect the maturation or bioactivity of a variety of MHC class I alleles. However, it should be noted that a number of studies have shown that motifs contained within the cytoplasmic tail modulate trafficking and recycling of surface localized mature MHC class I molecules (23–25). Inclusion of epitope tags at the C-terminus in these types of studies should be viewed with caution as their potential effects on interaction with the trafficking machinery are not known. Despite this cautionary note inclusion of these epitope tags allows for the detection of all MHC molecules irrespective of their conformations and folding state and enables direct comparisons of folded versus unfolded molecules within the same experiment. To analyze biosynthetic rates of MHC class I molecules we employed radiolabeling pulse chase protocols. An anti-epitope tag detection system can allow for the quantification of both mature and immature molecules within a single immunoprecipitation. Using immunoblotting

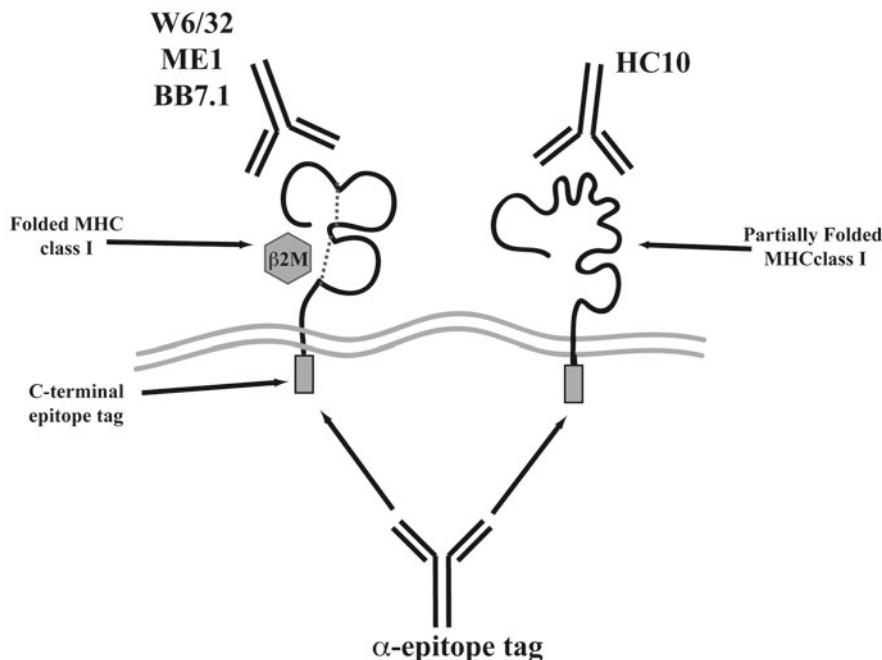


Fig. 1. Schematic outlining the use of conformational versus epitope tag specific antibodies to detect and monitor populations of MHC class I molecules. Conformationally specific antibodies such as W6/32 require appropriate folding to take place and will therefore detect only MHC class I molecules that have acquired a specific epitope. Some antibodies, such as HC10, are reactive against partially folded/unfolded MHC class I molecules and therefore require an appropriate epitope for antibody reactivity. It is possible that not all unfolded MHC class I molecules will be HC10 reactive. By adding an epitope tag at the C-terminus of the MHC class I heavy chain, the antibody reactivity is not biased to any one particular conformation. Employing this methodology in biosynthetic and degradative assays provides information on the entire population of MHC class I molecules.

and the same antibody strategy, we can determine protein associations throughout the biosynthetic process. As well as immunoblotting we also employ MHC class I specific antibodies in *fluorescence-activated cell sorting* (FACS) experiments to quantify steady-state surface expression of ectopically produced proteins or the effects of different pharmaceutical reagents on surface expression of different MHC class I alleles. These methodological approaches coupled with a pharmacological approach can aid in the analysis and understanding of both the biosynthetic and degradation process of this important group of proteins.

2. Materials

2.1. Common Reagents

1. Cell lines: HeLa or HEK293.
2. Eppendorf tubes or tissue culture grade 96-/24-/12-/6-well plates to incubate cells.
3. 1× PBS.

4. 1.5 M Tris-HCl pH 8.8.
5. 1 M Tris-HCl pH 6.8.
6. Dulbecco's phosphate buffered saline (DPBS).
7. DMSO.
8. Tissue culture media; RPMI or DMEM.

2.2. S^{35} Labeling and Pulse Chase of MHC Expressing Cells

1. Radioactive S^{35} methionine/cysteine.
2. Gloves, plastic shield, and plastic container designed to contain radioactive β -emissions.
3. Methionine/cysteine-free medium (DMEM or RPMI).
4. RPMI/DMEM supplemented with 10% fetal bovine serum.
5. Cyanogen bromide activated Sepharose 4B.
6. Protein A and/or Protein G sepharose beads.
7. Ionic detergents, e.g. 1% stock solution of NP40 or Triton X100 made up in double distilled water, 100 mM sodium chloride, and 10 mM Tris-HCl, pH 7.4. Non-ionic detergents such as digitonin powder.
8. Lysis buffer: 1% detergent, 1x complete protease inhibitors, 10 mM N-ethyl maleimide (NEM), 1 mM phenylmethane sulfonyl fluoride (PMSF).
9. Antibodies: conformationally specific antibodies such as W6/32, HC10, or anti-epitope tag antibodies.
10. Endoglycosidase H enzyme (Roche or NEB).
11. Fixative: 40% methanol, 10% acetic acid made up in double distilled water.
12. 100 mM Dithiothreitol solution made up in deionized water.
13. Gel drier, X-ray cassettes, and autoradiography film.

2.3. Immuno-precipitations

1. 20 mM NEM.
2. Lysis buffer containing either ionic detergents, e.g., 1% stock solution of NP40 or Triton X100 made up in double distilled water, 100 mM sodium chloride, and 10 mM Tris-HCl, pH 7.4 or non-ionic detergents such as digitonin powder, supplemented with protease inhibitors such as 1 mM PMSF and a complete protease cocktail (e.g., Roche, complete EDTA-free, catalogue number 11873580001) which targets serine and cysteine proteases and 10 mM NEM to prevent post-lysis oxidation events.
3. Cyanogen bromide activated Sepharose 4B.
4. Protein A and/or Protein G sepharose beads.
5. Antibodies: conformationally specific antibodies such as W6/32, HC10, or anti-epitope tag antibodies.

2.4. SDS**Polyacrylamide Gel Components**

1. 30% Acrylamide/bis-acrylamide mix (37.5:1).
2. Ammonium persulfate: 10% solution in deionized water (use within 1 week).
3. Tetramethylethylenediamine (TEMED).
4. Prestained molecular weight markers.
5. 3× SDS-PAGE sample buffer: 0.12 M Tris-HCl pH 6.8, 12% glycerol, 0.3% w/v SDS, 0.05% w/v Bromophenolblue.
6. Appropriate gel casting, blotting, and running apparatus (we use MiniPROTEAN 3, Bio-Rad).
7. Tris-Glycine SDS-Page running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS (see Note 1).

2.5. Immunoblotting Components

1. Western transfer buffer: 25 mM Tris, 192 mM glycine.
2. Nitrocellulose transfer membrane (Protran, Whatman).
3. 3MM chromatography paper (Whatmann).
4. Western Blocking Solution: 5% skim milk powder, 1× DPBS (see Note 2).
5. Western Washing Buffer: 1× DPBS, 0.1% tween-20.
6. Ponceau S Solution: 0.1%(w/v) Ponceau S in 5%(v/v) acetic acid.
7. Appropriate primary and HRP-conjugated secondary antibody (see Note 3).
8. Luminescent HRP detection reagent and X-ray film or appropriate computer capture detection system.

2.6. Flow Cytometry

1. FACS blocking solution: 1% FCS and 0.1% sodium azide.
2. Primary antibodies to be used in study diluted in FACS blocking solution and appropriate secondary antibodies if primary antibodies are not directly coupled to a fluorophore diluted in FACS blocking solution or DPBS.
3. Formalin: 1.9% solution in DPBS.
4. Flow cytometer with possibility of cell sorting.

2.7. Pharmaceutical Reagents

1. Cycloheximide, tunicamycin, deoxymannojirimycin, and MG132 can be made at 1,000 or 10,000× concentrations solutions in sterile tissue culture DMSO (Fig. 2).
2. Kifunensine: 1,000 or 10,000× concentration solutions in sterile tissue deionized water.
3. 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide): vortex solution prepared in serum-free medium and sonicate briefly if required. This solution will need to be filtered before use and can be used for up to 2 weeks after being made.

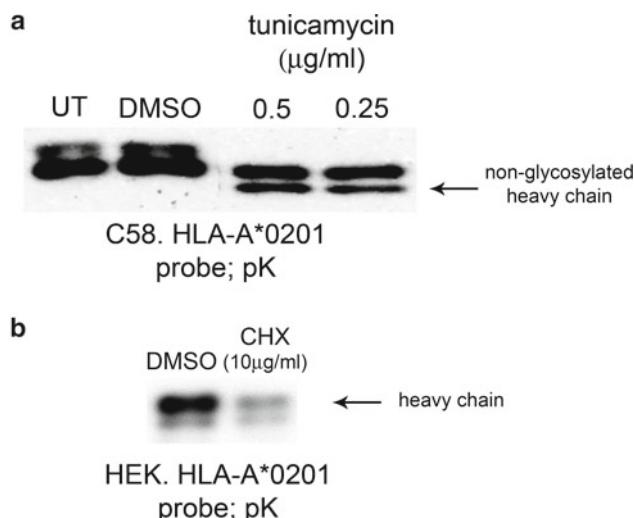


Fig. 2. Examples of pharmacological treatments in the analysis of MHC class I molecule biosynthesis and degradation using immunoblotting. (a) C58.HLA-A*0201 expressing cells were untreated (UT), treated with vehicle control (DMSO), or two different concentrations of the glycosylation inhibitor tunicamycin (0.25 and 0.5 μg) overnight, prior to 8% SDS-PAGE analysis and immunoblotting with the anti-epitope tag antibody pK. The appearance of a lower molecular weight band following tunicamycin treatment correlates with a non-glycosylated heavy chain. (b) Cycloheximide treatment of HEK.HLA-A*0201 cells results in the disappearance of HLA-A2 as synthesis of new HLA-A2 is inhibited and previously formed HLA-A2 is degraded.

4. Plate shaker.
5. Plate reader with 540 nm filter and 750 nm filter.
6. Total protein quantification reagent (Dc protein assay, Bio-Rad).

3. Methods

3.1. S^{35} Labeling and Pulse Chase of MHC Expressing Cells

1. Cells are grown and harvested whilst in log phase growth and normally used at a minimum of 1×10^6 cells per immunoprecipitation. Cells can be treated prior to harvesting with pharmacological agents disrupting the degradation process (see Subheading 3.5).
2. Incubate cells in cysteine/methionine-free medium at 1×10^7 cells/mL for 30 min at 37°C.
3. Add 100 μCi of S^{35} cysteine/methionine for 5–15 min (see Note 4).
4. Briefly pellet cells and remove all media into a suitable receptacle for disposal.

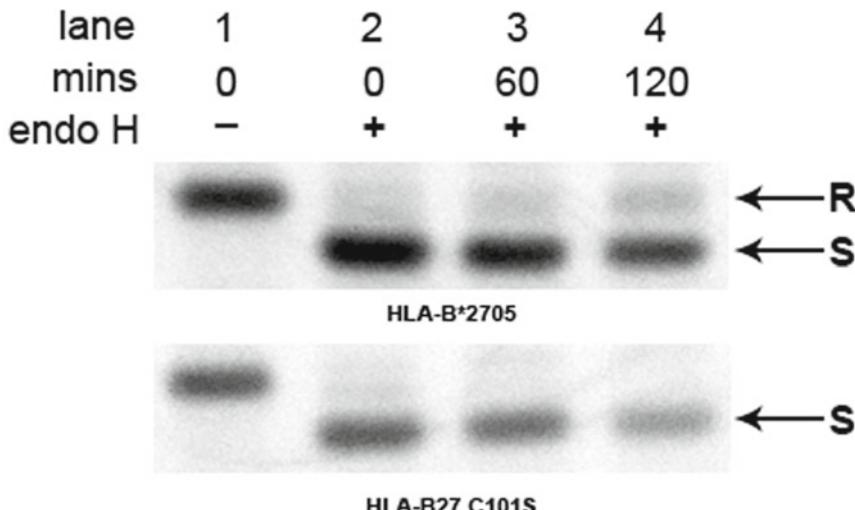


Fig. 3. An example of pulse chase and the use of Endo H digestion in monitoring MHC class I biosynthesis using an anti-Sv5 C-terminal epitope tag detection antibody. Here we demonstrate how the MHC class I molecule HLA-B*2705, which has been tagged at the C-terminus (*top panel*), can acquire endo H resistance (R) over time (min) compared to a mutant HLA-B27.C101S (*bottom panel*) molecule that is unfolded, cannot transit out off the ER and therefore the oligosaccharide modifications remain sensitive (S) to Endo H digestion. C58 rat thymoma cell lines expressing HLA-B27 and HLA-B27.C101S were serum starved for 30 min, labeled for 10 min with S^{35} cysteine/methionine, and then chased for 0, 60, and 120 min. Equal number of cells were lysed and immunoprecipitated with the anti-Sv5 tag antibody pK. Immunoprecipitates were then incubated with 0.005 U endo H for 1 h at 37°C, prior to running on an 8% SDS-PAGE reducing gel.

5. Resuspend cells in media supplemented with 10% FCS and immediately remove 2 aliquots of 10^6 cells; these will act as the 0 time points of the chase (see Note 5).
6. Incubate cells at 37°C for the desired times and remove 1×10^6 cells at each time point.
7. Pellet cells by short pulse centrifugation and resuspend in 500 μ L cold detergent lysis buffer and incubate on ice for a minimum of 15 min.
8. Centrifuge lysed samples at $26,400 \times g$ for 15 min at 4°C.
9. Incubate supernatants with 50 μ L sepharose 4B/10 μ L protein A/10 μ L protein G and preclear on a rotating wheel for a minimum of 30 min at 4°C.
10. Immunoprecipitate supernatants with respective antibodies (0.5–1.0 μ g) along with 10 μ L of protein A and G at 4°C for a minimum of 1 h at 4°C.
11. Briefly pellet beads by short pulse centrifugation and discard supernatants. Wash the Protein A and G sepharose beads with 5 \times 500 μ L detergent used to perform the initial lysis.
12. Following the final wash, pellet beads and remove detergent. To each set of beads add 0.005 U of Endo H and incubate for 1 h at 37°C. One 0 min control sample is left untreated with Endo H as a control for enzyme activity (see Fig. 2).

13. Resuspend immunoprecipitates in 20 µL SDS-PAGE sample buffer.
14. Analyze each immunoprecipitate by SDS-PAGE electrophoresis. Prior to electrophoresis samples are reduced using 100 mM dithiothreitol for 5 min at 95°C (see Subheading 3.3, steps 1–6).
15. Once samples have run, SDS-PAGE gels are dismantled and incubated in 100 mL of fixative for 15 min at room temperature.
16. Two pieces of 3MM chromatography paper are cut to an appropriate size that can accommodate the SDS-PAGE gel. One piece of 3MM paper is soaked in water and placed on top of the second dry piece of 3MM paper. The SDS-PAGE gel is placed on top of the wet 3MM paper.
17. Gels are then placed in a suitable gel drier and dried for 2 h at 80°C.
18. Once dried, the gel is placed in an X-ray cassette with autoradiography film and placed at –80°C.

3.2. Immuno-precipitations

1. Harvest 1×10^7 cells and wash once with 20 mL 1× PBS.
2. Incubate cells on ice for 20 min with ice-cold 20 mM NEM (see Note 6).
3. Pellet cells by short pulse centrifugation and lyse in 1.5 mL detergent lysis buffer (see Subheading 2.3, item 2 for detergent lysis buffer selection) on ice for 15 min.
4. Centrifuge lysates at $26,400 \times g$ for 15 min at 4°C.
5. Incubate supernatants with 50 µL sepharose 4B/10 µL protein A/10 µL protein G and preclear on a rotating wheel for a minimum of 30 min at 4°C.
6. Immunoprecipitate supernatants with respective antibodies (0.5–1.0 µg) along with 10 µL of protein A and G at 4°C for a minimum of 2 h at 4°C.
7. Briefly pellet beads by short pulse centrifugation and discard supernatants. Wash Protein A and G sepharose beads with 5 × 500 µL detergent used to perform the initial lysis.
8. Resuspend immunoprecipitates in 30 µL SDS-PAGE sample buffer.
9. Analyze each immunoprecipitate by SDS-PAGE electrophoresis. Once samples have run, dismantle SDS-PAGE gels and set up for immunoblotting (see Subheading 3.3).

3.3. Tris-Glycine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

1. Preparation and assembly of casting apparatus will vary depending on the system used. For a typical 7 × 10 cm gel with a 1.5 mm gel thickness make 10 mL of resolving gel mix. For separation of MHC class I molecules and other larger proteins such as co-precipitated chaperones an 8% is utilized (See Note 7).
2. For 10 mL of 8% resolving gel mix: 4.6 mL deionized water, 2.7 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris pH 8.8,

0.1 mL 10% SDS, 0.1 mL 10% APS, and 0.006 mL of TEMED. Mix well and immediately decant between the plates into the casting frame leaving room for the stacking gel with comb. Overlay with deionized water to allow gel polymerization. Once polymerization is complete remove the water overlaying the gel and dry the top of the gel (See Note 8 for safety information).

3. For 3 mL stacking gel to overlay on the resolve gel: 2.1 mL deionized water, 0.5 mL 30% acrylamide mix, 0.38 mL 1 M Tris pH 6.8, 0.03 mL 10% SDS, 0.03 mL 10% APS, and 0.003 mL of TEMED. Mix well and immediately decant into the casting frame overlaying the polymerized resolving gel. Insert the desired comb making sure there are no bubbles underneath or between the comb teeth. Once polymerization is complete gently remove the comb and wash out the wells with deionized water. The polymerized gel can be used immediately or stored at 4°C for a few days wrapped in moist paper towel to keep it from drying out.
4. Assembly of the gel within the running apparatus will vary according to model; however, with the MiniPROTEAN the chambers are filled with 1× Tris-Glycine Running buffer.
5. Load samples into the appropriate wells on the gel and run one well with an appropriate prestained molecular weight marker.
6. For a typical 7 × 10 cm MiniPROTEAN gel, run at 100 V for 80 min.
7. After separation is complete, remove the gel from between the glass plates and reassemble in a transfer cassette sandwiched between the nitrocellulose membrane and 3MM whatman paper. Place the transfer cassette in the blotting chamber, fill with transfer buffer, and run at 100 V for 90 min or until complete transfer of proteins to the membrane is achieved. Most rigs are cooled during the transfer either by placing ice blocks within them or running water through the transfer chamber.
8. Disassemble the rig and remove the nitrocellulose. Transfer efficiency can be tested by staining the nitrocellulose with Ponceau S. Wash the nitrocellulose in DPBS and incubate in block solution for 30 min at room temperature with agitation.
9. Incubate with the primary antibody. Antibody concentration and the time or temperature of the incubation will vary and must be experimentally determined.
10. Wash 2 × 15 min with western wash buffer.
11. Incubate with the HRP-conjugated secondary antibody. Secondary antibody concentration, time, and temperature of the incubation will vary between antibodies used although 30–60 min at room temperature with agitation is generally adequate.
12. Wash 3 × 15 min with western wash buffer.

13. Develop with chemiluminescent reagents. Luminescent detection of immunoreactive proteins will vary according to the reagents used. Either X-ray film or a specific cooled CCD camera system is the most commonly used methods for visualizing luminescence emitted from blots.

3.4. Fluorescence-Activated Cell Sorting

1. Count and resuspend cells in FACS blocking buffer. In a typical experiment $1\text{--}2 \times 10^5$ cells are used in each staining reaction. For most procedures staining can be performed within a V- or U-bottomed 96-well plate or in FACS tubes.
2. Pellet cells by spinning at $300 \times g$ for 5 min and resuspend in 50 μL primary antibody solution diluted in FACS blocking solution and incubate at 4°C with agitation for 30–60 min. After incubation cells are washed twice with 200 μL 1 \times DPBS.
3. If the primary antibody is not conjugated to a fluorophore, resuspend cells in 50 μL of an appropriate conjugated secondary antibody solution diluted in FACS block or DPBS. Selection of the secondary will depend on the species the primary is derived and the type of fluorophore required for the experiment. Optimal working concentrations for the secondary will vary and must be determined experimentally; however, a working concentration of 10 $\mu\text{g}/\text{mL}$ is a good starting point. For staining incubate cells with the secondary antibody for 30 min to 1 h at 4°C with agitation.
4. Wash cells twice with 200 μL 1 \times DPBS. After the final wash, fix cells with a final concentration of 1.9% formalin. Cells can be stored for short periods of time or visualized immediately on the FACS machine (see Note 9).

3.5. Treatment of Cells with Pharmacological Reagents and Normalizing SDS-PAGE Sample Loading

1. For testing the effects of pharmacological reagents on MHC class I biosynthesis and degradation grow cells to the desired density. For HeLa or HEK293 seed cells at a density of $2\text{--}3 \times 10^5/\text{well}$ in 500 μL of media in a 24-well plate 24 h prior to treatment. Just prior to adding the drugs change the media within each well. Include appropriate vehicle controls for all experiments.
2. Drug concentrations and administration times for each drug will vary between cell lines. Table 1 lists concentration and administration regimes we have successfully used in our studies in HeLa and HEK293 cells.
3. The cytotoxic effects of the different drugs can often complicate normalization of sample loading if it is based solely on cell counting. To overcome this difficulty we have also equalized sample loading by normalizing protein concentration (see step 4) or by quantifying cell metabolic activity using the MTT assay (see step 5).

Table 1
Pharmacological reagents and their concentration and administration times employed in cellular assays

Drug	Concentration	Administration time (h)	Note
Cycloheximide	10–100 µM	18–48	Fresh cycloheximide must be supplemented into each well at 24 h intervals
Tunicamycin	0.25–1 µg/mL	18	
Kifunensine	1–5 µg/mL	18	
Deoxymannojirimycin	0.5–1 mM	18	
MG132	0.1–0.5 µg/mL	18	

4. Normalizing sample loading by protein concentration:

- (a) Prepare cells lysates as described (see Subheadings 2.3, item 2 and 3.2); however, before addition of SDS-PAGE sample buffer reserve a 20 µL aliquot and test using the protein quantification assay according to the manufacturers' protocols (see Note 10).
- (b) Read samples at 750 nM using a plate reader. Determine sample concentrations by comparison to a standard curve made using BSA diluted in the same buffer used to prepare the cell lysates.

5. Normalizing sample loading by cell metabolic activity:

- (a) If normalizing sample loading using the MTT assay, for each experimental sample at least one additional equivalent sample must be set up in parallel which will be used in the MTT assay. Alternatively a small proportion of the cells from each sample can be reserved before cell lysis and used in the MTT assay.
- (b) Remove cell culture media and replace with MTT containing media (100 µL per 96-well or 500 µL per 24-well). Return cells to the tissue culture incubator for 1–3 h or until a visible purple precipitate forms.
- (c) Remove MTT containing media and add an equal volume of DMSO to each well. Agitate for at least 10 min until the precipitate goes into solution. Collect the samples and load into an optically clear flat-bottomed 96-well plate. Read the plate at 540 nM on a plate reader after blanking with DMSO. Under some circumstances the OD₅₄₀ will be saturated ($OD_{540} > 1.5$) and require that the MTT solution for that sample is diluted to measure its absorbance accurately.

- (d) Normalize samples by calculating the ratio of the OD₃₄₀ of each sample to a chosen reference sample and then adjust the loading volumes of lysates accordingly.

4. Notes

1. Tris-Glycine SDS-page running buffer can be made in-house or purchased as a 10× solution from a variety of companies. For consistency, we purchase premade 10× buffer from Bio-Rad.
2. Please consult the supplier's information to determine what blocking reagent is most appropriate for the antibody being used as some antibodies may require alternative blocking reagents such as BSA.
3. Selection of an appropriate HRP-conjugated secondary antibody is extremely important as some show nonspecific cross reactivities with certain sample types. This should be tested for each secondary used. For our own work, we commonly use the HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit from Pierce.
4. Incubation period with S³⁵ cysteine/methionine can vary from 2 to 15 min and largely depends on the number of cysteine and methionine residues expressed by proteins and can determine the strength of the radioactive signal incorporated.
5. During the pulse chase, two 0 min time point samples are removed. One sample is not treated with endoglycosidase H which should indicate the molecular weight of the heavy chain when glycosylated whilst the second 0 min sample should highlight the change in molecular weight when the heavy chain is deglycosylated by the activity of Endo H.
6. NEM is an alkylating agent and can be replaced with methyl methanethiosulfonate (MMTS). These reagents alkylate free thiols on cysteine residues and trap any transient disulfide bonded intermediates between two proteins. In the case of MHC class I heavy chains such a method is required to detect associations with oxidoreductases such as ERp57.
7. The percentage of polyacrylamide used in the SDS-Page gel will vary according to the size of the proteins being visualized with higher percentage gels (12–14%) being used if β2m is also being detected on the immunoblot.
8. Acrylamide is a neurotoxin and protocols relating to its use should always be performed with gloves and observe the appropriate health and safety procedures.
9. It is important to note that cells used in FACS analysis should not be fixed before they are stained for surface MHC unless it

has been previously established that the antibodies being used are still reactive with fixed antigens. We have successfully used W6/32 and ME1 with samples fixed with 3.8% PFA prior to staining. However, a number of other antibodies we have tested lose cross reactivity or specificity on fixed samples.

10. For protein quantification, we have used the Bio-Rad Dc protein quantification kit. Our selection of this reagent is based on its compatibility with samples containing a variety of detergents including triton, NP40, and SDS at concentrations normally used to prepare cell lysates.

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References

1. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329:506
2. Bjorkman PJ, Parham P (1990) Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu Rev Biochem* 59:253
3. Falk K, Rotzschke O, Rammensee HG (1990) Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* 348:248
4. Rotzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammensee HG (1990) Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 348:252
5. Lewis JW, Elliott T (1998) Evidence for successive peptide binding and quality control stages during MHC class I assembly. *Curr Biol* 8:717
6. Williams AP, Peh CA, Purcell AW, McCluskey J, Elliott T (2002) Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity* 16:509
7. Cresswell P (2005) Antigen processing and presentation. *Immunol Rev* 207:5
8. Antoniou AN, Powis SJ, Elliott T (2003) Assembly and export of MHC class I peptide ligands. *Curr Opin Immunol* 15:75
9. Harris MR, Yu YY, Kindle CS, Hansen TH, Solheim JC (1998) Calreticulin and calnexin interact with different protein and glycan determinants during the assembly of MHC class I. *J Immunol* 160:5404
10. Ford S, Antoniou A, Butcher GW, Powis SJ (2004) Competition for access to the rat major histocompatibility complex class I peptide-loading complex reveals optimization of peptide cargo in the absence of transporter associated with antigen processing (TAP) association. *J Biol Chem* 279:16077
11. Hughes EA, Hammond C, Cresswell P (1997) Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc Natl Acad Sci U S A* 94:1896–1901
12. Stagg HR, Thomas M, van den Boomen D, Wiertz EJ, Drabkin HA, Gemmill RM, Lehner PJ (2009) The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. *J Cell Biol* 186:685
13. Burr ML, Cano F, Svobodova S, Boyle LH, Boname JM, Lehner PJ (2011) HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. *Proc Natl Acad Sci U S A* 108(5): 2034–2039
14. Park B, Lee S, Kim E, Chang S, Jin M, Ahn K (2001) The truncated cytoplasmic tail of HLA-G serves a quality-control function in post-ER compartments. *Immunity* 15:213
15. Molinari M, Galli C, Piccaluga V, Pieren M, Paganetti P (2002) Sequential assistance of

- molecular chaperones and transient formation of covalent complexes during protein degradation from the ER. *J Cell Biol* 158:247
16. Antoniou AN, Lenart I, Giuliano DB (2011) Pathogenicity of Misfolded and Dimeric HLA-B27 Molecules. *Int J Rheumatol* 2011: 486856
 17. Welihinda AA, Tirasophon W, Kaufman RJ (1999) The cellular response to protein misfolding in the endoplasmic reticulum. *Gene Expr* 7:293
 18. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14:9
 19. Sakaguchi K, Ono R, Tsujisaki M, Richiardi P, Carbonara A, Park MS, Tonai R, Terasaki PI, Ferrone S (1988) Anti-HLA-B7, B27, Bw42, Bw54, Bw55, Bw56, Bw67, Bw73 monoclonal antibodies: specificity, idiotypes, and application for a double determinant immunoassay. *Hum Immunol* 21:193
 20. Parham P, Bodmer WF (1978) Monoclonal antibody to a human histocompatibility alloantigen, HLA-A2. *Nature* 276:397
 21. Stam NJ, Spits H, Ploegh HL (1986) Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J Immunol* 137:2299
 22. Antoniou AN, Ford S, Taurog JD, Butcher GW, Powis SJ (2004) Formation of HLA-B27 homodimers and their relationship to assembly kinetics. *J Biol Chem* 279:8895
 23. Santos SG, Antoniou AN, Sampaio P, Powis SJ, Arosa FA (2006) Lack of tyrosine 320 impairs spontaneous endocytosis and enhances release of HLA-B27 molecules. *J Immunol* 176:2942
 24. Schaefer MR, Williams M, Kulpa DA, Blakely PK, Yaffee AQ, Collins KL (2008) A novel trafficking signal within the HLA-C cytoplasmic tail allows regulated expression upon differentiation of macrophages. *J Immunol* 180: 7804
 25. Gruda R, Achdout H, Stern-Ginossar N, Gazit R, Betser-Cohen G, Manaster I, Katz G, Gonen-Gross T, Tirosh B, Mandelboim O (2007) Intracellular cysteine residues in the tail of MHC class I proteins are crucial for extracellular recognition by leukocyte Ig-like receptor 1. *J Immunol* 179:3655

Chapter 9

Studying Ubiquitination of MHC Class I Molecules

Marian L. Burr, Jessica M. Boname, and Paul J. Lehner

Abstract

The covalent attachment of ubiquitin to a protein is one of the most common post-translational modifications and regulates diverse eukaryotic cellular processes. Ubiquitination of MHC class I was first described in the context of viral proteins which target MHC class I for degradation in the endoplasmic reticulum and at the cell surface. Study of viral-induced MHC class I degradation has been extremely instructive in elucidating cellular pathways for degradation of membrane and secretory proteins. More recently, ubiquitination of endogenous MHC class I heavy chains which fail to achieve their native conformation and undergo endoplasmic-reticulum associated degradation has been demonstrated.

In this chapter we describe methods for identification of endogenous ubiquitinated MHC class I heavy chains by MHC class I-immunoprecipitation and ubiquitin-specific immunoblot or by metabolic labeling and immunoprecipitation.

Key words: MHC class I, Ubiquitin, Ubiquitin E3 ligase, Proteasome, Lysosome

1. Introduction

Ubiquitination is a versatile post-translational modification that targets proteins for degradation by the proteasome or lysosome and has additional non-proteolytic functions. Ubiquitination requires the 76 amino acid ubiquitin molecule to be activated by an E1 enzyme and then transferred to an E2-ubiquitin conjugating enzyme. The ubiquitin E3 ligase catalyzes the transfer of ubiquitin from the E2 to the target protein, thus conferring substrate specificity to the ubiquitination reaction.

The most commonly used method for determining whether a protein is ubiquitinated *in vivo* is to immunoprecipitate the protein of interest from cell lysates followed by immunoblotting to detect ubiquitin–protein conjugates. The ubiquitinated protein is most commonly visualized as a high-molecular weight smear (Fig. 1). An alternative approach is to immunoprecipitate the protein from radiolabeled cells which may reveal a “ladder” of higher molecular

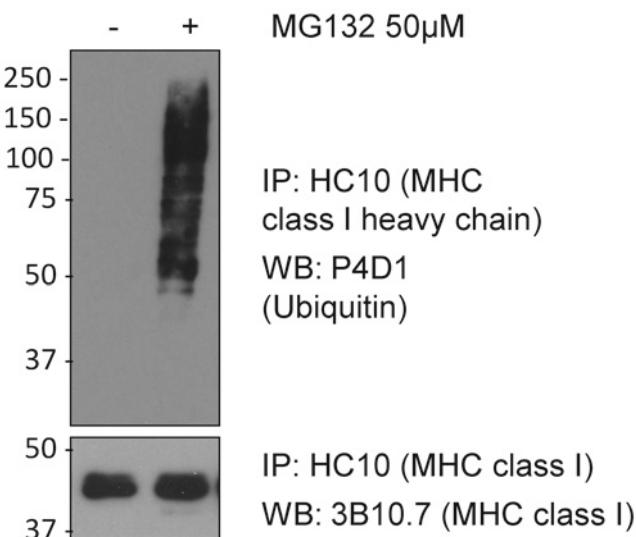


Fig. 1. Anti-ubiquitin immunoblot demonstrating polyubiquitinated MHC class I in β 2m-depleted HeLa cells. 4×10^6 HeLa cells stably expressing β 2m shRNA were incubated \pm MG132 50 μ M for 5 h prior to lysis in 1% Triton X-100. Non- β 2m bound MHC class I heavy chains were immunoprecipitated using HC10 antibody, separated by SDS-PAGE and probed for ubiquitin using P4D1 antibody (see Subheading 3.1).

weight species reflecting the conjugation of one or more ubiquitin moieties to the target protein (Fig. 2). The identity of these species can be confirmed by reprecipitating with a ubiquitin-specific antibody. Radiolabeling may allow elucidation of the kinetics of ubiquitination and deubiquitination and the ubiquitin bands are more readily discernible facilitating differentiation of the mono- and poly-ubiquitination reactions (1).

When and where does MHC class I ubiquitination occur? Under physiological conditions, MHC class I heavy chains which are unable to fold correctly and bind to β 2-microglobulin (β 2m) are targeted for ubiquitination and dislocation from the endoplasmic reticulum (ER) to the cytosol for proteasomal degradation by the ER-resident E3 ligase HRD1 (2, 3).

Ubiquitination of MHC class I has also been demonstrated in the presence of viral proteins which exploit cellular ubiquitin-dependent degradation pathways in order to downregulate MHC class I expression and evade immune detection. These include the human cytomegalovirus-encoded proteins US2 and US11 (4–7), the mouse γ -herpesvirus 68 encoded E3 ligase mK3 (8–11), and the Kaposi's sarcoma herpes virus (KSHV) E3 ligases K3 and K5 (12–16). US2, US11, and mK3 mediate dislocation of MHC class I heavy chains from the ER to the cytosol for proteasome-mediated degradation (8, 17, 18), whereas K3 and K5 target MHC class I later in the secretory pathway for endolysosomal degradation (12, 19, 20). mK3, K3, and K5 all have ubiquitin E3 ligase activity and

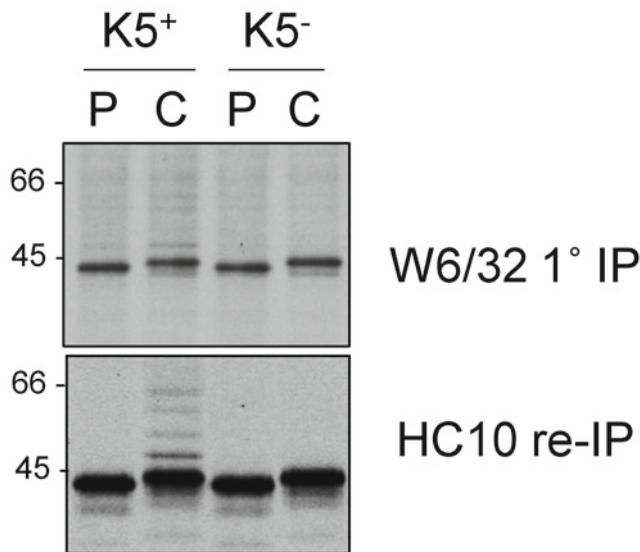


Fig. 2. Visualization of ubiquitinated MHC class I by radioimmune precipitation. HeLa cells \pm the viral E3 ubiquitin ligase K5 were pulse labeled for 10 min (P) and chased for 45 min (C) as described in the protocol (see Subheading 3.2). The primary W6/32 immunoprecipitation and the HC10 reimmunoprecipitation are shown. 10 days exposure to X-ray film.

ubiquitinate residues in the MHC class I cytoplasmic tail (10, 12, 13, 16). Conversely, US2 and US11 are not thought to have intrinsic ligase activity. US2 recruits the cellular E3 ligase Trc8 to mediate MHC class I ubiquitination and degradation while the E3 ligase involved in US11-mediated MHC class I degradation has not been identified (7).

Detecting ubiquitinated MHC class I can be challenging. Ubiquitination is a dynamic process and at any one time only a small proportion of the MHC class I in the cell will be ubiquitinated. For example, the rapid MHC class I degradation induced by viral proteins makes it difficult to trap and visualize ubiquitinated species. It is therefore critical to establish conditions which maximize ubiquitinated MHC class I levels. This involves identifying factors promoting ubiquitination and determining the likely route of degradation with lysosomal or proteasomal inhibitors. An additional consideration for detecting ubiquitinated MHC class I is the predicted conformation of the ubiquitinated heavy chain, i.e., whether or not it is likely to be in complex with β 2m and peptide, as this will affect the choice of antibody for immunoprecipitation.

In this chapter we describe the protocol for the detection of endogenous ubiquitinated MHC class I in β 2m-depleted cells by MHC class I immunoprecipitation and ubiquitin-specific immunoblot (2). In the second part we discuss metabolic labeling and immunoprecipitation, an approach we have utilized to identify ubiquitinated MHC class I in the presence of viral proteins (7, 8, 12, 14, 16).

2. Materials

2.1. Cell Culture

1. HeLa cells.
2. Growth medium: RPMI-1640 or Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin.
3. MG132 (Z-leucyl-leucyl-leucyl-CHO): 10 mM stock in DMSO, aliquoted and stored at -80°C.
4. Interferon- γ . Stock 400 U/µl in sterile water. Aliquotted and stored at -80°C.
5. Trypsin-EDTA solution: 0.5 g porcine trypsin, 0.2 g EDTA.

2.2. Sample Preparation

Prepare all buffers in deionized water (dH₂O).

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
2. Tris-buffered saline (TBS): 10 mM Tris, 150 mM NaCl, pH 7.4.
3. Phenylmethylsulfonyl fluoride (PMSF): 50 mM stock in isopropanol. Use at 0.5 mM (see Note 1).
4. Iodoacetamide (IAA): 500 mM stock in dH₂O. Use at 10 mM.
5. Roche complete EDTA-free protease inhibitor cocktail: Dilute one tablet in 1 ml dH₂O to make a 50× solution.
6. N-Ethylmaleimide (NEM): 1 M stock in ethanol. Use at 10 mM.
7. Lysis/immunoprecipitation buffer: 1% Triton X-100 in TBS, 0.5 mM PMSF, 10 mM IAA (or NEM), and Roche complete protease inhibitor.
8. SDS-denaturing buffer: 1% SDS in TBS, Roche complete protease inhibitor.
9. SDS lysis buffer: 1% SDS in TBS, 5 mM DTT, Roche complete protease inhibitor, Benzonase nuclease (Sigma).

2.3. Immunoprecipitation

1. IgG-Sepharose beads and Protein A-Sepharose beads. Wash before use and resuspend at 50%v/v slurry in TBS (see Note 2).
2. MHC class I and ubiquitin-specific antibodies.
3. 2× SDS sample loading buffer: 20%v/v glycerol, 100 mM Tris-HCl pH 6.8, 4%w/v SDS, 200 mM DTT, 0.01%w/v Bromophenol Blue (see Note 3).
4. Wash buffer: 0.1% Triton X-100 in TBS.

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Acrylamide stock solution: 30% acrylamide/0.8% bis-acrylamide.
2. Resolving gel buffer: 1.5 M Tris-HCl, 0.4% w/v SDS, pH 8.8.
3. Stacking gel buffer: 0.5 M Tris-HCl, 0.4% w/v SDS, pH 6.8.
4. N, N, N', N'-tetramethyl-ethylenediamine (TEMED).
5. 10% Ammonium persulfate (APS).
6. Water-saturated butanol.
7. 10% Polyacrylamide minigel; precast gels are available commercially or make it (see Subheading 3.1.5, step 1).
8. Gel electrophoresis chamber, transfer apparatus and power supply.
9. Filter paper.
10. Pre-stained molecular weight protein standards.
11. Tris-glycine gel running buffer: 20 mM Tris, 154 mM glycine, 0.1% SDS.
12. PVDF membrane.
13. Extra-thick filter paper (Bio-Rad).
14. Transfer buffer: 25 mM Tris, 192 mM Glycine, 20% methanol, 0.037% SDS.
15. Membrane denaturing solution: 6 M guanidinium chloride, 20 mM Tris-HCl pH 7.5, 1 mM PMSF (freshly added), 5 mM β-mercaptoethanol (freshly added).

2.5. Immunoblotting

1. Blocking buffer: 5% (w/v) BSA in TBS, 0.05% (v/v) Tween-20.
2. Wash solution (TBS-T): TBS, 0.05/0.1% Tween-20.
3. P4D1 antibody.
4. Horseradish-peroxidase (HRP)-conjugated secondary antibody.
5. Luminol-based chemiluminescent developing reagent (e.g., West Pico chemiluminescent (ECL) substrate (Pierce)).
6. X-ray film and developing cassette.

2.6. Metabolic Labeling

1. Starve medium: RPMI-1640 without glutamine, cysteine, or methionine (SIGMA R7513), 20 mM HEPES, 5% dialyzed FBS, and 2 mM glutamine (see Note 4).
2. Labeling medium: 100 µl/sample of prestarve medium with 10 µl of L-(³⁵S)-cysteine/(³⁵S)-methionine (see Note 5).
3. Chase medium: RPMI with 10% non-dialyzed FBS and 20 mM HEPES (see Note 6).
4. Gel fix solution: 10% acetic acid/20% methanol in water. 2% glycerol can be added to prevent gels cracking as they dry.

3. Methods

3.1. MHC Class I

Immunoprecipitation and Ubiquitin-Specific Immunoblot

This method involves immunoprecipitation of MHC class I from cell lysates, separation of precipitated proteins by SDS-PAGE gel electrophoresis, and immunoblot for ubiquitin to detect ubiquitinated MHC class I species.

Three different strategies for MHC class I immunoprecipitation will be described:

1. Lysis under non-denaturing conditions and immunoprecipitation of conformational MHC class I complexes using W6/32 or anti- β 2m antibody (21) or of free MHC class I heavy chains using HC10 antibody (22). A caveat with this strategy is that, depending on the detergent used, the ubiquitin signal detected on immunoblot could be due to co-purification of other ubiquitinated proteins rather than ubiquitinated MHC class I itself.
2. Denaturing lysis in 1% SDS to disrupt protein–protein interactions and immunoprecipitation of denatured MHC class I heavy chains using HC10 antibody. This allows more confident determination of MHC class I ubiquitination, though the MHC class I cannot be precipitated in a conformation-specific manner after lysis in SDS.
3. This problem can be circumvented by performing the primary lysis and immunoprecipitation under non-denaturing conditions with a conformation-specific antibody, eluting MHC class I from the beads in SDS which will disrupt protein–protein interactions and then reimmunoprecipitating with an antibody which recognizes denatured MHC class I (described in Subheading 3.2).

The protocol will be described for HeLa cells grown in a 6-well plate format but can be easily adapted to other cell types.

3.1.1. Treatment of Cells Prior to Harvest

1. Maintain HeLa cells in growth medium in an incubator at 37°C with 5% CO₂.
2. Optional: add interferon- γ to cells 24 h before harvest. Dilute to 100–200 U/ml in cell culture media (see Note 7).
3. Optional: add proteasome inhibitor to cells 4–5 h before harvest. Dilute MG132 to 50 μ M in cell culture media (see Notes 8–10). This will enhance detection of ubiquitinated MHC class I undergoing proteasome-dependent degradation. Alternatively, if degradation is occurring via a lysosomal route then detection of ubiquitinated MHC class I species may be enhanced by treatment with lysosomal inhibitors such as chloroquine (100 μ M), concanamycin A (50 nM), or bafilomycin A1 (200–400 nM) (12, 14, 15).

3.1.2. Cell Harvest

Substantially more cells are required to detect ubiquitinated MHC class I than are needed to detect just MHC class I itself. The number required will depend on the cell type and the proportion of MHC class I that is ubiquitinated in a particular experimental setting. Aiming for 1 mg total protein in 1 ml lysate is a reasonable starting point. This equates to about 4 million HeLa cells which is sufficient to readily detect ubiquitinated MHC class I after β 2m-depletion.

1. Cells should be just subconfluent at the time of harvest. This will yield approximately 1 million HeLa cells per well of a 6-well plate.
2. Wash with 1 ml PBS.
3. Add 500 μ l trypsin-EDTA solution and incubate until cells begin to detach (see Note 11).
4. Add 900 μ l growth medium (containing FBS) to neutralize trypsin and mix by pipetting. Transfer cell suspension to a universal tube.
5. Count cells and normalize to give equivalent numbers across all samples (see Note 12).
6. Centrifuge at $400 \times g$ for 5 min at 4°C to pellet cells.
7. Aspirate supernatant and wash cell pellet by resuspending in 10 ml ice-cold PBS. Centrifuge for 5 min at $400 \times g$ at 4°C and aspirate supernatant (see Note 13).
8. Resuspend in 1 ml PBS and transfer to a microcentrifuge tube. Pellet cells and aspirate supernatant.

3.1.3. Cell Lysis

It is essential to keep the samples cold throughout the lysis and immunoprecipitation in order to limit the activity of deubiquitininating enzymes. Keep samples on ice or in a cold room throughout and use a temperature-controlled centrifuge set to 4°C. Addition of IAA or NEM to the lysis buffer will inactivate most deubiquitininating enzymes via alkylation of their active site cysteine residue.

1. Resuspend cell pellet in 1 ml Triton X-100 lysis/immunoprecipitation buffer. Mix well by pipetting or vortexing for 10 s. Alternatively lyse cells under denaturing conditions in 100 μ l 1% SDS (see Note 14).
 2. Incubate for 30 min on ice.
 3. Centrifuge at 14,000 $\times g$ for 15 min at 4°C to pellet nuclei and cell debris.
 4. Transfer 950 μ l post-nuclear supernatant to a fresh 1.5 ml microcentrifuge tube.
-
1. Preclear: Add 20 μ l of prewashed IgG-Sepharose beads to lysates and incubate on an end-over-end rotator at 4°C for 1 h (see Note 15).

2. Centrifuge at $14,000 \times g$ for 5 min to pellet beads and remaining cell debris.
3. Transfer supernatant to a fresh microcentrifuge tube. Leave 50 μ l residual supernatant to avoid carrying IgG-Sepharose over to the immunoprecipitation (see Note 16).
4. Add MHC class I antibody to the precleared supernatant and incubate for 1 h on a rotator at 4°C (see Notes 17 and 18).
5. Add 25 μ l of pre-washed Protein A-Sepharose beads (50% v/v suspension in TBS).
6. Incubate on a rotator at 4°C for 2 h (can incubate overnight).
7. Centrifuge at $10,000 \times g$ for 1 min at 4°C to pellet beads. Discard supernatant.
8. Wash beads once with 1 ml lysis buffer and three times with 1 ml ice-cold wash buffer as follows: add 1 ml buffer, vortex to resuspend, pellet beads at $10,000 \times g$ for 1 min, discard supernatant.
9. After final wash, carefully aspirate wash buffer from bead pellet.
10. Elute protein in 25–50 μ l of 2×SDS sample buffer and heat to 95°C for 5 min.
11. Centrifuge at $10,000 \times g$ for 1 min to pellet beads and use supernatant for SDS-PAGE analysis.

3.1.5. SDS-PAGE

1. Make a 10% polyacrylamide minigel:
 - (a) Combine 4 ml water, 2.5 ml resolving gel buffer, and 3.5 ml acrylamide solution. Add 50 μ l 10% APS and 7.5 μ l TEMED and mix. Immediately pour between glass plates of assembled minigel apparatus avoiding the introduction of air bubbles.
 - (b) Add a layer of water-saturated butanol above the gel and leave to polymerize for 30 min.
 - (c) Wash off the butanol, remove excess water using filter paper, and add stacking gel mixture prepared by mixing (in this order) 3 ml water, 1.25 ml stacking gel buffer, 0.75 ml acrylamide solution, 50 μ l 10% APS, and 5 μ l TEMED.
 - (d) Insert well comb and leave to polymerize for 15 min.
2. Load 20 μ l supernatant (obtained in Subheading 3.1.4 step 11) per lane on an 8–10% polyacrylamide gel.
3. Run pre-stained molecular weight markers in a free lane.
4. Run gel according to the manufacturer's instructions.

3.1.6. Transfer of Ubiquitinated Proteins to Membranes

High molecular mass polyubiquitinated proteins do not transfer readily from gels to membranes. Wet transfer is a reliable method for transfer of large proteins (23). Alternatively, we find “semi-dry” transfer using the Bio-Rad Trans-Blot system to be effective for transfer of polyubiquitinated MHC class I.

1. Separate glass plates and remove stacking gel. Carefully detach the gel from the plate and place in transfer buffer (see Note 19).
2. Wet PVDF membrane in methanol for 10 s and then soak in transfer buffer.
3. Soak two sheets of extra-thick filter paper (cut to the same size as the gel) in transfer buffer and assemble as per manufacturer’s instructions (see Note 20).
4. Transfer at 25 V for 70–90 min (see Note 21).

3.1.7. Denaturation of Ubiquitinated Proteins on Membrane

Denaturation of the PVDF membrane in 6 M guanidine renders the ubiquitin epitopes more accessible to the antibody (24). We find this enhances the detection of ubiquitinated MHC class I when blotting with P4D1 antibody (see Note 22).

1. Immediately after transfer, place the PVDF membrane in 10 ml membrane denaturing solution. The membrane will become transparent.
2. Incubate on a rocker for 30 min at 4°C.
3. Wash membrane thoroughly in 0.05% TBS-T—three to four quick washes.

3.1.8. Immunoblotting

Do not allow the PVDF membrane to dry out during the immunoblotting procedure.

1. Add 10 ml blocking buffer. Incubate overnight on a rocker at 4°C.
2. Incubate with P4D1 antibody 1:1,000 in blocking buffer for 1 h at room temperature (see Note 23).
3. Wash three times for 10 min in 0.05%TBS-T.
4. Incubate with anti-mouse-HRP 1:10,000 in 3% BSA/0.05% TBS-T for 1 h at room temperature.
5. Wash four times for 5 min in 0.1% TBS-T.
6. Develop with West Pico chemiluminescent (ECL) substrate (Pierce). Ubiquitinated MHC class I appears as a high molecular-mass smear (Fig. 1).

3.2. Radioimmune Precipitation of Ubiquitinated MHC Class I

Follow local rules for the safe handling of radioactivity throughout this procedure.

1. Harvest 2×10^6 cells per sample into a universal tube as described in Subheading 3.1.2 and wash with 20 ml warm PBS (see Note 24).

2. Resuspend the cell pellet in 1 ml starve medium and incubate in a 37°C water bath for 20 min (see Note 25).
3. Pellet cells at $400 \times g$ for 5 min at room temperature or above (see Note 26).
4. Remove the starve medium without disturbing the pellet (see Note 27).
5. Gently resuspend the cell pellet in 100 μl prewarmed labeling medium (see Note 28) and incubate at 37°C for 10 min.
6. Add 2 ml “chase” medium, swirl to mix, and immediately remove 1 ml of cell suspension to 10 ml ice-cold PBS in a universal tube. This is your “pulse” sample (see Note 29).
7. Incubate remaining sample at 37°C for 30–90 min, agitating at 30 min intervals to prevent cells adhering to the container.
8. Harvest the “chase” sample by removing 1 ml of cell suspension into 10 ml ice-cold PBS.
9. Incubate cells on ice for 5 min.
10. Pellet cells at $400 \times g$ for 5 min at 4°C.
11. Discard the supernatant without disturbing the cell pellet.
12. Resuspend cell pellet in 1 ml ice-cold lysis buffer and transfer samples to a 1.5 ml microcentrifuge tube on ice. Incubate on ice for 20 min.
13. Pellet nuclei and debris by centrifugation at $14,000 \times g$ for 15 min at 4°C and transfer 950 μl of post-nuclear supernatant to a fresh microcentrifuge tube on ice (see Note 30).
14. Perform pre-clear, primary MHC class I immunoprecipitation and wash beads as described in Subheading 3.1.4 steps 1–9 (see Note 31).
15. Elute MHC class I by adding 100 μl of SDS-denaturation buffer to washed beads and heat to 70°C for 10 min. Pulse tubes in the microfuge to remove condensation from the lid, then add 1 ml of lysis/immunoprecipitation buffer to sequester the SDS.
16. Pellet beads at $10,000 \times g$ for 1 min and transfer 1 ml of supernatant to fresh microfuge tubes on ice (see Note 32).
17. Reimmunoprecipitation—add the antibody to be used for the reimmunoprecipitation and 20 μl of Protein A-Sepharose and incubate on a rotator at 4°C overnight (see Note 33).
18. Wash beads and elute in 2 \times SDS sample buffer as described in Subheading 3.1.4, steps 7–11.
19. Load 20 μl /lane onto a 10% polyacrylamide gel and run as per manufacturer’s instructions as described in Subheading 3.1.5.
20. Treat gel in gel fix solution for 15 min. Dry on an air-dryer and expose (see Note 34, Fig. 2).

3.3. Conclusion

Determination of whether or not a protein is ubiquitinated *in vivo* may be challenging. The protocols we have described rely on detection of endogenous ubiquitin–MHC class I conjugates. If these methods fail then expression of epitope-tagged ubiquitin in cells (e.g., His6, HA-, or myc-tagged) may enhance the sensitivity for detection of ubiquitination. MHC I–ubiquitin conjugates can be isolated by affinity purification or immunoprecipitation of epitope-tagged ubiquitin followed by MHC class I immunoblot (or vice versa). Please see references for protocols (24, 25). Other methods which may provide indirect evidence for MHC I ubiquitination include stabilization of MHC class I on expression of ubiquitin mutants, on inactivation of a temperature-sensitive E1 mutant, or by depletion of ubiquitinating enzymes (14, 16, 26). Once ubiquitination has been determined then the site of ubiquitination and ubiquitin chain-linkage may be established through expression of ubiquitin mutants, linkage-specific antibodies targeted to lysine-48 or lysine-63 polyubiquitin chains (27), MHC class I mutagenesis, and proteomic approaches (1, 10, 16, 28). Purification of ubiquitinated proteins for proteomic analysis has been facilitated by the development of antibodies which recognize the diglycine remnant left at sites of ubiquitination after trypsin digestion (28, 29) and tandem-repeated ubiquitin-binding entities (TUBES) which bind to polyubiquitinated proteins and inhibit proteasomal degradation and deubiquitination (30).

4. Notes

1. Aliquots of IAA and PMSF can be stored at -20°C; however, avoid repeat freeze–thaw cycles which will result in loss of activity. NEM should be made fresh each time.
2. Sepharose beads must be washed free of preservative prior to use. We rehydrate and wash the beads overnight in 50 ml TBS rotating at 4°C. The beads are pelleted by centrifugation (without a brake to minimize the loss of beads with each spin), washed a further two times in TBS, and stored at 4°C in 50 ml TBS containing 0.1% azide to prevent bacterial growth. For an experiment, the required volume of beads is transferred to a microcentrifuge tube, washed three times with 1 ml Triton-X-100 wash buffer, and resuspended in TBS as a 50% volume:volume slurry ready for use.
3. Store aliquots at -20°C. Avoid repeat freeze–thaw cycles to prevent loss of DTT activity.
4. We complete the 100 ml bottle of cysteine/methionine free medium with 10 ml of dialyzed FBS, 2 ml of 1 M HEPES, and 1 ml of 200 mM glutamine and make 5–10 ml aliquots of the

prestarve medium which we store at -20°C for up to 1 year. We will refreeze and reuse the prestarve medium once but do not recommend refreezing a third time.

5. We use EasyTagTM EXPRESS35S Protein Labeling Mix from Perkin Elmer (NEG772). It has a high specific activity (>1,000 Ci/mmol) and is available in a variety of sizes. It is a mixture of (³⁵S)-methionine and L-(³⁵S)-cysteine harvested from *E. coli* grown in the presence of $^{35}\text{SO}_4$ with an added stabilizer. We routinely store our label at 4°C but for long-term storage it can also be stored in aliquots at -20°C. Other forms of (³⁵S)Cys/Met can be used but we find this a very cost effective solution which yields good results.
6. The chase medium can be supplemented with excess cold L-cysteine (7.2 mg/10 ml medium, add fresh) and methionine (100× stock methionine: 45 mg/ml in dH₂O, -20°C), but if sufficient chase medium is added (a minimum 10× excess) then addition of extra cold amino acids is unnecessary.
7. Interferon-γ treatment upregulates MHC class I expression and may enhance the detection of ubiquitinated MHC class I (12).
8. The peptide aldehyde protease inhibitors MG132 and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) are cheap and usually sufficiently effective to demonstrate ubiquitinated MHC class I. They primarily inhibit the chymotrypsin-like activity of the proteasome but also inhibit other cellular cysteine proteases (31). Lactacystin is a more specific proteasome inhibitor but is significantly more expensive. In practice, the main problem with these inhibitors is toxicity which limits the time and dose to which cells can be exposed. The most effective non-toxic dose will need to be determined for each cell type used. In HeLa cells 40–50 μM MG132 for up to 5 h is effective without causing significant toxicity. We often see a reduction in steady-state levels of MHC class I after treatment with proteasome inhibitors which may be due to transcriptional inhibition or possibly to limiting peptide supply (32). It should be noted that MG132 and ALLN also inhibit some lysosomal proteases (31) and extended treatment with MG132 may inhibit lysosomal degradation via depletion of free ubiquitin (33).
9. Stock solutions of proteasome inhibitor in DMSO can be stored at -20 to -80°C. Ensure that stocks are sufficiently concentrated to avoid adding large amounts of DMSO to cell culture media. An equivalent amount of DMSO should be added to control cells not treated with proteasome inhibitor to control for nonspecific DMSO effects.
10. MG132 and ALLN are reversible and therefore in situations where MHC class I is degraded rapidly, for example, in the

presence of US2 or US11, they should be added to the neutralizing medium, PBS washes, and lysis buffer. Alternatively use an irreversible proteasome inhibitor such as Lactacystin.

11. MHC class I is trypsin resistant.
12. Alternatively samples can be normalized according to the total protein concentration of the lysate before proceeding to preclear.
13. 10 mM NEM can be added to the neutralizing medium and PBS washes to inhibit the activity of deubiquitinating enzymes.
14. Denaturation of the cell extract before immunoprecipitation, by lysing in SDS, disrupts protein–protein interactions, and so enables specific detection of ubiquitinated MHC class I itself. Denaturing lysis has the added benefit of inactivating deubiquitinating enzymes.

Add 100 µl 1% SDS lysis buffer containing 1 µl (~250 U) of Benzonase to cell pellet and resuspend by vortexing. Keep lysis buffer at room temperature or above to avoid precipitation of the SDS. Incubate lysate for 10 min at room temperature to allow the Benzonase to act and then heat to 90°C for 10 min to denature the extract. Benzonase degrades released nucleic acids and prevents the sample becoming viscous. It is active from 0 to 42°C and retains 100% activity in 1% SDS.

Add 1 ml 1% Triton-X-100 immunoprecipitation buffer to the sample to sequester the SDS before proceeding to centrifugation (to avoid denaturing the immunoprecipitation antibody).

15. The preclear removes proteins that bind nonspecifically to beads or antibody from the lysates.
16. Carry-over of proteins bound nonspecifically to beads from the pre-clear to the immunoprecipitation will lead to background signal on ubiquitin blot or radiolabel. It is therefore better to leave a larger volume of residual supernatant rather than risk disrupting the bead pellet.
17. The choice of antibody will be determined by the particular MHC class I allele(s) being studied and the expected conformation at the time of ubiquitination. Conformational HLA-A/-B/-C heavy chains in complex with β2-microglobulin can be immunoprecipitated with W6/32, a commercially available mouse monoclonal antibody (21), or with an anti-β2m antibody (Dako rabbit polyclonal works well). Allotype-specific conformational MHC class I antibodies are also available. Non-β2m bound MHC class I heavy chains can be immunoprecipitated with HC10, HCA2, and 3B10.7. These “non-conformational” MHC class I antibodies must be used if the cell extract has been denatured in SDS. HC10 recognizes most B and C alleles but is poorly reactive with many HLA-A alleles (22). HCA2 reacts preferentially with HLA-A alleles but also

recognizes several B alleles. 3B10.7, a rat monoclonal antibody, has a broad specificity for HLA-A, -B, and -C alleles.

18. The optimal amount of antibody for immunoprecipitation is highly antibody-dependent; however, a range of 1–5 µg per 1 ml sample is typical. We find that 1 µg of purified W6/32, HC10, or anti-β2m antibody in 1 ml lysate containing ~1 mg total protein works well to give a maximal pulldown of MHC class I.
19. Pre-wetting the gel in transfer buffer allows it to be detached from the plate more easily and renders it less liable to break. Insert a spacer under one end and gently peel the gel off the plate.
20. Remove air bubbles from between the gel, membrane, and filter paper layers by rolling a pipette over the surface of the filter paper.
21. Monitor during transfer to ensure that the transfer apparatus does not overheat. The risk of overheating can be minimized by ensuring that the filter paper is thoroughly soaked in transfer buffer, not transferring more than two minigels concurrently and setting a maximum current limit of 400 mA (Bio-Rad recommend a limit of 5.5 mA/cm² for minigels).
22. Alternative methods of denaturation include autoclaving the membrane in deionized water for 30–40 min. See references for further details (34, 35).
23. A wide-range of ubiquitin-specific antibodies are commercially available. To date we have had the most success in detecting polyubiquitination by immuoblot using P4D1, a mouse monoclonal antibody with specificity for polyubiquitin-protein conjugates, free polyubiquitin chains, and free ubiquitin.
24. Cells should NOT be in G0 phase or they will not label well. We routinely passage our cells the day before labeling to ensure they are metabolically active. Different cells express variable levels of MHC class I; however, 1 million cells per lane usually yields good levels of signal. If both a pulse and a chase sample are required, start with 2 million cells for each cell type/treatment.
25. The duration of the starve period can be varied. Indeed, some authors argue that any period of amino acid starvation can induce an ER stress response. We find that a 20 min starve works well in HeLa cells without inducing a stress response. A starve becomes most important when very short labeling periods are used.

If proteasomal or lysosomal inhibitors are required we usually add these to the starve medium, to the labeling solution, and to the chase medium.

26. It is important to keep the cells metabolically active, so avoid spinning the cells down at low temperatures. Our non-temperature controlled benchtop centrifuge can reach temperatures of almost 37°C if run at full speed at for 5–10 min prior to pelleting the cells, although a temperature-controlled centrifuge set to 37°C is a better option.
27. Pouring off the supernatant and then removing the last few drops with a p200 pipette tip (without putting the tube upright again) works well to remove the majority of the medium without disturbing the cell pellet. Alternatively, a p200 tip on the end of a p1000 tip can also be used to remove the 1 ml of prestarve medium.
28. When pipetting solutions containing significant quantities of radiolabel, we use aerosol resistant tips to minimize contamination of our pipettes. Care should also be taken not to create aerosols when resuspending the cells.
29. The pulse time can be varied anywhere from 1 min to 16 h depending on the metabolic activity of the cells and the level of ubiquitination seen on MHC class I. Viral inducers of MHC I ubiquitination target newly synthesized MHC class I and so polyubiquitination can be visualized with short labeling and chase times. Degradation of endogenous MHC class I heavy chains in the absence of viral proteins is much slower and detecting ubiquitination may require a chase of 3–4 h.
30. By leaving 5% residual supernatant the chance of disturbing the pellet is reduced resulting in cleaner immunoprecipitations.
31. As an alternative to preclearing the supernatant, the protein A beads can be pre-incubated with a cell lysate from unlabelled cells prior to immunoprecipitation. Non-specific binding will therefore be non-radiolabeled proteins and will not contribute to background signal.
32. To assess the efficiency of the primary IP, 20 µl of 6× SDS loading buffer can be added to the beads and the remaining supernatant. After heating to 95°C for 5 min, a fraction can be analyzed by SDS-PAGE.
33. For the reimmunoprecipitation, use an antibody that recognizes a denatured MHC class I epitope, e.g., HC10. This step reduces the background signal and enhances the detection of high molecular weight ubiquitinylated MHC class I species (Fig. 2). The identity of these species can be confirmed by reimmunoprecipitation with an ubiquitin-specific antibody. Note that in our hands, P4D1 does not immunoprecipitate monoubiquitinated MHC class I heavy chains (16).
34. Dried gels can be exposed to a Phosphorimager screen overnight but often the ubiquitinylated MHC class I bands will take

longer to visualize. We often expose over a weekend to see the ubiquitination pattern and then for 2–4 weeks against film to get a publication quality image.

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References

- Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J* 25(8):1635–1645. doi:[7601056](#)
- Burr ML, Cano F, Svobodova S, Boyle LH, Boname JM, Lehner PJ (2011) HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. *Proc Natl Acad Sci U S A* 108(5):2034–2039. doi:[1016229108](#)
- Hughes EA, Hammond C, Cresswell P (1997) Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc Natl Acad Sci U S A* 94(5):1896–1901
- Shamu CE, Story CM, Rapoport TA, Ploegh HL (1999) The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J Cell Biol* 147(1):45–58
- Shamu CE, Flierman D, Ploegh HL, Rapoport TA, Chau V (2001) Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. *Mol Biol Cell* 12(8):2546–2555
- Furman MH, Loureiro J, Ploegh HL, Tortorella D (2003) Ubiquitylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum. *J Biol Chem* 278(37):34804–34811. doi:[10.1074/jbc.M300913200](#)
- Stagg HR, Thomas M, van den Boomen D, Wiertz EJ, Drabkin HA, Gemmill RM, Lehner PJ (2009) The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. *J Cell Biol* 186(5):685–692. doi:[jcb.200906110](#)
- Boname JM, Stevenson PG (2001) MHC class I ubiquitination by a viral PHD/LAP finger protein. *Immunity* 15(4):627–636. doi:[S1074-7613\(01\)00213-8](#)
- Wang X, Connors R, Harris MR, Hansen TH, Lybarger L (2005) Requirements for the selective degradation of endoplasmic reticulum-resident major histocompatibility complex class I proteins by the viral immune evasion molecule mK3. *J Virol* 79(7):4099–4108. doi:[79/7/4099](#)
- Wang X, Herr RA, Chua WJ, Lybarger L, Wiertz EJ, Hansen TH (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J Cell Biol* 177(4):613–624. doi:[jcb.200611063](#)
- Wang X, Herr RA, Rabelink M, Hoeben RC, Wiertz EJ, Hansen TH (2009) Ube2j2 ubiquitinates hydroxylated amino acids on ER-associated degradation substrates. *J Cell Biol* 187(5):655–668. doi:[jcb.200908036](#)
- Hewitt EW, Duncan L, Mufti D, Baker J, Stevenson PG, Lehner PJ (2002) Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation. *EMBO J* 21(10):2418–2429. doi:[10.1093/emboj/21.10.2418](#)
- Cadwell K, Coscoy L (2005) Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science* 309(5731):127–130. doi:[309/5731/127](#)
- Duncan LM, Nathan JA, Lehner PJ (2010) Stabilization of an E3 ligase-E2-ubiquitin complex increases cell surface MHC class I expression. *J Immunol* 184(12):6978–6985. doi:[jimmunol.0904154](#)
- Rhodes DA, Boyle LH, Boname JM, Lehner PJ, Trowsdale J (2010) Ubiquitination of lysine-331 by Kaposi's sarcoma-associated herpesvirus protein K5 targets HFE for lysosomal degradation. *Proc Natl Acad Sci U S A* 107(37):16240–16245. doi:[1003421107](#)
- Boname JM, Thomas M, Stagg HR, Xu P, Peng J, Lehner PJ (2010) Efficient internalization of

- MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. *Traffic* 11(2):210–220. doi:[TRA1011](#)
17. Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384(6608):432–438. doi:[10.1038/384432a0](#)
 18. Wiertz EJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL (1996) The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84(5):769–779. doi:[S0092-8674\(00\)81054-5](#)
 19. Coscoy L, Ganem D (2000) Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc Natl Acad Sci U S A* 97(14):8051–8056. doi:[10.1073/pnas.140129797](#)
 20. Ishido S, Wang C, Lee BS, Cohen GB, Jung JU (2000) Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J Virol* 74(11):5300–5309
 21. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14(1):9–20. doi:[0092-8674\(78\)90296-9](#)
 22. Stam NJ, Vroom TM, Peters PJ, Pastoors EB, Ploegh HL (1990) HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. *Int Immunol* 2(2):113–125
 23. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76(9):4350–4354
 24. Bloom J, Pagano M (2005) Experimental tests to definitively determine ubiquitylation of a substrate. *Methods Enzymol* 399:249–266. doi:[S0076-6879\(05\)99017-4 \(pii\)](#)
 25. Laney JD, Hochstrasser M (2011) Analysis of protein ubiquitination. *Curr Protoc Protein Sci* Chapter 14:Unit1415. doi:[10.1002/0471140864.ps1405s66](#)
 26. Kikkert M, Hassink G, Barel M, Hirsch C, van der Wal FJ, Wiertz E (2001) Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. *Biochem J* 358(Pt 2):369–377
 27. Newton K, Matsumoto ML, Wertz IE, Kirkpatrick DS, Lill JR, Tan J, Dugger D, Gordon N, Sidhu SS, Fellouse FA, Komives L, French DM, Ferrando RE, Lam C, Compaan D, Yu C, Bosanac I, Hymowitz SG, Kelley RF, Dixit VM (2008) Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell* 134(4):668–678. doi:[S0092-8674\(08\)00959-8](#)
 28. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, Sowa ME, Rad R, Rush J, Comb MJ, Harper JW, Gygi SP (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 44(2):325–340. doi:[S1097-2765\(11\)00675-7](#)
 29. Xu G, Paige JS, Jaffrey SR (2010) Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat Biotechnol* 28(8):868–873. doi:[nbt.1654](#)
 30. Hjerpe R, Aillet F, Lopitz-Otsoa F, Lang V, England P, Rodriguez MS (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep* 10(11):1250–1258. doi:[embor2009192](#)
 31. Lee DH, Goldberg AL (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 8(10):397–403. doi:[S0962-8924\(98\)01346-4](#)
 32. Mimnaugh EG, Chen HY, Davie JR, Celis JE, Neckers L (1997) Rapid deubiquitination of nucleosomal histones in human tumor cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and the cellular stress response. *Biochemistry* 36(47):14418–14429. doi:[10.1021/bi970998j](#)
 33. Melikova MS, Kondratov KA, Kornilova ES (2006) Two different stages of epidermal growth factor (EGF) receptor endocytosis are sensitive to free ubiquitin depletion produced by proteasome inhibitor MG132. *Cell Biol Int* 30(1):31–43. doi:[S1065-6995\(05\)00226-X](#)
 34. Mimnaugh EG, Neckers LM (2005) Measuring ubiquitin conjugation in cells. *Methods Mol Biol* 301:223–241. doi:[1-59259-895-1:223](#)
 35. Swerdlow PS, Finley D, Varshavsky A (1986) Enhancement of immunoblot sensitivity by heating of hydrated filters. *Anal Biochem* 156(1):147–153. doi:[0003-2697\(86\)90166-1](#)

Chapter 10

Evaluation of Viral Interference with MHC Class I-Restricted Antigen Processing and Presentation Using a Flow Cytometry-Based Approach

**Daniëlle Horst, Maaike E. Ressing, Arend Mulder,
and Emmanuel J.H.J. Wiertz**

Abstract

The peptide content of MHC class I molecules present at the cell surface is monitored by surveilling CD8⁺ cytotoxic T cells. In case of a viral infection, a proportion of the MHC class I molecules will carry peptides derived from viral proteins. This allows the CD8⁺ T cells to recognize and eliminate virus-infected cells. This highly sensitive detection system of the host is counteracted by viruses, which have acquired functions to downregulate cell surface expression of MHC class I molecules. In this chapter, we describe a flow cytometry-based method to identify viral gene product(s) responsible for evasion from MHC class I-restricted antigen presentation. To this end, cells are transiently transfected using polyethylenimine (PEI) as a transfection reagent, followed by cell surface staining with MHC class I-specific monoclonal antibodies. Once viral proteins responsible for MHC class I downregulation have been identified, their mechanism of action can be characterized. Identification and characterization of virus-encoded MHC class I inhibitors augments our understanding of virus–host interactions and often provides new insights into antigen processing and presentation pathways, including related cellular processes such as protein trafficking and degradation.

Key words: Immune evasion, Major histocompatibility complex class I, Human leukocyte antigen class I, Antigen presentation, Antigen processing, Transient transfection, Cell surface staining, Flow cytometry, Viral infection, Transporter associated with antigen processing

1. Introduction

The MHC class I-restricted antigen presentation pathway is an important component of the host immune defense against viral infections. Viruses rely on the protein synthesis machinery of their host; consequently, viral replication requires the production of

many viral proteins in the host cell. As part of normal protein turnover, a proportion of these viral proteins is degraded by cytosolic proteasomes. The resulting peptides are translocated into the ER lumen by the transporter associated with antigen processing (TAP). Within the ER lumen, these peptides are loaded onto newly synthesized MHC class I molecules. Subsequently, the MHC class I/peptide complexes travel to the cell surface for surveillance by CD8⁺ cytotoxic T lymphocytes (CTLs). Upon recognition of a viral (foreign) peptide, the infected cell will be eliminated by the CTL, thereby restricting viral infection.

To increase chances for survival and replication, viruses appear to have evolved sophisticated strategies to prevent elimination by the immune system. Especially large DNA viruses like herpesviruses, poxviruses, and adenoviruses carry a multitude of immune evasion functions, and also other viruses like HIV hamper immune detection. The MHC class I-restricted antigen presentation pathway is a frequent target for viral interference, with almost every step being affected by dedicated viral proteins (Fig. 1) (1–3). These strategies vary from inhibiting synthesis of MHC class I molecules to hampering trafficking of MHC class I/peptide complexes and inducing their degradation by either cytosolic proteasomes or lysosomal proteases. Furthermore, viruses interfere with the peptide supply for MHC class I binding: they can inhibit proteasome function or block TAP-mediated peptide transport into the ER. Finally, certain viruses target the final step, preventing CTL recognition of MHC class I molecules at the cell surface.

To screen whether a virus interferes with MHC class I-restricted antigen presentation, flow cytometry can be used as a fast and specific method. For this, cells should be infected with the virus-of-interest and stained for cell surface MHC class I expression. Since each virus requires a specific infection protocol, a procedure for viral infection is not described in this chapter. To distinguish virus-infected cells from non-infected control cells, it is recommended to use a virus carrying or encoding a fluorescent marker, e.g., GFP. Alternatively, infected cells can be visualized by double-staining with virus-specific and MHC class I-specific antibodies. Often, viral proteins that are expressed at early stages of infection remain intracellular. In such cases, infected cells can be fixed and permeabilized to facilitate intracellular staining of the viral proteins following surface MHC class I staining.

If the virus downregulates MHC class I molecules at the cell surface, the next step is to identify the viral gene product(s) responsible. A screening method that is particularly useful in this context relies on expression of individual viral genes by transient transfection. After transfection, cells are stained for cell surface expression of MHC class I molecules and analyzed by flow cytometry. In this chapter, we describe a transient transfection protocol based on the use of polyethylenimine (PEI) as a transfection reagent. PEI has

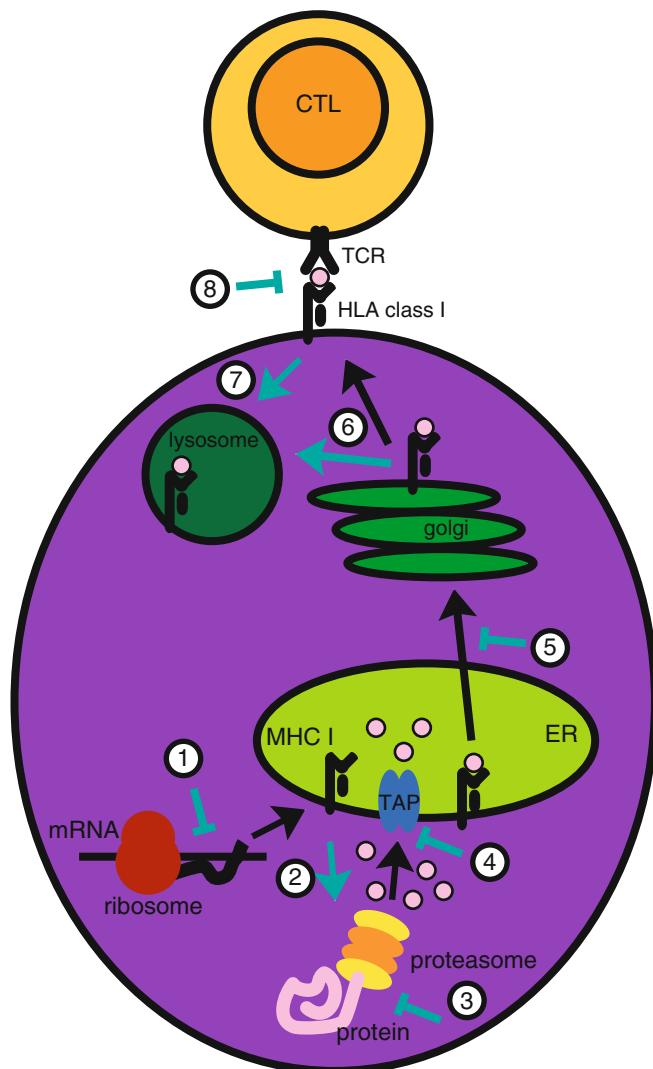


Fig. 1. Viral interference with MHC class I-restricted antigen presentation. Viruses subvert virtually every step of the MHC class I-restricted antigen presentation pathway: (1) inhibition of MHC class I synthesis, (2) degradation of MHC class I by cytosolic proteasomes, (3) blockage of proteasomal degradation, (4) obstruction of peptide transport into the ER via TAP, (5) inhibition of MHC class I trafficking, (6) redirection of MHC class I to lysosomes, (7) increase in endocytosis and lysosomal degradation of cell surface MHC class I, and (8) interference with recognition of cell surface MHC class I by T cell receptors of CTLs.

several advantages over other transfection agents and methods; it is very inexpensive compared to most commercial “ready to use” transfection reagents, it can be used in serum-supplemented medium, and is less harmful to the cells than electroporation. Furthermore, PEI transfections are easy to perform and usually are quite efficient, although the transfection efficiency depends on the cell line used. We recommend using a vector co-expressing the protein-of-interest and a fluorescent marker to easily distinguish

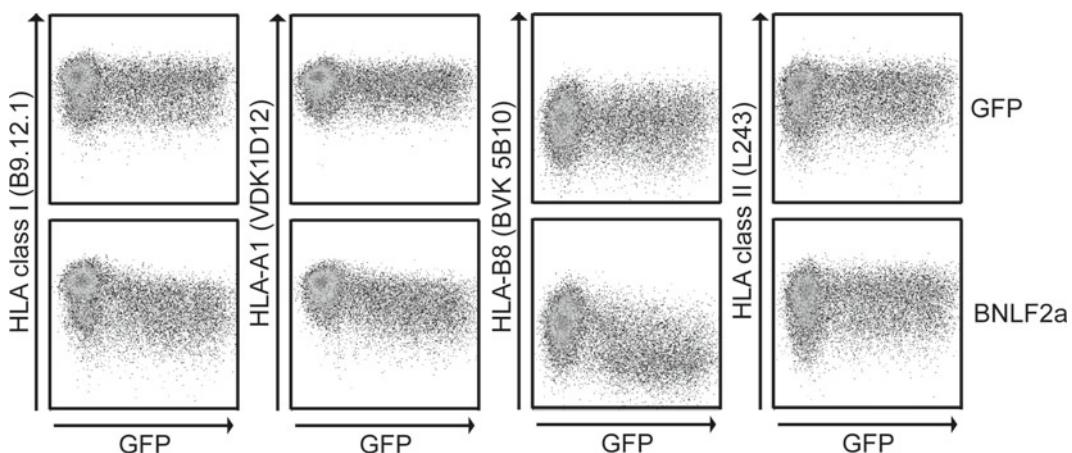


Fig. 2. Downregulation of cell surface MHC class I expression by the EBV TAP inhibitor BNLF2a. MJS cells were transiently transfected using PEI as a transfection reagent, resulting in expression of the control protein GFP (“GFP”) or co-expression of the Epstein–Barr virus-encoded TAP inhibitor BNLF2a and GFP (“BNLF2a”). After 24 h, cells were stained for cell surface expression of total HLA class I (mAb B9.12.1 (17)), HLA-A1 (mAb VDK1D12 (19)), HLA-B8 (mAb BVK 5B10 (19)), and HLA class II (mAb L243). Subsequently, the cells were analyzed by flow cytometry using CellQuest Pro (BD Biosciences) software.

transfected from non-transfected control cells. In addition, an indication of expression levels of the viral protein-of-interest can be obtained, since these usually correlate to the levels of the fluorescent reporter, especially when the viral protein and the reporter are expressed from the same transcript through an Internal Ribosomal Entry Site (IRES) or a picornaviral 2A-like sequence.

Additionally, we describe a protocol for surface staining of MHC class I molecules. The extent to which the expression of different MHC class I alleles is downregulated by a particular viral immune evasion protein may vary (Fig. 2). In addition to viral interference with surface display of MHC class I molecules, modulation by viral gene products may occur at other levels, not detectable with FACS analysis. For example, murine cytomegalovirus (MCMV) gp34 inhibits the recognition of MHC class I/peptide complexes by T cell receptors on CD8⁺ T cells (4). This category of MHC class I inhibitors will go unnoticed by the flow cytometry-based screening method described in this chapter, but can be identified using CTL recognition assays or TCR-like antibodies as described elsewhere in this volume (see Chapters 14, 20–22).

After identification of a viral protein interfering with MHC class I-restricted antigen presentation, cell lines stably expressing this viral protein can aid investigations into its mechanism of action. Approaches that may be used for this purpose have been described in detail in this volume (see Chapters 5, 6, 8, and 11). Metabolic labeling experiments can be extremely useful, for instance, to examine whether the immune evasion protein interferes with MHC class I synthesis or trafficking, or whether it induces degradation of MHC class I molecules via proteasomes in the cytosol or by

endosomal proteases ([5–7](#)) (Chapter [8](#)). Another frequent target of viral immune evasion proteins is the peptide transporter TAP ([1, 8](#)). Assays to analyze TAP function have been described by Jongsma and Neefjes (Chapter [5](#)) and have been used previously to successfully identify viral TAP inhibitors, e.g., the UL49.5 protein encoded by certain varicelloviruses and the BNLF2a protein of Epstein–Barr virus ([9–11](#)).

In conclusion, the MHC class I antigen presentation pathway represents a frequent target for viral immune evasion strategies. In this protocol, a flow cytometry-based method is described that may be used to scrutinize viruses for interference with MHC class I-restricted antigen processing and presentation. Once downregulation of MHC class I molecules has been detected at the cell surface of cells infected with the virus-of-interest, individual viral gene products may be screened to identify the orf(s) responsible for the observed phenotype. In this screen, a lentiviral expression vector is used for transient, simultaneous expression of the viral gene products and a fluorescent reporter. Using the same vector, lentiviruses can be produced for those viral proteins for which MHC class I downregulation is observed. This facilitates rapid establishment of stable cell lines instrumental for the final step: elucidation of the mechanisms underlying the observed interference with MHC class I-restricted antigen presentation.

2. Materials

2.1. Transient Transfection Using PEI

1. Cells expressing MHC class I (see Note 1).
2. 12-Well plate for cell culture (see Note 2).
3. Culture medium without antibiotics: complete culture medium lacking antibiotics. Medium used depends on the cell line-of-interest.
4. Plasmid expressing the gene-of-interest (see Note 3).
5. PEI solution: dissolve PEI powder (Polyethylenimine “Max”, (nominally Mw 40,000*)—High Potency Linear PEI (Equivalent to Mw 25,000 in Free Base Form) Polysciences, Inc.) to a concentration of 1 mg/ml in high purity water. This might take a couple of hours during which the solution should be shaken vigorously and can be facilitated by heating the solution to 80°C. Allow the solution to cool to room temperature, adjust the pH to 7.4 with HCl, and filter-sterilize it. Aliquot the PEI solution and store at –80°C. The working aliquot can be stored temporary at 4°C.
6. 150 mM NaCl, filter-sterilized. Store at room temperature.
7. Polypropylene tubes.

2.2. Cell Surface Staining and Flow Cytometry

1. PBS.
2. PBA: PBS with 0.5% BSA and 0.02% sodium azide (see Notes 4 and 5). Store at 4°C.
3. 1% Paraformaldehyde: PBS with 1% paraformaldehyde, prepare freshly before usage.
4. Primary antibody (see Note 6). Dilute in PBA to appropriate concentration (see Note 7) and store at 4°C.
5. Fluorescently labeled secondary antibody. Dilute in PBA to appropriate concentration (see Note 7) and store at 4°C in the dark to prevent bleaching of the fluorophore.
6. 15 ml Tubes.
7. 96-Well plate, V-bottom.
8. FACS tubes.
9. Flow cytometer and CellQuest Pro (BD Biosciences) software.

3. Methods

3.1. Transient Transfection Using PEI

1. Seed cells (~100,000–200,000/well) in a 12-well plate in 1 ml of culture medium without antibiotics. Cells should be around 70% confluent the next day.
2. Incubate overnight in CO₂ incubator (see Note 8).
3. Refresh the culture medium without antibiotics before the transfection.
4. Prepare the transfection mixture in polypropylene tubes (see Note 9). The amounts indicated below have been optimized for MJS cells.
 - (a) Add 0.8 µg of DNA (see Note 10) to 80 µl of 150 mM NaCl, vortex carefully during addition of the DNA.
 - (b) Add 5.2 µl of PEI to 80 µl of 150 mM NaCl, vortex carefully during addition of the PEI.
 - (c) Add the PEI solution to the DNA solution and vortex for 10 s.
 - (d) Incubate the transfection mixture at room temperature for 15–30 min.
5. Add the transfection mixture dropwise to the cells.
6. Gently shake the culture plate to ensure the transfection mixture is equally distributed in the medium.
7. Incubate for 24–72 h in CO₂ incubator (see Note 11).

3.2. Cell Surface Staining and Flow Cytometry

Work according to the local restrictions for working with transfected cells. All steps should be performed at 4°C/on ice unless indicated otherwise.

1. Collect the cells in a 15 ml tube (100,000 cells/staining) (see Note 12).
2. Wash the cells with PBS.
3. Add PBA to the cell pellet (100 µl PBA/staining).
4. Pipet 100 µl of the cell suspension per well of a 96-well plate (V-bottom).
5. Spin down the cells ($200 \times g$, 2 min).
6. Discard the supernatant.
7. Resuspend the cells using a vortex (maximum speed).
8. Add 20 µl of the primary antibody solution (see Note 13).
9. Mix gently using a vortex.
10. Incubate for 30 min.
11. Wash the cells twice with PBA.
12. Add 20 µl of the fluorescently labeled secondary antibody solution (see Note 14).
13. Mix gently using a vortex.
14. Incubate for 30 min in the dark.
15. Wash the cells twice with PBA.
16. Add 100 µl 1% paraformaldehyde (see Note 15).
17. Incubate for 15 min in the dark.
18. Transfer cells to a FACS tube.
19. Analyze the cells using a flow cytometer (see Note 16).

4. Notes

1. We regularly use Mel JuSo (MJS) cells (12); these cells can be transfected with high efficiency and express both MHC class I and MHC class II molecules at the cell surface. Moreover, MJS expresses HLA-A1 and -B8 molecules, which are relatively dependent on antigenic peptides for proper surface expression. For these reasons, the MJS cells have been particularly useful for studies on viral immune evasion (9–11, 13). Other cells can be used provided that they express MHC class I and can be efficiently transfected.
2. When a 6-well plate is used, the amounts mentioned in Subheading 3.1 should be doubled.

3. To facilitate analysis, it is recommended to use a plasmid that co-expresses the gene-of-interest and GFP or some other fluorescent marker. We regularly use plasmids derived from the lentiviral vector pLV-CMV-IRES-eGFP (14). This vector gives high expression levels in MJS and HeLa cells, amongst others. Additionally, this vector can be used to produce lentiviruses, allowing fast generation of cell lines stably expressing the gene-of-interest (15).
4. BSA blocks non-specific antibody binding and sodium azide prevents internalization of surface antigens.
5. Sodium azide is highly toxic, handle with care and according to the local restrictions.
6. For most applications, mAbs with pan reactivity for MHC class I molecules (i.e., irrespective of the HLA typing) will suffice, such as W6/32 (16) or B9.12.1 (17). In the example of Fig. 2, we show the additional use of mAbs reactive with distinct alloantigens.
7. Optimal concentration of the antibodies should be determined by titration.
8. Alternatively, cells can be seeded immediately before the transfection.
9. Optimal ratios of DNA and PEI should be determined in a pilot experiment and are cell line dependent.
10. Appropriate controls should be taken along in each experiment; e.g., negative control protein that has no effect on MHC class I levels, positive control protein that is known to down-regulate cell surface MHC class I levels.
11. Medium might be replaced by medium with antibiotics in the meantime, but this is not required.
12. Appropriate controls should be taken along in each experiment; e.g., secondary antibody only, isotype control, antibody against a cell surface protein that is not affected by the protein-of-interest.
13. For intracellular staining, the cells should be fixed and permeabilized before addition of the primary antibody.
14. In case a directly conjugated primary antibody is used, steps 12–15 should be omitted.
15. Alternatively, cells can be fixed before the antibody staining. However, certain antibodies lose reactivity after fixation, so this should be tested first.
16. The methods described in this paper may also be used to evaluate viral evasion of MHC class II-restricted antigen presentation (13), as well as viral subversion of other forms of adaptive and innate immunity (18), provided antibodies against the potential cellular target proteins are available.

Acknowledgements

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References

1. Horst D, Verweij MC, Davison AJ, Ressing ME, Wiertz EJ (2010) Viral evasion of T cell immunity: ancient mechanisms offering new applications. *Curr Opin Immunol* 23(1):96–103
2. Horst D, Ressing ME, Wiertz EJ (2011) Exploiting human herpesvirus immune evasion for therapeutic gain: potential and pitfalls. *Immunol Cell Biol* 89(3):359–366
3. Hansen TH, Bouvier M (2009) MHC class I antigen presentation: learning from viral evasion strategies. *Nat Rev Immunol* 9(7):503–513
4. Doom CM, Hill AB (2008) MHC class I immune evasion in MCMV infection. *Med Microbiol Immunol* 197(2):191–204
5. Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384(6608):432–438
6. Wiertz EJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL (1996) The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84(5):769–779
7. Reusch U, Muranyi W, Lucin P, Burgert HG, Hengel H, Koszinowski UH (1999) A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. *EMBO J* 18(4):1081–1091
8. Griffin BD, Verweij MC, Wiertz EJ (2010) Herpesviruses and immunity: the art of evasion. *Vet Microbiol* 143(1):89–100
9. Koppers-Lalic D, Reits EA, Ressing ME, Lipinska AD, Abele R, Koch J, Marcondes Rezende M, Admiraal P, van Leeuwen D, Bienkowska-Szewczyk K, Mettenleiter TC, Rijsewijk FA, Tampe R, Neefjes J, Wiertz EJ (2005) Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing. *Proc Natl Acad Sci U S A* 102(14):5144–5149
10. Hislop AD, Ressing ME, van Leeuwen D, Pudney VA, Horst D, Koppers-Lalic D, Croft NP, Neefjes JJ, Rickinson AB, Wiertz EJ (2007) A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. *J Exp Med* 204(8):1863–1873
11. Koppers-Lalic D, Verweij MC, Lipinska AD, Wang Y, Quinten E, Reits EA, Koch J, Loch S, Marcondes Rezende M, Daus F, Bienkowska-Szewczyk K, Osterrieder N, Mettenleiter TC, Heemskerk MH, Tampe R, Neefjes JJ, Chowdhury SI, Ressing ME, Rijsewijk FA, Wiertz EJ (2008) Varicellovirus UL 49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP. *PLoS Pathog* 4(5):e1000080
12. van Ham SM, Tjin EP, Lillemoer BF, Gruneberg U, van Meijgaarden KE, Pastoors L, Verwoerd D, Tulp A, Canas B, Rahman D, Ottenhoff TH, Pappin DJ, Trowsdale J, Neefjes J (1997) HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* 7(12):950–957
13. Ressing ME, van Leeuwen D, Verreck FA, Gomez R, Heemskerk B, Toebees M, Mullen MM, Jarde茨ky TS, Longnecker R, Schilham MW, Ottenhoff TH, Neefjes J, Schumacher TN, Hutt-Fletcher LM, Wiertz EJ (2003) Interference with T cell receptor-HLA-DR interactions by Epstein-Barr virus gp42 results in reduced T helper cell recognition. *Proc Natl Acad Sci U S A* 100(20):11583–11588
14. Vellinga J, Uil TG, de Vrij J, Rabelink MJ, Lindholm L, Hoeben RC (2006) A system for efficient generation of adenovirus protein IX-producing helper cell lines. *J Gene Med* 8(2):147–154
15. Horst D, Favoloro V, Vilardi F, van Leeuwen HC, Garstka MA, Hislop AD, Rabu C, Kremmer E, Rickinson AB, High S, Dobberstein B, Ressing ME, Wiertz EJ (2011) EBV protein BNLF2a exploits host tail-anchored protein integration machinery to inhibit TAP. *J Immunol* 186(6):3594–3605
16. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group

- A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14(1):9–20
17. Malissen B, Rebai N, Liabeuf A, Mawas C (1982) Human cytotoxic T cell structures associated with expression of cytolysis. I. Analysis at the clonal cell level of the cytolysis-inhibiting effect of 7 monoclonal antibodies. *Eur J Immunol* 12(9):739–747
18. van Gent M, Griffin BD, Berkhoff EG, van Leeuwen D, Boer IG, Buisson M, Hartgers FC, Burmeister WP, Wiertz EJ, Ressing ME (2011) EBV lytic-phase protein BGLF5 contributes to TLR9 down regulation during productive infection. *J Immunol* 186(3):1694–1702
19. Mulder A, Kardol MJ, Arn JS, Eijsink C, Franke ME, Schreuder GM, Haasnoot GW, Doxiadis II, Sachs DH, Smith DM, Claas FH (2010) Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. *Mol Immunol* 47(4):809–815

Chapter 11

Determining the Activity of the Transporter Associated with Antigen Processing in the Compartments of the Secretory Pathway

Esther Ghanem and Sebastian Springer

Abstract

Peptide-receptive MHC class I molecules and the TAP (transporter associated with antigen processing) peptide transporter are known to leave the ER and cycle through the *cis* side of the Golgi apparatus. The amount, and the extent of the activity, of TAP in post-ER compartments is likely to vary between different cell types. Here we describe a convenient microscopic assay to determine it.

Key words: TAP, Golgi, Saponin, Fluorescently labeled peptide

1. Introduction

MHC class I molecules are loaded with antigenic peptides prior to acquiring endoglycosidase H resistance in the medial Golgi (1). Traditionally, peptide loading has been assumed to take place in the endoplasmic reticulum (ER), but peptide-receptive class I molecules are known to leave the ER and travel through the early secretory pathway to the ER–Golgi intermediate compartment and to the *cis*-Golgi, from where they are retrieved to the ER (2, 3). The recent description of the direct channeling of freshly generated peptides into the class I pathway (4) suggests that antigen presentation may by spatially compartmentalized.

The TAP (transporter associated with antigen processing) is the main peptide transport activity into the ER in mammalian cells, and it is required for loading most class I allotypes with high-affinity peptides (5, 6). Via the TAP-associated protein, tapasin, and together with the chaperone proteins, ERp57 and calreticulin, it

binds to class I to form the peptide loading complex (PLC) (7). The TAP transporter, and possibly the PLC, at least partially accompany class I on its cycle through the *cis*-Golgi (8, 9).

To assess peptide loading in these and other intracellular locations, we have developed an assay for peptide transport into organelles that uses a fluorescent peptide and is based on microscopy as a readout. It is based on the work of the group of John Yewdell, who were the first to bind fluorescent peptides to peptide-receptive class I in cells (10) and on the work of Jacques Neefjes, who devised the first ATP-dependent assay for TAP (11), and it was first used by us to determine TAP activity in the Golgi apparatus of COS and RMA cells (9).

One specialty of the assay is that the translocated peptide is captured by a matching class I allotype to prevent it from leaking from the lumen of the respective organelle. Here, we describe a form of the assay that uses the murine class I molecule H-2K^b and the ovalbumin peptide, SIINFEKL (also called the OVA peptide (10)). In principle, any other allotype/peptide combination should be amenable (see Notes 1 and 2).

The assay should be adaptable to most murine and human cell lines and primary cells that grow in suspension or on solid support.

2. Materials

All solutions are made with ultrapure water, and reagents are of the highest grade available unless specifically mentioned.

2.1. Cell Culture and Transfection

1. Cells can be obtained from the appropriate culture collections such as ATCC or DSMZ. COS cells were used in this protocol (DSMZ) (see Note 3).
2. Media and supplements should be chosen as appropriate for the cells or cell lines. For the COS cells in this paper, standard DMEM (Dulbecco's modified Eagle's medium) with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine were used.
3. Cells are grown in an atmosphere of 5% CO₂ at 37°C.
4. Transfection medium: 120 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 0.15 mM CaCl₂, 25 mM HEPES, pH 7.2, 2 mM EGTA, 2 mM ATP, and 5 mM oxidized glutathione.
5. Plasmids encoding the class I allotype construct are prepared using maxipreparation kits from QIAGEN.
6. The plasmid construct encoding a fusion of human beta-2 microglobulin (β₂m) to the N terminus of the murine class I

allotype H-2K^b in the pEGFP-N1 vector (Clontech) was described in ref. 9. It is available from the authors (see Note 1).

7. Polylysine solution: 0.1% L-polylysine (Sigma) in water, sterile filtered.
8. Microscopy coverslips.
9. Electroporator and cuvette.

2.2. TAP Assay

1. Phosphate-buffered saline (PBS): 1.5 mM KH₂PO₄, 2.5 mM KCl, 10 mM Na₂HPO₄, 130 mM NaCl.
2. PBS-glycine: 100 mM glycine in PBS.
3. Fluorescently labeled peptide: For the choice of the peptide, see Note 13. The peptide used here was synthesized and purified by Biosyntan (Berlin, Germany). Fluorescently labeled peptides are usually quite hydrophobic and may need heating and/or sonication to dissolve in water even at low concentrations. The concentration of a solution can be measured by the absorption of the fluorophor. In the protocol here, the extinction coefficient of TAMRA is 65,000 M⁻¹ cm⁻¹ at 555 nm.
4. Saponin (Sigma).
5. 10× ATP regeneration mix: 1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase (Sigma) in 10 mM Tris-Cl pH 7.5.
6. Apyrase (ATPase, Sigma). The stock solution is 100 µg/ml.
7. The ICP47 protein is a soluble cytosolic protein encoded by the herpes simplex virus HSV-1 that inhibits human TAP in a competitive fashion (12). See also Note 14.

2.3. Fixation and Microscopy

1. 3% Formaldehyde (paraformaldehyde): A formalin (37% formaldehyde) solution is diluted with 12 parts of PBS. Filter through a 0.45 µm syringe filter. Store at 4°C and discard after one week.
2. Mowiol: 2.4 g of Mowiol 4-88 (Fluka via Sigma-Aldrich) is combined with 6 g of glycerol and 6 ml of water and stirred in a small glass beaker for 2 h. 14 ml of PBS are added, the beaker is covered, and the mixture is heated to 50°C and stirred overnight. The solution is then filtered through a 0.45 µm syringe filter and stored at -20°C.
3. Mouse monoclonal GM130 antibody (BD Biosciences, Nr. 610823).
4. Secondary antibody goat-anti-mouse IgG conjugated with Alexa Fluor 647 (Dianova, Hamburg, Germany, Nr. 115-605-062) or AF 633.
5. Confocal laser scanning microscope.

3. Methods

3.1. Transfection of Cells with H-2K^b-GFP Fusion Construct (See Note 4)

1. Sterilize microscopy coverslips by dipping them in 70% ethanol and passing them through a flame. Place them into tissue culture six-well plates. To each well, then add 1 ml cell culture medium. (For suspension cells, see Note 5.)
2. Use cells that are growing logarithmically at about 50% cell density. Trypsinize cells if required, and count approximately 10^6 cells (see Note 2). Sediment them by centrifugation at $200 \times g$. Wash the cells by gently resuspending them in PBS and sedimenting them again.
3. Discard the supernatant and resuspend cells in 100 μ l transfection medium.
4. Prepare a microcentrifuge tube with 10 μ g plasmid DNA in a maximum volume of 10 μ l. Add the cell suspension to this tube and mix by pipetting gently, without forming air bubbles.
5. Transfer to an electroporation cuvette with 2 mm gap width, and bring the cells to the bottom of the cuvette by gently tapping it onto the bench.
6. Electroporate using cell-specific settings (see Note 6). For COS cells, use two pulses of 500 V, 200 μ s.
7. Leave cells to sit in the electroporation cuvette for 10 min on ice. Then, add 500 μ l of prewarmed (37°C) medium and mix gently using the pipette. Transfer a few drops to each of the wells of the six-well plates that contain the coverslips (see Note 7).
8. Incubate for 24 h at 37°C in an atmosphere of 5% CO₂ to allow the cells to adhere to the glass and to express the transfected class I molecule.

3.2. Transport Assay (See Note 8)

1. Working on one well after another, withdraw the medium from the wells with a pipette or a vacuum tube fitted with a pipette tip. Add 1 ml PBS per well at room temperature, being careful not to pipette any liquid directly onto the cells.
2. Place the plates on ice, withdraw the PBS, and wash the cells once with 1 ml ice-cold PBS-glycine per well.
3. Add 500 μ l of cold saponin solution (concentration 0.005–0.05% in PBS, see Note 9) and incubate on ice for 3 min.
4. Withdraw the saponin and wash the cells with 1 ml cold PBS on ice.
5. Add as required (see Note 8 for the necessary controls):
 - (a) 50 μ l of the 10× ATP regenerating system
 - (b) 50 μ l of the apyrase stock solution

- (c) 50 µl of the 500 µM stock of the ICP47 peptide (see Note 10) in a total volume of 500 µl PBS. Incubate at 20–37°C (see Note 11) for 15 min.
6. Transfer the plate to room temperature and immediately add SIINFEKL-TAMRA peptide to 2 µM final concentration. Mix gently using the pipette and incubate for 1–2 min at 20°C.
1. Following the incubation, wash the cells twice with 1 ml each of PBS at 20°C.
2. Withdraw the PBS and add 1 ml of 3% paraformaldehyde in PBS. Incubate (to fix the cells) for 10 min at 20°C.
3. Add a mixture of monoclonal mouse anti-GM130 which was diluted 1/100 and secondary goat-anti-mouse Alexa Fluor 647/633 coupled antibody which was diluted 1/200 in PBS. Incubate in a moisturized chamber at room temperature for 40 min (see Note 12).
4. Withdraw the antibody mixture and wash twice with PBS.
5. Mount the coverslips onto microscopy slides with one drop of Mowiol. Incubate for 48 h at room temperature in a dark chamber to allow the Mowiol to set.

3.4. Detection

Observation of the cells should take place preferentially with a confocal laser scanning microscope using settings for the crosstalk-free detection of the fluorophores used.

4. Notes

1. It is a good idea to choose an allotype of class I for the assay that is not highly dependent on the presence of tapasin for surface expression. Highly tapasin-dependent allotypes are conformationally unstable and may be less inclined to bind peptide in the assay; when transfected into a cell, they may also be unable to reach the desired compartments if they are held back in complex with tapasin in the ER or if they are unable to fold properly without the assistance of tapasin. The murine molecule H-2K^b, used in this protocol, works well in our hands for the murine and human cell lines that we have tested.
2. In wild-type cells, murine class I molecules reach almost every compartment of the exocytic and endocytic secretory pathway, including endosomes and lysosomes, and may be used to trap transported peptide there (authors' unpublished observations). For human allotypes, peptide-receptive molecules are usually more stringently localized in the early secretory pathway, and

it might be necessary to induce peptide-receptive molecules in the endocytic pathway (if desired) by performing a low-pH treatment of the intact cells, leading to peptide loss and internalization of class I.

3. Make sure the cells are free of mycoplasma since in mycoplasma infection, disturbances of the secretory pathway may occur in some cells. Mycoplasma tests by PCR are now commercially available.
4. For the transfection, use any protocol previously established for the cell type. Transduction with lentiviruses may also work for the expression, while vaccinia expression is not recommended since it cannot be excluded that it may lead to disturbances of the secretory pathway. In the example here, electroporation is used, since it gives consistent results with a quick protocol.
5. Suspension cells should not be mounted on slides directly after transfection but only just before the assay. Following transfection, they are grown in individual wells of six-well plates for 24 h. Coverslips are then coated with 100 µl of polylysine solution for 10 min, washed three times with 100 µl of water, and air-dried. Suspension cells are centrifuged at 200 × g, 5 min at room temperature, and approx. 2×10^5 cells are resuspended in 100 µl of PBS and seeded on the L-polylysine-coated coverslips for 15 min at 37°C.
6. For the Eppendorf electroporator used in our laboratory, the following settings can be used: COS cells two pulses of 500 V, 200 µs; Raji cells 250 V, 40 µs; HeLa cells two pulses of 435 V, 400 µs.
7. The amount of cells transferred to each well has to be determined experimentally for each cell line. It is best to try different amounts of cell suspension in each experiment. After 24 h incubation, those samples should be processed further that have reached a confluence of 50–70%.
8. In each assay, use the following control samples:
 - (a) No saponin (unpermeabilized cells): Only the stain of peptide bound to the cell surface only should be visible. Allows the distinction of extra- and intracellular peptide binding.
 - (b) Apyrase instead of ATP (no energy): no stain inside any organelle should be visible. Controls for the dependence of transport on ATP.
 - (c) ICP47: Specifically inhibits human (and primate) TAP and thus controls for any non-TAP peptide transport activity in the sample. Not usable in murine cells since ICP47 does not inhibit rodent TAP.

- (d) No peptide: To assess crosstalk between the channels in microscopy.
 - (e) Untransfected cells: To control for the binding of peptide to intracellular structures other than the transfected MHC class I (for example, to endogenous class I or to histones in the nucleus).
9. The optimal concentration of saponin must be determined experimentally. The lowest concentration that leads to permeabilization of the plasma membrane should be used to avoid damage to the internal membranes.
 10. ICP47 is a competitive inhibitor of TAP, and for full activity, it needs to prebind to TAP before the peptide is added. The protocol takes this into account.
 11. The optimal temperature for incubation with ATP or apyrase has to be determined experimentally. For the COS cells in this protocol, it is 37°C.
 12. The mix of primary and secondary antibody is used to accelerate the protocol. It has to be determined experimentally depending on the cell type. A traditional staining method (first the primary, then wash with PBS, then the secondary) may be used if the class I-peptide complex is sufficiently stable.
 13. The peptide used as a fluorescent label should match the allotype used to trap it and it needs to be modified with a fluorophore at a side chain such that no interference to peptide binding occurs and that the complex of class I with peptide is stable for several hours; this must be tested in a binding assay. In the example here, the SIINFEKL peptide for H-2K^b is modified on the lysine side chain, which is known to point out of and away from the binding groove, with TAMRA (5(6)-carboxytetramethylrhodamine) succinimidyl ester.
 14. An active 35-amino acid fragment of ICP47, which is used in this assay, can be obtained by peptide synthesis ([13](#)). It needs to be HPLC purified. The ICP47 peptide is readily soluble in water, and the stock concentration is 500 µM.

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References

1. Townsend A, Bodmer H (1989) Antigen recognition by class I—restricted cytotoxic T lymphocytes. *Annu Rev Immunol* 7:601–624
2. Hsu VW, Yuan LC, Nuchtern JG, Lippincott SJ, Hä默ling GJ, Klausner RD (1991) A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules. *Nature* 352(6334):441–444
3. Garstka M, Borchart B, Al-Balushi M, Praveen PV, Kuhl NM, Majoul I, Duden R, Springer S (2007) Peptide-receptive MHC class I molecules cycle between endoplasmic reticulum and cis-Golgi in wild type lymphocytes. *J Biol Chem* 282(42):30680–30690
4. Lev A, Princiotta MF, Zanker D, Takeda K, Gibbs JS, Kumagai C, Waffarn E, Dolan BP, Burgevin A, Van Endert P, Chen W, Bennink JR, Yewdell JW (2010) Compartmentalized MHC class I antigen processing enhances immunosurveillance by circumventing the law of mass action. *Proc Natl Acad Sci U S A* 107(15):6964–6969
5. van Endert P (2011) Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell Mol Life Sci* 68(9):1553–1567
6. Beismann-Driemeyer S, Tampe R (2004) Function of the antigen transport complex TAP in cellular immunity. *Angew Chem Int Ed Engl* 43(31):4014–4031
7. Peaper DR, Cresswell P (2008) Regulation of MHC class I assembly and peptide binding. *Annu Rev Cell Dev Biol* 24:343–368
8. Paulsson KM, Kleijmeer MJ, Griffith J, Jevon M, Chen S, Anderson PO, Sjogren HO, Li S, Wang P (2002) Association of tapasin and COPI provides a mechanism for the retrograde transport of major histocompatibility complex (MHC) class I molecules from the Golgi complex to the endoplasmic reticulum. *J Biol Chem* 277(21):18266–18271
9. Ghanem E, Fritzsche S, Al-Balushi M, Hashem J, Ghuneim L, Thomer L, Kalbacher H, van Endert P, Wiertz E, Tampe R, Springer S (2010) The transporter associated with antigen processing (TAP) is active in a post-ER compartment. *J Cell Sci* 123(Pt 24):4271–4279
10. Day PM, Esquivel F, Lukszo J, Bennink JR, Yewdell JW (1995) Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity* 2(2):137–147
11. Neefjes JJ, Momburg F, Hammerling GJ (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* 261(5122):769–771
12. Fruh K, Ahn K, Djaballah H, Sempe P, van Endert PM, Tampe R, Peterson PA, Yang Y (1995) A viral inhibitor of peptide transporters for antigen presentation. *Nature* 375(6530):415–418
13. Galocha B, Hill A, Barnett BC, Dolan A, Raimondi A, Cook RF, Brunner J, McGeoch DJ, Ploegh HL (1997) The active site of ICP47, a herpes simplex virus-encoded inhibitor of the major histocompatibility complex (MHC)-encoded peptide transporter associated with antigen processing (TAP), maps to the NH₂-terminal 35 residues. *J Exp Med* 185(9):1565–1572

Chapter 12

Biochemical Large-Scale Identification of MHC Class I Ligands

Daniel J. Kowalewski and Stefan Stevanović

Abstract

The large-scale identification of MHC class I presented peptides is indispensable for gaining insight into the fundamental rules of immune recognition as well as it is an invaluable tool in identifying potential targets for the immunotherapy of disease. In this chapter we briefly review the existing strategies for the analysis of MHC ligandomes and provide an in-depth protocol for the immunoaffinity purification of MHC class I presented peptides from primary tissues or cells.

Key words: MHC class I, pMHC, Antigen presentation, Ligandome analysis, Immunoaffinity purification, Mass spectrometry

1. Introduction

The MHC class I ligandome, i.e., the entirety of peptides presented by MHC class I molecules on a single cell, represents a showcase of this cells' protein content presented for scrutiny by CD8⁺ T cells. Thus, the ligandome is the level at which the immune system monitors cells for alterations indicating pathological processes. It is of great interest to thoroughly characterize ligandomes in health and disease in order to pinpoint such alterations and thereby identify potential targets of immune recognition. Such targets constitute the cornerstone of targeted immunotherapy. Furthermore, the large-scale identification of MHC class I ligands is the basis for establishing MHC class I peptide binding motifs as described in this book by Trautwein et al. Being the endpoint of antigen presentation, the MHC ligandome is resultant from a number of factors like gene expression, protein turnover, and composition of the antigen

processing machinery. Consequently, it is a highly dynamic and complex entity comprising thousands of different peptide species in copy numbers ranging from 1 to 10,000 copies per cell (1). The current strategy of choice for the large-scale analysis of the MHC ligandome is the isolation of MHC ligands followed by liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) based peptide sequencing.

Several strategies for the isolation of MHC class I ligands have been described in the past. The elution of ligands from the surface of viable cells under mild acidic conditions (2) allows for repeated steps of elution from the same batch of cells and thus increases the yield. The major drawback of this method is the high amount of co-eluted contaminating peptides, which are not derived from MHC molecules (3). A second method employs the transfection of cells with soluble MHC molecules lacking the transmembrane domain (4). In this approach assembled peptide:MHC complexes (pMHC) are secreted into the culture medium and can be readily isolated by immunoprecipitation thus enabling yields of MHC ligands in the milligram range. A huge disadvantage of this strategy is the potential distortion of the ligandome caused by the use of recombinant techniques. Both, the qualitative and the quantitative constitution of the ligandome might be altered by a potentially permuted binding specificity of the transfected MHC molecule and by its overexpression. Furthermore this strategy is not applicable to the isolation of MHC ligands from primary tissue. The third approach and focus of this chapter is the immunoaffinity purification of pMHC from the lysate of cells using MHC-specific monoclonal antibodies (5). This method does not allow for repeated or continuous extraction of MHC ligands as the above-mentioned strategies do, but is restricted to the amount of peptides present at the time of cell lysis. On the upside this method is applicable to primary tissues, excels in purity of the isolated ligands, and allows for the allele-specific isolation of ligands from samples displaying a range of different MHC allotypes. This method has been used in our group for many years and was instrumental in the elucidation of allele-specific motifs (6), setting up and expanding the MHC databank SYFPEITHI (7), as well as identifying naturally presented T cell epitopes in the context of autoimmune disease, viral infection, and malignancy (8–10).

Here we describe the basic protocol for MHC class I precipitation from cells and primary tissue using immunoaffinity purification. Slight modifications adapting the protocol to the respective sample type and quantity are included and suggestions for the instrumentation and methods for the LC-MS/MS analysis are being made.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store solutions at 4°C unless indicated otherwise.

2.1. Affinity Chromatography

1. MHC class I specific monoclonal antibodies. See Table 1 for an overview of antibodies routinely used in our lab for the immunoaffinity purification of human MHC class I molecules.
2. Cyanogen bromide- (CNBr-) activated Sepharose (GE Healthcare).
3. Reactivating solution for CNBr-activated Sepharose: 1 mM HCL.
4. Antibody coupling buffer: 0.5 M NaCl, 0.1 M NaHCO₃, pH 8.3. Add about 100 ml water to a 1 L graduated cylinder. Weigh out 29.2 g NaCl and 8.4 g NaHCO₃ and transfer to the cylinder. Add water to a volume of 900 ml. Mix and adjust pH (at 4°C) with HCl. Add water up to 1 L.
5. Blocking solution for CNBr-activated Sepharose: 0.2 M glycine. Weigh out 15 g of glycine and prepare a 1 L solution as in the previous step, but without adjusting the pH.
6. Low pressure chromatography columns and tubing: Depending on the amount of sample, different column sizes have to be utilized. For sample volumes (tissue/cell pellet) of up to 3 ml use spin columns equipped with screw caps with Luer-Lok adaptors and 900 µl column volume (Pierce Protein Research Products (Thermo Fisher Scientific)). For sample volumes of 3–10 ml use low pressure glass chromatography columns of 1.0 × 10 cm size with 8 ml volume and 0.79 cm² cross-sectional area (Bio-Rad). For even higher sample volumes use adequate

Table 1
Monoclonal antibodies suited for the immunoaffinity purification of human MHC class I molecules

Clone	Isotype	Specificity	References
W6/32	IgG _{2a}	HLA-A, -B, -C	(11)
L243	IgG _{2a}	HLA-DR	(12)
BB7.2	IgG _{2b}	HLA-A*02	(13)
B1.23.2	IgG _{2b}	HLA-B, -C	(14)
GAP A3	IgG _{2a}	HLA-A*03	(15)

column sizes with similar cross-section-to-volume ratios. Use two-way stopcocks with female Luer to male Luer fitting (Bio-Rad). Use polypropylene Luer adapters with 1/8" lock rings and 1/8" Teflon tubing (Cole-Parmer).

7. Peristaltic pump with adjustable flow rate of 1–5 ml/min.
8. Spectrophotometer.
9. Vacuum Centrifuge/Lyophyllisator.
10. 1× DPBS.
11. 0.02% (w/v) NaN₃/DPBS.
12. 0.2% Trifluoroacetic acid (TFA).

2.2. Sample Preparation

1. Tissue or cell pellet. Use fresh or frozen samples (see Note 1). Additives (e.g., DMSO) have to be removed by repeated washing before the lysis protocol can be applied.
2. 100× Phosphatase inhibitor cocktail (see Note 2): 200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate, 400 mM sodium tartrate, 200 mM β-glycerophosphate, 200 mM sodium pyrophosphate. Add HCl until all the ingredients are completely dissolved. Store in aliquots of 330 µl at –20°C. Alternatively, use commercially available products, e.g., PhosSTOP (Roche).
3. 2× Solubilization buffer: Add 33 ml DPBS, 400 mg CHAPS (see Note 3), 1 “Complete Protease Inhibitor” tablet (Roche), and 660 µl phosphatase inhibitor cocktail (100×) to a beaker. Stir until completely dissolved.
4. Potter-Elvehjem tissue homogenizer.
5. Ultrasonic homogenizer with at least 150 W of ultrasonic power.
6. Sterile syringe filters with 0.22 µm pore size.
7. Centrifugal filter units: Amicon Ultra 10 kDa NMWL with volumes of 0.4, 4, 15 ml, depending on sample volume (Millipore).
8. ZipTipµ_{-C18} pipette tips (Millipore).
9. ZipTip desalting solution: 1% (v/v) formic acid (FA) in LC-MS grade water.
10. ZipTip elution solution: 1% (v/v) FA, 50% (v/v) acetonitrile (ACN) in LC-MS grade water.

2.3. Mass Spectrometry

1. LC-MS/MS System, e.g. UltiMate 3000 RSLC nano (Dionex) on-line coupled to a LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific).
2. Ultrasound bath.
3. LC-MS grade Acetonitrile.

4. Nano LC C18 columns, e.g. Dionex Acclaim PepMap RSLC C18 columns.
5. Proteome Discoverer software (Thermo Fisher Scientific) and the Mascot search engine (server 2.2, Matrix science).

3. Methods

The immunoaffinity purification of HLA-ligands spreads over 2 days. The sample homogenization and the preparation of the affinity columns take place on day 1. The affinity chromatography is executed overnight, followed by elution, isolation, pre-concentration, and LC-MS/MS analysis of the HLA-ligands on day 2 (see Fig. 1).

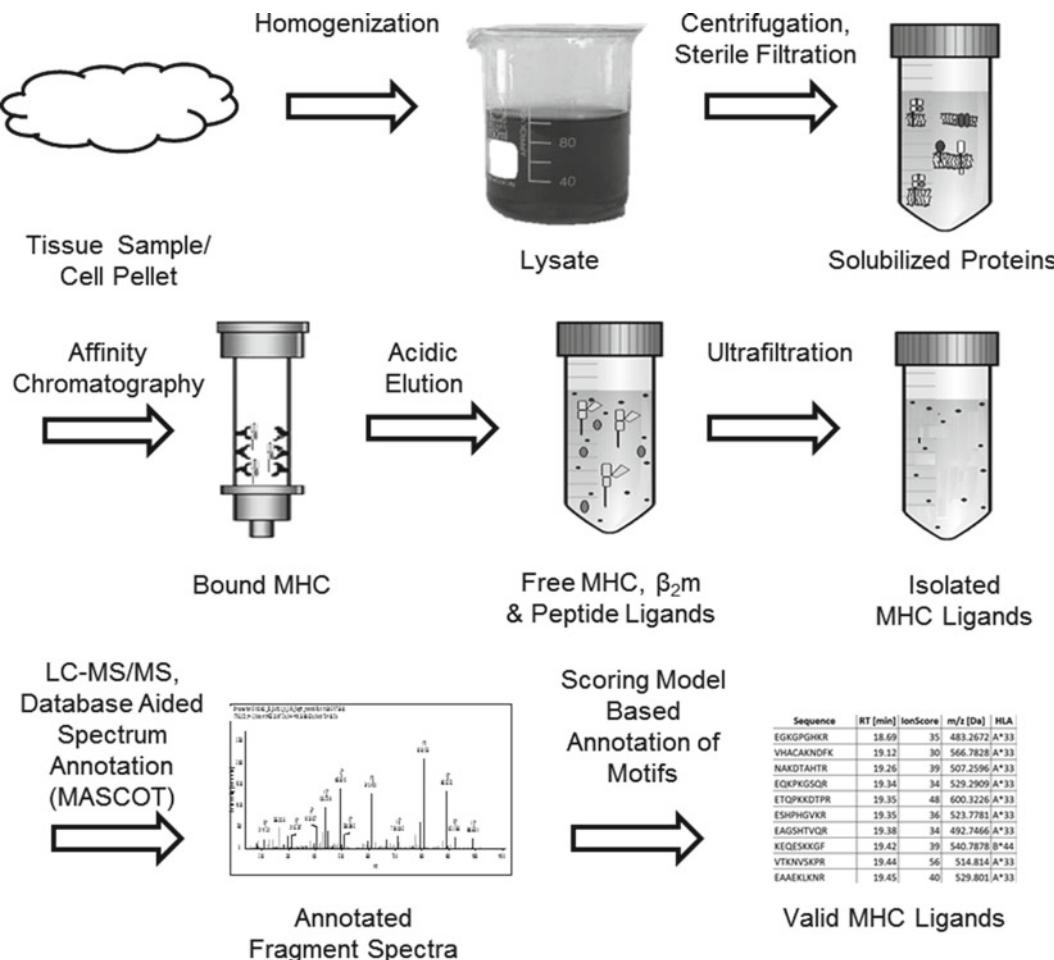


Fig. 1. Schematic overview of the experimental steps for the large-scale identification of MHC ligands using immunoaffinity purification.

3.1. Day 1: Preparation of the Affinity Chromatography Column Matrix

Carry out all procedures at room temperature unless indicated otherwise. The stationary phase of the affinity columns is prepared in batches. The batch size can be specifically adjusted to the sample volume by using 1 mg of antibody coupled to 40 mg of CNBr-activated sepharose per 1 g/1 ml of tissue/cell pellet, respectively (see Note 4). Alternatively, larger batches can be prepared and stored in aliquots. The following steps describe the preparation of 840 mg of stationary phase sufficient for the immunoaffinity purification of HLA-ligands from 20 g/ml of sample. During the incubation times perform the preparation of the sample for the immunoaffinity purification as described in Subheading 3.2.

1. Weigh out 800 mg of CNBr-activated sepharose and transfer it to a 50 ml falcon tube. Add 1 mM HCl up to 45 ml and rotate slowly for 30 min.
2. Spin down the sepharose at lowest speed for 5 min with the brake deactivated and discard the supernatant.
3. Resuspend the sepharose in 10 ml of antibody coupling buffer. Add 40 mg of antibody of the desired specificity and fill up to 45 ml with antibody coupling buffer.
4. For determining the coupling efficiency, extract 100 µl of this suspension, spin down the sepharose, and measure the absorbance of the supernatant at 280 nm.
5. Slowly rotate the coupling mix for 120 min.
6. At $t=120$ min, extract another 100 µl of the coupling suspension and determine the absorbance of the supernatant as described in step 4. For sufficient coupling efficiency ($\geq 90\%$) the absorbance at $t=120$ min should be 10% or less of the absorbance determined in the initial measurement. (In case the coupling efficiency is not high enough the reaction may be continued until the desired efficiency is reached.)
7. When the desired coupling efficiency is reached, spin down the antibody-coupled sepharose at lowest speed without brakes and remove the supernatant.
8. Add to the sepharose 0.2 M glycine up to 45 ml and rotate slowly for 60 min.
9. Discard the supernatant as in step 7 and add PBS up to 45 ml for washing of the sepharose. Invert the suspension five times, spin down the sepharose, and remove the supernatant. Repeat this step a second time.
10. Add to the sedimented antibody-coupled sepharose 0.02% (w/v) NaN_3 /PBS up to 20 ml and thoroughly resuspend by repeated aspiration and dispensing with a 1,000 µl pipette. Aliquot into 1 ml aliquots containing 1 mg of antibody coupled to 40 mg of sepharose. These aliquots can be stored at 4°C for at least 3 months.

3.2. Day 1: Preparation of the Tissue Sample/ Cell Pellet for the Immunoaffinity Purification

Carry out all the procedures at 4°C in a cooling chamber. Pay attention to always keep the sample sufficiently covered with lysis buffer. Avoid loss of sample by rinsing the utilized containers and instruments with small volumes of lysis buffer if necessary. The initial step of the protocol differs for tissue sample and cell pellet.

1. (a) Disintegration of tissue sample:

Determine the mass of the tissue sample. Immediately afterwards transfer it to a Petri dish, cover it with 1 volume of 2× solubilization buffer, and cut the tissue into very thin slices using a scalpel. Chop the tissue slices into small pieces and transfer them into the Potter-Elvehjem homogenizer. Rinse the Petri dish with 1 volume of 1× solubilization buffer and transfer the suspension into the homogenizer. Homogenize the sample thoroughly on ice (see Note 5). Transfer the suspension to a beaker. Rinse the homogenizer with 1 volume of 1× solubilization buffer. Stir for 60 min.

(b) Lysis of cell pellet:

Determine the volume of the cell pellet and add 1 volume of 2× solubilization buffer. Transfer the pellet into a beaker and stir slowly until it is completely resuspended. Rinse the vial which contained the cell pellet twice with 1 volume of 1× solubilization buffer and transfer the suspension to the beaker. Stir at 4°C for 60 min.

2. Homogenization of the lysate: Apply pulsed sonification with 150 W of ultrasonic power and 30% pulse length for 3 min on ice (see Note 5). Stir at 4°C for 60 min.

3. Centrifugation: Spin down remaining tissue fragments and debris in the lysate at $2,000 \times g$ and 4°C for 20 min. Carefully collect the supernatant.

4. Ultracentrifugation:

(a) Transfer the supernatant into an ultracentrifuge tube of appropriate volume (see Note 6).

(b) Rinse the previously used tube carefully with 1× solubilization buffer and use this suspension to fill the ultracentrifuge tube to the brim.

(c) Balance the ultracentrifuge tubes exactly and centrifuge at $150,000 \times g$ and 4°C for 70 min.

(d) Collect the supernatant and transfer it directly to a syringe sterile filter with 0.22 µm pore size.

(e) Rinse the ultracentrifuge tubes repeatedly with small volumes of 1× solubilization buffer (see Note 7).

5. Sterile filtration: filter the lysate through the syringe sterile filter into a sterile beaker. This filtered lysate is employed in the affinity chromatography.

3.3. Day 1–2: Affinity Chromatography

Perform all the steps at 4°C in a cooling chamber. Multiple affinity columns packed with stationary phases containing antibodies of different specificities may be connected in series for specific immunoaffinity purification of several MHC allotypes. Note that increasing the number of columns also increases the void volume of the chromatography system. The void volume should not exceed the total lysate volume in order to avoid the need for further dilution of the sample. For experiments with more than 3 columns it is recommended to sequentially implement up to 2 columns in subsequent affinity chromatography steps of 10 h length. Make sure to evaluate the ideal sequence of specificities for these experiments (see Note 8).

1. Transfer one aliquot of antibody-coupled sepharose per 1 g/ml of tissue/cell pellet, respectively, into the chromatography column (see Note 4). For immunoaffinity purifications with several specificities use separate columns for each antibody. Set up and connect the columns and the pump and flush the system linearly (i.e., from solvent supply to waste) with PBS at a flow rate of 4 ml/min for 30 min. Check the entire system for leakage during flushing.
2. Stop the pumping and replace the PBS with the lysate. Aspirate the system linearly with the lysate at a flow rate of 2 ml/min until it completely displaces the PBS in the void volume of the system, but avoid any loss of lysate to the waste (see Note 9). Stop the pumping.
3. Close the cycle by transferring the outlet from the waste to the beaker containing the lysate. Gently stir the lysate on a magnetic stirrer and recirculate the lysate through the system overnight at a flow rate of 2 ml/min.
4. The next morning, linearize the system and flush it with PBS at a flow rate of 4 ml/min. Collect the remaining lysate in the beaker and in the void volume of the system and store it at -20°C until the success of the immunoaffinity purification is assured. Continue to flush the system with PBS for 30 min.
5. Flush the system with ddH₂O for 60 min at a flow rate of 4 ml/min.
6. Let the column run dry, close the stopcock, and disconnect the column from the system.

3.4. Day 2: Elution of the Affinity Columns, HLA-Ligand Isolation, and Preparation for LC-MS Analysis

Carry out all the steps at 4°C in a cooling chamber. Use LC-MS grade reagents only. Wash the required tubes and centrifugal filter units thoroughly with 0.2% TFA before usage (see Note 10). Use Hamilton syringes or polyethylene glycol (PEG) reduced pipette tips for handling the solutions. Depending on the amount of stationary phase used in the affinity chromatography, different sizes of centrifugal filter units (Amicon Ultra) have to be utilized: For

≤60 mg of stationary phase use filter units with 0.4 ml volume, for 61–500 mg of stationary phase use 4 ml filter units, for >500 mg of sepharose use 15 ml filter units.

1. Elution of the affinity column:

- (a) Cover the dried column matrix with 0.2% TFA (approx. 40 µl of TFA per 40 mg of stationary phase). Only in the first elution step further add 1 µl of 10% TFA per 40 mg of column matrix in order to quickly lower the pH.
 - (b) Incubate the resuspended column matrix on a shaker for 20 min.
 - (c) Open the stopcock and transfer the eluate directly into a centrifugal filter unit by applying pressure to the column with an empty 50 ml syringe.
 - (d) Close the stopcock.
 - (e) Repeat this first step eight times.
2. Ultrafiltration of the eluate: Centrifuge the filter unit at 4°C and maximum speed for 30 min. The retentate contains proteins of >10 kDa, mainly α- and β-chains of MHC molecules, and might be used for further analysis. The filtrate contains the MHC ligands, which have to be desalted and pre-concentrated prior to LC-MS/MS analysis.
 3. Desalting and pre-concentration of peptides in the filtrate:
 - (a) Wash the ZipTip_μ_{Cl₈} by aspirating and dispensing to waste of 10 µl ZipTip elution solution. Repeat ten times.
 - (b) Equilibrate the ZipTip by ten cycles of aspirating and dispensing to waste of 10 µl of desalting solution.
 - (c) Bind the peptides to the ZipTip matrix by 30 cycles of aspirating and dispensing of the filtrate (see Note 11).
 - (d) Wash the bound peptides by five cycles of aspirating and dispensing 10 µl of desalting solution.
 - (e) Elute the ZipTip into 5 µl of elution solution directly into an autosampler vial by ten cycles of carefully aspirating and dispensing the solution without introducing air.
 - (f) Evaporate the elution solution under vacuum to complete dryness. Store the remaining filtrate at -20°C for potential repetition of the peptide pre-concentration.

3.5. Day 2: LC-MS/ MS-Based Analysis of the MHC-Derived Peptides

Thus far this protocol theoretically yields 2 µg of (mainly) MHC-derived peptides (see Note 12). For the LC-MS/MS-based analysis of the recovered peptides we can only make suggestions, as different instrumentations call for different standard operating procedures. The following steps are adapted for the use of a nanoLC-2D system (Eksigent) online coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific) hybrid mass spectrometer.

1. Resuspend the dried peptides in 5 µl 0.2% TFA using an ultrasound bath.
2. Inject the complete sample into the LC-system using the pulled loop µl-pickup mechanism. Use 0.2% TFA as loading fluid.
3. Employ a precolumn in front flush mode to trap the peptides. Wash the trapped peptides with loading fluid.
4. For the chromatographic separation utilize a column of 25 cm length and 75 µm inner diameter (I.D.) packed with C₁₈ matrix with 3 µm particle size.
5. Apply a gradient ramping from 0 to 30% ACN in 80 min at a flow rate of 300 nL/min.
6. For the mass spectrometric analysis, employ a method fragmenting the five most intense precursor ions (“top 5 method”) in the mass range 400–1,000 *m/z* using collision-induced dissociation (CID).
7. Employ computerized annotation of the fragment spectra using the Proteome Discoverer software and the Mascot search engine searching the respective taxonomy in the Uniprot database (<http://www.uniprot.org>). Set the precursor mass tolerance to 5 ppm and the fragment mass tolerance to 0.8 Da. Do not limit the search by enzymatic specificity and do not apply spectrum grouping. Set the oxidation of methionine as a variable modification.
8. Filter the results of the database search for Mascot scores ≥ 20, peptide ranks ≤ 3. Manually examine the filtered peptides for the occurrence of peptide motifs of the respective allotype (go to SYFPEITHI database (<http://www.syfpeithi.de>) for a listing of defined peptide motifs). Discard peptides which do not contain the correct motifs and/or display wrong peptide lengths for the respective allotype.
9. Manually check the fragment spectra of motif-containing peptides for validity. Discard invalid annotations. The remaining peptides constitute valid naturally processed and presented MHC ligands. The false discovery rate can be determined by random synthesis of identified ligands and their subsequent LC-MS/MS analysis. The retention times and fragment spectra of the synthetic peptides have to precisely match those of their naturally occurring counterparts.

The comprehensive analysis of MHC class I ligandomes calls for repeated LC-MS/MS analysis of ligands derived from the same experiment, as these highly complex samples cannot be completely analyzed in single runs.

4. Notes

1. For immediate application of the protocol cover fresh samples with solubilization buffer ($2\times$) instantaneously after recovery to inhibit proteolysis and dephosphorylation. Alternatively flash freeze fresh samples in liquid nitrogen and store at -80°C for future application of the protocol.
2. Use phosphatase inhibitor cocktail only if the analysis of phosphorylated MHC ligands falls within the scope of your research. Note that the analysis of phosphorylated MHC ligands requires their enrichment prior to analysis and calls for specific mass spectrometric methods as reviewed in ref. 16.
3. Detergent presents a potential source of contamination for the mass spectrometric analysis. CHAPS is a zwitterionic detergent with a defined molecular weight of 614.88 Da. It does not contain a PEG modification. Residual CHAPS in samples does therefore not interfere with mass spectrometrical analysis as much as other detergents do. Nevertheless several steps in this protocol are aimed at removing detergent (and other contaminants) from the sample (ZipTip pre-concentration, precolumn trapping).
4. The amount of 1 mg of antibody per 1 g/1 ml of sample refers to a standard immunoaffinity purification of HLA class I ligands from human samples using the antibodies listed in Table 1. MHC class I expression levels of the analyzed tissue/cell type as well as specificity and affinity of the implemented antibody have to be taken into consideration when applying this protocol to samples of different origin using antibodies not listed in Table 1.
5. Disintegration of certain samples (e.g., fibrotic tissue) requires vigorous homogenization. To efficiently cool the homogenizer, submerge it in a cooling bath consisting of 25% (w/w) NaCl in ice.
6. The volume of the tube should exceed the lysate volume by no more than 25% in order to limit the dilution effect. This is because ultracentrifuge tubes generally have to be filled to the brim for centrifugation under vacuum. In case the ultracentrifugation step cannot be performed (e.g., if the lysate volume is too small), distribute the lysate into 2 ml reaction cups and centrifuge at maximum speed at 4°C for 100 min.
7. Take care to completely recover the lipid-rich fraction found on top of the lysate after ultracentrifugation as it contains the bulk membrane proteins. Use a 1,000 μl pipette with cut-off tips to specifically aspirate this fraction.

8. Example: Specific isolation of an MHC class I allotype (from an MHC typed sample) can be performed, even if there is no allotype-specific antibody at your disposal. Use an excess of specific antibodies in a first affinity chromatography step to deplete MHC molecules of other MHC allotypes from the sample. Then use a pan-specific anti-MHC class I antibody (e.g., W6/32) for purification of the remaining MHC molecules of the allotype of interest.
9. In the case of tissue preparations the influx of the lysate into the system can often be easily tracked because of its red color caused by myoglobin and hemoglobin. The displacement of PBS by clear cell lysate has to be estimated considering the flow rate of the pump and the void volume of the system.
10. The washing steps with 0.2% TFA remove easily elutable PEG and other contaminants (e.g., residual glycerol from the centrifugal filter units), which would interfere with the LC-MS/MS analysis.
11. For better sample recovery it is recommended to reduce the volume of the filtrate to approx. 100 µl by vacuum centrifugation prior to ZipTip pre-concentration. Alternatively lyophilize the filtrate and resuspend the peptides in 100 µl 0.2% TFA by vigorous vortexing and application of ultrasound. Use this suspension for ZipTip pre-concentration.
12. Given the amount of sample and its MHC expression were high enough and the immunoaffinity purification was successful.

References

1. Stevanovic S, Schild H (1999) Quantitative aspects of T cell activation-peptide generation and editing by MHC class I molecules. *Semin Immunol* 11(6):375–384. doi:[10.1006/smim.1999.0195](https://doi.org/10.1006/smim.1999.0195) S1044-5323(99)90195-1 (pii)
2. Storkus WJ, Zeh HJ 3rd, Salter RD, Lotze MT (1993) Identification of T-cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J Immunother Emphasis Tumor Immunol* 14(2):94–103
3. Torabi-Pour N, Nouri AM, Saffie R, Oliver RT (2002) Comparative study between direct mild acid extraction and immunobead purification technique for isolation of HLA class I-associated peptides. *Urol Int* 68(1):38–43. doi:[uin68038](https://doi.org/10.1159/000068038) (pii)
4. Prilliman K, Lindsey M, Zuo Y, Jackson KW, Zhang Y, Hildebrand W (1997) Large-scale production of class I bound peptides: assigning a signature to HLA-B*1501. *Immunogenetics* 45(6):379–385
5. Van Bleek GM, Nathenson SG (1990) Isolation of an endogenously processed immunodomi-
- nant viral peptide from the class I H-2K^b molecule. *Nature* 348(6298):213–216. doi:[10.1038/348213a0](https://doi.org/10.1038/348213a0)
6. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351(6324):290–296. doi:[10.1038/351290a0](https://doi.org/10.1038/351290a0)
7. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S (1999) SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50(3–4):213–219. doi:[90500213.251](https://doi.org/10.1007/s00213-000-0213-251) (pii)
8. Falk K, Rotzschke O, Stevanovic S, Gnau V, Sparbier K, Jung G, Rammensee HG, Walden P (1994) Analysis of a naturally occurring HLA class I-restricted viral epitope. *Immunology* 82(3):337–342
9. Wahlstrom J, Dengjel J, Persson B, Duyar H, Rammensee HG, Stevanovic S, Eklund A, Weissert R, Grunewald J (2007) Identification of HLA-DR-bound peptides presented by

- human bronchoalveolar lavage cells in sarcoidosis. *J Clin Invest* 117(11):3576–3582. doi:[10.1172/JCI32401](https://doi.org/10.1172/JCI32401)
10. Schirle M, Keilholz W, Weber B, Gouttefangeas C, Dumrese T, Becker HD, Stevanovic S, Rammensee HG (2000) Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. *Eur J Immunol* 30(8):2216–2225. doi:10.1002/1099-1522(200008)30:8<2216::AID-IMMU2216>3.0.CO;2-7 (pii) 10.1002/1521-4141(2000)30:8<2216::AID-IMMU2216>3.0.CO;2-7
11. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14(1):9–20. doi:[0092-8674\(78\)90296-9](https://doi.org/10.1016/0092-8674(78)90296-9) (pii)
12. Goldman JM, Hibbin J, Kearney L, Orchard K, Th'ng KH (1982) HLA-DR monoclonal anti-bodies inhibit the proliferation of normal and chronic granulocytic leukaemia myeloid progenitor cells. *Br J Haematol* 52(3):411–420
13. Parham P, Brodsky FM (1981) Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum Immunol* 3(4):277–299
14. Rebai N, Malissen B (1983) Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies. *Tissue Antigens* 22(2):107–117
15. Berger AE, Davis JE, Cresswell P (1982) Monoclonal antibody to HLA-A3. *Hybridoma* 1(2):87–90
16. Eyrich B, Sickmann A, Zahedi RP (2011) Catch me if you can: mass spectrometry-based phosphoproteomics and quantification strategies. *Proteomics* 11(4):554–570. doi:[10.1002/pmic.201000489](https://doi.org/10.1002/pmic.201000489)

Chapter 13

Establishing MHC Class I Peptide Motifs

Nico Trautwein and Stefan Stevanović

Abstract

Major histocompatibility complex (MHC) class I peptide motifs are used on a regular basis to identify and predict MHC class I ligands and CD8⁺ T-cell epitopes. This approach is above all an invaluable tool for the identification of disease-associated epitopes. As a matter of fact, the vast majority of T-cell epitopes discovered during the past two decades was identified by means of epitope prediction. Here we describe the steps which are necessary to establish MHC class I peptide motifs and to compose a reliable scoring matrix for epitope prediction. As an example, a scoring matrix for the prediction of HLA-B*35-presented T-cell epitopes will be developed by examining the characteristics of 76 naturally presented HLA ligands.

Key words: MHC class I, Peptide motif, Anchor, Auxiliary anchor, Preferred residue, Scoring matrix

1. Introduction

Major histocompatibility complex (MHC) class I molecules play an important role in cellular immunology with impact on the growing fields of individualized medicine and cancer immunotherapy (1, 2). The main purpose of these protein complexes is to present peptides of intracellular origin to T-cells for immunosurveillance. Only a specific subset of peptides is showcased in such a way. Peptide precursors are mainly produced by the proteasome and are transported into the *endoplasmatic reticulum* (ER) by the *transporter associated with antigen processing* (TAP). Further trimming by ER proteases yield peptides which are transported to the plasma membrane after being bound to MHC complexes (3–5). All of these steps are dependent on the peptide sequence. Hence peptides presented by a certain MHC allotype usually share common characteristics referred to as MHC peptide motif (6). These motifs usually determine the preferred peptide length and specific amino acid residues in certain positions called anchors (7). In most cases the

peptides comprise nine amino acids and the anchors are the second and the C-terminal amino acid (8). Here we show how the amino acid distribution of naturally processed and presented ligands of a specific MHC allotype can be exploited for the elucidation of the corresponding peptide motif (9). We further describe the procedural steps to confirm the accuracy and validity of such a motif. Figure 1 illustrates the consecutive steps which lead to the definition of a peptide motif and to setting up a scoring matrix for epitope prediction.

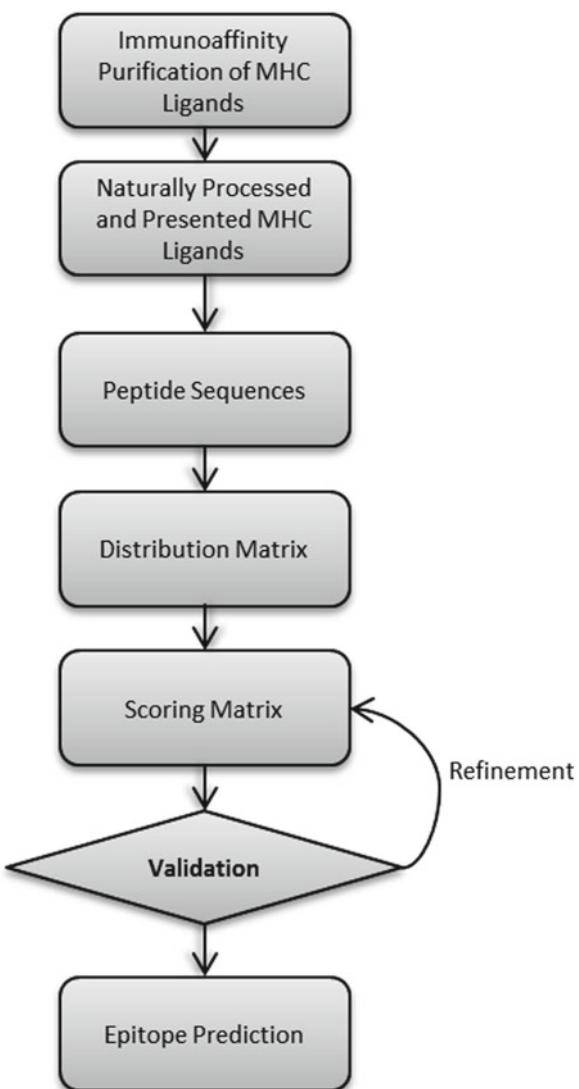


Fig. 1. Workflow for establishing MHC class I peptide binding motives.

2. Methods

1. Isolate and identify at least 50 naturally presented MHC ligands of the desired MHC allotype (see Note 1).
2. Create a curated list of these ligands that contains only valid MHC ligands (see Note 2).
3. Sort the peptides according to their length in order to identify the peptides with the most abundant length. Very often, the preferred length will be nine amino acids. The following steps will only be carried out for length variants which make up more than 10% of the entire peptide repertoire.
4. Create a distribution matrix based on the relative frequency of amino acids in the different positions. An example is depicted in Table 1.
5. Based on this amino acid distribution matrix a scoring matrix is assigned. A scoring matrix based on the frequency of amino acids in each position of the investigated MHC class I ligands represents the simplest example of such a matrix. Thus, Table 1 can directly be used as a scoring matrix. It will serve as a benchmark for all the other scoring matrices which will be compared with respect to accuracy and sensitivity.
6. There are numerous strategies to create a scoring matrix, including those based on automated systems (for example, machine learning, support vector machines, artificial neural networks (10, 11)). We will describe a procedure that can be followed without the need of bioinformatics. This method is to assign points for the different amino acids in the different positions (12). Obviously this can be done in various ways. As a guideline the following approach is suggested: Peptides with similar chemical properties are combined in one group (see Note 3). If the relative frequency of one group in a certain position exceeds 80% it is declared an anchor and 10 points are awarded. If a group constitutes 50–80% in one position it is declared an auxiliary anchor and 4–6 points are assigned. If a group constitutes 10–50% of amino acids in a specific position, 1 up to 3 points can be awarded. These amino acids are called preferred residues. This is only one exemplary strategy to award points, which can be altered on a logical basis (see Note 4). A typical scoring matrix is shown in Table 2.
7. Defining anchor residues: Identify highly abundant residues in your distribution matrix. Investigate whether chemically closely related amino acids are also overrepresented. As shown in Table 1 leucine, phenylalanine, and tyrosine dominate in position 9 (P9). Together these three amino acids occur in P9 of 93.42% of the investigated MHC ligands. Because phenylalanine

Table 1
Amino acid distribution matrix of 76 HLA-B*35 ligands

Pos/AA	1	2	3	4	5	6	7	8	9
A	7.89	6.58	7.89	7.89	7.89	10.53	3.95	11.84	0
C	0	0	0	0	0	0	0	1.32	0
D	1.32	1.32	10.53	11.84	9.21	10.53	0	0	0
E	3.95	0	9.21	18.42	5.26	9.21	7.89	2.63	0
F	13.16	0	6.58	1.32	1.32	2.63	1.32	2.63	15.79
G	0	0	0	5.26	0	1.32	0	2.63	0
H	9.21	0	1.32	5.26	3.95	5.26	7.89	1.32	0
I	5.26	0	7.89	3.95	22.37	13.16	13.16	6.58	2.63
K	0	0	2.63	7.89	0	7.89	3.95	10.53	0
L	15.79	0	13.16	7.89	14.47	9.21	7.89	9.21	31.58
M	11.84	0	0	0	0	5.26	5.26	0	3.95
N	7.89	0	6.58	2.63	1.32	1.32	3.95	0	0
P	0	89.47	0	7.89	9.21	2.63	5.26	0	0
Q	2.63	0	2.63	2.63	1.32	1.32	6.58	5.26	0
R	3.95	0	3.95	7.89	5.26	6.58	7.89	9.21	0
S	2.63	0	7.89	5.26	6.58	3.95	2.63	5.26	0
T	9.21	0	2.63	2.63	1.32	5.26	3.95	17.11	0
V	2.63	2.63	13.16	0	6.58	2.63	13.16	11.84	0
W	0	0	1.32	1.32	1.32	0	1.32	0	0
Y	2.63	0	2.63	0	2.63	1.32	3.95	2.63	46.05

The columns represent the respective position of amino acids (AA) within the peptide sequence (shown here only for 9mer peptides). The numbers indicate the preference for amino acids of the respective positions indicated as percentage of occurrence. For example, 89.47% of all ligands carry proline (P) in position 2. Values above 10% are given in **bold**.

is subdominant compared to leucine, it is assigned 8 points. Leucine is assigned 10 points. Methionine and isoleucine share some characteristics with leucine and therefore these amino acids are put in one group. Because this group is less abundant, only 6 points are awarded. The other anchor is proline in position 2 (P2). A vast majority of the investigated ligands (89.47%) contain this residue in P2. Thus 10 points are assigned to this amino acid in P2.

- Defining auxiliary anchors: This is carried out analogously to the definition of anchor residues described in step 7. A group of chemically related amino acids is defined as an auxiliary anchor if its relative frequency in a specific position lies between

Table 2
Scoring matrix for HLA B*35

AA/Pos	1	2	3	4	5	6	7	8	9
A	0	0	0	1	0	1	0	1	0
C	0	0	0	0	0	0	0	0	0
D	0	0	1	1	1	1	0	0	0
E	0	0	1	1	0	0	0	0	0
F	1	0	0	0	0	0	0	0	8
G	0	0	0	0	0	0	0	0	0
H	1	0	0	0	0	0	0	0	0
I	0	0	0	0	2	1	1	0	6
K	0	0	0	1	0	0	0	0	0
L	1	0	1	0	1	1	0	1	10
M	1	0	0	0	0	0	0	0	6
N	0	0	0	0	0	0	0	0	0
P	0	10	0	0	0	0	0	0	0
Q	0	0	0	0	0	0	0	0	0
R	0	0	0	1	0	0	0	0	0
S	0	0	0	0	0	0	0	0	0
T	0	0	0	0	0	0	0	1	0
V	0	0	1	0	0	0	1	1	0
W	0	0	0	0	0	0	0	0	0
X	0	0	0	0	0	0	0	0	0
Y	0	0	0	0	0	0	0	0	10

50% and 80% (see Note 4). In the present example (see Table 1) no auxiliary anchors can be defined according to these criteria.

9. Defining preferred residues: All non-anchor positions are investigated for the occurrence of preferred residues. If the relative frequency of a specific amino acid exceeds 10% in one position it is considered a preferred residue.
10. The next step is to evaluate the proposed scoring matrices and to compare them. Confidence in a new matrix is achieved if known ligands are found reliably in their source proteins. Table 3 compares the two exemplarily developed matrices according to this aspect.

Table 3
Prediction of HLA-B*35 ligands from the sequences of their source proteins

Length	Protein ID	Sequence	Position	B*35 Percent		B*35 Point	
				Rank	Top 2%	Rank	Top 2%
394	ARP2_HUMAN	FPEHIFPAL	27–35	1	++	1	++
245	BZLF1_EBVB9	LPCVLWPVPL	44–52	3	+	1	++
907	GP350_EBVB9	TPNATSPTL	566–574	3	++	2	++
1,128	DNBL1_EBVB9	YPLREVATL	30–39	3	++	2	++
245	BZLF1_EBVB9	LPQGQLTAY	56–64	1	++	1	++
866	NAA15_HUMAN	TPLEEAIKF	652–660	1	++	2	++
323	AK1C3_HUMAN	TPALIALRY	251–259	1	++	1	++
292	MLEC_HUMAN	TAVALLRLL	10–18	19	–	4	+
122	SAA3_HUMAN	RPAGLPEKY	114–122	1	++	1	++
776	CUL1_HUMAN	LPSELERSY	555–563	1	++	2	++
500	IC1_HUMAN	LPSDTRLVL	281–289	2	++	2	++
463	JMJD4_HUMAN	QPKEELLQQQL	445–453	2	++	2	++
7,570	DYST_HUMAN	NPVELKALY	302–310	1	++	1	++
1,035	Q53FM3_HUMAN	LVNEPLAAY	855–863	39	–	20	+
256	OBF1_HUMAN	LPHQPLATY	52–60	1	++	1	++
1,217	SMC3_HUMAN	LPQEAFEKY	949–957	1	++	2	++
599	APC7_HUMAN	LPSEIEVKY	132–140	1	++	1	++
851	STAT2_HUMAN	LPVDIRQYL	27–35	1	++	1	++
601	2AAB_HUMAN	MAWLVDHVY	460–468	25	–	21	–
278	CNPY3_HUMAN	MPEPASRCL	4–12	2	++	2	++
1,000	K0528_HUMAN	MPFP AHLTY	474–482	1	++	1	++
5,890	AHNK_HUMAN	MPKFSMPGF	882–890	–	–	24	++
239	EGLN3_HUMAN	MPLGHIMRL	1–9	1	++	1	++
414	COT2_HUMAN	MPLHVAPLL	262–270	1	++	1	++
394	NDRG1_HUMAN	MPSASMTRL	315–323	2	++	2	++
250	TNF13_HUMAN	MPSHPDRAY	200–208	1	++	1	++
317	PPR3C_HUMAN	MPVVDVAMRL	20–38	1	++	1	++
626	SAMH1_HUMAN	NPIDHVSFY	513–521	3	++	3	++
737	PLOD2_HUMAN	NPVDWKEKY	536–544	1	++	5	++

(continued)

Table 3
(continued)

Length	Protein ID	Sequence	Position	B*35 Percent		B*35 Point	
				Rank	Top 2%	Rank	Top 2%
165	UBD_HUMAN	NPYDSVKKI	25–33	6	–	4	–
1,063	MYO1C_HUMAN	NPYRDLQIY	88–96	1	++	1	++
92	RUXE_HUMAN	QPINLIFRY	16–24	1	++	1	++
298	SDCB1_HUMAN	RPFERTITM	193–201	1	++	2	++
289	IPYR_HUMAN	SPFHDIPIY	30–38	1	++	1	++
1,801	PAR14_HUMAN	TPDEIDHVF	92–100	1	++	1	++
314	O10A6_HUMAN	TPLLNLLIY	282–290	1	++	1	++
733	NIBL1_HUMAN	VPLDERIVF	50–58	4	++	3	++
128	NDUB6_HUMAN	VPVWIIHYY	77–85	1	++	1	++
444	TBB5_HUMAN	YPDRIMNTF	159–167	2	++	2	++
488	ANXA7_HUMAN	YPQPPSQSY	105–113	1	++	5	++
1,318	MTP_EBVB9	YPAPCISGY	434–442	4	++	7	++
173	TP4A1_HUMAN	RPAPVEVTY	6–14	1	++	2	++
543	EHD2_HUMAN	LPLEEHYRF	34–42	2	++	4	++
394	ARP2_HUMAN	FPEHIFPAL	27–35	1	++	1	++
245	BZLF1_EBVB9	LPCVLWPVL	44–52	3	+	1	++
245	BZLF1_EBVB9	TPDPYQVPF	14–22	6	–	5	+
404	EAD_EBVB9	LPLDLSVIL	286–294	1	++	1	++
479	A7UMS0_EBVG	NPGTLSSLL	430–438	3	++	2	++
391	HERP1_HUMAN	APAPIHNQF	211–219	3	++	4	++
1,709	SN_HUMAN	APISLHSVY	974–982	4	++	12	++
141	POMP_HUMAN	APLKLQMELF	75–83	4	–	2	+
165	DEST_HUMAN	APLKS KMIY	109–117	1	++	1	++
419	BZW1_HUMAN	DPFKDIILY	269–277	1	++	1	++
151	CHCH2_HUMAN	EPARP DITY	91–99	1	++	1	++
709	Q59EJ3_HUMAN	EPTAAAIA Y	243–251	1	++	1	++
456	OST48_HUMAN	FPDKPITQY	225–233	1	++	1	++
476	CBPE_HUMAN	FPDLD RIVY	191–199	1	++	1	++
446	TBB6_HUMAN	FPDRIMNTF	159–167	1	++	1	++

(continued)

Table 3
(continued)

Length	Protein ID	Sequence	Position	B*35 Percent		B*35 Point	
				Rank	Top 2%	Rank	Top 2%
119	NDUC2_HUMAN	FPEEDKKTY	100–108	1	++	1	++
394	ARP2_HUMAN	FPEHIFPAL	27–35	1	++	1	++
1,058	UBA1_HUMAN	FPNAIEHTL	637–645	1	++	1	++
328	BUB3_HUMAN	FPNKQGYVL	188–196	2	++	1	++
739	VCAM1_HUMAN	FPRDPEIEM	403–411	7	++	7	++
292	CNOT8_HUMAN	FPSIYDVKY	189–197	1	++	1	++
285	CNOT7_HUMAN	FPVIYDVKY	189–197	1	++	1	++
445	DCA13_HUMAN	HPEKLATVL	75–83	2	++	1	++
824	ADAM8_HUMAN	HPERVSYVL	53–61	4	++	1	++
2,364	SPTB2_HUMAN	HPESERISM	1,663–1,671	11	++	3	++
485	Q6N087_HUMAN	HPISEEELL	233–241	5	++	3	++
2,511	FAS_HUMAN	HPLGDIVAF	1,351–1,359	2	++	1	++
531	TCPZ_HUMAN	HPRIITEGF	115–123	1	++	1	++
954	B7Z8Z6_HUMAN	HPVSREWTL	613–621	4	++	2	++
904	MCM2_HUMAN	HPVSREWTL	563–571	4	++	2	++
437	PLIN2_HUMAN	IARNLTQQL	313–321	38	–	32	–
740	DCLK1_HUMAN	IPATITERY	382–390	1	++	1	++
457	Q53GX4_HUMAN	IPIGIDKAL	198–205	1	++	1	++
730	HNRPM_HUMAN	IPNEIIHAL	189–197	1	++	1	++
223	NTM1A_HUMAN	LPDEIYHVY	210–218	1	++	1	++
4,022	VP13B_HUMAN	LPDPPIIHL	2,853–2,861	1	++	1	++
769	ITB2_HUMAN	LPDTLKVTY	374–382	1	++	1	++
375	Q4KKW2_HUMA	LPFLANSIL	172–180	3	++	3	++
Total				71	++	74	++
				2	+	4	+
				8	–	3	–

Epitope prediction was performed using an extended data set of 81 natural HLA ligands, applying the reference matrix shown in Table 1 (designated as “B*35 Percent”) or the scoring matrix shown in Table 2 (designated as “B*35 Point”). If a peptide is ranked among the top scoring 1% within the respective protein sequence, it is awarded a (++)). If it is ranked among the top 2%, it is assigned a (+). A (–) is assigned if the peptide rank is below the top 2%

11. Evaluating and refining the scoring matrices is the most important step in establishing a new peptide motif. One way to refine your prediction matrix is to use another set of input peptides: In the present example the anchor amino acid in position 2 is proline (see Table 1). In the first cycle this amino acid showed up in 89.47% of all cases in position 2. One could claim that the residual 10.53% are derived from peptides not eluted from HLA molecules. In order to refine the scoring matrix one could only use peptides with proline in position 2 for the next cycle. This will not change the main anchors but will potentially alter the preferred residues, which will lead to modified ranking of predicted ligands. Of course the same procedure can be applied to other positions as well. Another way to alter the scoring is to redefine the amino acid groups. For example tyrosine, leucine, methionine, isoleucine, and phenylalanine are usually not grouped together, because methionine, leucine, and isoleucine claim less space in the HLA binding groove than the bulky aromatic residues phenylalanine and tyrosine. In the present example, however, all five hydrophobic residues are prominent in the C-terminal position and therefore are grouped together.
12. After developing and evaluating different modified scoring matrices, choose the top performing one to predict new ligands.

3. Notes

1. For a brief review of strategies for the isolation of MHC ligands and the protocol for immunoaffinity purification of MHC class I ligands used for the example at hand, see Chapter 12 by Kowalewski et al. in this volume.
2. To validate MHC class I derived peptides they have to be checked for several criteria: Firstly they have to be of appropriate length for MHC ligands. It is suggested to use only peptides with 8–12 amino acids. Secondly the occurrence of specific amino acids at the C-terminal position (which always serves as an anchor position in MHC class I restricted peptides) has to be evaluated. MHC class I ligands usually do not possess glycine, serine, or acidic amino acids in the C-terminal position. Furthermore, peptides derived from known contaminations or artifacts have to be excluded (13). If some preliminary information about the MHC motif of interest already exists, it can be implemented in the verification of the isolated peptides.
3. Amino acids are usually grouped according to their chemical properties and size. Such grouping may vary between MHC

ligand pools of different allotypes. For example, aspartic acid and glutamic acid are often grouped together. However, there are several cases where only one of these amino acids is accepted in an anchor position (e.g., HLA-B*44 exclusively allows glutamic acid in position 2; HLA-C*04 exclusively allows aspartic acid in position 3). Sometimes even amino acids with considerably different properties may occupy the very same anchor position. There are, for example, cases (HLA-B*13:02, -B*15:01), in which glutamine and leucine constitute the P2 anchor position (see ref. 12).

4. For example, rare amino acids such as methionine or tryptophan can be assigned 1 point in a point-based scoring matrix, even if their respective frequencies do not reach the 10% threshold in a specific position. This exception is based on the low natural frequency of these rare amino acids.

References

1. Rammensee HG et al (2002) Towards patient-specific tumor antigen selection for vaccination. *Immunol Rev* 188:164–176
2. Singh-Jasuja H, Emmerich NP, Rammensee HG (2004) The Tubingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy. *Cancer Immunol Immunother* 53(3):187–195
3. Germain RN, Margulies DH (1993) The biochemistry and cell biology of antigen processing and presentation. *Annu Rev Immunol* 11:403–450
4. Serwold T et al (2002) ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419(6906):480–483
5. Germain RN (1995) The biochemistry and cell biology of antigen presentation by MHC class I and class II molecules. Implications for development of combination vaccines. *Ann N Y Acad Sci* 754:114–125
6. Falk K et al (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351(6324):290–296
7. Rammensee HG, Falk K, Rotzschke O (1993) Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 11:213–244
8. Bouvier M, Wiley DC (1994) Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science* 265(5170):398–402
9. Rammensee HG, Friede T, Stevanović S (1995) MHC ligands and peptide motifs: first listing. *Immunogenetics* 41(4):178–228
10. Donnes P, Elofsson A (2002) Prediction of MHC class I binding peptides, using SVMHC. *BMC Bioinformatics* 3:25
11. Soam SS, Bhasker B, Mishra BN (2011) Improved prediction of MHC class I binders/non-binders peptides through artificial neural network using variable learning rate: SARS corona virus, a case study. *Adv Exp Med Biol* 696:223–229
12. Rammensee H et al (1999) SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50(3–4):213–219
13. Keller BO et al (2008) Interferences and contaminants encountered in modern mass spectrometry. *Anal Chim Acta* 627(1):71–81

Chapter 14

Quantitating MHC Class I Ligand Production and Presentation Using TCR-Like Antibodies

Brian P. Dolan

Abstract

Accurately determining the number of peptide–MHC class I complexes on the cell surface is necessary when evaluating cellular processes or pharmaceuticals that alter the antigen presentation machinery. Here I describe a quantitative flow cytometry application for determining the number of peptide–MHC complexes on the surface of cells grown in tissue culture that express an endogenous protein from which the peptide is derived. The procedure requires a monoclonal antibody with the ability to distinguish MHC class I molecules presenting the peptide of interest from other peptide–MHC complexes. Fluorescence signal measured on antibody-labeled cells can be compared to fluorescent-calibrated beads to determine the relative number of antibodies bound to the cell surface and hence the number of specific peptide–MHC complexes expressed by the cell. As new monoclonal antibodies with TCR-like specificity for peptide–MHC complexes are created, this method will be helpful in quantifying the exact numbers of complexes generated by cell types and relating these numbers to physiological outcomes of T cell activation.

Key words: Antigen presentation, MHC class I, Quantitative flow cytometry

1. Introduction

Destroying the cells of the body that have become diseased as a result of intracellular infection or oncogenic transformation while sparing healthy cells is the goal of CD8⁺ T cells. In order to distinguish between healthy and diseased cells, CD8⁺ T cells rely on the binding between the antigen-specific T cell receptor (TCR) and the cognate peptide-major histocompatibility complex class I protein complex (hereafter pMHC) expressed on the surface of the cell undergoing immune surveillance. MHC class I proteins can bind a variety of 8–13 amino acid peptides derived by precursor proteins that have been degraded by cellular proteolytic processes (1–3). Because the antigenic peptide is the lynchpin in the immune reaction, an understanding of how peptides are generated and presented is

of upmost importance in the drive to either enhance CD8⁺ T cell responses to intracellular infections and oncogenic transformations, and conversely, dampening the immune responses for autoimmune diseases.

In order to determine if therapeutic interventions can modulate T cell responses there needs to be an accurate method for quantifying the number of antigen-specific pMHCs on the surface of cells being studied. A number of studies have used biochemical methods for isolating and quantifying peptides from cells in order to infer the number of peptides bound to a given MHC I molecule (4–8). However, biochemical methods often require large amounts of both time and material to accurately determine the amount of a given peptide present in a sample population, not to mention the requirements of expensive instrumentation. Therefore, a method for rapidly quantifying pMHC numbers on a relatively small number of antigen presenting cells would be advantageous.

Flow cytometry is a technique that uses fluorescently labeled antibodies to identify the presence of antigens on cells and is especially useful when cells are in far shorter supply than what is required for traditional biochemical analysis. Additionally, flow cytometry can identify subsets of cells within a total population that either express or do not express specific cellular markers. Sample analysis is also quite fast. Flow cytometry can also be employed to quantify the amount of a given antigen presence by inferring the number of antibodies bound to the cell from the fluorescence signal measured by the instrument. Therefore flow cytometry can be a valuable tool to quantify the number of pMHC on a given cell population (9–11).

Here I describe a detailed procedure for quantifying pMHC on the surface of cells using monoclonal antibodies with T cell-like specificity (TCRm), that is antibodies which can distinguish between MHC class I molecules bound to a specific peptide from MHC molecules occupied by a different peptide. We used the first such TCRm to be described, 25D-1.16, which recognized the ovalbumin peptide SIINFEKL bound to murine K^b molecules (12). In recent years there have been many other TCRms published that recognize a variety of antigenic peptides bound to either human (13–17) or mouse (18) MHC class I molecules and the protocol described here should be of interest to those researchers working with and further developing TCRms, especially as the clinical applications of these reagents becomes more feasible.

2. Materials

Follow standard practices for sterile tissue culture when working with cell lines prior to quantification. Follow institutional safety guidelines when applicable. A basic understanding of the use and

care of flow cytometry is needed even if experiments will be conducted by trained personal in a core facility.

2.1. Antibodies and Antibody Labeling

1. TCRm purified monoclonal antibodies in PBS should be stored at -20 to 4°C (see Note 1). Antibody producing cell lines are generally made available by the investigators who created them and it is best to contact investigators about such cell lines.
2. Commercial kit for fluorescently labeling antibody stored according to the manufacturer's recommendation (Invitrogen, Pierce, etc.) (see Note 2).
3. Necessary equipment not supplied with manufacturers' fluorescent labeling kits.
4. Spectrophotometer that can measure absorbance at 280 nm and the absorbance maximum of the fluorescent dye used.

2.2. Cells and Antibody Staining

1. Appropriate tissue culture media for cell lines being used (includes sterile media for cell line passaging).
2. Materials and reagents needed for examination of primary cells isolated from animals or patient samples (follow standard institutional guidelines for working with such materials).
3. Peptide-antigen positive and negative cell lines.
4. Phosphate-buffered saline (PBS).
5. BSS/BSA: Filter sterilized balanced salt solution (BSS) or PBS, containing 0.1% bovine serum albumin.
6. 2–4% Paraformaldehyde.
7. Round bottom 96-well plates.

2.3. FACS Analysis

1. Flow cytometer equipped with the appropriate lasers and filter sets for proper excitation of the chosen fluorescent dye.
2. Fluorescence calibration beads such as FITC-calibration beads from Spherotech or QuantumTMMESF beads from Bang's Laboratories.
3. Appropriate analytical software that can determine if the mean fluorescent intensity (MFI) of a given population of cells for the fluorochrome of interest.

3. Methods

3.1. Antibody Labeling

1. All labeling of antibodies with fluorescent dyes should be conducted according to manufacturer's protocols as such protocols have been designed for the specific dye formulations provided.
2. Store labeled antibody solutions at 4°C. DO NOT FREEZE.

3.2. Determine the Fluorescence to Protein Ratio

1. Record the spectral absorbance at 280 nm and at the absorbance maximum for the dye used (494 nm for FITC).
2. Calculate the antibody concentration using the following formula where 203,000 is the Molar extinction coefficient for IgG and 0.2 is the correction factor for FITC (other dyes will have a different correction factor):

Antibody Concentration (M)

$$= \left[\text{Abs}_{280} - (\text{Abs}_{494} \times 0.2) \right] / 203,000$$

3. Calculate the degree of labeling with the following formula where 68,000 is the molar extinction coefficient for FITC:

Moles of FITC per moles of protein

$$= \text{Abs}_{494} / (68,000 \times \text{Antibody concentration})$$

4. If the ratio is less than 1, the antibody labeling reaction should be repeated (see manufacturers “Troubleshooting” section of the labeling directions).

3.3. Determine the Optimal Antibody Dilution

1. Plate 10^5 antigen positive or negative cells/well of a 96-well plate 11 times.

2. Wash cells by adding BSS/BSA, centrifuging the plate at $\sim 700 \times g$ for 1 min at 4°C , and *gently* flick plate to remove liquid without disturbing the pelleted cells.

3. Make 100 μl dilutions of the labeled antibody in BSS/BSA as follows:

1:100, 1:200, 1:300, 1:400, 1:600, 1:800, 1:1,200, 1:1,600, 1:2,400, 1:3,200 (see Note 3).

4. Add between 30 and 50 μl of antibody solution (or BSS/BSA lacking any antibody) to both antigen positive and negative cells. Pipet cells up and down 2–4 times in the antibody solution to ensure they are properly resuspended.

5. Incubate on ice or in the refrigerator for 30–45 min. Protect the plate from light.

6. Wash cells by adding 200 μl of BSS/BSA to cells and centrifuging as in Subheading 3.3, step 2.

7. *If cell fixation is necessary:* Resuspend cells in 50 μl of PBS and add 50 μl 2% paraformaldehyde to the cells. Incubate at room temperature for 15 min (protected from light) and then wash 2 \times with BSS/BSA.

8. Transfer cells to appropriate tubes for flow cytometry analysis (if necessary, some flow cytometers are compatible with 96-well plates).

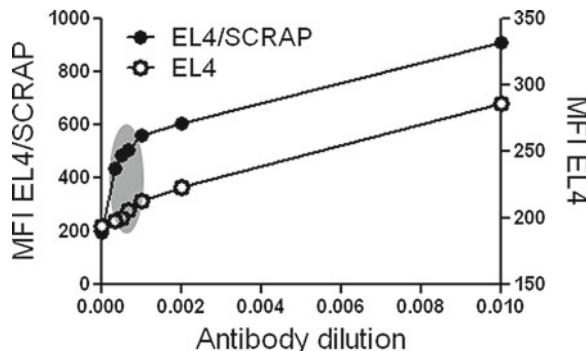


Fig. 1. Determining optimal TCRm concentration. EL4 (open circle, right axis MFI values) or EL4/SCRAP cells (black circles, left axis) which over express the SIINFEKL peptide (20) were stained with the FITC-coupled TCRm 25D-1.16 at indicated dilutions of stock antibody (~0.2 mg/ml) for 30 min on ice and analyzed by flow cytometry. The optimal concentration (denoted by a shaded ellipse) occurs where the MFI fold-increase over background is maximal, in this case approximately 1:1,500, and near the plateau of the curve.

9. Analyze by flow cytometry and determine with mean fluorescence intensity (MFI) of both the antigen positive and negative population for each staining. Plot the MFI as a function of antibody concentration.
10. Determine the optimal antibody dilution which should ideally be the dilution just below the plateau for antigen positive cells and where background staining of antigen negative cells is minimal (see Fig. 1).

1. Make a solution of fluorescence equivalent beads by combining ~1 drop of each bead labeled with a particular molar quantity of dye included in the kit. In general, each kit contains 4–5 different concentrations of dye and an unlabeled control.
2. Analyze by flow cytometry the mixture of beads using the same settings from Subheading 3.3, steps 2–9 (and ideally, directly before or after analyzing cells).
3. Determine the MFI for each “peak” of beads in the sample (see Fig 2a) and plot the MFI as a function of molar fluorescence equivalents for that particular bead (see Fig. 2b).
4. It may be necessary to remove the beads with the highest concentration of dye depending on the sensitivity of the flow cytometer used.

3.4. Determine the Fluorescence Equivalent Range of the Flow Cytometer

3.5. Quantifying pMHC on Cells

1. Stain antigen positive and negative cells in triplicate using the optimal antibody dilution conditions determined in Subheading 3.3, step 10.
2. Analyze by flow cytometry as in Subheading 3.3, steps 9–10 and determine the average MFI for use in the calculation below.

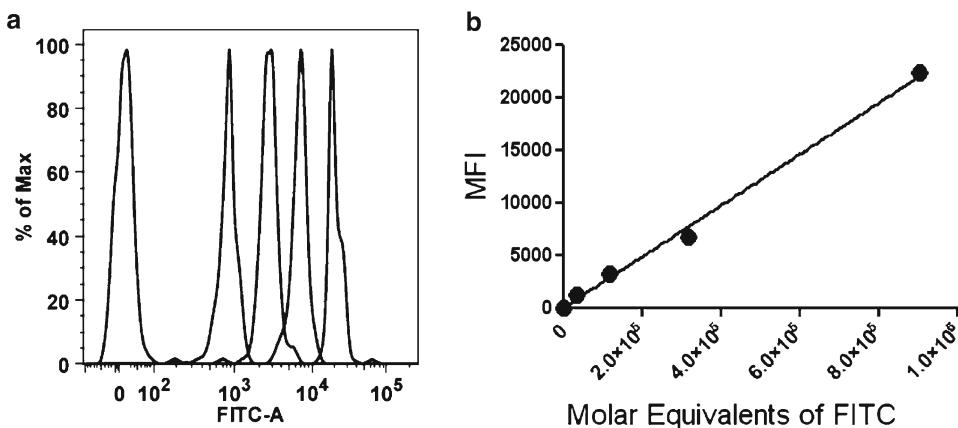


Fig. 2. Converting MFI signal to molar amounts of bound dye. Beads containing different concentrations of FITC (and negative control beads) were mixed together in equivalent amounts and analyzed by flow cytometry. (a) The MFI of each individual peak was plotted as a function of the corresponding molar equivalent of FITC dye (b) and the regression analysis plotted. The slope of this line would be used in Subheading 3.5 to convert the MFI of each antibody-labeled sample to the number of pMHC, and thus the number of pMHC, on the cell surface.

3. Prepare and analyze fluorescent beads as in Subheading 3.4.
4. Plot the regression analysis of fluorescence beads to determine the slope of the line of best fit (m).
5. Determine the number of pMHC on cells using the following formula (see Note 4):

$$\text{pMHC} = (\text{MFI of antigen positive cells} / m / F / P \text{ ratio}) - (\text{MFI of antigen negative cells} / m / F / P \text{ ratio})$$

where m is the slope of the line of best fit from Subheading 3.5, step 4 and F/P ratio is the fluorescence to protein ratio obtained from Subheading 3.2, step 3.

4. Notes

1. Accurately quantifying pMHC will be most influenced by the antibody used. It is therefore important to keep the following considerations in mind:
 - Antibody solutions must be of high quality and do not provide high background when applied to antigen-negative cells.
 - Be sure that all traces of unreactive dye are removed, which can be accomplished through simple dialysis.

- Use purified antibody solutions rather than hybridoma supernates or ascites fluid as these solutions cannot be used for direct coupling of dyes.
2. In general, the fluorescent dye you choose should meet some basic criteria, such as the ease to work with, the availability of calibrated beads, and the relative intensity of the dye. Manufacturers such as Molecular Probes and Pierce can supply ready to use kits optimized for binding and purifying antibodies and should be used. Some dyes, such as Alexa 647, are spectacular for detecting low levels of antigen, however, as of yet there is no commercially available source of calibration beads to convert signal to molar amounts of dye. Substitute dye-coupled beads (such as APC) which can be used to determine linear ranges, are not suitable for the determining concentrations because the alternative dyes have different molar extinction coefficients.
 3. In addition be sure to accurately determine the concentration of antibody needed (Subheading 3.3), too much antibody can drastically throw off your results. Other considerations for staining include time and volume. It may be necessary to stain cells for longer periods of time or in greater volumes of antibody solution than described here to attain optimal results. If so, maintain continuity between experiments.
 4. To accurately determine the pMHC counts on a given cell type, it will be necessary to repeat experiments several times over the course of a week or more. It is imperative that the instrument be in the same configuration during this time period so that recorded values are consistent. Calibration beads should always be included in each analysis, even if the conversion equation (Subheading 3.5) has been previously determined. Theoretically, a change in laser power, compensation, or other parameter should not affect the number of pMHC determined empirically, but in practice such changes can alter the calculated values. To be confident in the numbers reported, especially differences between cell populations or experimental conditions, one should perform all experiments together and in a short time frame.
 5. TCR-like antibodies such as 25D-1.16 can have a higher affinity of peptide–MHC complexes than the corresponding TCR (19), but the high avidity afforded by T cells combined with the signaling cascade resulting in T cell activation, means that T cells can detect far fewer peptide–MHC complexes on the cell surface compared to antibodies. Therefore in instances when peptide–MHC density is low, such as primary cells which express low levels of MHC class I or cross-presenting dendritic cells which rely on engulfing low levels of antigen, it may be difficult to detect peptide–MHC complexes on the cell

surface via antibody staining. In general, at least a 250–500 pMHC difference between experimental and control cells is needed in order to quantify pMHC with antibodies, although in many instances this number can range into the thousands. Therefore this method may not be able to accurately quantify pMHC at very low levels, though such levels are sufficient to activate T cells.

Acknowledgment

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References

1. Sijts EJ, Kloetzel PM (2011) The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell Mol Life Sci* 68:1491–1502
2. Del Val M, Iborra S, Ramos M, Lazaro S (2011) Generation of MHC class I ligands in the secretory and vesicular pathways. *Cell Mol Life Sci* 68:1543–1552
3. van Endert P (2011) Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell Mol Life Sci* 68:1553–1567
4. Fortier MH, Caron E, Hardy MP, Voisin G, Lemieux S, Perreault C, Thibault P (2008) The MHC class I peptide repertoire is molded by the transcriptome. *J Exp Med* 205:595–610
5. Lemmel C, Weik S, Eberle U, Dengjel J, Kratt T, Becker HD, Rammensee HG, Stevanovic S (2004) Differential quantitative analysis of MHC ligands by mass spectrometry using stable isotope labeling. *Nat Biotechnol* 22:450–454
6. Pang KC, Wei JQ, Chen W (2006) Dynamic quantification of MHC class I-peptide presentation to CD8+ T cells via intracellular cytokine staining. *J Immunol Methods* 311:12–18
7. Tan CT, Croft NP, Dudek NL, Williamson NA, Purcell AW (2011) Direct quantitation of MHC-bound peptide epitopes by selected reaction monitoring. *Proteomics* 11:2336–2340
8. Villanueva MS, Fischer P, Feen K, Pamer EG (1994) Efficiency of MHC class I antigen processing: a quantitative analysis. *Immunity* 1:479–489
9. Princiotta MF, Finzi D, Qian SB, Gibbs J, Schuchmann S, Buttigereit F, Bennink JR, Yewdell JW (2003) Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity* 18:343–354
10. Wherry EJ, Puorro KA, Porgador A, Eisenlohr LC (1999) The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J Immunol* 163:3735–3745
11. Wolf BJ, Princiotta MF (2010) Viral and bacterial minigenes products are presented by MHC class I molecules with similar efficiencies. *Mol Immunol* 48:463–471
12. Porgador A, Yewdell JW, Deng Y, Bennink JR, Germain RN (1997) Localization, quantitation, and *in situ* detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6:715–726
13. Kim S, Li L, McMurtrey CP, Hildebrand WH, Weidanz JA, Gillanders WE, Diamond MS, Hansen TH (2010) Single-chain HLA-A2 MHC trimers that incorporate an immunodominant peptide elicit protective T cell immunity against lethal West Nile virus infection. *J Immunol* 184:4423–4430
14. Verma B, Hawkins OE, Neethling FA, Caseltine SL, Largo SR, Hildebrand WH, Weidanz JA (2010) Direct discovery and validation of a peptide/MHC epitope expressed in primary human breast cancer cells using a TCRm monoclonal antibody with profound antitumor properties. *Cancer Immunol Immunother* 59:563–573
15. Makler O, Oved K, Netzer N, Wolf D, Reiter Y (2010) Direct visualization of the dynamics of antigen presentation in human cells infected with cytomegalovirus revealed by antibodies mimicking TCR specificity. *Eur J Immunol* 40:1552–1565

16. Epel M, Carmi I, Soueid-Baumgarten S, Oh SK, Bera T, Pastan I, Berzofsky J, Reiter Y (2008) Targeting TARP, a novel breast and prostate tumor-associated antigen, with T cell receptor-like human recombinant antibodies. *Eur J Immunol* 38:1706–1720
17. Nunoya J, Nakashima T, Kawana-Tachikawa A, Kiyotani K, Ito Y, Sugimura K, Iwamoto A (2009) Short communication: generation of recombinant monoclonal antibodies against an immunodominant HLA-A*2402-restricted HIV type 1 CTL epitope. *AIDS Res Hum Retroviruses* 25:897–904
18. Lev A, Takeda K, Zanker D, Maynard JC, Dimberu P, Waffarn E, Gibbs J, Netzer N, Princiotta MF, Neckers L, Picard D, Nicchitta CV, Chen W, Reiter Y, Bennink JR, Yewdell JW (2008) The exception that reinforces the rule: crosspriming by cytosolic peptides that escape degradation. *Immunity* 28:787–798
19. Mareeva T, Lebedeva T, Anikeeva N, Manser T, Sykulev Y (2004) Antibody specific for the peptide-major histocompatibility complex. Is it T cell receptor-like? *J Biol Chem* 279: 44243–44249
20. Dolan BP, Li L, Veltri CA, Ireland CM, Bennink JR, Yewdell JW (2011) Distinct pathways generate peptides from defective ribosomal products for CD8+ T cell immunosurveillance. *J Immunol* 186:2065–2072

Chapter 15

Biochemical Analysis of Naturally Processed Antigenic Peptides Presented by MHC Class I Molecules

Takayuki Kanaseki and Nilabh Shastri

Abstract

Immune surveillance of infected or tumor cells by CD8⁺ T cells requires that MHC class I molecules present a diverse repertoire of peptides on the cell surface. Even a few copies of individual peptides among this mixture are sufficient for recognition by the antigen receptors of appropriate CD8⁺ T cells. Here we describe a method for biochemical analysis of the naturally processed peptides in living cells. The peptide mixture in cell extracts is fractionated using reverse phase high performance liquid chromatography and detected by the activation of CD8⁺ T cell hybridomas. The results provide information on the structure and amount of the peptides and yield insights into the mechanisms that generate the naturally processed peptides.

Key words: Antigen presentation, Antigen processing, MHC, CD8⁺ T cells

1. Introduction

The antigen processing pathway yields a peptide bound to the MHC I molecule (pMHC I) on the cell surface (1). Foreign pMHC I serve as potential ligands for the CD8⁺ T cell repertoire. Many different proteases, chaperones, transporters, and adapters are involved in generating the pMHC I complexes. However, the particular peptide presented by the MHC I molecule is a homogenous species, containing 8–10 amino acids and usually a consensus motif defined for each polymorphic MHC I molecule (2).

The naturally processed peptides presented by MHC I molecules were first identified more than 20 years ago (3, 4). Since then, numerous antigenic peptides derived from various self- and non-self-proteins have been identified (5). Identification of these peptides is important not only to establish the precise structure of the antigenic entity and its protein source but also to ensure that appropriate T cells are detected when the peptides are used to

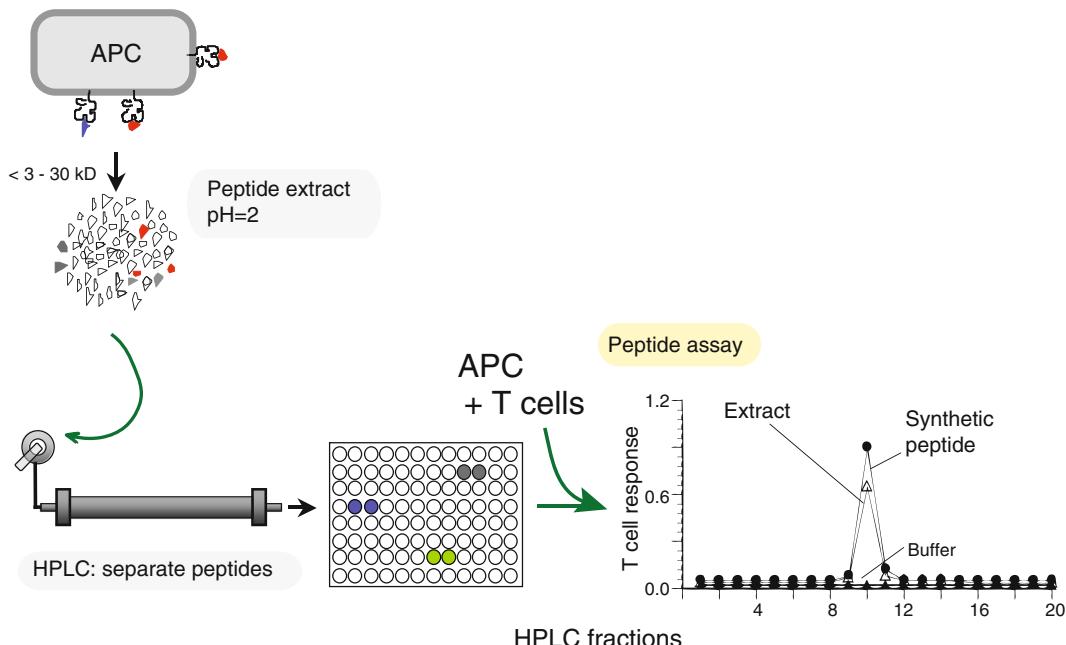


Fig. 1. Biochemical analysis of naturally processed peptides. Cells are homogenized in acid and filtered through a 3–30 kDa molecular filter. The filtrate containing the complex peptide mixture is then fractionated by reverse phase HPLC. The fractions, collected in 96-well plates, are dried and resuspended in buffer. Appropriate APC and T cells are added to the wells and after overnight culture, the T cell response is measured by the conversion of the *lacZ* substrate to a colored product. In the schematic shown, the naturally processed peptide in extracts of OVA expressing cells co-elutes with the synthetic SIINFEKL (SL8) peptide. Mock injections of buffer alone are carried out prior to each run to ensure that the activity in the test fractions is not due to spurious cross-contamination between samples.

produce tetramer reagents for binding T cell receptors. Even a single amino acid addition or deletion can have major impact on the ability of the peptide to bind to the relevant MHC molecule as well as being recognized by the T cell clones.

Here we describe a method for identifying the naturally processed peptides in antigen-presenting cells (Fig. 1). This method can be used to detect antigenic activity in cell extracts and to identify the processed peptide if synthetic peptide analogs are available. Synthetic peptide analogs can also be used to construct standard curves and to estimate the number of copies of the corresponding antigenic peptide present in the cells. Typically a cell produces less than ten to thousands of copies of the antigenic peptide that are recognized by appropriate receptors of CD8⁺ T cells.

This biochemical method is adapted from the original methods described by the Rammensee group (4). The analysis is most easily and reproducibly carried out using a *lacZ* inducible, CD8⁺ T cell hybridomas, natural or transfected cells expressing the antigenic peptide (APC), and an assay for detecting exogenous peptide activity. More specialized applications of this method used to analyze proteolytic intermediates are described elsewhere (6, 7).

2. Materials and Equipment

2.1. Common Reagents and Lab Equipment

1. PBS.
2. 10 cm Dishes.
3. 10% Formic acid.
4. Water bath.
5. 10 or 30 kDa cut-off filter (Microcon YM-10 or 30).
6. 96-Well plates.

2.2. Cellular Source of the Antigenic Peptides

Cells naturally presenting the antigen, such as virus infected cells or tumor cells can be used as source of peptides. Alternatively, we use the pcDNA1 vector to express the relevant antigen encoding genes in mammalian cells using standard methods. The expression of the appropriate MHC I molecule ensures that the cells serve as APCs and contain the processed antigenic peptide (8, 9).

2.3. T Cells and APCs for Exogenous Peptide Assay

1. As a model system, we use the B3Z T cell hybridoma to analyze the antigenic peptides derived from ovalbumin (OVA). B3Z is CD8⁺ T cell hybridoma with a TCR specific for the OVA peptide SIINFEKL (SL8 or its analogs). B3Z contains the *Escherichia coli* lacZ reporter gene under the control of NFAT element of IL-2 gene. Typically, B3Z produces lacZ in response to <1 pM of SL8 presented by H-2K^b on K^b expressing L-cells (see Notes 1 and 2). The generation of CD8⁺ T cell hybridomas has been discussed elsewhere in this series.
2. Mouse L-cells stably expressing the relevant K^b MHC I molecule (called K89 cells) are used as antigen presenting cells in exogenous peptide stimulation assays.

2.4. Cell Culture

1. The B3Z and K89 are cultured at 37°C in 5% CO₂ incubator with RPMI1640 (Invitrogen) supplemented with 10% fetal calf serum (HyClone), 0.1% 2-mercaptoethanol, 1% MEM sodium pyruvate, and 1% Penicillin–Streptomycin–Glutamine.

2.5. Reverse Phase High-Performance Liquid Chromatography

A typical system for reverse phase high-performance liquid chromatography (RP-HPLC) consists of

1. Agilent 1100 series including degasser (G1322A), quat pump (G1311A), VWD (G1314A), and manual injector (G1328B) with 500 µl injection loop.
2. 2.1×250 mm, 5 µm, C18 RP-HPLC column (Vydac, #218TP52).
3. Buffer A: 0.1% trifluoroacetic acid (TFA) in H₂O (ultra pure water (>18 MΩ cm at 25°C, Millipore)) (see Note 3).
4. Buffer B: 0.1% TFA in acetonitrile (see Note 3).

5. Thermo Scientific Savant SPD2010 SpeedVac Concentrator for drying the samples in microfuge tubes or directly in 96-well plates.

2.6. B3Z Assay

1. CPRG: Dilute 91 mg of chlorophenolred β -d-galactopyranoside (CPRG), 1.25 ml of NP40, and 9 ml of 1 M MgCl₂ in 1 l of PBS. CPRG solution must be protected from light and stored at 4°C (see Note 4).
2. Synthetic peptides: We use the synthetic peptides with >95% purity. Impurities generated during synthesis, can confuse results if they migrate in different fractions and also stimulate T cells. 1 mM stocks dissolved in appropriate solvents are stored at -20°C (see Note 5).
3. 96-well plate reader.

3. Methods

All procedures are carried out at room temperature unless otherwise specified.

3.1. Preparation of Peptide Samples from Transiently Transfected Cells

1. Transiently transfect the host cells with the precursor construct (see Note 6). We use FuGENE6 (Roche) following manufacturer's instructions. Typically, 1.2×10^6 host cells are plated in a 10-cm dish and cultured overnight. Dribble the mixture of 6 μ g of DNA (antigen encoding gene), 18 μ l of FuGENE6, and 600 μ l of serum-free medium into the cells in a 10-cm dish. Culture for 2 days.
2. Harvest the cells (typically, $\sim 5 \times 10^6$), spin down at $800 \times g$ for 2 min, and wash the cells with PBS.
3. Add 10% formic acid (1.0×10^7 cells/ml) and if necessary, a "martyr" peptide (2 μ M, see Note 7) to the cell pellet.
4. Mix thoroughly by pipetting and incubate the mixture for 5 min in a boiling water bath.
5. Spin down at $10,000 \times g$ for 15 min, and pass the supernatant through 10 or 30 kDa cut-off filter by centrifugation at $10,000 \times g$ for 1 h.
6. Store the flow-through on ice or frozen at -20°C until loaded into RP-HPLC.

3.2. Running Samples on RP-HPLC

1. The solvent gradient used for peptide elution must be optimized according to a particular RP-HPLC system and the antigenic peptide. Each peptide has a distinct elution profile depending upon the column, the amino acid sequence, and the elution conditions. For example, using a C18 column, the

solvent gradient for fractionating SL8 peptide is 0–5 min, 23% B; 5–49 min, 23% B–45% B; 49–52 min, 45% B–77% B at a flow rate of 0.25 ml/min (buffer A + buffer B = 100%). We collect the fractions by the total number drops (five drop per well of 96-well plate).

2. Inject the sample onto the RP-HPLC column and collect fractions in a 96-well plate. Dry the fractions to remove the solvents in a vacuum centrifuge overnight at room temperature (see Notes 8 and 9).
1. (Optional) Resuspend the fractions by adding 30 μ l PBS to each well. Incubate the 96-well plate at 37°C for 3 h (see Note 10).
2. Meanwhile, prepare complete RPMI containing B3Z (1×10^6 cells/ml) and antigen presenting cells (K89, 5×10^5 cells/ml).
3. Add 170 μ l of B3Z/K89 mixture per a well of 96-well plate. Culture overnight at 37°C.
4. Spin down the 96-well plates at $800 \times g$ for 2 min to collect the cells at the bottom of the wells. Flick off the plates to discard the supernatant. Add 100 μ l of CPRG reagent to each well (see Note 11). Incubate the plates at room temperature for up to 4 h and measure the absorbance with a 96-well plate reader at 575–595 nm (and at 655 nm as the reference) (see Note 12).
5. Identify the antigenic peptide activity in the sample by comparing the active fractions with corresponding fractions observed with synthetic peptides. The overall amount of the antigenic peptide can also be estimated by comparison of the activity with standard curves of synthetic peptides run in parallel in the same experiment.

4. Notes

1. Theoretically, any (antigenic peptide) can be used instead of the OVA-derived SL8 peptide, provided the sensitivity of the cognate T cell is high enough (~pM range) for that peptide. For example, we have successfully assayed the WI9 peptide encoded by Y-chromosome, Uty gene and presented by H-2D^b MHC I or the QL9 peptide encoded by oxoglutarate dehydrogenase (OGDH) gene and presented by the H-2L^d MHC I molecules.
2. We sometimes observe a gradual loss of antigen-sensitivity of the T cell hybridoma after prolonged time in culture. The antigen-sensitivity can be restored by cloning the hybridoma by limiting dilution, and picking the most sensitive subclone or by

simply thawing another vial of frozen stocks. Keeping the T-cell sensitivity high (typically < 1 pM of SL8) is critical for clear results.

3. Given the high sensitivity of the T cells, it is possible to detect contaminant or carry-over peptides. Extreme care should be taken to prepare the solvents in high-quality glassware.
4. The CPRG reagent should be stored at 4°C and prepared fresh if spontaneous hydrolysis to chlorophenolred is observed. Generally, the CPRG reagent can be stored for up to several weeks.
5. To identify the antigenic activity in the RP-HPLC fractions synthetic peptides corresponding to the expected peptide and potential analogs should also be analyzed. For example, peptides with additional N- or C-terminal residues.
6. It is also useful to generate the host cells stably expressing the precursor construct by a resistant gene expression and drug selection.
7. To ensure possible degradation of the antigenic peptide, we find it useful to add a high concentration of an irrelevant “martyr” peptide as a competitor.
8. Also, run 10 fmol each of the synthetic peptides prepared in Note 7, and identify the fraction numbers specific to the precursor and antigenic peptides.
9. To ensure the accuracy of measurements, always run a “mock” sample that consists of buffer A alone (0% B) before and after the samples and assay the fractions in parallel. These runs should not yield any activity demonstrating absence of contamination and peptides remaining in the column from previous runs. Injection of excessive amounts of synthetic peptides can permanently ruin the HPLC column.
10. Shake the plate horizontally on the bench to agitate the sample with PBS and avoid spilling.
11. Ideally, CPRG should be added all at once to all of the wells. We generally add CPRG by a multichannel pipette (12 wells at once) and repeat as quickly and accurately as possible.
12. Incubation time depends on the peptide concentration in the samples and the sensitivity of the T cell hybridoma. Incubations range from a few minutes to overnight, but in general, 2–3 h is enough. The absorbance of the 96-well plates should be measured well before the reactions are saturated.

References

1. Shastri N, Cardinaud S, Schwab SR, Serwold T, Kunisawa J (2005) All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol Rev* 207:31–41
2. Falk K, Rötzschke O, Stevanovic S, Jung G, Rammensee H-G (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290–296
3. Van Bleek GM, Nathenson SG (1990) Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature* 348:213–216
4. Rötzschke O, Falk K, Wallny HJ, Faath S, Rammensee HG (1990) Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. *Science* 249(4966):283–287
5. Rammensee HG, Bachmann J, Stevanovic S (1997) MHC ligands and peptide motifs. Landes Bioscience, Austin, TX
6. Kunisawa J, Shastri N (2003) The group II chaperonin TRiC protects proteolytic intermediates from degradation in the MHC class I antigen processing pathway. *Mol Cell* 12(3):565–576
7. Kunisawa J, Shastri N (2006) Hsp90alpha chaperones large proteolytic intermediates in the MHC class I antigen processing pathway. *Immunity* 24:523–534
8. Falk K, Rötzschke O, Rammensee HG (1990) Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* 348(6298):248–251
9. Malarkannan S, Goth S, Buchholz DR, Shastri N (1995) The role of MHC class I molecules in the generation of endogenous peptide/MHC complexes. *J Immunol* 154(2):585–598

Chapter 16

Identifying Source Proteins for MHC Class I-Presented Peptides

Nathalie Vigneron, Wenbin Ma, Alexandre Michaux,
and Benoît J. Van den Eynde

Abstract

Identification of antigenic peptides recognized by cytolytic T lymphocytes (CTL) is a prerequisite for the development of targeted cancer immunotherapy approaches. This chapter provides a global approach for the identification of peptides recognized by CTL. It implies the identification of the HLA molecule presenting the peptide as well as the design and screening of a cDNA library derived from the tumor cells.

Key words: Cytolytic T lymphocytes, Peptide identification, cDNA library, Chromium release assay

1. Introduction

Cytolytic T lymphocytes (CTL) recognize 8–11 amino-acid long peptides derived from the degradation of cellular proteins and presented at the cell surface by MHC class I molecules. Because CTL play a key role in the surveillance against cancers, the identification of class I antigenic peptides specifically expressed on tumors is crucial for the development of efficient immunotherapy protocols. Here, we describe an expression cloning approach for the identification of antigenic peptides recognized by CTL.

Anti-tumor CTL can be isolated from the blood or tumor infiltrates of cancer patients (1–3). This is usually done in vitro, by stimulating lymphocytes with autologous tumor cells, a process that leads to the amplification of a T-cell population able to lyse the tumor cells. These anti-tumor T cells can then be further cloned and studied. Before starting the peptide identification procedure, it is worth determining whether the antigen recognized by the CTL clone represents a promising candidate for the development of immunotherapeutic vaccines. Firstly, the CTL should recognize

a tumor-specific antigen that is not present on normal cells. Testing the lytic activity of the CTL towards NK-sensitive cell line K562 and normal autologous cells such as EBV-B cells, proximal tubular epithelial cells (PTEC), or fibroblast should therefore be evaluated (as described in Subheading 3.1.1). Secondly, the CTL should recognize an antigen present on a large variety of tumor lines. Indeed, CTL recognizing only one tumor generally target mutated peptides, which are unique to a patient and therefore, not usable in large-scale immunotherapeutic trials. Because the identification of the antigen likely requires the screening of a cDNA library, it is useful to test the ability of the T cells to secrete cytokines such as tumor necrosis factor (TNF) or gamma-interferon (γ IFN), which are easily detectable and will help the screening procedure.

2. Materials

All solutions should be prepared with ultrapure water. When using radioactivity, diligently follow the recommended handling precautions and waste regulations.

2.1. CTL Activation Assays

2.1.1. Chromium Release Assay

1. Target cells: tumor cells or EBV-B cells expressing the relevant HLA molecule.
2. T cells.
3. Complete Iscove's modified Dulbecco's medium (IMDM): Iscove's modified Dulbecco's medium (Life Technologies, Carlsbad, CA, USA) supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), penicillin (100 U/ml), and streptomycin (100 μ g/ml).
4. CTL medium: complete IMDM, supplemented with 10% human serum.
5. Fetal bovine serum (FBS, Hyclone research grade Fetal Bovine Serum, Thermo Scientific, Waltham, MA, USA).
6. $\text{Na}_2^{51}\text{CrO}_4$ (^{51}Cr , Perkin Elmer Life Sciences, Waltham, MA, USA).
7. 96-Well V-bottom plate (Greiner Bio one Frickenhausen, Germany).
8. Triton X-100 (Sigma-Aldrich, St Louis, MO, USA).
9. γ -Counter.

2.1.2. TNF Release Assay

1. Complete RPMI medium: RPMI medium (Life Technologies) supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), penicillin (100 U/ml), and streptomycin (100 μ g/ml).

2. CTL medium: complete IMDM medium supplemented with 10% human serum.
3. FBS (Thermo Scientific).
4. Interleukin 2 (R&D systems): reconstitute at 10⁵ U/ml in sterile phosphate buffer saline (PBS) 0.1% BSA. Store aliquots at -20 to -80°C.
5. 96-Well plate flat-bottom (Greiner Bio one).
6. TNF-sensitive line WEHI 164 cl13 (4) (see Note 1).
7. 1 M LiCl, filtered on a 0.2 µm filter (Sigma-Aldrich).
8. Actinomycin D (Sigma-Aldrich): 25 mg of actinomycin D is resuspended in 2.5 ml dimethyl sulfoxide (DMSO), then diluted to 1 mg/ml in RPMI.
9. Recombinant lymphotoxin (rTNF-β) (R&D systems, Minneapolis, MN, USA) (see Note 2).
10. PBS: 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4. Sterilize on a 0.2 µm filter and keep at 4°C.
11. MTT: [3,(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] (Promega, Madison, WI, USA): Resuspend 1 g of MTT powder in 400 ml PBS. Filter-sterilize using a 0.2 µm filter. Keep the solution for 1 month at 4°C in a light-protected container.
12. SDS-DMF lysis solution: To 1 L of water, add 500 g sodium dodecyl sulfate (SDS) and dissolve the SDS overnight at 65°C. The next day add water to obtain a final volume of 1.667 L. To 600 ml of this 30% SDS solution, add 300 ml DMF (*N,N*-dimethyl formamide), 18 ml anhydrous acetic acid, and 2.3 ml HCl 1 M. Verify that the pH is 4.7. Keep the solution at 37°C.
13. Spectrophotometer.

2.2. Production of a cDNA Library

1. mRNA purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA)
2. Superscript Choice System for cDNA Synthesis (Life Technologies).
3. [α -³²P] dCTP (10 mCi/ml) (PERKIN ELMER Life Sciences).
4. Oligo (dT) primer containing a NotI site at its 5' end [5'-ATA AGAATGCGGCCGCTAACTA(T)₁₈VN (V=G, A, or C; N=G, A, T, or C)].
5. 5× First-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) (Superscript Choice System for cDNA Synthesis, Life Technologies).
6. 0.1 M DTT stock solution (Store aliquots at -20°C).
7. dNTP (10 mM each dATP, dCTP, dGTP, dTTP) (Superscript Choice System for cDNA Synthesis, Life Technologies).

8. Superscript II Reverse transcriptase (200 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
9. 5 \times Second-strand buffer (100 mM Tris–HCl pH 6.9, 450 mM KCl, 23 mM MgCl₂, 0.75 mM β -NAD⁺, 50 mM (NH₄)₂SO₄) (Superscript Choice System for cDNA Synthesis, Life Technologies).
10. *Escherichia coli* DNA ligase (10 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
11. *E. coli* DNA polymerase I (10 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
12. *E. coli* Rnase H (2 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
13. T4 DNA polymerase (5 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
14. 0.5 M EDTA.
15. UltraPure Phenol:Chlorophorm:Isoamyl alcohol (25:24:1, v/v) (Life Technologies).
16. Cold ethanol (-20°C).
17. 7.5 M NH₄OAc (Ammonium acetate). Autoclave.
18. HindIII primers: 5'-AGCTTCCCGGG-3' and 5' PO4-CCGGGA-3'.
19. 5 \times Adapter buffer (330 mM Tris–HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP) (Superscript Choice System for cDNA Synthesis, Life Technologies).
20. T4 DNA ligase (1 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
21. T4 polynucleotide kinase (10 U/ μ l) (Superscript Choice System for cDNA Synthesis, Life Technologies).
22. 5 \times T4 DNA ligase buffer (250 mM Tris–HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG 8000) (Superscript Choice System for cDNA Synthesis, Life Technologies).
23. DEPC-treated water: Add 1 ml of diethylpyrocarbonate (DEPC) to 1 L distilled water, mix well, and leave under agitation for 12 h at 37°C. Autoclave. Let it cool down to room temperature prior use.
24. 10 mM ATP: a 100 mM stock of ATP (Thermo Fisher Scientific) is diluted to 10 mM in ultrapure water.
25. Restriction enzyme Not I (New England Biolabs, Ipswich, MA, USA).
26. Restriction NEBuffer 3 (New England Biolabs).
27. TAE buffer: 40 mM Tris Acetate, 1 mM EDTA pH 8 (see Note 3).

28. SeaKem LE Agarose (Lonza, Basel, Switzerland).
29. Yeast tRNA 1 µg/µl (Superscript Choice System for cDNA Synthesis, Life Technologies).
30. SYBR Gold Nucleic Acid Gel Stain (Life Technologies) and blue-light transilluminator.
31. cDNA size fractionation columns (Superscript Choice System for cDNA Synthesis, Life Technologies).
32. TEN buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 25 mM NaCl), autoclaved.
33. 1 mm Cuvette (Bio-Rad, Hercules, CA, USA).
34. Gene Pulser MXcell Electroporation System (Bio-Rad).
35. Lysogeny broth (LB): Mix 10 g Tryptone, 5 g Yeast Extract, 10 g NaCl to 1 L of water. Supplement with 15 g Agar if necessary. Adjust at pH 7 and autoclave.
36. Ampicillin 25 mg/ml (Sigma-Aldrich).
37. Nunc Bio-Assay Dishes 245 × 245 mm (cat 240835, VWR/NUNC Thermo Scientific), large plates.
38. Petri dish 100 mm (VWR/NUNC Thermo Scientific), standard plates.
39. LB 20% glycerol. Autoclave.
40. Expression vector containing NotI and Hind III restriction sites in the proper orientation.
41. *E. coli*: DH5α or MegaX DH10B

2.3. Transient Transfection and T-Cell Activation Assay

2.3.1. Transfection of COS-7

1. COS-7 cells.
2. Complete Dulbecco's modified Eagle medium (DMEM): DMEM supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Life Technologies).
3. CTL Medium as in Subheading 2.1.2.
4. Interleukin 2: as in Subheading 2.1.2.
5. 10 mg/ml Diethylaminoethyl-Dextran (DEAE-Dextran) (GE Healthcare) in water. Filter-sterilize on a 0.2 µm filter and keep aliquots at 4°C or -20°C.
6. 10 mM Chloroquine (Sigma-Aldrich) in water. Filter-sterilize on a 0.2 µm filter and keep aliquots at 4°C or 20°C.
7. NU-serum, devoid of DNase (Becton Dickinson, Franklin Lakes, NJ, USA).
8. 96-Well plate V-bottom (Greiner Bio one).
9. Culture grade DMSO (Sigma-Aldrich).
10. PBS: 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4. Filter-sterilize on a 0.2 µm filter and keep at 4°C.

**2.3.2. Transfection
of HEK-293 Cells**

1. HEK-293 cells.
2. Media as in Subheading 2.3.1.
3. Optimem (Life Technologies).
4. 96-Well plate flat-bottom (Greiner Bio one).
5. 96-Well plate V-bottom (Greiner Bio one).
6. Lipofectamine (Life Technologies).

**2.4. Cloning
the Positive Pool
of Bacteria**

1. Lysogeny broth (LB): as in Subheading 2.2.
2. Ampicillin 25 mg/ml.
3. 96-Well round-bottom plates (Greiner Bio one).
4. Nunc Bio-Assay Dishes 245 × 245 mm (cat 240835, VWR/
NUNC Thermo Scientific), large plates.
5. Resuspension buffer: 50 mM Tris-Cl, pH 8.0, 10 mM
EDTA.
6. Lysis buffer: 0.2 M NaOH/1%SDS.
7. Potassium acetate buffer: 3 M CH₃CO₂K pH 4.8 with acetic
acid.
8. Isopropanol (Thermo Fisher Scientific).
9. Absolute ethanol (Thermo Fisher Scientific).
10. LB 20% glycerol.
11. RNase A (Qiagen).

**2.5. Identification
of the Peptide-Coding
Sequence**

1. 10 mM dNTP (2.5 mM each dATP, dGTP, dCTP, dTTP)
(Clontech, Mountain View, CA, USA).
2. PFU DNA polymerase (Thermo scientific).
3. 10× PFU buffer with MgSO₄ (Thermo scientific).
4. Adequate primers.
5. Taq polymerase Clontech, Mountain View, CA, USA.
6. pcDNA3.1 TOPO/TA expression kit (Life Technologies).
7. Lysogeny broth (LB): as in Subheading 2.2.
8. Ampicillin 25 mg/ml.
9. One Shot TOP10 Chemically Competent *E. coli* (Life
Technologies).

**2.6. Identification
of the Peptide
Recognized
by the T Cell**

1. Idem Subheading 2.1.1.
2. All peptides are synthesized on solid phase using Fmoc for
transient N-terminal protection and characterized by mass
spectrometry. The lyophilized peptides are diluted at a final
concentration of 20 mg/ml in DMSO and stored at -80°C.

3. Methods

3.1. CTL Activation Assays

3.1.1. Chromium Release Assay (Adapted from (5))

The activity of anti-tumor CTL can be measured using different types of assays. The so-called chromium release assay is helpful to study the ability of the T cells to lyse various tumor cell lines. Alternatively, cytokines released by the CTL can be assessed by ELISA or using a colorimetric cell-based cytotoxicity bioassay, e.g. to measure TNF production (as described in Subheading 3.1.2).

The principle of this assay is to measure the ability of the CTL to lyse different target cells. Target cells are labeled with radioactive ^{51}Cr and incubated with the CTL. The radioactivity measured in the co-culture supernatant gives an estimation of the percentage of target cells lysed by the CTL.

1. Collect 2×10^6 target cells and centrifuge the cells at $240 \times g$ for 5 min in a 15 ml conical polypropylene tube.
2. Carefully aspirate the supernatant and resuspend the cells in 50 μl complete IMDM containing 50% FBS and 100 μCi ^{51}Cr . Incubate at 37°C for 1 h, flipping the tube every 15 min to optimize ^{51}Cr incorporation.
3. Wash the cells in 10 ml complete IMDM and centrifuge the cells at $240 \times g$ for 5 min. Repeat these washes four times and resuspend the cells in 1 ml complete IMDM containing 10% FBS.
4. Count the labeled cells and resuspend them at a cell density of 10,000 cells per ml in complete IMDM containing 10% FBS.
5. Count the T cells and spin them down for 5 min at $240 \times g$. Resuspend the cell pellet in CTL medium at the appropriate density for the assay (generally 300,000 cells per ml to start the assay with an effector-to-target ratios of 30:1). Various dilutions of the amount of effector cells are generally planned. In a 96-well V-bottom plate, add 100 μl of the T cells dilutions in duplicate wells. As control of spontaneous and maximal ^{51}Cr release, 100 μl of medium and 100 μl of medium supplemented with 1% triton-X 100 are, respectively, added to three wells.
6. Add 100 μl of labeled target cells (corresponding to 1,000 cells) to the CTL and controls. Spin the plates for 5 min at $168 \times g$ and incubate for 4 h at 37°C in an 8% CO₂ incubator.
7. After 4 h of incubation, spin the plates for 5 min at $168 \times g$, and carefully collect 100 μl of the supernatant. Avoid disturbing the pellet to limit the diffusion of membrane-associated radioactivity in the culture supernatant. Radioactivity contained in the supernatant is then evaluated using a γ -counter.
8. The specific lysis can be calculated as follows: ((Experimental cpm – Spontaneous release cpm)/(Maximal release cpm – Spontaneous release cpm)) × 100 (see Note 4).

3.1.2. TNF Release Assay

TNF production is measured by testing sample cytotoxicity for TNF-sensitive cell line WEHI 164cl13 (4, 6). The optimal effector-to-target ratio needs to be defined for each T-cell-target combination. Usually, 5,000–40,000 target cells per well are incubated together with 5,000–20,000 T cells.

1. In a 96-well flat-bottom plate, mix the CTL and target cells at the appropriate effector-to-target ratio in 200 µl CTL medium supplemented with 25 U/ml IL2. Incubate the co-culture overnight at 37°C in an 8% CO₂ incubator.
2. After 20 h of co-culture, centrifuge the plate at 240×g for 5 min, collect the supernatants, and transfer 50 µl into a new 96-well flat-bottom plate. The supernatant can also be diluted in CTL medium for more precise results. Additionally, prepare a standard curve of recombinant lymphotxin (50 µl per well) using concentrations ranging from 200 to 0 pg/ml.
3. Resuspend WEHI 164 clone 13 at a cell density of 6×10⁵ cells/ml in complete RPMI medium containing 5% FBS, 40 mM LiCl, and 2 µg/ml Actinomycin D (7). Add 50 µl of the WEHI mix to the 50 µl of supernatant and controls. Incubate 20 h at 37°C in a 5% CO₂ incubator.
4. The next day, the number of live WEHI cells is measured in a colorimetric assay using the tetrazolium MTT, which is bioreduced into soluble formazan crystals by living cells.
 - (a) Add 50 µl of a solution containing 2.5 mg/ml MTT in phosphate-buffered saline to the wells containing the WEHI 164 clone 13 cells.
 - (b) After 3 h of incubation at 37°C, lyse the cells and dissolve the formazan crystals by adding 100 µl of an SDS/DMF lysis solution. Incubate the plate overnight in a 37°C incubator (dedicated to that purpose since DMF is toxic to cells).
 - (c) Measure the optical density at 570 nm, which is proportional to the amount of living cells in the well. As a reference, the absorbance at 650 nm is also measured (see Note 5).

3.2. Identification of the HLA Molecule Presenting the Antigenic Peptide

In order to identify the HLA class I molecule presenting the peptide, available allogeneic tumors sharing one or more HLA class I molecules with the autologous tumor cells are tested for their ability to activate the CTL (cf. Subheading 3.1). In addition, blocking CTL recognition using specific anti-HLA class I antibodies can also be performed by adapting the CTL activation assay as follows:

1. Prior to the target and T-cell co-cultures, incubate the target cells in the presence of 10 µg/ml of blocking anti-HLA antibodies (8) (see Notes 6 and 7).

2. After 30 min incubation, add effectors at the appropriate cell density and resume the T-cell activation assay (as in Subheading 3.1).

Allogeneic tumor lines can also be transiently transfected (as described in Subheading 3.4.2) with candidate HLA cDNA and tested for their ability to stimulate TNF release by the CTL (see Notes 8 and 9).

3.3. Production of a cDNA Library

In many cases, if the antigenic peptide does not correspond to a known tumor antigen, it is necessary to prepare a cDNA library derived from the CTL-sensitive tumor cells, preferably the autologous tumor cells (Fig. 1). DNA from pools corresponding to about 100 different cDNA will then be transiently transfected, together with the cDNA encoding HLA, into highly transfectable antigen-presenting cells (COS-7 or HEK-293, for example). Transfected cells will then be tested for their ability to activate cytokine release by the CTL. In our hands, directional cDNA libraries were often successfully generated using the SuperScript Choice System for cDNA Synthesis (Life Technologies) (9, 10). We recently adapted the manufacturer protocol to avoid the use of radioactivity. This was made possible by the use of a nanodrop spectrophotometer as well as the SYBR Gold staining dye, which both enable the detection of nanogram amounts of double-strand DNA. However, the drawback of this new approach is that the yield of first-strand cDNA synthesis cannot be evaluated during the procedure. Here, we will describe both procedures.

3.3.1. RNA Extraction

Total RNA (tRNA) can be extracted from tumor cells using the guanidine isothiocyanate procedure (11) or RNA extraction kits such as RNeasy (Qiagen) or Nucleospin RNA (Macherey-Nagel). High-quality mRNA is then purified after processing through two successive oligo(dT)-cellulose columns (mRNA purification kit; GE healthcare).

3.3.2. First-Strand Synthesis

1. In a sterile RNase-free Eppendorf tube, transfer 1 µg of the oligo(dT) primer containing a NotI site (see Note 10). Add 5 µg of mRNA diluted in DEPC water to obtain a final volume of 7 µl.
2. Heat for 10 min at 70°C then quickly chill on ice.
3. Centrifuge briefly. Add 4 µl of 5× first-strand buffer, 2 µl DTT 0.1 M, and 1 µl dNTP 10 mM. If the yield of the first-strand synthesis has to be verified, add 1 µl of [α -³²P] dCTP (10 µCi/µl). Otherwise add 1 µl of DEPC-treated water. Vortex gently, centrifuge briefly, and place the tube at 37°C for 2 min to equilibrate the temperature (see Note 11).
4. Add 5 µl SuperScript II reverse transcriptase (200 U/µl). Mix gently and incubate at 37°C for 1 h.

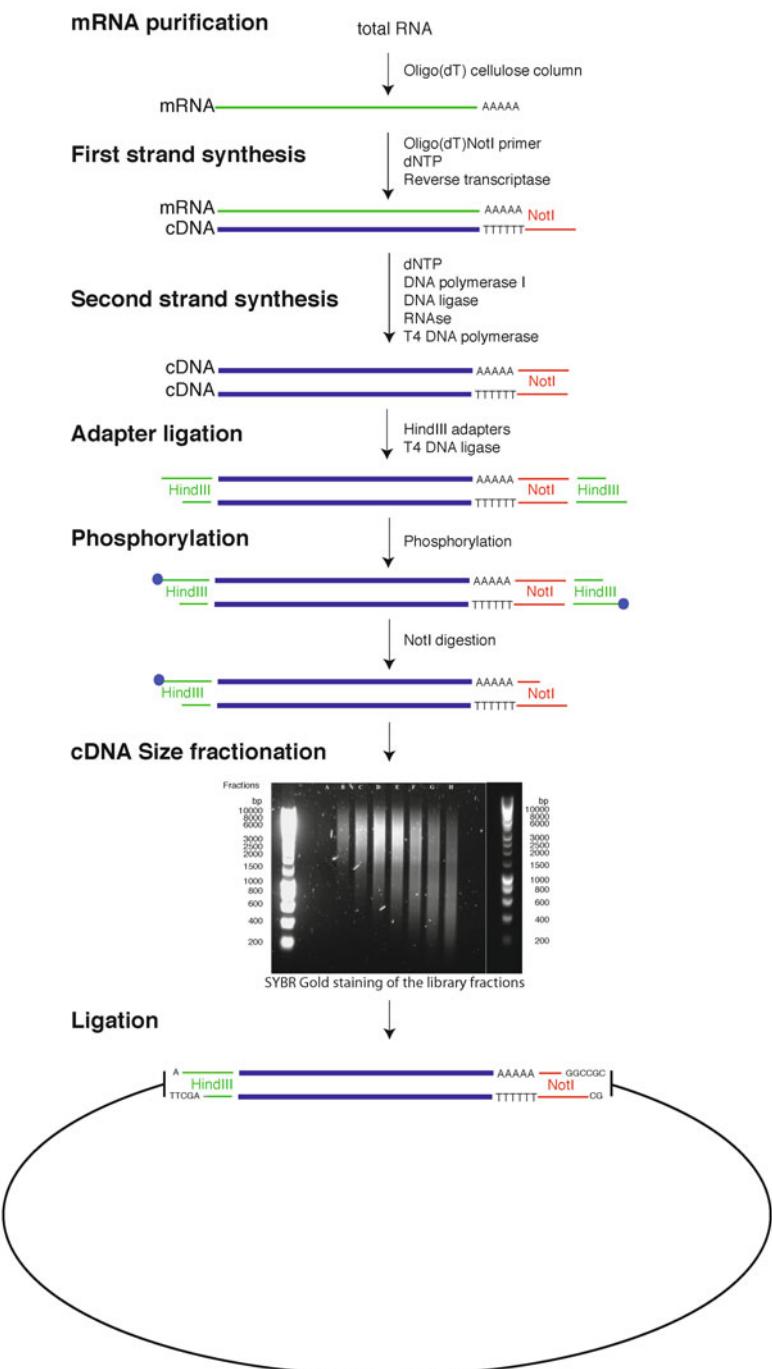


Fig. 1. Schematic representation of the procedure used to construct a directional cDNA library.

5. Place the tube on ice.
6. If the yield of the first-strand synthesis is measured using $[\alpha\text{-}^{32}\text{P}]$ dCTP, remove 2 μl of the reaction. Calculate the yield of the first-strand reaction as described in Note 12.

3.3.3. Second-Strand Synthesis

1. To the DNA obtained in Subheading 3.3.2, add on ice (and in the following order) 93 μ l DEPC water, 30 μ l of the 5 \times second-strand buffer, 3 μ l dNTP 10 mM, 1 μ l *E. coli* DNA ligase (10 U/ μ l), 4 μ l *E. coli* DNA polymerase I (10 U/ μ l), and 1 μ l *E. coli* RNase H (2 U/ μ l). Vortex gently and incubate the reaction at 16°C for 2 h.
2. Add 2 μ l of T4 polymerase (5 U/ μ l) and incubate 16°C for 5 min.
3. To stop the reaction, chill the tube on ice and add 10 μ l EDTA 0.5 M.
4. Add 150 μ l of a phenol/chlorophorm/isoamyl alcohol solution (25:24:1). Vortex thoroughly and separate the phases by centrifuging at room temperature for 5 min at 14,000 $\times g$.
5. Carefully remove 140 μ l of the upper, aqueous layer into a new tube and precipitate the DNA by adding 70 μ l of NH₄OAc 7.5 M and 500 μ l cold absolute ethanol (-20°C). Vortex thoroughly and centrifuge immediately at room temperature for 20 min at 14,000 $\times g$.
6. Carefully aspirate the supernatant and overlay the pellet with 500 μ l of cold 70% ethanol (-20°C). Centrifuge at 14,000 $\times g$ for 2 min and remove the supernatant.
7. Dry the pellet for 10 min at 37°C in a speed-vacuum.

3.3.4. Annealing of the HindIII Adapters (See Note 13)

1. In an Eppendorf tube, add 5 μ g of each of the HindIII DNA oligomers and adjust the volume to 8 μ l with sterile water. Add 2 μ l of adapter buffer. Incubate 10 min at 65°C.
2. Transfer the solution at room temperature for 2 h to allow both DNA strands to hybridize to each other.

3.3.5. Ligation of the HindIII Adapters to the Double-Stranded cDNA

1. Place on ice the cDNA pellet obtained in Subheading 3.3.3 and add, in the following order, 20 μ l DEPC-treated water, 8 μ l of 5 \times adapter buffer, 10 μ l of the annealed HindIII adapters from Subheading 3.3.4, 7 μ l DTT 0.1 M, and 5 μ l T4 DNA ligase (1 U/ μ l) in a final volume of 50 μ l. Mix gently and incubate at 16°C overnight (minimum 16 h).
2. Inactivate the T4 DNA ligase by heating the reaction at 70°C for 10 min.
3. Place the reaction on ice.

3.3.6. Phosphorylation of the cDNA Ligated to HindIII Adapters

1. To the cold DNA solution obtained in Subheading 3.3.5, add 3 μ l of T4 polynucleotide kinase (10 U/ μ l) and 1 μ l of 10 mM ATP.
2. Mix gently and incubate for 30 min at 37°C.
3. Heat the reaction 10 min at 70°C to inactivate the kinase.
4. Place the reaction on ice.

*3.3.7. NotI Digestion
of the Phosphorylated
Product*

1. To the phosphorylated product, add 10 µl buffer 3 (NEB) and water to obtain a final volume of 90 µl. Add 10 µl of enzyme NotI (10,000 U/ml). Incubate the reaction at 37°C for 1 h.
2. Inactivate the enzyme by heating for 10 min at 70°C.
3. Place the reaction on ice.

*3.3.8. Size Fractionation
of the DNA Fragments
by Chromatography*

1. Place the chromatography column on an appropriate support and remove the top cap, and then the bottom cap, to let the excess liquid drain.
2. Add 800 µl of TEN buffer to the top of the column and let it drain completely. The flow rate should not be significantly slower than 20 min/ml.
3. Repeat three more times the washes with 800 µl TEN buffer to completely remove the ethanol used to store the column.
4. Label 20 sterile Eppendorf tubes that will be used to collect the samples exiting the column.
5. Add 50 µl of TEN buffer to cDNA sample from Subheading 3.3.7 and drop the entire sample on the top frit of the column.
6. Collect the total effluent into one tube (tube 1).
7. Add 100 µl TEN buffer to the column and collect the effluent into another tube (tube 2).
8. Let the column drain completely.
9. Add another 100 µl TEN buffer to the column and collect each single drop into a different tube (tubes 3–20). Keep adding 100 µl aliquots of TEN buffer until 18 drops be collected.
10. Measure the volume of each drop using a pipette and record it. Use a fresh tip for each fraction to avoid cross-contamination. Because the first fractions eluting from the column contain the longer cDNA fragments, those fractions will be used to produce a high-quality cDNA library. The fractions eluting over 600 µl should therefore be discarded.
11. If the sample was labeled with ^{32}P , measure the amount of DNA in each fraction by counting the sample in the tritium channel of a scintillation counter. Calculate the corresponding amount of cDNA in each tube using the equation in Note 12.
12. If no radioactivity was used in the first-strand reaction, then collect 1 µl of each tube and measure its absorbance at 280 nm with a nanodrop spectrophotometer.
13. Each ligation will be performed using 40–50 ng DNA. If the amount of DNA in a fraction is not sufficient, then add cDNA from the subsequent fraction until the 50 ng of DNA required for the reaction is reached.

14. To evaluate the global size of the fragments contained in the different fractions, load 2 μ l of each fraction on a 1% agarose gel stained with 1/10,000 SYBR Gold stock solution. Let the gel migrate for 1 h in the dark at 120 V. Visualize the stained DNA using a blue-light transilluminator. If 32 P was used, dry the gel and visualize the radioactive fragments according to the protocol described in Note 14.

*3.3.9. Ligation of the DNA to an Expression Vector
(See Note 15)*

1. To each 40–50 ng of the DNA obtained in Subheading 3.3.8, add 100 ng of dephosphorylated vector (see Note 16), 5 μ l of yeast tRNA (1 μ g/ μ l), half a volume of 7.5 M NH_4OAc , and 2 volumes of absolute ethanol (-20°C). Precipitate overnight at -20°C . Plan an extra tube as a control for vector self-ligation by performing the ligation reaction in the absence of insert.
2. Eliminate the supernatant after centrifugation at $14,000 \times g$ for 10 min (4°C).
3. Wash the pellet in 0.5 ml 70% ethanol (-20°C).
4. Discard the supernatant after centrifugation at $14,000 \times g$ for 10 min (4°C).
5. Air-dry the pellet for 20 min.
6. Resuspend each dry pellet in 40 μ l of a mix containing 30 μ l water, 8 μ l 5 \times T4 ligase buffer, and 2 μ l T4 ligase (1 U/ μ l).
7. Incubate for 3 h at room temperature.
8. After the ligation, pool the tubes corresponding to one original fraction from section 3.3.8.10 (with X being the number of tubes used to pool the fraction).
9. Precipitate the DNA by adding 1/2 volume of 7.5 M NH_4OAc and 2 volumes of absolute ethanol (-20°C). Precipitate for 15 min at -80°C then 15 min on dry ice.
10. Eliminate the supernatant after centrifugation at $14,000 \times g$ for 10 min (4°C).
11. Wash the pellet in 0.5 ml 70% ethanol (-20°C).
12. Eliminate the supernatant after centrifugation at $14,000 \times g$ for 10 min (4°C).
13. Air-dry the pellet for 20 min.
14. Resuspend the fractions in X*10 μ l of water.

*3.3.10. Transformation of *E. coli**

In order to increase the number of colonies, bacteria with the highest transformation efficiency, such as Max efficiency DH5 α or MegaX DH10B, should be used.

1. To 20 μ l of electrocompetent cells add 2 μ l of the ligation mix, which correspond to 20 ng of vector.
2. Electroporate in a 1 mm cuvette using the following parameters: 2 kV/25 μ F/200 Ω .

3. Immediately add 1 ml of LB medium to the bacteria and pool the suspensions corresponding to one fraction in one 50 ml polypropylene tube. Incubate for 1 h at 37°C in LB medium.
4. Spread various amounts of each pool of bacteria on large-LB agar plates containing 100 µg/ml ampicillin (2.5, 1.2, and 0.6 ml can be used in order to obtain different densities of bacteria on the plates). At this stage, the total content of the bacteria suspension should be spread on plates.
5. To calculate the transformation efficiency, spread 100 µl of a 1:1, 1:10, and 1:100 diluted culture on standard-LB agar plate containing 100 µg/ml ampicillin.
6. The next day, count the number of colonies on each standard plate to evaluate the total number of colonies obtained (see Note 17). No or very few colonies should be found on the self-ligation control.
7. In order to verify the quality of the library, pick 24 colonies from each fraction. Amplify them in 2 ml LB containing 100 µg/ml ampicillin and extract the plasmid DNA using a miniprep extraction kit according to the manufacturer protocol. Digest the extracted plasmids with HindIII and NotI. Measure the frequency of insertion and the mean length of the insert for each fraction (see Note 18).
8. If single colonies can be visualized on the large-LB agar plates, then proceed to Subheading 3.3.11. If single colonies cannot be visualized, then harvest the plates corresponding to each fraction with 10 ml LB. Centrifuge at 1,050 ×*g* for 15 min.
9. Resuspend each bacteria pellet in 2 ml LB glycerol 20% and freeze them at -80°C.
10. Measure the density of the bacteria stock by seeding various dilutions on standard LB plates containing 100 µg/ml ampicillin (as in step 4).
11. When the density of bacteria in the frozen stock is estimated, spread the required amount bacteria on large-LB agar plates (containing 100 µg/ml ampicillin) in order to grow approximately 100 bacteria per 2 cm².

3.3.11. Extraction of the DNA from the Bacteria Culture

1. On the bacteria plate draw squares of 1 cm² and count the number of bacteria in several of these squares to estimate the mean number of bacteria per cm².
2. Using a sterile scalpel, cut pieces of agar of a size corresponding to approximately 100 colonies of bacteria.
3. Transfer these pieces of agar into 50 ml Falcon tubes containing 15 ml of LB medium and 100 µg/ml ampicillin.
4. Grow the bacteria for 4 h at 37°C.

5. Keep an aliquot of the bacteria (100 µl) and freeze it in 1 volume of LB containing 20% glycerol.
6. Pellet the remaining bacteria and extract the plasmid DNA using a miniprep extraction kit according to the manufacturer protocol. The complete screening of a cDNA library may require up to 1,500 pools of 100 bacteria. Plasmid DNA extraction is the tedious part. It can be pursued concurrently with the start of the screening.

3.4. Transient Transfection and T-Cell Activation Assay

3.4.1. Transfection of COS-7

Identification of the cDNA encoding the antigenic peptide is generally done by transfecting COS-7 or HEK-293 cells (see Note 19) with DNA from a plasmid construct encoding the HLA molecule presenting the peptide (generally cloned in an expression vector such as pCDNA3 or pCDNA1) and DNA from the various pools of cDNA included in the library. Transfection of these two cell lines can be performed as described below.

1. The day before transfection, 15,000 COS-7 cells are seeded in a flat-bottom, 96-well plate.
2. Incubate overnight at 37°C in an 8% CO₂ incubator.
3. The day of transfection dilute the DNA encoding the HLA at a concentration of 2.6 µg/ml in DMEM containing 10% NU-serum. Add 45 µl in each well of a V-bottom 96-well plate. Add 15 µl of DNA from the various cDNA pools of the library.
4. In a 15 ml tube, prepare a solution containing 10% NU-serum, 200 µM chloroquine, and 800 µg/ml DEAE-dextran. Add 60 µl of this solution to the various DNA mixes. Mix well and add 50 µl of these transfection solutions in duplicate wells onto the COS-7 cells.
5. Incubate for 4 h at 37°C in an 8% CO₂ incubator.
6. After these 4 h gently remove the transfection mix and add 50 µl of a solution of PBS containing 10% DMSO. Incubate 2 min, remove, and add 200 µl DMEM containing 10% FBS. Incubate for 24 h at 37°C in an 8% CO₂ incubator.
7. The next morning, carefully remove the supernatant and add 10,000 T cells in 200 µl CTL medium containing 25 U/ml IL2. Incubate for 20 h at 37°C in an 8% CO₂ incubator.
8. After 20 h, the amount of IFNγ or TNF in the co-culture supernatant is analyzed by ELISA or as described in Subheading 3.1.2.

3.4.2. Transfection of HEK-293 Cells

1. The day before transfection, HEK-293 cells (30–40,000/well) are seeded in a 96-well flat-bottom plate in a final volume of 200 µl. Incubate overnight at 37°C in an 8% CO₂ incubator.

2. The day of transfection dilute the DNA encoding the HLA at a concentration of 2.6 µg/ml in Optimem medium. Add 45 µl in each well of a V-bottom 96-well plate and 15 µl of DNA from the various cDNA pools of the library.
3. In a separate tube, prepare a solution of Optimem containing 160 µl of lipofectamine per ml. Add an equal volume of the lipofectamine solution to the DNA solution. Incubate for 45 min at room temperature.
4. Carefully remove the supernatant of the HEK-293 culture and add gently 50 µl of the DNA/lipofectamine mixture. Incubate the cells for 4 h at 37°C in an 8% incubators
5. After 4 h gently add 200 µl IMDM 10%FBS. Incubate overnight at 37°C in an 8% CO₂ incubator.
6. The next morning, carefully remove the supernatant and add 10,000 T cells in 200 µl CTL medium containing 25 U/ml IL2. Incubate for 20 h at 37°C in an 8% CO₂ incubator.
7. After 20 h, the amount of IFNγ or TNF in the co-culture supernatant is analyzed by ELISA or as described in Subheading 3.1.2.

3.5. Cloning the Positive Pool of Bacteria

When a pool of cDNA able to activate the CTL is identified in the library, it is still necessary to verify that it does so in an HLA-restricted manner. COS-7 or HEK-293 cells are transfected as above, but the cDNA encoding the HLA molecule is omitted. Indeed, cytokine production could be originating from the transfection of the antigen-presenting cells with the gene encoding the TNF-alpha protein itself or proteins such as the p65 subunit of the NF-kappaB, the transcription factor involved in the expression of TNF-alpha. Once a confirmed positive pool is identified, it must be subcloned (as described below) and DNA from individual colonies contained in the pool will be tested by transfection as described above.

1. Thaw the frozen stock of bacteria corresponding to the positive cDNA pool.
2. Dilute half of it into 2.5 ml LB medium.
3. Incubate at 37°C for 90 min.
4. Spread on a large-LB agar plate containing 100 µg/ml ampicillin. Various dilutions can be made in order to grow single colonies. Grow the bacteria overnight at 37°C.
5. The next morning, pick colonies from the plate and seed each of them into one well of a 96-well round-bottom plate containing 250 µl LB medium supplemented with 100 µg/ml ampicillin. In order to screen at least one copy of each cDNA from the pool, about 500 colonies will have to be picked up for each original pool.
6. Grow the plate overnight at 37°C (do not shake).

7. Freeze 50 µl of bacteria as a back-up in LB 20% glycerol.
8. Centrifuge the plates $1,050 \times g$ for 5 min.
9. Discard the supernatant and shake the plate on an orbital shaker to dissociate the pellet.
10. Add 50 µl of resuspension buffer and shake the plate.
11. Add 100 µl of lysis solution and shake the plate until bacteria are completely lysed.
12. Add 50 µl of potassium acetate buffer.
13. Shake the plates for 15 min.
14. Cover the plate with a SealPlate sealing film and centrifuge at $1,500 \times g$ for 5 min.
15. During the centrifugation, prepare plates containing 120 µl isopropanol.
16. Transfer 120 µl of supernatant in the new plates containing isopropanol.
17. Mix by pipetting and cover with a 96-well plate plastic lid.
18. Keep the plates at -20°C for 20 min.
19. Centrifuge the plate at $1,050 \times g$ for 25 min.
20. Discard the supernatant and rinse the wells with 200 µl absolute ethanol.
21. Centrifuge at $1,050 \times g$ for 5 min.
22. Discard the supernatant and dry the plates in the hood for 30 min.
23. Add to the pellet 50 µl water containing 50 µg/ml Rnase. Leave the plate at room temperature for 30 min to resuspend the pellet.
24. Store the plate at -20°C until further use.
25. Transfect DNA from individual colonies as in Subheading 3.4.

3.6. Identify the Peptide Coding Sequence in the Positive cDNA

When the cDNA encoding the peptide has been identified (see Note 20), shorter size fragments obtained either by PCR or after digestion of the cDNA with restriction enzymes are cloned into expression vectors. In our hand, the cloning of PCR products (amplified using a thermostable DNA polymerase with proofreading activity to decrease the rate of error during the PCR amplification) has been successfully done using the pcDNA3.1/V5-His TOPO TA Expression Kit (Life Technologies). The constructs are then tested by transfection as described in Subheading 3.4 to check their ability to activate the CTL.

3.7. Identify the Peptide Recognized by the T Cell

Once the region of the protein containing the antigenic peptide has been defined, peptides bearing adequate HLA-binding motifs are synthesized and tested for their ability to sensitize autologous EBV-B cells to lysis by the CTL. This is done according to the

protocol described in Subheading 3.1.1 with the following adaptation.

1. After labeling the cells as described in Subheading 3.1.1, steps 1–3, resuspend the target cells at a cell density of 20,000 cells/ml.
2. Prepare the peptide dilutions by dispensing, in duplicate wells of a 96-well V-bottom plate, 50 µl of peptides solutions of concentrations ranging from 60 µM to 1 pM.
3. Add 50 µl of labeled target cells (1,000 cells per well) to each well containing the peptides. As control, cells are also added to 50 µl medium without peptide. Incubate the cells with the peptide for 45 min at 37°C.
4. Add 10,000 CTL in 100 µl and resume the chromium release assay as described in Subheading 3.1.1, steps 6 and 7.

4. Notes

1. WEHI 164 clone13 cells are grown in complete RPMI supplemented with 5%FBS. Cells are grown in non-tissue culture Petri dishes (Greiner Bio one). Cells should not be grown at a cell density higher than 5×10^5 cells per ml.
2. Actinomycin D is very toxic and as a powder, it should be carefully handled in a pharmaceutical containment hood and according to manufacturer instructions.
3. TAE buffer is usually prepared as a 50× stock solution for laboratory use. In a 2-L bucket, add 484 g of Tris base to 1 L of ultrapure water. Dissolve under agitation and add 114.2 ml anhydrous acetic acid and 200 ml EDTA 0.5 M pH 8. Add water for a final volume of 2 L. Check that the solution is at pH 8, after it has cooled down.
4. In order for the result of the test to be the most reliable, the spontaneous release should not exceed 15–20% of the maximal release.
5. Alternatively, the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) can also be used.
6. Antibodies such as W6/32 (anti-HLA class I (12)), 4E (anti-HLA-B and -C (13)), B1.23.2 (anti-HLA-B and -C, -A24, -A31, and -A32 (14)), BB7.2 (HLA-A2 (15)), MA2.1 (HLA-A2, -B17 (16)), P2.1 (HLA-A2, -A28 (17)) ME1 (anti-HLA-B7, -B27, -Bw22, -Bw42 (18)), GAP-A3 (anti-HLA-A3 (19)), and C7709A2.6 (anti-HLA-A24 (20)) have been successfully used for this type of assay (9, 21).
7. When performing a chromium release assay in these conditions, always include incubation of the antibody with target cells alone to exclude antibody-dependent cell lysis in the absence of CTL.

8. Because of the strong polymorphism of the class I antigens within serologically homogeneous groups, it is recommended, when possible, to work with the HLA cDNA obtained from the autologous cell line.
9. If the presenting molecule cannot be definitely identified, it is still possible to transfet the cDNA library into COS-7 or HEK-293 together with two or three putative class I candidates.
10. The use of adapters containing an oligo(dT) priming site has the advantage that only poly(A)-containing RNA are reverse transcribed and that the mRNA is complete up to the poly(A) tail. However, reverse transcription of long RNA might not generate full-length cDNA (22). Other rare restriction sites such as SfiI can also be used.
11. For best results, the yield of first-strand cDNA synthesis should be above 10% and mostly depends on the quality and the amount of mRNA that is used (22).
12. To calculate the yield of the first-strand reaction, add 2 µl first-strand reaction to 43 µl of 20 mM EDTA (pH 7.5) and 5 µl Yeast tRNA. Spot 10 µl of diluted sample on 2-glass fiber filter. Dry one of the filters at room temperature (filter A). Wash the other filter 3× 5 min in a beaker containing 50 ml of ice-cold 10% TCA containing 1% sodium pyrophosphate. Wash it once more with 50 ml 95% ethanol at room temperature for 2 min. Dry the second filter at room temperature (filter B). Count both filters in standard scintillant to measure the ³²P in the reaction (A) and the incorporated ³²P (B).

The amount of cDNA synthesized can be calculated using the following equation (Superscript Choice System for cDNA Synthesis):

$$\text{Amount of ds cDNA (mg)} = \frac{(B \text{ cpm}) \cdot 200}{(A \text{ cpm} / 200) \cdot 3}$$

$$\text{The yield of first - strand synthesis (\%)} \text{ is } \frac{\text{cDNA (\text{ug})}}{\text{total RNA (\text{ug})}} \cdot 100.$$

13. Adapters containing other restriction sites can also be used.
14. Rinse the gel with 300 ml TCA 10% containing 1% sodium pyrophosphate and dry the gel in a vacuum dryer for 15 min. Overlay the gel with a sheet of photographic paper. Expose the film for 14 h at room temperature.
15. The type of expression vector chosen for the cloning of the cDNA library depends on the cells in which the library will be transfected. For example, the construct will be more efficiently replicated when plasmids bearing the SV40 origin, e.g. pcDNA1 or pcDNA3 (Life Technologies), are transfected in SV40 large T antigen expressing cells such as COS-6.57 or

HEK-293-T. Vectors containing the viral EBV origin of replication, e.g. pCEP4 (Life Technologies), should be used when transfecting cells expressing the Epstein–Barr virus nuclear antigen 1, such as HEK-293-EBNA.

16. Preparation of the expression vector. Digest 2 µg of expression vector using the enzymes NotI and HindIII according to the manufacturer protocol. To limit the possibility of self-recirculation, the digested vector will be dephosphorylated prior to the ligation. To do so, add 1/10 volume of 10× Antarctic Phosphatase Reaction Buffer to the digested vector. Add 1 µl of Antarctic Phosphatase (5 U) and mix. Incubate for 15 min at 37°C. Heat inactivate for 5 min at 65°C.
17. The total number of colonies to be screened should be higher than 10⁵.
18. The frequency of insert should be higher than 80% and their length should be comprised between 0.5 and 4 kb.
19. COS-7 have been often successfully used for the identification of peptides recognized by human CTL. However, in some cases, they cannot be used to screen a cDNA library because some CTL clones recognize COS cells either untransfected or transfected with the relevant HLA gene. This is likely related to the production of a peptide that resembles the antigenic peptide recognized by the CTL on the tumor cells.
20. Screening of the cDNA library can sometimes lead to the identification of several cDNA recognized by one CTL clone, due to the presence of peptide mimotopes encoded by these DNA sequences. Analyzing the expression profile of the gene encoding the peptide in parallel to the T-cell recognition assays will help identifying the peptide that is really recognized and processed by the tumor cells.

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References

1. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethé B, De Plaen E, Velu T, Coulie PG (2005) High frequency of anti-tumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. *J Exp Med* 201:241–248
2. Zarour H, De Smet C, Lehmann F, Marchand M, Lethé B, Romero P, Boon T, Renaud J-C

- (1996) The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100. *J Invest Dermatol* 107:63–67
3. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF, Sette A, Appella E, Rosenberg SA (1995) Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 154:3961–3968
 4. Espenik T, Nissen-Meyer J (1986) A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 95:99–105
 5. Boon T, Van Snick J, Van Pel A, Uyttenhove C, Marchand M (1980) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. II. T lymphocyte-mediated cytosis. *J Exp Med* 152:1184–1193
 6. Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119:203–210
 7. Beyaert R, Vanhaesebroeck B, Suffys P, Van Roy F, Fiers W (1989) Lithium chloride potentiates tumor necrosis factor-mediated cytotoxicity in vitro and in vivo. *Proc Natl Acad Sci U S A* 86:9494–9498
 8. Adotevi O, Mollier K, Neuveut C, Cardinaud S, Boulanger E, Mignen B, Fridman WH, Zanetti M, Charneau P, Tartour E, Lemmonier F, Langlade-Demoyen P (2006) Immunogenic HLA-B*0702-restricted epitopes derived from human telomerase reverse transcriptase that elicit antitumor cytotoxic T-cell responses. *Clin Cancer Res* 12(10):3158–3167. doi:[10.1158/1078-0432.CCR-05-2647](https://doi.org/10.1158/1078-0432.CCR-05-2647), 12/10/3158 [pii]
 9. Guéguen M, Patard J-J, Gaugler B, Brasseur F, Renauld J-C, Van Cangh PJ, Boon T, Van den Eynde B (1998) An antigen recognized by autologous CTL on a human bladder carcinoma. *J Immunol* 160:6188–6194
 10. Ma W, Germeau C, Vigneron N, Maernoudt A-S, Morel S, Boon T, Coulie PG, Van den Eynde B (2004) Two new tumor-specific antigenic peptides encoded by gene *MAGE-C2* and presented to cytolytic T lymphocytes by HLA-A2. *Int J Cancer* 109:698–702
 11. Davis LG, Dibner MD, Battey JF (1986) Guanidine isothiocyanate preparation of total RNA. In: Davis LG, Dibner MD, Battey JF (eds) Basic methods in molecular biology. Elsevier, New York, pp 130–135
 12. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell-surface antigens—new tools for genetic analysis. *Cell* 14:9–20
 13. Yang SY, Morishima Y, Collins NH, Alton T, Pollack MS, Yunis EJ, Dupont B (1984) Comparison of one-dimensional IEF patterns for serologically detectable HLA-A and B allo-types. *Immunogenetics* 19:217–231
 14. Rebai N, Malissen B (1983) Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies. *Tissue Antigens* 22:107–117
 15. Parham P, Brodsky FM (1981) Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum Immunol* 3:277–299
 16. McMichael AJ, Parham P, Rust N, Brodsky F (1980) A monoclonal antibody that recognizes an antigenic determinant shared by HLA-A2 and B17. *Hum Immunol* 1:121–129
 17. Parham P, Bodmer WF (1978) Monoclonal antibody to a human histocompatibility alloan-tigen, HLA-A2. *Nature* 276:397–399
 18. Ellis SA, Taylor C, McMichael A (1982) Recognition of HLA-B27 and related antigen by a monoclonal antibody. *Hum Immunol* 5(1):49–59. doi:[10.1016/0198-8859\(82\)90030-1](https://doi.org/10.1016/0198-8859(82)90030-1) [pii]
 19. Berger AE, Davis JE, Cresswell P (1982) Monoclonal antibody to HLA-A3. *Hybridoma* 1(2):87–90
 20. Lehmann F, Marchand M, Hainaut P, Pouillart P, Sastre X, Ikeda H, Boon T, Coulie PG (1995) Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur J Immunol* 25: 340–347
 21. Van den Eynde BJ, Gaugler B, Probst-Kerper M, Michaux L, Devuyst O, Lorge F, Weynants P, Boon T (1999) A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription. *J Exp Med* 190:1793–1799
 22. De Plaein E, Lurquin C, Lethé B, van der Bruggen P, Brichard V, Renauld J-C, Coulie P, Van Pel A, Boon T (1997) Identification of genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Methods* 12: 125–142

Chapter 17

Purification, Preparation, and Use of Chaperone–Peptide Complexes for Tumor Immunotherapy

Ayesha Murshid, Jianlin Gong, and Stuart K. Calderwood

Abstract

The molecular chaperone heat-shock protein 70 (Hsp70) possesses immune stimulatory properties that have been employed in the preparation of anticancer vaccines. Hsp70 binds antigenic peptides in the cytoplasm of cancer cells. Hsp70 thus serves as a convenient, non-discriminating transporter of antigens and can protect the peptides during internalization by APC and cross presentation to T lymphocytes. We describe a method for purifying Hsp70–peptide complexes that can be used to prepare molecular chaperone-based vaccines, involving sequential gel filtration, ion exchange, and affinity chromatography

Key words: Affinity chromatography, Chaperone, Heat-shock protein, HSP70, Lipopolysaccharide, Vaccine

1. Introduction

Intracellular heat-shock proteins (HSP) play an essential role in protein folding and quality control (1, 2). They apparently recognize topological features in unfolded proteins and can bind to a wide array of small peptides and unfolded polypeptide sequences (3). This property suggested that such proteins might be employed to bind polypeptides that contain within their sequence antigenic determinants (4). Indeed, this approach has been used to prepare anticancer vaccines using molecular chaperones such as HSP including Hsp70, Hsp90, Hsp110, and GRP170 (5–7). We have concentrated in this report on Hsp70-derived vaccines.

The Hsp70 family contains 12 members including cytoplasmic proteins such as hspa1, ER-resident protein Grp78/BiP, and mitochondrially localized mortalin (1, 8). Some of these Hsp70 proteins such as Hspa1a, Hspa1b, and Grp78 are strongly induced by stress while others are expressed constitutively (1). The Hsp70 family proteins appear to be deployed in the front line in the process

of housekeeping folding on ribosomes as well as during stress and their precise functions are likely similar, differences being more to do with contrasting transcriptional stimulus and cellular location (9). Biochemically purified Hsp70 used to prepare cancer vaccines will likely contain multiple Hsp70 family members (10–13).

Purification of Hsp70 can employ its strong, but reversible, association with adenosine nucleotides for affinity chromatography purposes (14). Hsp70 proteins all contain an N-terminal adenosine nucleotide-binding domain and a C-terminal peptide-binding domain. Hsp70 binds to its clients only when associated with ADP and binding is reversed when ATP is exchanged for ADP (9). HSP-interacting polypeptides are referred to as “clients” rather than substrates, as HSPs, strictly speaking, are not enzymes (3, 9). Avid binding to ADP has the convenient implication that Hsp70 can be purified in association with interacting peptides. The peptide-binding domain of Hsp70 can enclose 7-mer peptide sequences and it has been shown *in vivo* that Hsp70 binds preferentially to peptides of 8–26 mer (15). Thus Hsp70 seems an ideal molecule to chaperone processed peptides in the cytosol and transport them into antigen-presenting cells.

2. Materials

2.1. Source Materials for Hsp70

1. Human melanoma A375 cells or MC-38/MUC1 murine cells were used as source material for the cell-derived Hsp70 and grown in low-endotoxin medium (see Note 1). Alternatively, Hsp70 for metabolic studies was prepared from minced mouse liver essentially as described for cell lines below. Recombinant hsp70.1 protein was prepared from *Escherichia coli* bacteria BL-21 by standard methodology and purified as below (16).
2. 15 cm tissue culture dishes.
3. Both mammalian cell types were grown in Dulbecco’s Modified Eagle’s Medium mixed with 10% fetal bovine serum and supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.2. Common Equipment and Reagent

1. Dounce Homogenizer.
2. Eppendorf centrifuge.
3. Spectrophotometer (Beckmann Model DU-7).
4. Gradient maker.
5. Fraction Collector with drop counter (Bio Rad).
6. PD10 chromatography columns (GE Healthcare).
7. Sephadex G25 (Sigma) swollen in endotoxin-free double-distilled H₂O 24 h prior to use.

8. DE-52 diethyaminoethyl (DEAE) cellulose (Whatman) pre-equilibrated in buffer B overnight.
9. Adenosine diphosphate (ADP)-agarose (Sigma).
10. Dulbecco's phosphate-buffered saline (pH 7.4): Commercially obtained.
11. Liquid handling procedures: All liquid handling was carried out with plastic pipettes with low endotoxin ratings. Likewise all chemicals and liquids were low in endotoxins.

2.3. Hsp70 Purification

1. 20× Buffer B (2 L): 400 mM Tris base (96.8 g), 400 mM Sodium chloride (48 g NaCl), 2.0 mM EDTA (1.68 g), make up to 2 L with H₂O, and titrate to pH 7.5 with glacial acetic acid.
2. Glycerol.
3. Phenylmethylsulfonylfluoride (PMSF): 10 mg/ml in ethanol.
4. Pepstatin: 1 mg/ml in methanol.
5. Leupeptin: 10 mg/ml in H₂O.
6. Dithiothreitol Stock (0.5 M): 0.5 M Dithiothreitol (7.7125 g), make up to 100 ml with H₂O.
7. 300 mM MgCl₂ stock solution (100 ml): 300 mM MgCl₂ (2.856 g), make up to 100 ml with H₂O.
8. Lysis Buffer B (100 ml): Dilute 5 ml 20× buffer B in 100 ml H₂O, add 0.1 ml 0.5 M Dithiothreitol, 1 ml PMSF stock, 0.1 ml pepstatin stock, 0.1 ml leupeptin stock.
9. Buffer B elution (500 ml): Dilute 25 ml 20× buffer B in 500 ml H₂O, add 0.5 ml 0.5 M dithiothreitol, 5 ml PMSF stock, 0.5 ml pepstatin stock, 0.5 ml leupeptin stock.
10. Buffer B high salt (500 ml): Dilute 25 ml 20× buffer B in 100 ml H₂O, add 0.5 ml 0.5 M dithiothreitol, NaCl to 350 mM (9.9 g), 5 ml PMSF stock, 0.5 ml pepstatin stock, 0.5 ml leupeptin stock.
11. Buffer D (2 L): 100 ml 20× buffer B, 20 ml 300 mM stock MgCl₂, make up to 2 L with H₂O.
12. Buffer D high salt (0.5 M NaCl): 500 ml Buffer D, 0.5 M NaCl (15 g).
13. Buffer D + 1 mM GTP: 100 ml Buffer D, 1 mM GTP (52.3 mg), adjust to pH 7.5.
14. Buffer D + 3 mM ADP: 100 ml Buffer D, 3 mM ADP (128.16 mg), adjust to pH 7.5.

2.4. LPS Removal

1. Polymyxin-B-sepharose columns (END-X, associates of Cape Cod, Inc.)
2. 1% Sodium deoxycholate.
3. Limulus Amoebocyte Lysate (LAL) assay (BioWhittaker).

2.5. Hsp70 ATPase**Activity**

1. Malachite green: 0.081% w/v. Store at 4°C.
2. Polyvinyl alcohol: 2.3% w/v. Store at 4°C.
3. Ammonium heptamolybdate tetrahydrate: 5.7% w/v in 6 M HCl. Store at 4°C.
4. Malachite Green Reagent: Mix the Malachite green, polyvinyl alcohol (2.3% w/v), and ammonium heptamolybdate tetrahydrate with H₂O in the ratio 2:1:1:2.
5. 2.5 M Adenosine triphosphate (ATP).
6. Sodium Citrate: 34% in H₂O.

2.6. Lowry Assay

1. Copper-tartrate–carbonate (CTC): Add 20% sodium tartrate to a solution of 0.2% copper sulfate-tartrate to a final concentration of 0.1% copper sulfate, 0.2% potassium tartrate, and 10% sodium carbonate.
2. 10% sodium dodecyl sulfate (SDS).
3. 0.8 M sodium hydroxide.
4. 2 M Folin-Ciocalteu phenol reagent (Sigma).
5. Lowry Reagent A: Mix equal parts of the CTC, NaOH, SDS, and H₂O.
6. Lowry reagent B: Dilute 1 volume of Folin-Ciocalteu phenol reagent with five volumes of distilled water.
7. Bovine serum albumin.

2.7. Immunoblot Assay

1. Mini-gel protein electrophoresis apparatus with 200 v power source (BioRad, CA).
2. Western blotting, dry transfer apparatus for minigel (BioRad).
3. Polyvinylidene fluoride (PVDF) membranes (Millipore).
4. 2× Laemmli sample Buffer (Sigma Chemicals).
5. Tris glycine buffer, polyacrylamide solutions for electrophoresis, see (17).
6. Towbin's electrophoretic transfer buffer: Tris base 30.3 g, Glycine 144 g, Double-distilled water 1.0 L.
7. Anti-Hsp70 antibody (rabbit polyclonal Ab 46) was prepared in-house using a synthetic peptide from the C-terminal 20 amino acids of human Hsp70.1 (18). Antibodies were diluted 1/10,000 in phosphate-buffered saline for immunoblot.
8. Biotin/avidin/alkaline phosphatase reagents (Vector Laboratories Inc.)

3. Methods

3.1. Hsp70–Peptide Complex Purification by Ion Exchange and Immunoaffinity Chromatography

1. Grow 15 dishes of cells in 15 cm tissue culture dishes to confluence (see Note 1).
2. Scrape into 12 ml of Lysis buffer B (approx. 3 g of cells).
3. Lyse cells in 20 strokes of Dounce (tight pestle).
4. Spin for 30 min at 15,000 $\times g$ to sediment debris.
5. Elute through a 55 ml G-25 column pre-equilibrated with buffer B, collecting 2.0 ml fractions.
6. Measure OD 280 and combine fractions in the first peak.
7. Take a sample for protein assay by the Lowry method (see Subheading 3.2.4) and adjust protein concentration to 10 mg/ml.
8. Add glycerol to 5% and layer onto the 2 \times 20 cm DE52 column.
9. Wash column with 200 ml of buffer B.
10. Elute with a 20–350 mM NaCl gradient (600 ml total volume taking 8 ml fractions). Elution is carried out with a gradient maker, beginning with the low-salt buffer (elution buffer B) and introducing high-salt buffer (Buffer B high salt).
11. Measure OD 280 on fractions.
12. Assay cell lysate, flow through, and every fourth sample by 10% SDS-PAGE using Coomassie Blue staining and Hsp70 immunoblot (see Subheading 3.2.5).
13. Collect main Hsp70 peak for ADP-agarose affinity chromatography (see Note 2).
14. Adjust to 3.0 mM MgCl₂ from the 300 mM stock.
15. Apply sample to 10 ml ADP-agarose affinity column and leave for 1 h.
16. Hook up the column to a fraction collector and elute with, sequentially, 40 ml Buffer D (160 drop fractions), 20 ml Buffer D high salt (80 drop fractions), 20 ml buffer D (low salt wash), 20 ml Buffer D + 1 mM GTP (80 drop fractions), and 40 ml Buffer D + 3.0 mM ADP (80 drop fractions).
17. Hsp70 in the fractions is detected by immunoblot (see Subheading 3.2.5) and Hsp70-containing fractions are pooled and loaded onto a PD-10 column pre-equilibrated with ice-cold PBS. Hsp70 was eluted in the void volume.
18. Concentrations of Hsp70 in the preparation are determined by the Lowry protein assay (see Subheading 3.2.4).
19. Hsp70–peptide complexes are frozen at –80°C prior to use for tumor immunization.
20. Vaccine doses of 1.5–3.0 µg of Hsp70–peptide complex were employed *in vivo* as described (13).

3.2. Testing the Purified Hsp70

For use in vaccine preparation, it is important to ascertain that the Hsp70-peptide complexes are free from low-molecular-weight impurities. Hsp70 can bind avidly to endotoxins such as LPS (see Note 3). Therefore great care is taken to avoid introducing LPS into the purified Hsp70 preparations. After purification, the Hsp70 is tested for endotoxin contamination by the LAL assay purchased from BioWhittaker (Walkersville MD). In our work, contaminated preparations are routinely discarded. However it is possible to rescue the Hsp70 preparations by sequential elution through polymyxin-B-sepharose columns, until the preparations are low in endotoxins (see Subheading 3.2.1 and see Note 4).

In addition to endotoxins, purified Hsp70 may also contain other ions or molecules such as ADP or high salt that could affect the biological properties of the chaperone in an immune setting. It is therefore optimal to exchange purified Hsp70 into the final buffer used in experiment by gel filtration (see Subheading 3.2.2).

Some of the procedures described above could also lead to inactivation of the Hsp70. To test for this possibility, one Hsp70 biochemical function that can be readily assayed is ATPase activity. ATPase (phosphatase) activity can be measured by the Malachite Green colorimetric assay (see Subheading 3.2.3) (19). ATPase activity in suspect preparations is compared to that of standard, purified Hsp70 in order to check for inactivation. Ability of Hsp70 to bind peptide antigens may be impaired if ATPase activity is compromised.

3.2.1. LPS Removal from Hsp70 Preparations

1. 0.5 ml polymyxin-B-sepharose columns are washed sequentially with 10 ml of deoxycholate and 10 ml of phosphate-buffered saline.
2. 100 µl of Hsp70 from ATP agarose elution is loaded onto the polymyxin-B-sepharose column.
3. Column is eluted with 5 ml phosphate-buffered saline, taking 0.5 ml fractions.
4. Fractions are assayed for endotoxin contamination by the LAL assay.

3.2.2. Buffer Exchange of Purified Hsp70

1. PD-10 column is washed with 25 ml endotoxin-free phosphate-buffered saline.
2. Hsp70 fractions are loaded onto the column and eluted with 10 ml phosphate-buffered saline, taking 1.0 ml fractions.
3. Fractions are tested for Hsp70 by immunoblot on 5% SDS-PAGE gels (see Subheading 3.2.5). Hsp70 elutes in the void volume (fractions 1–3).

3.2.3. Hsp70 ATPase Activity (20)

1. Add 15 µl of the Hsp70 preparation under test (approx 1.0 µM) to an Eppendorf centrifuge tube.
2. Add 10 µl of the ATP solution.

3. After 30-min equilibration at 37°C, add 80 µl of malachite green reagent and incubate for 1 h.
4. Add 10 µl of 34% sodium citrate to halt the reaction.
5. Mix thoroughly and measure OD at 620 nm.
6. Compare absorbance to that of purified Hsp70.

3.2.4. Lowry Assay for Protein Concentration (21)

1. Take 20 µl aliquot of column eluate.
2. Add 20 µl of reagent A, mix, and stand for 10 min at room temperature.
3. Add 10 µl of reagent B and mix.
4. At 30 min, read the absorbance at 750 nm using a spectrophotometer.
5. Determine protein concentration by reference to a standard curve determined using bovine serum albumin concentrations from 5.0, 20, 50, 100, 200, to 250 µM BSA.

3.2.5. Immunoblot Assay for Purified Hsp70 in Column Fractions

1. Fractions are diluted 1:1 with 2× Laemmli buffer and boiled at 100°C for 5 min.
2. A 25 µl sample is loaded onto the gel sandwich for discontinuous SDS-PAGE, using 5% stacking gel and 10% separating gel, and subjected to electrophoresis until the Bromophenol Blue tracking dye reaches the bottom of the sandwich.
3. Separating gels are removed, soaked in transfer buffer, and proteins then transferred electrophoretically onto PVDF membranes for 30 min using standard conditions (18).
4. Membranes are blocked in 5% condensed milk and then incubated with anti-Hsp70 antibodies overnight with shaking at 25°C.
5. Hsp70 bands are detected using a tiered biotin–avidin–alkaline phosphatase amplification system.

4. Notes

1. If Hsp70 vaccine is to be prepared from tumors *in vivo*, tumor material is rapidly resected, dissected free of non-tumor tissues, and snap frozen in liquid nitrogen. Collected tumor tissue is then weighed, rapidly thawed, and lysed by Dounce as in step 2 in the Methods section. For procedures involving tissue culture cells, levels of Hsp70 can be increased by prior heat shock. Cells are exposed to 1 h at 43°C and allowed to recover for 6 h at 37°C. This treatment leads to an approximate doubling in intracellular Hsp70 levels.

2. Client proteins/peptides rapidly dissociate from Hsp70 in the presence of ATP while ADP locks the client in place in the peptide-binding domain. Purification of Hsp70-peptide antigen complexes is thus carried out using ADP-agarose. ATP levels in solution can be rapidly decreased by treatment with the bacterial ATPase *apyrase*.
3. Hsp70 can bind avidly to endotoxins such as *E. coli*-derived LPS (22, 23). It is thus advisable to test all Hsp70 vaccine preparations for endotoxins using the LAL test as in Subheading 3.2. In addition, as with all purified protein preparations, it is advisable to remove chemicals used in the purification by buffer exchange. This can be done conveniently by dialysis or gel filtration.
4. It should be noted that while polymyxin-B can bind avidly to *E. coli* LPS, it does not remove all types of endotoxin and, for example, LPS from *S. Minnesota* does not bind to this agent (24).

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References

1. Lindquist S, Craig EA (1988) The heat shock proteins. *Annu Rev Genet* 22:631–637
2. Calderwood SK, Murshid A, Prince T (2009) The shock of aging: molecular chaperones and the heat shock response in longevity and aging—a mini-review. *Gerontology* 55:550–558
3. Ellis RJ (2007) Protein misassembly: macromolecular crowding and molecular chaperones. *Adv Exp Med Biol* 594:1–13
4. Murshid A, Gong J, Stevenson MA, Calderwood SK (2011) Heat shock proteins and cancer vaccines: developments in the past decade and chaperoning in the decade to come. *Expert Rev Vaccines* 10:1553–1568
5. Kurotaki T, Tamura Y, Ueda G, Oura J, Kutomi G, Hirohashi Y, Sahara H, Torigoe T, Hiratsuka H, Sunakawa H, Hirata K, Sato N (2007) Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. *J Immunol* 179:1803–1813
6. Udon H, Srivastava PK (1993) Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178:1391–1396
7. Wang XY, Kazim L, Repasky EA, Subjeck JR (2003) Immunization with tumor-derived ER chaperone grp170 elicits tumor-specific CD8+ T-cell responses and reduces pulmonary metastatic disease. *Int J Cancer* 105:226–231
8. Kampinga HH, Hageman J, Vos MJ, Kubota H, Tangay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for

- the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 14:105–111
9. Bukau B (2005) Ribosomes catch Hsp70s. *Nat Struct Mol Biol* 12:472–473
10. Srivastava PK (2000) Heat shock protein-based novel immunotherapies. *Drug News Perspect* 13:517–522
11. Gong J, Zhang Y, Durfee J, Weng D, Liu C, Koido S, Song B, Apostolopoulos V, Calderwood SK (2010) A heat shock protein 70-based vaccine with enhanced immunogenicity for clinical use. *J Immunol* 184:488–496
12. Gong J, Zhu B, Murshid A, Adachi H, Song B, Lee A, Liu C, Calderwood SK (2009) T cell activation by heat shock protein 70 vaccine requires TLR signaling and scavenger receptor expressed by endothelial cells-1. *J Immunol* 183:3092–3098
13. Enomoto Y, Bharti A, Khaleque AA, Song B, Liu C, Apostolopoulos V, Xing PX, Calderwood SK, Gong J (2006) Enhanced immunogenicity of heat shock protein 70 peptide complexes from dendritic cell-tumor fusion cells. *J Immunol* 177:5946–5955
14. Welch WJ, Feramisco JR (1985) Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. *Mol Cell Biol* 5:1229–1237
15. Calderwood SK, Theriault JR, Gong J (2005) Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity. *Eur J Immunol* 35:2518–2527
16. Theriault JR, Adachi H, Calderwood SK (2006) Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J Immunol* 177:8604–8611
17. Hames DE, Rickwood D (1981) *Gel Electrophoresis of Proteins: A Practical Approach*. IRL Press, London
18. Stevenson MA, Calderwood SK (1990) Members of the 70-kilodalton heat shock protein family contain a highly conserved calmodulin-binding domain. *Mol Cell Biol* 10:1234–1238
19. Geladopoulos TP, Sotiroudis TG, Evangelopoulos AE (1991) A malachite green colorimetric assay for protein phosphatase activity. *Anal Biochem* 192:112–116
20. Chang L, Bertelsen EB, Wisen S, Larsen EM, Zuiderweg ER, Gestwicki JE (2008) High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. *Anal Biochem* 372:167–176
21. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 83:346–356
22. Gao B, Tsan MF (2004) Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. *Biochem Biophys Res Commun* 317:1149–1154
23. Triantafilou K, Triantafilou M, Dedrick RL (2001) A CD14-independent LPS receptor cluster. *Nat Immunol* 2:338–345
24. Watanabe J, Miyazaki Y, Zimmerman GA, Albertine KH, McIntyre TM (2003) Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *J Biol Chem* 278:42361–42368

Chapter 18

Recombinant Poxviruses: Versatile Tools for Immunological Assays

Nicholas A. Siciliano, Lan Huang, and Laurence C. Eisenlohr

Abstract

The study of antigen processing and presentation is critical to our understanding of the mechanisms that govern immune surveillance. A typical requirement of assays designed to examine antigen processing and presentation is the de novo biosynthesis of a model antigen. Historically, Vaccinia virus (VACV), a poxvirus closely related to Cowpox, has enjoyed widespread use for this purpose. Recombinant poxvirus-based expression has a number of advantages over other systems. Poxviruses accommodate the insertion of large pieces of recombinant DNA into their genome, and recombination and selection are relatively efficient. Poxviruses readily infect a variety of cell types, and they drive rapid and high levels of antigen expression. Additionally, they can be utilized in a variety of assays to study both MHC class I-restricted and MHC class II-restricted antigen processing and presentation. Ultimately, the numerous advantages of poxvirus recombinants have made the Vaccinia expression system a mainstay in the study of processing and presentation over the past two decades. In an attempt to address one shortcoming of VACV while simultaneously retaining the benefits inherent to poxviruses, our laboratory has begun to engineer recombinant Ectromelia viruses. Ectromelia virus, or mousepox, is a natural pathogen of murine cells and performing experiments in the context of a natural host-pathogen relationship may elucidate unknown factors that influence epitope generation and host response. This chapter describes several recombinant poxvirus system protocols used to study both MHC class I and class II antigen processing and presentation, as well as provides insight and troubleshooting techniques to improve the reproducibility and fidelity of these experiments.

Key words: Recombinant poxvirus systems, Recombinant Vaccinia systems, Recombinant Ectromelia systems, MHC class I and class II pathways, Antigen processing and presentation, In vitro and in vivo assays, Procedures and troubleshooting

1. Introduction

Vaccinia virus (VACV), a large double-stranded DNA poxvirus, has long-standing use as a recombinant expression system for the study of antigen processing and presentation (e.g., (1–5)). Recombinant Vaccinia virus (rVACV) expression systems have

several characteristics that make them powerful tools in this respect: (1) Production is relatively efficient and the recombinant viruses express high levels of proteins (6–9); (2) large inserts up to 25 kb can be placed into the VACV genome; (3) VACV infects many different cell types which makes it practical for use with established cell lines; (4) VACV, a vaccine strain for smallpox, can also be broadly utilized *in vivo* due to its ability to infect a variety of hosts; and (5) VACV is a highly stable, robust virus that retains infectivity at 4°C for months and can be stored at –80°C for years. One limitation of this system is the cytopathic nature of VACV. This can be minimized by employing Modified Vaccinia Ankara (MVA), a highly attenuated VACV adapted to growth in avian cells (10). Alternatively, one can block the late phase of the viral life cycle with the drugs cytosine β-D-arabinofuranoside (Ara-C) or cidofovir (Vistide®), thereby preventing DNA replication, expression of late genes, and subsequent lytic program (11, 12). A potential disadvantage of VACV recombinant systems, depending upon application, is the unnatural relationship between VACV and many cell lines or model organisms. The origin of VACV and its original natural host remains unknown, although it is almost certainly not man, mouse, or cow (13). Studying antigen processing and presentation in the context of a coevolved host–pathogen relationship could provide key insights into the mechanisms that govern MHC class I and class II epitope generation as well as the resulting T cell response. Under conditions where this may be important, one can turn to Ectromelia virus (ECTV), or mousepox, which has a high homology with VACV and is a natural mouse pathogen (14, 15) that has historically been used to study poxvirus pathogenesis, not epitope presentation (16). The generation of recombinant VACV has been thoroughly described (1, 2, 5). This chapter details the experimental assays used to examine antigen processing, presentation, and T cell reactivity, in conjunction with recombinant poxviruses. The focus is on assays that employ recombinant poxviruses to express model antigens in order to assess the production of MHC class I- and MHC class II-restricted determinants and the correlating epitope-specific CD8⁺ and CD4⁺ T cell responses.

2. Materials

2.1. General and Safety Considerations

Poxviruses have many advantages but several considerations must be factored in before working with either ECTV or VACV. Work with VACV and ECTV requires BSL2/BSL3 facilities and well-trained personnel. Poxviruses are very stable but proper storage and containment are essential for successful work with these pathogens. When possible, polypropylene (not polystyrene) tubes should be used to avoid cracking and potential leaking of virus. Also, when virus is frozen, viral particles tend to aggregate and require disruption

using a cup sonicator once thawed. Probe sonication will cause potentially hazardous aerosolization. VACV poses a risk to humans and has been known to cause laboratory-acquired infections in unimmunized lab workers (17). Accordingly, immunization of lab personnel and proper BSL2/3 pathogen training are highly recommended. ECTV does not cause infections in humans but induces significant pathogenesis and mortality in mice. For this reason, work with ECTV requires specific containment procedures. The stability of poxviruses makes it essential to take great care in handling and storage of the viruses and to avoid contamination of work surfaces. A diligent sterilization program including cleaning of virus work areas with 10% bleach is critical to minimize the risk when working with poxviruses.

2.2. Common Consumables and Lab Equipment

1. Tissue culture equipment: Laminar flow hood, plates, flasks, incubators, flat- and round-bottom 96-well plates, conical tubes, pipettes, multichannel pipettes, etc.
2. Dulbecco's Modified Eagle Media (DMEM).
3. RPMI 1640.
4. Fetal calf serum (FCS): Both high quality (e.g., Hyclone) and general use.
5. Assay Media: RPMI 1640 media containing 5.5×10^{-5} M 2-mercaptoethanol, 10% high-quality FCS, 10 $\mu\text{g}/\text{ml}$ gentamicin, and/or 1× Pen/Strep.
6. BSS/BSA: 0.15 M NaCl, 5 mM KCl, 1.22 mM MgSO₄, 1.3 mM K₂HPO₄, 0.74 mM KH₂PO₄ (mix K₂HPO₄ and KH₂PO₄ and adjust pH to 7.2), 10 mM HEPES (pH 7.2), 2.5 mM CaCl₂, 0.1% w/v BSA (see Note 1).
7. FACS Buffer: PBS, 1% BSA, 0.09% sodium azide.
8. FACS Buffer without azide: PBS, 1% BSA.
9. ACK buffer: 0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH to 7.2–7.4 using 1 N HCl, filter sterilize through a 0.22 μm filter and store at room temperature or commercial ACK lysis buffer.
10. Cup Sonicator (e.g., Branson Sonifier 450) (see Note 2).
11. Tissue grinders (homogenizers) (e.g., VWR, Thomas) or sterilized frosted microscope slides (Fisher 12-552-3).
12. Cell strainers (40 μm Nylon, sterile) (Falcon).
13. Dissecting tools (scissors, forceps).
14. Microscope.

2.3. Recombinant Poxvirus Generation

1. vRB12 VACV: The vRB12 strain is a mutant F13L deletion strain.
2. pRB21 VACV recombination plasmid expressing gene of interest.

3. FuGENE 6 transfection reagent (Roche 11 814 443 001).
4. BSC-1 cell line (ATCC, cat. no. CCL26) and CV-1 cell line (ATCC, cat. no. CCL70).
5. Low-melting-point (LMP) agarose: Make up a 1.8% solution in sterile water, autoclave, and melt in a microwave.
6. 2× Eagle Minimum Essential Medium (EMEM) with sodium bicarbonate and L-glutamine, without phenol red.
7. Infection medium: 1× EMEM with 2% high-quality FCS and 10 µg/ml gentamicin.
8. OPTI-MEM, 1× reduced serum medium.
9. Crystal violet: 0.1% solution in 20% EtOH.
10. Sterile 1.5 ml polypropylene Eppendorf tubes.

2.4. Chromium Release

1. Inbred mouse strains.
2. 0.5-ml insulin syringes.
3. MHC-matched target cells.
4. Alternative method of T-cell restimulation (other than poxvirus) (see Note 3).
5. Recombinant mouse IL-2 (Peprotech).
6. Beta 2-microglobulin (Scripps Labs).
7. 96-well round-bottom plates.
8. ^{51}Cr as sodium chromate in a sodium chloride solution.
9. 96-well plates for Liquid Scintillation Counter.
10. Plastic tubes (for samples if using a gamma counter).
11. Sealing tape for plate (Fisher).
12. Scintillation fluid.
13. 15-ml conical tubes and rack.
14. Tabletop centrifuge.
15. Infection Medium: DMEM, 5% FCS.
16. Multichannel pipette.
17. Triton X-100.
18. Rotator (for 15-ml conical tubes).
19. Liquid Scintillation Counter, gamma counter, or beta plate reader.

2.5. *In Vivo* Cytotoxicity

1. Cell strainers (40 µm Nylon, sterile) (Falcon 352340).
2. Glass homogenizers or sterilized frosted microscope slides.
3. Assay Media: RPMI 1640 media containing 5.5×10^{-5} M 2-mercaptoethanol, 10% high-quality FCS (Hyclone), 1× Pen/Strep.

4. 500 ml RPMI-1640.
5. 50 ml Hyclone Fetal bovine serum (FBS).
6. 5 mM stock of CFSE in provided DMSO (18 μ l in one bottle=5 μ M) (180 μ l in one bottle=0.5 μ M) (Invitrogen CellTracer #C34554).
7. Peptide epitopes: > 1 mg/ml in DMSO.
8. FACS Buffer: PBS, 1% BSA, 0.09% sodium azide.

2.6. Enzyme-Linked Immunospot

1. Enzyme-Linked Immunospot (ELISPOT) plates (i.e., 96-well nitrocellulose-backed microtiter plates).
2. Cytokine-specific primary antibody (e.g., Mouse IFN- γ ELISPOT Capture Antibody (BD 51-2525KZ)).
3. Labeled secondary cytokine-specific antibody (e.g., Mouse IFN- γ ELISPOT Detection Antibody (BD 51-1818KA)).
4. Detecting antibody or protein (e.g., Streptavidin–Horseradish Peroxidase (BD)).
5. DPBS without Ca²⁺/Mg²⁺.
6. Coating Buffer: Antibody (e.g., anti-IFN γ antibody) diluted in DPBS without Ca²⁺/Mg²⁺.
7. Dilution Buffer: DPBS without Ca²⁺/Mg²⁺ plus 10% FBS.
8. Wash Buffer: DPBS without Ca²⁺/Mg²⁺ plus 0.05% Tween 20.
9. ACK lysis buffer (BioWhittaker 10-548E).
10. Assay Media: Subheading 2.2, step 5.
11. Cytokine-secreting cells.
12. Human or mouse recombinant IL-2 (NIH AIDS Research and Reagents Program/Peprotech).
13. Developing Substrate: AEC substrate set (BD 551951).
14. Light microscope for counting spots or, ideally, a digital camera and spot-counting software.

2.7. Tetramer Analysis

1. Tetramer (streptavidin-linked and fluorochrome-conjugated PE or APC) (see Note 4).
2. ACK Buffer: Subheading 2.2, step 9.
3. Cell strainers (40 μ m Nylon, sterile) (Falcon 352340).
4. 96-well U-bottom plate or FACS tubes.
5. Fc Block (BD).
6. FACS Buffer: PBS, 1% BSA, 0.09% sodium azide.
7. Anti-CD8 antibody.
8. 2% paraformaldehyde.
9. Flow Cytometer.

2.8. Intracellular Cytokine Staining

1. PMA (ICN).
2. Ionomycin (Sigma).
3. Brefeldin A (Sigma).
4. Alternate form of restimulation (i.e., peptide).
5. Fluorochrome-conjugated anti-cytokine antibody (e.g., anti-IFN- γ).
6. Isotype-matched negative control antibody.
7. Fluorochrome-conjugated anti-CD8 antibody.
8. Staining Buffer: PBS, 3% heat-inactivated FCS, 0.09% sodium azide.
9. FACS Buffer without azide: PBS, 1% BSA (see Note 5).
10. Cytofix/Cytoperm solution (Pharmingen).
11. Perm/Wash solution (Pharmingen).
12. Flow cytometer.

2.9. Beta-Galactosidase Detection

1. Tissue culture needs (Flat- and round-bottom 96-well plates, conical tubes, pipettes, multichannel pipettes, etc.).
2. Antigen-presenting cells (APC) and T cell hybridomas.
3. Recombinant poxviruses and synthetic peptides.
4. Potassium ferrocyanide and Potassium ferricyanide.
5. 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal).
6. Fixative solution: 2% formaldehyde and 0.2% glutaraldehyde in water.
7. Substrate Solution: 5 mM Potassium ferrocyanide (100 μ l from 0.5 M stock), 5 mM Potassium ferricyanide (100 μ l from 0.5 M stock (light-sensitive compound)), 2 mM MgCl₂ (20 μ l from 1 M MgCl₂), and 1 mg/ml X-gal (1 ml from 10 mg/ml stock (made in DMSO)); bring up to 10 ml in PBS.
8. 4-methyl umbelliferyl b-D galactoside (MUG—33 mg/ml stock solution of MUG is made in DMSO and stored frozen at -20°C).
9. Fluorescent Substrate Solution: 100 mM 2-mercaptoethanol, 9 mM MgCl₂, 0.125% NP40, 3 mM MUG.
10. Plate reader with fluorescence capabilities.

3. Methods

3.1. Recombinant Poxvirus Generation

Recombinant VACV can be generated via simultaneous infection/transfection with an rVACV plasmid containing a gene of interest. Efficiency of recombination can be enhanced by using a selection marker such as disruption of the thymidine kinase (TK) locus and

subsequent treatment with BrdU (see Note 6). The TK mutant method of generating recombinants has been well described (18). Here, we describe an alternative method that allows for selection of recombinants based on plaque size (19–21). Performing recombination with an F13L deletion strain (vRB12) and a plasmid that both adds the gene of interest and restores the F13L gene (pRB21) (see Note 7) allows recombinants to be identified and selected by their large plaque size as compared to the small plaque size of the non-recombined F13L deletion strain.

1. 24 h before VACV infection/transfection, split a confluent culture of CV-1 cells and seed 2 ml/well in a six-well tissue culture plate. Incubate the cells at 37°C in a CO₂ incubator overnight. Cells should be roughly 70% confluent.
2. Prepare VACV by diluting a crude virus stock in BSS/BSA solution. The amount of virus should be adjusted to give a multiplicity of infection (MOI) of 0.05 plaque-forming units (pfu) per cell in a total volume of 500 µl/well.
3. Aspirate assay medium from the cultures and add the virus–BSS/BSA mixture. Incubate at 37°C for 1 h.
4. During the 1-h virus incubation, begin preparing the transfection components in sterile 1.5-ml polypropylene Eppendorf tubes. For each well to be transfected:
 - (a) Dilute 6 µl FuGENE in 94 µl serum-free OPTI-MEM and incubate at room temperature for 10 min.
 - (b) Transfer the OPTI-MEM/FuGENE mix to a tube containing 2–3 µg pRB21 with the gene of interest plasmid DNA, and mix gently by pipetting. Incubate at room temperature for 40 min.
5. Aspirate virus inoculum from the CV-1 cell cultures and wash the cells once with 2 ml serum-free EMEM. Add 2 ml infection medium per well.
6. Add the pRB21/FuGENE solution drop by drop to each well of CV-1 cells. Incubate for 48–72 h in a CO₂ incubator at 37°C.
7. Harvest the transfected/infected CV-1 cells from the well with a disposable rubber scraper. Collect the cell suspension in a sterile 1.5-ml polypropylene Eppendorf tube.
8. Release rVACV from cells with three successive cycles of freeze–thawing the harvested cells by transferring them from a dry ice/ethanol bath to a 37°C water bath followed by vortexing. The cell lysate can be stored at –80°C.
9. Plaque purification:
 - (a) Plate BSC-1 cells at a density of 4.0×10^5 cells/well in a 6-well plate, and allow to adhere overnight (for this initial plaquing, plating of BSC-1 cells at a higher density may result in better separation of plaques).

- (b) On day 0, remove media from BSC-1 cells, and add 450 ml sterile BSS/BSA.
 - (c) Infect BSC-1 cells with serial dilution of the CV-1 extract (although not essential, a brief spin at $3,000 \times g$ will pellet cell debris, leaving virus in suspension). Infect one well with a 1:10 dilution of the CV-1 lysate by adding 50 μ l CV-1 extract. Mix well, and transfer 50 μ l to the next well, for a 1:100 dilution of the lysate (usually 2–3 dilutions are enough). Place in the incubator for 2 h, rocking every 15–20 min.
 - (d) Carefully remove BSS/BSA and add mixture of 2 \times EMEM (supplemented with 10% FCS and 10 μ g/ml gentamicin), and sterile 1.8% LMP. For one well, this will be 0.75 ml 2 \times EMEM (prewarmed in a 45°C water bath)+0.75 ml LMP (melted and equilibrated to 45°C in a water bath), and allow to cool completely before returning to the incubator (if the overlay is not completely solid before returning to the incubator, it will remain semisolid and prevent proper plaquing).
10. Picking and labeling plaques: It is important to select recombinant plaques that are as isolated as possible for further purification. Small plaques must be avoided. Pick isolated large plaques once they can be identified (see Note 8).
- (a) Identify and circle three isolated large plaques, labeling the most isolated or most promising “A,” the next “B,” and third “C.” Pick the plaques, using a sterile 200–1,000 μ l pipette tip.
 - (b) Transfer plaques to sterile 1.5 ml Eppendorf tubes with 0.5 ml sterile BSS/BSA. Freeze–thaw–vortex, and sonicate (full strength, 50% time pulse) for 1 min.
 - (c) Repeat the plaquing procedure (step 9), using two dilutions for each plaque (usually 50 and 5 μ l are sufficient [A, B, and C]).
 - (d) Again, pick three plaques, and label similarly (thus second-round plaques will be named AA, AB, AC, BA, and so on, according to which wells yield the best plaques).
 - (e) Repeat for a third round (see Note 9). The virus can then be expanded and sequenced, or tested to confirm the integrity of the inserted gene.
11. Viral expansion:
- (a) Once a pure recombinant plaque is identified, approx 200 μ l of the disrupted plaque can be added directly to a 6-well plate of BSC-1 cells seeded at 4.0×10^5 cells/well.
 - (b) One day before infection, prepare a T-75 flask seeded with BSC-1 cells at 6.0×10^6 cells/flask.

- (c) After 2 days the well is scraped and harvested, as was done for the CV-1 cells (Step 8). After the three rounds of freeze/thaw/vortex and a brief spin, add most of the extract to fresh BSC-1 wells, to amplify the virus (saving a small amount as a backup).
- (d) This extract can be used to infect the T-75 flask seeded 1 day previously.
- (e) Harvest after 2 days of infection using a cell scraper, transfer to a sterile 50 ml polypropylene conical, and centrifuge for 5 min at $1,500 \times g$.
- (f) Remove the supernatant and resuspend in 1 ml BSS/BSA, freeze–thaw–vortex three rounds, sonicate, and spin at $1,500 \times g$.
- (g) Transfer the supernatant to a new tube, vortex gently for 20 s, aliquot in sterile 1.5 ml microcentrifuge tubes, and store at -80°C .

3.2. In Vitro Chromium Release Assay

The in vitro cytotoxic T-lymphocyte (CTL) assay provides information on the reactivity and effector function of epitope-specific CD8⁺ T cells. It has a long-standing history in the MHC class I antigen presentation field and remains one of the best options to perform this type of study (see Notes 10 and 11). To perform these experiments, MHC-matched (for the epitope of interest) cells are infected with the recombinant poxviruses expressing the protein under study. These infected cells serve as “targets” that are subsequently loaded with an indicator to measure CTL-specific cell-lysis (see Note 12).

1. 6–7 days prior to the day of the chromium release assay, CTLs should be harvested from mice of interest and the epitope-specific CTL population expanded by in vitro restimulation for 6–7 days (see Subheading 3.3.1, step 9).
2. Typically 5×10^5 MHC-matched target cells are infected with 5–10 pfu/cell of the recombinant poxvirus expressing the target protein of interest (see Note 13). In a 15 ml conical tube, add the appropriate amount of virus to BSS/BSA for a final volume of 200 μl . Then to the poxvirus-containing tubes, add 200 μl of a suspension containing target cells in BSS/BSA (set targets at a concentration of 2.5×10^6 cells/ml) (see Note 14).
3. Place cells into a rotator at 37°C for 1 h. Make sure that the rotator is set in a position such that the tubes are almost horizontal, so as to prevent the cells from pelleting at the bottom. After 1 h, add 2 ml Infection Medium to the tubes and continue to incubate for an additional 3 h.
4. At any time during the infection consider adding Brefeldin A (BFA) at 5 $\mu\text{g}/\text{ml}$ (see Notes 15–17).

5. Set up the effector population in 96-well plates. The CTL are plated in triplicate and four dilutions are set up for a total of four effector-to-target ratios (E:T) for each target to be tested (see Note 18). Using a multichannel pipette, add 150 μ l of effector cells to the top row of the plates and from these remove 50 μ l of cell suspension and add it to the next row which will already contain 100 μ l of Assay Media. Repeat this step for the two more rows, always starting from the row just above that into which you are adding the cells. This will result in 1:3 dilutions of the effector cells. Discard the 50 μ l from the last row. Place the plate in a 37°C incubator at 5% CO₂ to await the addition of target cells (see Note 19).
6. Set up another plate to assess spontaneous and maximal ⁵¹Cr release by target cells. For each target cell add 100 μ l of Assay Media to 5 wells and 100 μ l of a 1% Triton X-100 (in PBS) solution to another 5 wells. Place plate(s) into a 37°C incubator at 5% CO₂ to await the addition of target cells.
7. After the infection of the target cells is complete (4 h total, Subheading 3.2, step 2), pellet the infected cells by centrifugation at 600 $\times g$ for 5 min. Remove as much of the media as possible (see Note 20).
8. Target cells (see Note 21) will be labeled in a total volume of 25 μ l containing 50 μ Ci/5 $\times 10^5$ cells of ⁵¹Cr diluted in Infection Medium. The cells can be resuspended in the ⁵¹Cr by pipetting up and down with a p200 pipette. Place tubes back in rotator for 1 h.
9. After labeling, pellet cells as above and wash two times with 3 ml of cold PBS for each wash. It would be useful to remove the first supernatant (containing the high-activity ⁵¹Cr) by pipetting with a p200 and placing it into a shielded container. The wash supernatants will contain relatively low amounts of radioactivity and may therefore be decanted into a low-activity waste receptacle (according to institutional guidelines).
10. Resuspend the cells with the appropriate amount of Assay Medium. For example when using only one effector population, 2.7 ml by well of Assay Medium will be added to each target cell population, which allows for the addition of 100 μ l by well of target cell suspension to the wells containing effector cells and to the wells containing medium and Triton X-100. A multichannel pipette can be used.
11. Incubate cells in 37°C incubator at 5% CO₂ for 4 h.
12. Carefully remove plates after 4-h incubation as not to disturb the cell pellet at the bottom of each well. Transfer 50 μ l of supernatant to a new 96-well plate (specific for gamma counter) that already contains 50 μ l of scintillation fluid (see Note 22).

13. Calculate percent lysis by Percent Lysis = [(Experimental-Spontaneous)/(Maximal-Spontaneous)] × 100. The spontaneous lysis value is obtained by counting cpm from the target cells added to medium in the absence of CTL, and the maximal lysis value is obtained from target cells added to Triton X-100.

3.3. In Vivo and In Vitro MHC Class I-Restricted Assays

Using poxvirus priming as a starting point it is relatively easy to determine CD8⁺ T cell responses in vivo. The number of epitope-specific T cells generated can be determined by a number of different assays, including in vivo CTL, interferon (IFN)-γ ELISPOT (22), staining with MHC-I/peptide tetramers (23, 24), intracellular cytokine staining (ICS) (23), and CD107a degranulation assay. The number of MHC class I/epitope complexes expressed on a target cell can be deduced using the above assays or measured directly using Surface MHC/peptide complex staining. These assays are described below.

3.3.1. Priming Assay

1. Dilute recombinant poxviruses into BSS/BSA at a final volume of 200 µl. Usually anywhere from 10⁵ to 10⁷ pfu of VACV and 3 × 10³ of ECTV will be sufficient (see Note 23).
2. Prime mice intraperitoneally (i.p.), intravenously (i.v.), or foot-pad (f.p.) using insulin syringes (see Notes 24 and 25).
3. Mice are typically sacrificed between 7 and 14 days post infection depending on application, and the spleens are removed (see Note 26).
4. The spleen is homogenized using a tissue grinder or with sterile frosted microscope slides in 7 ml of Assay Medium and the resulting cells pelleted by spinning at 600 ×*g* for 5 min. Do all subsequent steps on ice.
5. Lyse red blood cells by adding 1 ml of ACK Buffer and incubate on ice for exactly 1 min. After 1 min immediately add 30 ml PBS.
6. Pellet cells at 600 ×*g* for 5 min at 4°C. Wash 1× with PBS.
7. Resuspend cells in Assay Medium and pipette through a cell strainer back into Assay Medium.
8. Count cells and set at appropriate concentration (usually 10⁷ cells/ml).
9. In vitro restimulation will probably be required as primary ex vivo CTL activity is not typically robust. To restimulate, use of a second method of epitope presentation is required. This negates expansion of poxvirus-specific responses that could mask responses to the desired epitope (see Note 27). A protocol for restimulation using peptide follows:
 - (a) Count splenocytes: If comparing different spleen populations, be sure to keep the total number of cells the same between populations.

- (b) For each priming condition, two-thirds of the total spleen cells will be used to generate epitope-specific CTL and one-third will be used to assess poxvirus-specific priming.
 - (c) Naïve spleen cells from uninfected mice are used as the restimulators for VACV-specific responses and will be added at a 1:3 ratio to responders. For VACV-specific responders, infect the appropriate number of restimulators with 1–5 pfu of wild-type poxvirus in a total volume of 400 µl of BSS/BSA by rotating the cells for 1 h at 37°C.
 - (d) After incubation, pellet cells and wash once with BSS/BSA, resuspend in the appropriate volume of assay media, and add to responder cells. Keep the poxvirus-specific CTLs in a T-25 flask in a final volume of 12 ml.
 - (e) For the epitope-specific CTL populations, pulse 0.01–10.0 µg/ml peptide onto irradiated MHC-matched cells, and rotate at 37°C for 1 h. Wash peptide-loaded cells once with BSS/BSA and add them to the CTL cultures at a ratio of 10:1 (CTL:restimulators) (see Note 28).
 - (f) Restimulation is for 6–7 days. In general, the best CTL activity is on day 6. Day 7 is slightly worse and after day 7 there is a sharp drop-off of CTL activity (see Note 29).
10. CTL activity can be assessed via Chromium Release Assay (see Subheading 3.2).

3.3.2. *In Vivo Cytotoxicity Assay*

Immunize mice with recombinant VACV (i.p./i.v./f.p.) or ECTV (f.p.) as described in Subheading 3.3.1, step 1. Continue with protocol below 2 weeks later.

1. Prepare naïve donor splenocytes from *naive* mice as described in Subheading 3.3.1, steps 4 thru 8.
2. Wash cells 2× with 20 ml serum-free RPMI, and pellet for 5 min at 600 g.
3. Discard sup and resuspend in ~20 ml serum-free RPMI per 10 spleens.
4. Remove any clumps by passing cells through cell sieve.
5. Count cells using trypan blue exclusion and dilute to 1×10^7 /ml in serum-free RPMI.
6. Split cells equally into three 50 ml conical tubes (for 3 populations) for CFSE labeling.
7. Add CFSE to cells at 0.1 µM (CFSE_{lo}) [5 µl of 0.5 mM in 25 ml], 0.5 µM (CFSE_{med}) [25 µl of 0.5 mM in 25 ml], and 2.5 µM (CFSE_{hi}) [12.5 µl of 5.0 mM in 25 ml] for three populations. Incubate for 10 min at 37°C and swirl constantly for sharp peaks (see Note 30).
8. Quench by adding equal volume of ice-cold FBS to each vial and incubating for 5 min on ice.

9. Pellet cells and wash 3× with Assay Media.
10. Resuspend each vial in 10 ml Assay Media and leave CFSE_{med} unpulsed or pulsed with a control peptide.
11. Pulse CFSE_{lo} and CFSE_{hi} with 10 µg/ml of experimental peptides for 1 h at 37°C (see Note 31).
12. Pellet cells and wash 2× with Assay Media.
13. Pellet cells and resuspend in 10 ml DPBS.
14. Obtain accurate cell counts, then combine equal numbers of CFSE_{lo}, CFSE_{med}, and CFSE_{hi} cells, and pellet in new tube.
15. Pellet cells and resuspend in DBPS to yield 5–10 × 10⁷ of each cell type per ml.
16. Inject 100 µl by tail vein into immunized mice giving 5–10 × 10⁶ of each cell type per mouse.
17. Remove spleens individually 3–4 h later into 5 ml FACS buffer.
18. Homogenize spleens with cell homogenizer or between frosted ends of super-frost microscope slides.
19. Pellet cells and wash 3× with 5 ml FACS buffer.
20. Resuspend each spleen in 10 ml FACS buffer with 2% PFA, pass through cell sieve, and analyze by flow cytometry (see Note 32).

3.3.3. Enzyme-Linked Immunospot Assay

Precursor frequency of T cells can often be quite low and ELISPOT is more sensitive than the above-mentioned *in vivo* assays (23). Statistically robust tetramer assays and ICS assays may require somewhat larger CTL precursor populations. The ELISPOT assay works best for CTL populations that are taken from a mouse at least 14 days post poxvirus priming. CTL collected prior to this time tend to produce a high number of nonspecific IFN-γ spots due to residual immune responses and/or persisting virus (ECTV).

1. Prime mice and prepare spleen cells as described in Subheading 3.3.1.
2. Plate with the anti-cytokine antibody (usually 10–40 µg/ml in 1× DPBS without Ca²⁺/Mg²⁺). Use 100 µl of coating solution in each well. Wrap the plate in parafilm and incubate overnight at 4°C (see Note 33).
3. On experiment day, remove the coating buffer and wash the plates 3× with DPBS without Ca²⁺/Mg²⁺. Add 100 µl Assay Media to cells and place into a 37°C incubator at 5% CO₂ for 2 h.
4. Remove spleens, homogenize as described in Subheading 3.3.1, step 4, and pellet the cells by centrifugation at 600 × g for 5 min. Keep cells on ice for this and subsequent steps.
5. Lyse red blood cells by adding 2 ml ACK buffer/spleen for 1 min in 15 ml conical. Immediately after 1 min at room temp,

top off tube with Assay Medium. Pellet cells, resuspend in 5 ml Assay Medium, and count cells.

6. Harvest MHC-matched stimulator cells from naïve mice. These cells can be infected with a recombinant poxvirus expressing the required epitope (see Note 34) or pulsed with peptide (see Note 35). Whether peptide pulsing or poxvirus infecting, do this at 37°C for 1 h.
7. Wash and resuspend stimulator cells in Assay Media. A good starting point is 2.5×10^6 cells per ml (2.5×10^5 cells per 100 µl). Remove Assay Medium from plates that are in the incubator and add 100 µl of cell suspension into each well (see Note 36).
8. Set the concentration of the spleen cells to do the appropriate dilutions. For epitope-specific responses 2.5×10^6 /ml (2.5×10^5 /100 µl), E:T 1:1, is a good starting concentration and for poxvirus-specific responses, 5×10^4 /100 µl, E:T 5:1, is a good starting point. At least 2 five-fold dilutions should be done for each spleen population. Add 100 µl to wells that already contain stimulator cells.
9. Incubate cells at 37°C for 18–24 h.
10. Remove media and lyse the remaining cells by adding 200 µl/well ddH₂O for 10 min.
11. Wash cells 5× with Wash Solution 200 µl/well.
12. Add 50 µl/well secondary biotinylated cytokine-specific antibody diluted in Dilution buffer (4 µg/ml in 50 µl) and incubate at room temperature for 2 h.
13. Remove antibody/dilution buffer and wash plate 3× with Wash Solution.
14. Add HRP-avidin (use 10 µg/ml in 50 µl) and incubate at room temperature for 1 h.
15. Wash plate 5× with Wash Solution.
16. Add 50 µl/well AEC Developing Substrate. One drop of AEC chromogen (BD 51-2578KC) per 1 ml of AEC Substrate buffer (BD 51-2577KC) (both supplied in AEC Substrate kit, BD 551951). 6 drops into 6 ml to develop one full 96-well plate. Once spots have developed (3–5 min) (see Note 37), wash plates with water to stop the reaction and allow plates to dry.
17. Count spots and determine CTL frequencies (see Note 38).

3.3.4. Surface MHC/ Peptide Complex Staining

Examination of MHC class I epitope presentation from recombinant poxviruses can be easily quantified through the use of antibodies that recognize the MHC/epitope complex at the cell surface. Most popular for this purpose has been the 25-D1.16 monoclonal antibody specific for the Ova_{257–264}/H-2K^b complex. Indeed, this antibody performs so well that many laboratories,

including ours, have inserted this epitope into their antigens of choice via genetic engineering. Below is the basic surface staining protocol to be used in conjunction with this antibody and recombinant poxviruses expressing the Ova_{257–264} (SIINFEKL) epitope. It is a simple assay but one that yields valuable data regarding the number of MHC/SIINFEKL complexes presented on a cell's surface. Accurate quantitative number of MHC/SIINFEKL complexes per cell can be obtained by calibrating with antibody-conjugated beads (25, 26).

1. Infect target cells expressing appropriate MHC class I molecule with recombinant poxviruses expressing an epitope of interest (e.g., SIINFEKL).
2. Count cells and Place 5×10^5 cells in a 5 ml FACS tube.
3. Resuspend cells in 50 μ l of Fc Block (see Note 39).
4. Incubate for 15 min either on ice or at 4°C.
5. Wash cells 1× with FACS Buffer.
6. Cells should be incubated in a total of 50 μ l staining buffer with a predetermined optimal concentration of a fluorochrome-conjugated MHC/epitope complex-specific antibody (e.g., 25-D1.16).
7. Staining should take place for 20–30 min at 4°C, keeping the samples in the dark.
8. After the incubation is complete, wash the cells two times with FACS Buffer, pellet cells by centrifugation at about $600 \times g$, and remove supernatant.
9. Wash 1× and resuspend in 400 μ l 2% paraformaldehyde (PFA).
10. Run samples through a Flow Cytometer.

3.3.5. Tetramer Staining

Tetramer analysis is a convenient method to determine the number of epitope-specific CTLs that arise after priming with a recombinant poxvirus. Performing a surface and/or intracellular stain in conjunction with this assay is recommended (see Note 40).

1. Prime mice and prepare spleen cells as described in Subheading 3.3.1 and dilute cells to an appropriate concentration (usually 10^7 cells/ml).
2. Plate 10^6 cells/well of a 96-well v-bottom plate. Spin plate at $600 \times g$ and remove supernatants.
3. Resuspend cells in 50 μ l/well of Fc Block (see Note 39).
4. Incubate for 15 min either on ice or at 4°C.
5. Wash cells 1× with FACS Buffer.
6. After washing, resuspend pellet in 50 μ l PBS containing diluted tetramer (see Note 41). Do all subsequent steps keeping cells in the dark.

7. Incubate either at room temperature or on ice for 30 min (see Note 42).
8. Wash cells 1× with FACS Buffer.
9. Add other surface marker antibodies diluted in FACS buffer (see Note 43).
10. Incubate on ice for 30 min.
11. Wash 1× and resuspend in 400 µl 2% PFA.
12. Run samples through a Flow Cytometer.

3.3.6. Intracellular Cytokine Staining

For ICS, an excellent protocol is available on the BD Biosciences Web site www.bdbiosciences.com and is summarized for use with poxvirus systems below.

1. Prime mice and prepare spleen cells as described in Subheading 3.3.1 and dilute cells to an appropriate concentration (usually 10^7 cells/ml).
2. Samples should be set up for stimulation such that there is a set of cells for experimental, positive, and negative controls (see Note 44).
3. The experimental set of cells should be pulsed with 10^{-6} to 10^{-10} M of appropriate peptide for 4–6 h at 37°C (see Note 45). For the positive control, stimulate with PMA (50 ng/ml) and ionomycin (700 ng/ml) for 4 h at 37°C. Lastly, for the negative control, use an irrelevant peptide or perform a mock stimulation without any peptide present. 10 µg/ml of BFA must be added to each set of conditions for the last 3 h of stimulation.
4. Count cells and place 10^6 cells/well in a 96-well round-bottom plate.
5. Once all sets of cells have been stimulated, staining for a particular cytokine can proceed.
6. Cells should be incubated in a total of 50 µl staining buffer with a predetermined optimal concentration of a fluorochrome-conjugated, cell surface monoclonal antibody, such as anti-CD8 antibody.
7. Staining should take place for 20–30 min at 4°C, keeping the samples in the dark.
8. After the incubation is complete, wash the cells two times with FACS Buffer without azide, pellet cells by centrifugation at about $600 \times g$, and remove supernatant.
9. Fix and permeabilize cells by thoroughly resuspending them in 100 µl of Cytofix/Cytoperm solution and incubating on ice for 10–20 min in the dark.
10. Wash cells two times with Perm/Wash solution, pellet, and remove supernatant.

11. Resuspend cells in the Perm/Wash solution containing either the anti-cytokine antibody or the isotype-matched antibody (negative control) at the proper concentration.
12. Incubate for 30 min on ice in the dark.
13. After incubation, wash cells 2× with Perm/Wash solution.
14. Wash cells 1× with FACS buffer without azide.
15. Fix cells with 2% PFA (add 100 µl/well).
16. Analyze by flow cytometry.

3.4. MHC Class II-Restricted T Cell Responses

Analysis of MHC class II-restricted T cell responses employing recombinant poxviruses is often more challenging than MHC class I-restricted T cell responses. MHC class II-restricted responses are generally studied using traditional methods such as in vitro proliferation assays and quantification of IL-2 released by activated T cells or T cell hybridomas. Some of these assays require lengthy in vitro culture periods, and are therefore incompatible with the use of cytopathic poxviruses. Such problems can be alleviated if poxviruses are rendered replication incompetent (see Note 46). Modified immunization protocols can be employed to generate in vivo CD4⁺ T cell responses (see Note 47). Hence, it may be necessary to modify antigens and/or employ modified priming protocols to generate in vivo CD4⁺ T cell responses.

3.4.1. Cytotoxic T Cell Assays

Primary CD4⁺ T cell cultures generally have a lower cytolytic capacity than CD8⁺ T cells owing to reduced perforin expression as well as lower precursor frequency of Fas ligand-expressing cells (27). Assays can be performed using the in vivo cytotoxicity protocol described above with modification. A modified chromium release assay can be used for in vitro assays. Longer incubation periods are required for CD4-mediated killing of target cells. Since longer assay periods may allow for measurable CD4⁺ T cell killing, the cytopathic nature of poxviruses may pose problems. Hence, assays are done in a similar fashion as MHC class I-restricted CTL assays with an exception that co-incubation of effector and target cells is extended, yet sufficiently shorter than conventional MHC class II-restricted assays, which avoids nonspecific lysis of target cells by poxviruses. MHC class II-restricted CTL assays are performed as follows:

1. Target cells (at 2×10^7 cells/ml density) are infected with poxvirus (5 pfu/cell) for 1 h at 37°C in BSS/BSA in 15 ml conical tubes.
2. Spin down the cells, resuspend in 5 ml Assay Medium, and incubate for an additional 4 h at 37°C.
3. Load cells with ^{51}Cr as described in Subheading 3.2.

4. Mix 1×10^4 radiolabeled cells and effector cells at desired E:T ratios in 96-well round-bottom plates.
5. Incubate overnight at 37°C and harvest supernatant. The percentage-specific lysis is calculated as described in Subheading 3.2, step 13.

3.4.2. Beta-Galactosidase-Inducible T Cell Hybridomas

T cell hybridomas that express β-galactosidase following antigen-driven stimulation are perhaps the most convenient system (see Note 48) to assess MHC Class I- and Class II-restricted responses. Splenocytes or T cell clones are fused with the BWZ.36 cell line that is stably transfected with the Nuclear Factor-Activated T cell (NFAT) enhancer element-β-galactosidase construct. Thus, β-galactosidase is specifically produced in response to antigen/MHC complexes (28) (see Note 49). Quantification of activated T cell hybridomas using fluorescent substrates is preferred over the previous method of microscopic enumeration because it is quick, simple, and avoids potential bias in counting. A variety of substrates can be used to measure T hybridoma activation. We use MUG in our efforts to quantitate T hybridoma activation (see Note 52). Assays are performed as follows:

1. Infect APC with recombinant poxviruses at 5 pfu/cell in BSS/BSA for 1 h at 37°C.
2. Wash cells once with Assay Medium and plate 1×10^5 cells in 100 µl Assay Medium in a 96-well flat-bottom plate (note: clear bottom).
3. Add 1×10^5 T cell hybridoma in 100 µl Assay Medium (see Note 50).
4. Incubate plate at 37°C overnight.
5. Add MUG substrate at 1:1,000 in MUG buffer.
6. Add 50 µl/well.
7. Incubate plate at 37°C for 1 h (see Notes 51 and 52).
8. Read plate at 360 nm excitation and 460 nm emission on a fluorescence plate reader.
9. Return plate to 37°C for an additional hour.
10. Repeat Step 8.

4. Notes

1. PBS + 0.1% BSA may be substituted for BSS/BSA.
2. Due to safety concerns, when sonicating poxviruses a cup sonicator is safer because a probe sonicator will aerosolize the virus.

3. Poxviruses should not be used to restimulate epitope-specific T cells due to cross-reactivity and high background that will result in the assay if poxvirus-infected cells are used as targets.
4. Currently, tetramers can be obtained from the NIAID Tetramer Facility, Emory University Vaccine Center at Yerkes, or purchased from Beckman Coulter Inc.
5. FACS buffer for Intracellular cell staining (Subheading 3.3.5) does not contain sodium azide as it is toxic to the cells. For all other applications, FACS buffer may contain 0.09% Sodium Azide.
6. Selection can be further enhanced by inclusion of a marker such as a chromogenic substrate. We recommend the use of the beta-glucuronidase (β -glucuronidase) gene in concert with the X-gluc substrate (beta-galactosidase and X-gal are more popularly utilized for this purpose but recombinant VACV expressing beta-galactosidase (β -galactosidase) can cause nonspecific background in assays employing certain types of T cell hybridomas that also express β -galactosidase).
7. This method allows for recombinants to be selected based on their ability to generate large plaques indicative of a WT VACV phenotype restored via recombination. Small plaque size, reflecting compromised replication, can be conferred through deletion of the F13L VACV envelope protein.
8. Be sure to pick around the edges of the plaque, and carefully scrape the bottom of the plate with the tip. VACV grows as a cell-associated virus spreading from cell to cell via membrane contact. Therefore, the majority of the virus in a plaque will be around the edges of the plaque, where there are cells actively producing virus, not at the center of the plaque where there are few cells remaining.
9. Usually, picking three plaques/round is sufficient, and the labeling system indicates which plaques are most desirable. Thus, plaque ABA would be more promising than BBA.
10. Over the years, our lab has tested several nonradioactive methods for measuring cytolysis. The chromium release assay remains unsurpassed in its ability to detect specific killing in an *in vitro* assay.
11. Although chromium release remains the gold standard for *in vitro* killing assays, other assays can also be employed to this end. One such assay employs epitope-specific T cell hybridomas transfected with β -galactosidase that provide an alternative readout to chromium release. One example is the β -galactosidase (*LacZ*)-transfected T hybridoma cells engineered in the Shastri laboratory (Berkeley, CA) (28) and the use of this system is described later in Subheading 3.4.2. The β -galactosidase-based

assays for class I-restricted responses are identical to those described for class II assays.

12. A population of CTL specific for the epitope of interest can be generated by priming mice and restimulating *in vitro* or by using CTL cell lines. A limitation of this assay is the time required from the beginning of viral infection of targets to the harvesting of culture supernatants. If performed efficiently, this assay will take about 10–11 h. Time considerations must be observed because longer incubation times may result in greater spontaneous lysis and variable results owing to the cytopathic nature of VACV or ECTV at the later stages of replication.
13. If MHC-matched target cells are not readily available one can coinfect with a poxvirus expressing the appropriate MHC molecule or transfect/transduce the appropriate MHC molecule into the target cell. When infecting with more than one recombinant poxvirus, limit the total MOI to 15 pfu/cell. If using naïve splenocytes as matched targets, infection of murine splenocytes with VACV is inefficient and can require high doses of virus, whereas ECTV readily infects murine splenocytes. Thus, using a recombinant ECTV provides an advantage in this scenario.
14. For convenience, one can set up the tubes containing recombinant poxvirus in BSS/BSA the night before the assay and store the tubes at 4°C until the next morning, at which time target cells can be added to begin the infection.
15. One can also use poxvirus infection of targets to study the virus-mediated aspects of protein processing and presentation in the specific context of a poxvirus infection. Using this method one can assess such things as the impact of targeting a protein of interest to different cellular compartments, making mutations within a protein of interest, and by altering the poxvirus promoter strength driving the foreign gene, one can examine the effects of protein expression level on antigen processing and presentation. Additionally, the assay allows one to investigate the pathways and proteases that are required for the production of particular antigens.
16. BFA is added to the cells in order to inhibit transport of MHC class I/peptide complexes to the cell surface (29). Using BFA is an easy way to gauge the efficiency of presentation in cases where maximal presentation of epitopes is reached at relatively early time points in the course of the poxvirus infection. For rapidly processed epitopes, considerable differences in presentation efficiency may be masked without the addition of BFA simply because the rate of presentation of all epitopes is so rapid as to reach maximal levels by the time the CTL are added to the targets. The appropriate time to add BFA needs to be

determined experimentally. In our hands 110 min post infection is a good starting point. In cases where agents need to be added to the target cells that may be harmful to T cells (i.e., BFA), the concentration of BFA can be lowered up to five-fold during co-incubation. Lastly, another area in which BFA may be useful is in ensuring equal infection of target cells. Use of poxvirus-specific CTL to confirm equal infection is complicated by the magnitude of the anti-poxvirus response; this often results in similar killing, even at different E:T ratios. Addition of BFA at various times post infection may limit presentation such that a titration of specific lysis is observed with different E:T ratios.

17. BFA needs to be stored properly (-20°C) and validated for each experiment, since it can lose its potency over time.
18. While we do four E:T ratios, some labs do as many as eight.
19. When target cells are being added to the 96-well plates it is best to add the cells to the Triton X-100-containing wells last as to avoid any splashing of the detergent. Even an imperceptible amount of Triton X-100 on the tip of a pipette will yield high lysis in the next wells plated out. When setting up the plates that will include the Triton X-100 samples, it is helpful to keep the Triton X-100 wells separated from the assay media-containing wells. We do this by establishing a two-row gap between our spontaneous and maximal lysis groups.
20. The total volume in which the cells are resuspended for ^{51}Cr -labeling will have an impact on the efficiency of labeling. Therefore it is important to keep the volume equal from target to target. Carefully suctioning all of the liquid from the last wash (just prior to addition of ^{51}Cr) is critical for achieving equivalent volumes. It is often necessary to suction each tube a second time, to allow PBS on the walls of the tube to flow to the bottom.
21. Count target cells before plating to adjust them to the proper cell density. This is probably not necessary as long as all the tubes have been handled similarly. In addition, the total amount of chromium released (lysis by Triton X-100) will indicate whether the cells/well for each target were equivalent. The calculation of E:T ratio is actually arbitrary when using bulk populations of CTL from spleen because the number of true, epitope-specific cells is not known. One exception is when comparing infected to uninfected targets; in this case cell loss during washes may occur to differing degrees due to early cytopathic effects of VACV infection. In this case it will be necessary to count target cells before plating out.
22. Some scintillation fluids can neutralize poxviruses. We have success with Optiphase (Wallac), which is effective in killing VACV used in the assays. Be sure to wash the multichannel

pipette tips with water between effector dilutions so as not to cross contaminate samples. Seal the plates with tape and shake at low speed for 20 min. After mixing read plates in counter. If using a conventional gamma counter, the protocol is modified such that 100 µl of supernatant should be transferred to the appropriate tubes and chased with 100 µl of 10% bleach for counting.

23. It is important to evaluate recombinant poxviruses for infectivity to determine possible differences in virulence between unique recombinant viruses.
24. Mice can be primed intraperitoneally (i.p.), intradermal/footpad (f.p.), or intravenously (i.v.). Since ECTV is a murine tropic virus, has a high rate of in vivo replication, and causes significant pathogenesis, extra care in determining dose and route must be exercised. For most assays, f.p. (ECTV) and i.p. (VACV) priming will yield sufficient responses. But for assays where priming efficiency must be equivalent between mice (i.e., VACV in vivo assays) we generally obtain more consistent results when the mice are primed i.v. through the tail vein with VACV.
25. Keep inoculum volumes low: 30 µl works well for f.p. injections. Use insulin syringes for low-volume injections. Additionally, primed mice must be caged separately from unprimed mice. This will prevent cross contamination/infection that may result from virus leakage or shedding (minimal with VACV).
26. Spleens from mice infected with VACV or ECTV are typically harvested at 14 days post infection. Immunocompetent mice are typically able to clear VACV from the spleens by Day 14 post infection, but ECTV can persist. Additionally, ECTV is lethal in susceptible strains of mice (Balb/c) within 7–10 days post infection, so alternate measures such as treatment with cidofovir at day 5 post infection must be utilized when using Balb/c mice. Spleens can be harvested earlier than 14 days, but this can lead to increased background in certain assays and care must be taken particularly with ECTV-infected animals regarding the presence of persisting virus.
27. One method is the use of a synthetic peptide pulsed onto irradiated MHC-matched presenting cells. Cells can be irradiated with 10,000 cGy in a cesium source. Another method is the addition of 1.0 µg/ml or 0.1 µg/ml peptide (0.1 µg/ml is sufficient when working with Ova_{257–264}) directly to harvested splenocyte cultures for 6 days in the presence of 20–40 U/ml of rIL-2. Alternatively, one can express the antigen via a heterologous viral system (e.g., influenza or adenovirus) to infect and present antigen. Using poxvirus to express antigen in targets as well as prime T cells is not advised due to the significant poxvirus-specific background this experimental format will generate.

28. The optimal concentration of peptide pulsed onto the restimulator cells will need to be determined. We have had success with going as low as 0.01 µg/ml peptide. We recommend initially doing a dose response with 10.0 µg/ml, 1.0 µg/ml, 0.1 µg/ml, and 0.01 µg/ml peptide.
29. Long-term maintenance of CTL populations (longer than a week) requires the addition of rIL-2 (20–40 U/ml) and/or T-STIM™ (BD 354115).
30. The 0.5 mM CFSE stock is generated by adding 180 µl of DMSO to 50 µg of CFSE. The 5.0 mM CFSE stock is generated by adding 18 µl of DMSO to 50 µg of CFSE.
31. The CFSE_{lo} and CFSE_{hi} peaks can be pulsed with a poxvirus-specific epitope and the epitope of interest. Percent killing of the poxvirus epitope pulsed peak can provide a control for the priming efficiency of a recombinant poxvirus.
32. Cells must be resuspended in DPBS containing at least 2% PFA before being run on a flow cytometer in order to neutralize live poxvirus that may be present in cells from infected tissues. This is of particular concern with the murine tropic ECTV which replicates to high levels in mice.
33. All antibody concentrations should be optimized for the particular antibody being used.
34. If using poxvirus to infect stimulator cells, one must include a wild-type poxvirus to determine any cytokine secretion by the CTL that is poxvirus specific. This method is useful because it allows one to assess the level of priming for each animal used in the assay. Corrections for uneven priming can be made once the primary data have been obtained (30). For infections 1–5 pfu/cell is sufficient.
35. Optimal concentrations for peptide pulsing will need to be determined but can range from 0.01 µg/ml to 10.0 µg/ml.
36. The optimal density of stimulators per well needs to be determined experimentally for each system.
37. AEC substrate cannot be left on the plate for longer than 10 min or over-development will occur.
38. This process can be automated. There are complete ELISPOT counting setups that are commercially available. The AID ELISPOT reader system from Autoimmun Diagnostika GmbH, Germany, and AELVIS GmbH, and Immuno-Spot 5.0 software for ELISPOT analysis are some options. AELVIS GmbH, Germany, offers an ELISPOT analysis service worldwide for complete analysis of ELISOPT plates.
39. In place of BD Fc Block reagent, one can use 2.4G2A hybridoma supernatant (contains MAbs against Fcγ receptor) and normal goat serum at a 2.5:1 ratio.

40. Some tetramer-positive cells may not be cytolytic or otherwise functional (31, 32). Therefore, in order to properly evaluate T cell functionality it is important to also measure cytokine secretion of the tetramer-positive population via intracellular staining.
41. The optimal concentration of tetramer must be determined for each preparation of tetramer used.
42. Incubation temperature must be determined experimentally for a particular assay. Also, as a reminder sodium azide cannot be used if incubating at room temp.
43. Antibody concentration must be optimized. One should remember to at least include anti-CD8 antibody.
44. For these experiments a number of in vitro methods for stimulating cytokine production have been described. Common methods include the use of phorbol esters (PMA) and calcium ionophores. As for a negative control, activated cells should be stained with an irrelevant, isotype-matched antibody. Also note, it has been reported that cell activation with PMA alone causes a transient loss of CD4 expression from the surface of mouse T cells (33). Additionally, cell activation with PMA and a calcium ionophore together can cause a greater and more sustained decrease in CD4 and to a lesser extent CD8 from the surface of mouse thymocytes, and mouse, and human peripheral T lymphocytes.
45. The optimal concentration must be determined for each peptide used.
46. This can be achieved by psoralen-UV treatment of poxviruses (34, 35). Alternatively, less cytopathic MVA (10) can be used in these assays. We have not tested these possibilities in our laboratory. We prefer to employ assays of shorter duration to evaluate MHC class II-restricted responses. In vitro ^{51}Cr -release assays can be performed if MHC class II-restricted cytotoxic T cell clones are available. Perhaps, the most convenient method is to employ T cell hybridomas that express β -galactosidase following antigen-driven stimulation, a method devised by Shastri et al. (28). Additionally, it may be essential to use sucrose-purified poxviruses while assaying MHC class II-restricted responses (36). Crude preparations of poxviruses may contain contaminating exogenous antigens that will complicate the interpretation of experimental results.
47. Subcutaneous or footpad immunizations with poxviruses emulsified in Freund's adjuvant have been reported to generate efficient CD4 $^{+}$ responses (37). Antigens fused with an endosome sorting signal from LAMP (37) and invariant chain (37) have been shown to prime CD4 $^{+}$ T cells efficiently in vivo following immunization with recombinant poxviruses.

48. In vitro chromium release assays utilizing CD4⁺ T cells can be performed, but maintaining CD4⁺ T cell lines and additional properties of CD4 killing make this assay challenging. When available, we prefer to use T cell hybridomas that express β-galactosidase and the MUG assay to measure MHC Class II/epitope complexes expressed on the surface of target cells.
49. Recombinant poxviruses with the standard β-galactosidase selection markers cannot be used in conjunction with β-galactosidase-inducible T cell hybridomas owing to high levels of background from VACV-infected APC. ARA-C or cidofovir, compounds that inhibit VACV DNA synthesis, can be used to block β-galactosidase expression if the β-galactosidase is driven by a late VACV promoter. However, we get highly variable results using this compound, and as mentioned previously, we recommend the generation of recombinant poxviruses using alternate selection markers such as β-glucuronidase which does not cleave β-galactosidase substrates (10, 18).
50. Though most of the T cell hybridomas that we have tested respond well at the indicated APC and T hybridoma ratios, it is useful to determine appropriate ratios for individual T cell hybridomas. Depending on the density of specific peptide/MHC complexes presented by the APCs and the T Cell Receptor affinity for the complex, T cell hybridoma responses may attain saturation quickly. Hence, it is advisable to double dilute APC and assess T hybridoma activation to obtain graded responses.
51. Fluorescent substrates are light sensitive. Thus, it is necessary to protect assay plates from direct light. It is also necessary to keep samples devoid of bubbles as bubbles may interfere with assay reading. Brief centrifugation of assay plates prior to reading is recommended.
52. Other substrates such as 0.15 mM chlorophenol red b-galactopyranoside (CPRG), 5 mM o-nitrophenol-b-galactopyranoside (ONPG), or 1 mM FDG can also be used with absorption wavelengths 595, 415, or 495 nm, respectively, and 635 nm as the reference wavelength. MUG and CPRG seem to be the most sensitive substrates in these assays.

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References

1. Bennink JR, Yewdell JW, Smith GL, Moller C, Moss B (1984) Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. *Nature* 311 (5986):578–579
2. Yewdell JW, Anderson R, Cox JH, Eisenlohr LC, Esquivel F, Lapham C, Restifo NP, Bennink JR (1993) Multiple uses of viruses for studying antigen processing. *Semin Virol* 4:109–116
3. Morrison LA, Lukacher AE, Braciale VL, Fan DP, Braciale TJ (1986) Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J Exp Med* 163(4):903–921
4. Jaraquemada D, Marti M, Long EO (1990) An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II-restricted T cells. *J Exp Med* 172(3):947–954
5. Moss B (1991) Vaccinia virus: a tool for research and vaccine development. *Science* 252:1662–1667
6. Weir JP, Moss B (1984) Regulation of expression and nucleotide sequence of a late vaccinia virus gene. *J Virol* 51(3):662–669
7. Davison AJ, Moss B (1989) Structure of vaccinia virus late promoters. *J Mol Biol* 210(4): 771–784
8. Davison AJ, Moss B (1989) Structure of vaccinia virus early promoters. *J Mol Biol* 210(4): 749–769
9. Moss B (1990) Regulation of vaccinia virus transcription. *Annu Rev Biochem* 59: 661–688
10. Carroll MW, Moss B (1997) Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a non-human mammalian cell line. *Virology* 238(2): 198–211
11. De Clercq E (2002) Cidofovir in the treatment of poxvirus infections. *Antiviral Res* 55(1):1–13
12. Taddie JA, Traktman P (1993) Genetic characterization of the vaccinia virus DNA polymerase: cytosine arabinoside resistance requires a variable lesion conferring phosphonoacetate resistance in conjunction with an invariant mutation localized to the 3'-5' exonuclease domain. *J Virol* 67(7):4323–4336
13. Baxby D (1981) Jenner's smallpox vaccine. The Riddle of the Origin of Vaccinia Virus, Heinemann, London
14. Esteban DJ, Buller RM (2005) Ectromelia virus: the causative agent of mousepox. *J Gen Virol* 86(Pt 10):2645–2659
15. Fenner F (1949) Mouse-pox; infectious ectromelia of mice; a review. *J Immunol* 63(4):341–373
16. Jackson RJ, Ramsay AJ, Christensen CD, Beaton S, Hall DF, Ramshaw IA (2001) Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J Virol* 75(3): 1205–1210
17. Centers for Disease Control and Prevention (CDC) (2008) Laboratory-acquired vaccinia exposures and infections—United States, 2005–2007. *MMWR Morb Mortal Wkly Rep* 57(15):401–404
18. Buller RM, Smith GL, Cremer K, Notkins AL, Moss B (1985) Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* 317(6040):813–815
19. Blasco R, Moss B (1992) Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J Virol* 66(7):4170–4179
20. Husain M, Moss B (2001) Vaccinia virus F13L protein with a conserved phospholipase catalytic motif induces colocalization of the B5R envelope glycoprotein in post-Golgi vesicles. *J Virol* 75(16):7528–7542
21. Blasco R, Moss B (1995) Selection of recombinant vaccinia viruses on the basis of plaque formation. *Gene* 158(2):157–162
22. Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, Zavala F (1995) Quantification of antigen specific CD8+ T cells using an ELISPOT assay. *J Immunol Methods* 181(1):45–54
23. Murali-Krishna K, Altman JD, Suresh M, Sourdice DJ, Zajac AJ, Miller JD, Slansky J, Ahmed R (1998) Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8(2):177–187
24. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274(5284):94–96
25. Motta I, Lone YC, Kourilsky P (1998) In vitro induction of naive cytotoxic T lymphocytes with complexes of peptide and recombinant MHC class I molecules coated onto beads:

- role of TCR/ligand density. *Eur J Immunol* 28(11):3685–3695
26. Wherry EJ, Puorro KA, Porgador A, Eisenlohr LC (1999) The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J Immunol* 163(7):3735–3745
 27. Williams NS, Engelhard VH (1996) Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. *J Immunol* 156(1):153–159
 28. Sanderson S, Shastri N (1994) LacZ inducible, antigen/MHC-specific T cell hybrids. *Int Immunol* 6(3):369–376
 29. Yewdell JW, Bennink JR (1989) Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science* 244(4908):1072–1075
 30. Wherry EJ, McElhaugh MJ, Eisenlohr LC (2002) Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J Immunol* 168(9):4455–4461
 31. Gallimore A, Glicher A, Godkin A, Tissot AC, Pluckthun A, Elliott T, Hengartner H, Zinkernagel R (1998) Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* 187(9):1383–1393
 32. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdice DJ, Suresh M, Altman JD, Ahmed R (1998) Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188(12):2205–2213
 33. Picker LJ, Singh MK, Zdravetski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC (1995) Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86(4):1408–1419
 34. Jin Y, Shih WK, Berkower I (1988) Human T cell response to the surface antigen of hepatitis B virus (HBsAg). Endosomal and nonendosomal processing pathways are accessible to both endogenous and exogenous antigen. *J Exp Med* 168(1):293–306
 35. Tsung K, Yim JH, Marti W, Buller RM, Norton JA (1996) Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. *J Virol* 70(1):165–171
 36. Earl PL, Moss B (1993) Purification of vaccinia virus. Greene and Wiley-Interscience, New York
 37. Sanderson S, Frauwirth K, Shastri N (1995) Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. *Proc Natl Acad Sci U S A* 92(16):7217–7221

Chapter 19

Bioinformatics Identification of Antigenic Peptide: Predicting the Specificity of Major MHC Class I and II Pathway Players

**Ole Lund, Edita Karosiene, Claus Lundsgaard,
Mette Voldby Larsen, and Morten Nielsen**

Abstract

Bioinformatics methods for immunology have become increasingly used over the last decade and now form an integrated part of most epitope discovery projects. This wide usage has led to the confusion of defining which of the many methods to use for what problems. In this chapter, an overview is given focusing on the suite of tools developed at the Technical University of Denmark.

Key words: Immune, Epitope, MHC, HLA, Class I, Class II, Antigen processing, Proteasome, TAP, Visualization, Bioinformatics, Prediction, Web server.

1. Introduction

Experimental methods for analyzing antigenic peptide generation, transport, and binding to Major Histocompatibility Complex (MHC) class I molecules are expensive and time consuming. While bioinformatics methods can never replace experiments in the laboratory, they may in a highly cost-effective manner guide the experimental efforts in a direction that increases the likelihood of discovering immunologically important responses. At the Technical University of Denmark, we have over the last decade developed a number of methods for predicting which part of an antigen most likely is presented to the immune system. A complicating factor is that the MHC molecules associated with response to foreign antigens are encoded at several loci. Furthermore, these genes are the most polymorphic in the human genome and thousands of different alleles are known. Many of these alleles encode different variants of MHC molecules having different peptide binding specificities.

However, it is possible to cluster alleles with similar specificities into functional groups called supertypes, first described by Sette and Sidney (1). The pioneering methods for predicting binding to MHC class I molecules such as BIMAS (2) and SYPEITHI (3) helped initiate the field of immunological bioinformatics, but these methods have since been surpassed by newer methods like the ones described in this chapter, and we propose that experimental efforts may be minimized by basing the experiments on these newer methods.

2. Binding of Peptides to MHC

In recent years numerous methods for predicting binding to MHC molecules have been proposed. These methods can broadly be divided into two classes: one being the allele-specific and one being the pan-specific methods. Allele-specific methods are constructed for a given allele, and can interpolate between different ligands and give predictions for peptides for which no binding data are available. An obvious limitation by these methods is that predictions can only be made for alleles for which a number of binding data is already available. This requirement has been circumvented by the so-called pan-specific methods, which can also interpolate between different MHC alleles and thus make predictions for alleles for which no known binders are available. This strongly increases the number of alleles for which predictions can be obtained, from the few hundreds for which binding data is available to the more than 3,000 for which the protein sequence is known.

The accuracy of methods for MHC peptide binding prediction depends critically on the available data characterizing the binding specificity of the MHC molecules. This makes it very difficult for the nonexpert user to choose the most suitable method for predicting binding to a given MHC molecule. To complicate things even further, it has been demonstrated that consensus methods defined as combinations of two or more different methods led to improved prediction accuracy.

3. Prediction of MHC Class I Peptide Binding

To benefit from the consensus approach and to guide the nonexpert user on selecting the most appropriate binding prediction method for a given MHC class I molecule, we have recently developed the *NetMHCcons* method. The method is available at <http://www.cbs.dtu.dk/services/NetMHCcons>.

The method integrates predictions from three well-established prediction methods (*NetMHC* (4, 5), *NetMHCpan* (6, 7), and

Fig. 1. Submission site of *NetMHCcons* server. Two submission types are handled—a list of peptides or protein sequence(s). The server provides a possibility for the user to choose MHC molecules in question from a list of alleles or alternatively upload a full-length MHC protein sequence of interest. The user has a choice of setting the threshold for defining strong and weak binders based on predicted affinity (IC50) or %Rank. The output can be sorted based on predicted binding affinity as well as filtered on the user-specified thresholds.

PickPocket (8)) and allows the user in an automatic manner to obtain the most accurate predictions for any given MHC class I molecule of known protein sequence. The three methods included in *NetMHCcons* are state of the art and have performed well in recent benchmarks (9–14). For MHC class I alleles with well-characterized binding specificity, the method is defined as a combination of the *NetMHC* and *NetMHCpan* methods, and for alleles with unknown binding specificity, the method is defined in terms of the *NetMHCpan* method combined with *PickPocket*. For details on the method and its benchmark performance refer to (15).

The submission site of the server can be seen in Fig. 1.

1. Select method. By default, the consensus method (*NetMHCcons*) is selected but each of the three individual prediction methods can be run separately.
2. Select Allele(s). To aid in navigation, the alleles listed by default are limited to the human supertype representatives, but all alleles from different human/animal loci can be selected under “Select species/loci” (the list of selectable alleles is limited to alleles with well-characterized binding specificity when using the *NetMHC* method). In the MHC allele selection field, multiple alleles can be selected but the selection is limited to 20 alleles per submission. Multiple alleles can also be inputted as a comma-separated list. For the pan-specific methods (*NetMHCcons*, *NetMHCpan*, and *PickPocket*) the user can upload a file containing the protein sequence of an MHC class I molecule that is not among the available, selectable alleles, and the method will perform peptide binding predictions for this molecule.
3. Provide input sequence. The input can either be in peptide raw text or protein FASTA format. In peptide format, each line is assumed to be a separate peptide. All peptides must be of equal length. In FASTA format, the sequence of each protein must be preceded by a line beginning with a “>.” When FASTA input is used, multiple different epitope lengths from 8 to 11 residues can be selected.
4. Select output formatting. By default the output is sorted by the residue number, but the user can choose to sort the output by the predicted binding affinity. Predictions for all the input peptides given are by default but by setting “Filter output” to “Yes,” only the peptides predicted to bind stronger than the defined thresholds are given in the output. The output can optionally be saved to a file readable by spreadsheet applications for further processing by the user.
5. Press submit.
6. Wait for the server to produce output. The output from the server consists of a list of peptides, each associated with three prediction values: 1-log50k(aff), Affinity, and %Rank. The 1-log50k value is the raw score provided by the prediction method, and is related to the predicted binding affinity value as $1-\log(\text{Aff})/\log(50,000)$. The %Rank score gives % rank of the prediction score to a set of 200,000 random natural 9mer peptides. Thresholds can be selected for which peptides to report as strong binders (SB) and weak binders (WB). The peptides are labeled as a strong binder if the %Rank score or the binding affinity is below the specified thresholds for the strong binders. Likewise, peptides are labeled as weak binders if the %Rank or the binding affinity is above the thresholds of strong binders, but below the specified threshold for the weak binders.

References to other well-performing methods for prediction of MHC class I binding can be found in one of the several reviews that have been written on the subject including a recent one from our group (14).

3.1. Prediction of MHC

Class II Peptide Binding

For class I, alignment-free methods like the ones described earlier can readily be applied, since the binding motif is well characterized and most natural peptides that bind MHC class I are of the same length. For MHC class II, the situation is quite different due to the great variability in the length of natural MHC-binding peptides. This variation in ligand length makes alignment a crucial and integrated part of estimating the MHC-binding motif and predicting peptide binding. During the last decade, large efforts have been invested in developing data-driven prediction methods for MHC class II peptide binding. For an overview of these refer to one of the many reviews written on the theme including the one written by our group (16).

The binding of a peptide to a given MHC class II molecule is predominantly determined by the amino acids present in the peptide-binding core. However, peptide residues flanking the binding core (the so-called peptide flanking residues, PFR) do also to some degree affect the binding affinity of a peptide (17–19). Most published methods for MHC class II binding prediction focus on identifying the peptide-binding core only, ignoring the effects on the binding affinity of PFRs. In the work by (19) it was demonstrated that the additional information provided by the PFR leads to significantly improved predictions.

Two high-performing methods for MHC class II binding prediction developed by our group are *NetMHCII* (19) and *NetMHCIIpan* (20, 21). The *NetMHCII* method is allele-specific and allows for peptide–MHC binding predictions to a set of 14 HLA-DR, six HLA-DQ, six HLA-DP, and two mouse H2 class II alleles. *NetMHCIIpan* is HLA-DR pan specific, allowing for prediction of peptide binding to all HLA-DR molecules with a known protein sequence. Several benchmark studies have demonstrated these methods to be high performing and state of the art (22–25).

1. Select input sequences. Both methods accept input either as individual peptides in raw text format or as protein sequence(s) uploaded in FASTA format (see earlier). If protein sequences are uploaded, the user can specify the peptide length and predictions are made for each overlapping peptide of the specified length. Multiple MHC alleles can be specified.
2. Customize search. The input to (and output from) the *NetMHCIIpan* method is very similar to that of *NetMHCII*. Only does the *NetMHCIIpan* method (as was the case for MHC class I methods described earlier) allow the user to upload a file containing the protein sequence of an HLA-DR

molecule that is not among the available, selectable alleles, and the method will perform binding predictions for this molecule. Likewise the user can define the prediction score threshold values used to classify prediction as strong and weak binders. Also can the output from the *NetMHCIIpan* server be saved to a file readable by most spreadsheet applications for further processing by the user.

3. Select output formatting. By default the output is sorted by the residue number but the output can also be sorted by affinity. Predictions for all peptides are by default given but by setting a “Threshold,” only the peptides predicted to bind stronger than the defined threshold (in 1-log50k units) are given in the output.
4. Press Submit.
5. Wait for output. As for the MHC class I prediction server described earlier, the output from the MHC class II prediction servers consists of a list of peptides, each associated with the predicted binding core and three prediction values: 1-log50k(aff), Affinity, and %Rank. The 1-log50k value is the raw score provided by the prediction method, and is related to the predicted binding affinity value as $1 - \log(\text{Aff}) / \log(50,000)$. The %Rank score gives % rank of the prediction score to a set of 200,000 random natural peptides. Peptides are labeled as a strong binder if the binding affinity is below 50 nM. Likewise, peptides are labeled as a weak binder if the binding affinity is below 500 nM.

4. MHCMotif-Viewer: Browsing and Visualization of MHC Class I and Class II Binding Motifs

The number and binding specificity diversity of MHC molecules can be overwhelming for most users. To help get an overview, we have developed the *MHCMotifViewer* server (<http://www.cbs.dtu.dk/biotools/MHCMotifViewer/>). The homepage is shown in Fig. 2.

1. Select species/loci. By clicking on “Human alleles,” different loci can be selected. For other species the user is taken directly to a list of alleles.
2. Select allele. Clicking on one of the thumbnail pictures will create a larger logo for that allele. This is shown for HLA-A*01:03 in the right panel of Fig. 2. On the x-axis the nine positions in the binding motif are given. The height of the columns of letters at each position corresponds to the predicted contribution to binding on that position calculated according to the formula developed by Kullback–Leibler (26). The amino acids for which their frequency differs the most

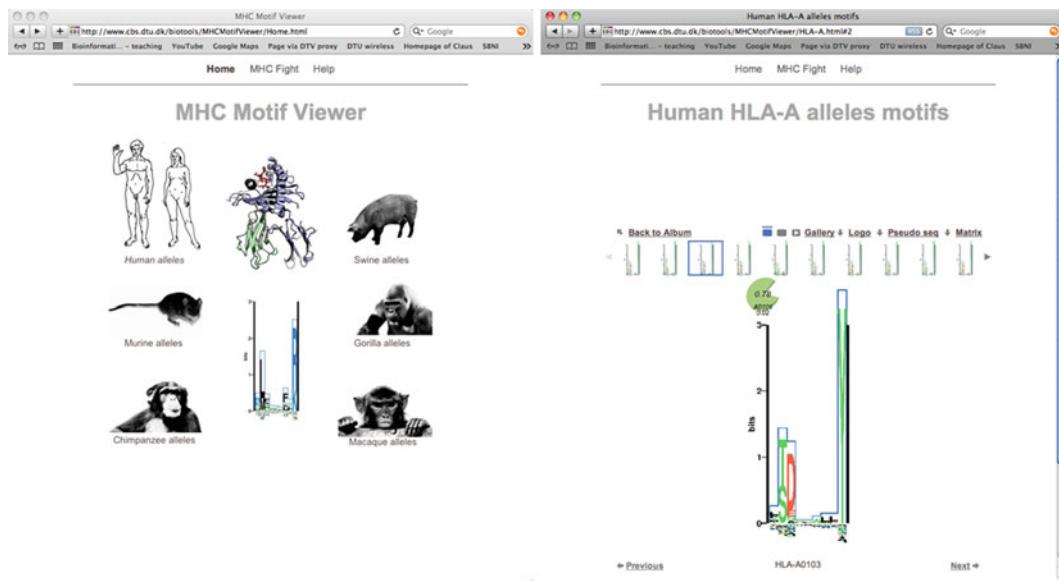


Fig. 2. The *MHCMotifviewer* server. Left panel shows the homepage of the *MHC Motif Viewer* server where the organism can be selected. Human, murine, chimpanzee, swine, gorilla, and macaque alleles can be browsed. In the right panel an allele from the Human HLA-A loci (HLA-A*01:03) is selected and its motif is displayed as the sequence logo representation.

from the background frequency for that amino acid in proteins in general are shown with the highest letters. The overrepresented amino acids are shown above the x-axis, and the underrepresented ones below.

The binding motif of up to four different alleles can be shown side by side by clicking on “MHC Fight.” By default, all four alleles are the same, but by clicking on the blinking cursor, the allele name can be changed by deleting (part of) the name using the backspace key and typing the new name. By holding the cursor over the “K” button, the display will shift between showing a Kullback–Leibler (K), and a Sequence frequency (S)-based logo. In a sequence frequency-based logo the relative height of each letter within a column is proportional to the frequency of the corresponding amino acid at that position. A more detailed explanation can be found in (27).

5. HLArrestrctor: Patient-Specific HLA Restriction Elements and Optimal Epitopes Within Peptides

Considering the many different peptides that can be generated, even from a small target protein, and the extensive polymorphism of the presenting MHC molecules, identifying pathogen-specific, HLA-restricted T cell epitopes can be an immense experimental

task. To reduce this complexity, one could conveniently exploit a commonly used approach of T cell epitope discovery: testing overlapping peptides (OLP) with a length of 15–18 amino acids in IFN γ release, ELISpot, or flow cytometric intracellular staining assays. Given a positive peptide it is, however, not a simple task to find the actual stimulatory peptide (minimal epitope) and the presenting HLA restriction element. By way of example, a 15mer peptide tested positive in a patient with six different HLA class I molecules could potentially be explained by any one of the possible $22 * 6 = 132$ 8–11mer HLA combinations. To lower this experimental burden, we have developed an immunoinformatics method, *HLArestrctor* (www.cbs.dtu.dk/services/HLArestrctor) (28), which has been tailored to support CTL epitope discovery in individual subjects. As inputs, the method requires the amino acid sequence of the positive peptide(s) and the HLA type of the individual in question (high-resolution HLA typing, e.g., HLA-A*01:01, and preferably for all relevant loci, e.g., for HLA-A, -B, -C for HLA class I-restricted CTL responses). Using these inputs, *HLArestrctor* creates all possible 8, 9, 10, and 11mer peptides from the target peptide(s), predicts their binding to all the HLA molecules in question, and generates an output file consisting of the most likely peptide/HLA combination(s). Peptide/HLA tetramers is one of the most efficient means to validate T cell epitopes, and *HLArestrctor* can also be viewed as a tool for efficient design of specific peptide/HLA tetramers. The vehicle behind the *HLArestrctor* is the *NetMHCpan* method, and the Webpage interface bears a high resemblance to the interfaces for *NetMHCpan*, *NetMHCIIpan*, and *NetMHCcons*.

1. Select input sequences. Multiple peptide sequences can be uploaded in FASTA format.
2. Select HLA alleles. The host HLA allele names can be selected or typed in.
3. Select lengths of epitopes. The lengths of the predicted minimal epitopes can be specified.
4. Select prediction threshold. Threshold values defining how the prediction scores are interpreted can be specified in terms of threshold values for strong and weak binding peptides.

With default settings, the server will scan all possible 8, 9, 10, and 11mer peptides from the target peptide(s) for binding to all HLA alleles of the host and report peptides with %Rank score less than or equal to 0.5 or affinity stronger than 50 nM as strong binders, and peptides with %Rank score less than or equal to 2 or affinity stronger than 500 nM as weak binders.

6. Interpreting the Output from the Prediction Servers

All the prediction servers described here provide three prediction scores for each peptide, as well as a label classifying the peptides into groups of strong and weak binders. For the end user, these prediction values are meant to serve as a guide to make rational peptide selections for epitope discovery and/or interpretation of immune responses. This opens for questions on how to define relevant thresholds relating prediction values to likelihoods of a peptide being a T cell epitope. It is becoming apparent that not all MHC molecules present peptides at the same binding threshold (29, 30). The two distinct prediction values (affinity and %Rank) are included to capture these intrinsic differences between MHC molecules in terms of binding threshold for presentation of peptides. Large benchmark studies have demonstrated that the vast majority of known CTL epitopes are characterized by having a %Rank score less than or equal to 2 or an affinity stronger than 500 nM (28, 31, 32). These numbers are hence used as default values for the definition of weak binding peptides for all MHC class I prediction methods. For MHC class II the situation is less clear. While it is clear that the prediction values correlate strongly with the measured binding affinity, few studies have investigated the direct correlation between %Rank score, predicted affinity values, and the likelihood of a peptide being immunogenic. The default values for the classification of peptides as weak and strong binders are hence poorly justified for MHC class II, and the relationship to the likelihood of being immunogenic is at the best poorly investigated. However, for both MHC class I and class II it is clear that using the prediction score to rank peptides provides a highly cost-effective tool to guide the experimental efforts in a direction that increases the likelihood of discovering immunologically important responses.

7. The MHC Class I Antigen Presentation Pathway

As part of the protein recycling machinery, proteins in our cells are cut into shorter peptides by the proteasome. These peptides may bind to the Transporter associated with Antigen Processing (TAP) and be transferred to the Endoplasmic Reticulum (ER). Inside the ER, peptides may be further trimmed, bind the MHC class I molecules, and be transported along with it to the cell surface. If the peptide is of nonself origin, the peptide–MHC complex may bind to a T Cell Receptor (TCR) on a cytotoxic T cell, which will then initiate an immune response. More detailed descriptions of and

references to these processes can be found in other chapters of this book. The three most essential of the above steps (cleavage by the proteasome, transport by TAP, and binding to MHC class I) have been modeled by bioinformatics methods that can predict which peptides from a given protein/organism are most likely to be presented to the immune system.

8. NetChop: Proteasomal Cleavages (MHC Class I Ligands)

A method has been developed, which predicts proteasomal cleavage sites. The method is called *NetChop* (33), and a server is available at <http://www.cbs.dtu.dk/services/NetChop/>.

1. Select prediction method. Two different versions of the method exist: “C term 3.0” and “20S 3.0.” They differ by the sets of data they have been trained on. While *NetChop 20S 3.0* has been trained on *in vitro* constitutive proteasome protein digests, *NetChop C term 3.0* has been trained on natural MHC class I ligands. The rationale for the latter is that the proteasome most likely has generated the ligand’s C-terminal ends. *NetChop C term 3.0* predicts the C-terminal end of CTL epitopes with a higher specificity than *NetChop 20S 3.0* (has fewer false positives). The main reason for this is that since it is trained on natural ligands, it predicts a combination of MHC class I binding, TAP transport efficiency, and proteasomal cleavage.
2. Select input sequence. The input to the server is proteins or peptide fragments in FASTA format (see earlier). The method assigns a score in the range 0–1 to each residue in the input sequence. The higher the score, the more likely it is that the proteasome cleaves after this residue. Note that the score refers to cleavage of the peptide bond on the C-terminal side of the residue to which the score is assigned.
3. Select prediction threshold.

By default, 0.5 is used as the threshold for predicted proteasomal cleavage. In the output, scores above the threshold are assigned an “S” in the C (cleavage) column, while lower scores are assigned a “.”.

9. NetCTL and NetCTLpan: Integrated Class I Antigen Presentation

Two methods that integrate predictions of proteasomal C-terminal cleavage, TAP transport efficiency, and MHC class I binding for the overall prediction of MHC class I presentation called *NetCTL*

and *NetCTLpan* have been developed by our group. The *NetCTL* method (34) is available at <http://www.cbs.dtu.dk/services/NetCTL/>. For prediction of proteasomal cleavage, it uses *NetChop C term 3.0* (see above). Predictions of TAP transport efficiency are based on the weight matrix-based method described by Peters et al. (35). For predictions of MHC class I binding, *NetMHC* (see above) is used.

1. Select input sequence. The input to the server is proteins or peptide fragments in FASTA format (see earlier).
2. Select Allele/supertype. The user must specify for which of the 12 MHC class I supertypes the predictions should be performed (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, or B62; for a definition of supertypes see (1)). *NetCTL* integrates the individual scores from *NetChop*, the TAP matrix, and *NetMHC* into one, overall score. To allow for comparison between different MHC class I supertypes, the rescaled affinity is used (see (34) for details on how the rescaled affinity is calculated).
3. Select weighting of processing steps. As default, the relative weight of C-terminal cleavage is 0.15, while it is 0.05 for TAP transport efficiency. The default weights have been found to result in optimal performance, but can be changed by the user.
4. Select prediction threshold. The user can also specify which threshold to use for defining a CTL epitope. By default it is 0.75.
5. Select sorting of output. Lastly, the user can specify how the 9mers of the input sequence should be sorted in the output. In the default “no sort” option, the 9mers are listed according to the order in which they appear in the input sequence. Alternatively, they can be sorted according to the combined score, MHC binding, proteasomal cleavage, or TAP. For each 9mer sub-peptide in the input sequence, the output will list the predicted affinity and the prediction scores of proteasomal cleavage, TAP binding, and finally a combined score. If the combined score is above the selected threshold for defining an epitope, it is marked by an “E.”

NetCTLpan is an extended and improved version of *NetCTL*, which is available at <http://www.cbs.dtu.dk/services/NetCTLpan/> and described in detail in (30). The C-terminal proteasomal cleavage and TAP transport efficiency are predicted as for the *NetCTL* method, while MHC class I binding is based on the *NetMHCpan* method. While *NetCTL* only allows for predictions of peptides restricted by one of the 12 MHC class I supertypes, *NetCTLpan* allows for predictions of CTL epitopes binding any MHC class I molecule for which the protein sequence is known. As for the above-described pan prediction methods, it is additionally possible

to paste in or upload a file containing the protein sequence of an MHC class I molecule that is not among the available, selectable alleles, and the method will perform CTL epitope predictions for this molecule. *NetCTLpan* furthermore performs predictions for 8–11mers. The Webpage interface of *NetCTLpan* bears a high resemblance to the interfaces of *NetCTL*. One difference is that it is possible to select a threshold that the combined score must exceed for the predictions to be displayed in the output page. By default, this threshold is -99.9, which results in all predictions being displayed. In the output page, the same values are listed as in the *NetCTL* output. Additionally, the %Rank value is given (see above for definition of the %Rank value).

References

- Sette A, Sidney J (1999) Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 50(3–4):201–212
- Parker KC, Bednarek MA, Coligan JE (1994) Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152(1):163–175
- Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S (1999) SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50(3–4):213–219
- Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M (2008) NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. *Nucleic Acids Res* 36(Web Server issue):W509–W512. doi:[gkn202](#) (pii) [10.1093/nar/gkn202](#)
- Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O (2003) Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. *Protein Sci* 12(5):1007–1017. doi:[10.1110/ps.0239403](#)
- Hoof I, Peters B, Sidney J, Pedersen LE, Sette A, Lund O, Buus S, Nielsen M (2009) NetMHCpan, a method for MHC class I binding prediction beyond humans. *Immunogenetics* 61(1):1–13. doi:[10.1007/s00251-008-0341-z](#)
- Nielsen M, Lundegaard C, Blicher T, Lamberth K, Harndahl M, Justesen S, Roder G, Peters B, Sette A, Lund O, Buus S (2007) NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. *PLoS One* 2(8):e796. doi:[10.1371/journal.pone.0000796](#)
- Zhang H, Lund O, Nielsen M (2009) The PickPocket method for predicting binding specificities for receptors based on receptor pocket similarities: application to MHC-peptide binding. *Bioinformatics* 25(10):1293–1299. doi:[10.1093/bioinformatics/btp137](#)
- Peters B, Bui HH, Frankild S, Nielson M, Lundegaard C, Kostem E, Basch D, Lamberth K, Harndahl M, Flerl W, Wilson SS, Sidney J, Lund O, Buus S, Sette A (2006) A community resource benchmarking predictions of peptide binding to MHC-I molecules. *PLoS Comput Biol* 2(6):e65. doi:[06-PLCB-RA-0058R2](#) (pii) [10.1371/journal.pcbi.0020065](#)
- Lin HH, Ray S, Tongchusak S, Reinherz EL, Brusic V (2008) Evaluation of MHC class I peptide binding prediction servers: applications for vaccine research. *BMC Immunol* 9:8. doi:[1471-2172-9-8](#) (pii) [10.1186/1471-2172-9-8](#)
- Zhang GL, Ansari HR, Bradley P, Cawley GC, Hertz T, Hu X, Jojic N, Kim Y, Kohlbacher O, Lund O, Lundegaard C, Magaret CA, Nielsen M, Papadopoulos H, Raghava GP, Tal VS, Xue LC, Yanover C, Zhu S, Rock MT, Crowe JE Jr, Panayiotou C, Polycarpou MM, Duch W, Brusic V (2011) Machine learning competition in immunology—Prediction of HLA class I binding peptides. *J Immunol Methods*. doi:[S0022-1759\(11\)00255-9](#) (pii) [10.1016/j.jim.2011.09.010](#)
- Zhang H, Lundegaard C, Nielsen M (2009) Pan-specific MHC class I predictors: a benchmark of HLA class I pan-specific prediction methods. *Bioinformatics* 25(1):83–89. doi:[10.1093/bioinformatics/btn579](#)

13. Zhang L, Udaka K, Mamitsuka H, Zhu S (2011) Toward more accurate pan-specific MHC-peptide binding prediction: a review of current methods and tools. *Brief Bioinform.* doi:[bbr060](https://doi.org/10.1093/bib/bbr060) (pii) [10.1093/bib/bbr060](https://doi.org/10.1093/bib/bbr060)
14. Lundsgaard C, Hoof I, Lund O, Nielsen M, Lundsgaard C, Hoof I, Lund O, Nielsen M (2010) State of the art and challenges in sequence based T-cell epitope prediction. *Immunome Res* 6(Suppl 2):S3. doi:[1745-7580-6-S2-S3](https://doi.org/10.1186/1745-7580-6-S2-S3) (pii) [10.1186/1745-7580-6-S2-S3](https://doi.org/10.1186/1745-7580-6-S2-S3)
15. Karosiene E, Lundsgaard C, Lund O, Nielsen M (2011) NetMHCcons: a consensus method for the major histocompatibility complex class I predictions. *Immunogenetics.* doi:[10.1007/s00251-011-0579-8](https://doi.org/10.1007/s00251-011-0579-8)
16. Nielsen M, Lund O, Buus S, Lundsgaard C (2010) MHC class II epitope predictive algorithms. *Immunology* 130(3):319–328. doi:[10.1111/j.1365-2567.2010.03268.x](https://doi.org/10.1111/j.1365-2567.2010.03268.x)
17. Godkin AJ, Smith KJ, Willis A, Tejada-Simon MV, Zhang J, Elliott T, Hill AV (2001) Naturally processed HLA class II peptides reveal highly conserved immunogenic flanking region sequence preferences that reflect antigen processing rather than peptide-MHC interactions. *J Immunol* 166(11): 6720–6727
18. Lovitch SB, Pu Z, Unanue ER (2006) Amino-terminal flanking residues determine the conformation of a peptide-class II MHC complex. *J Immunol* 176(5):2958–2968. doi:[10.1007/s00251-005-2958-0](https://doi.org/10.1007/s00251-005-2958-0) (pii)
19. Nielsen M, Lund O (2009) NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics* 10:296. doi:[10.1186/1471-2105-10-296](https://doi.org/10.1186/1471-2105-10-296)
20. Nielsen M, Lundsgaard C, Blicher T, Peters B, Sette A, Justesen S, Buus S, Lund O (2008) Quantitative predictions of peptide binding to any HLA-DR molecule of known sequence: NetMHCIIPan. *PLoS Comput Biol* 4(7):e1000107. doi:[10.1371/journal.pcbi.1000107](https://doi.org/10.1371/journal.pcbi.1000107)
21. Nielsen M, Justesen S, Lund O, Lundsgaard C, Buus S (2010) NetMHCIIPan-2.0—Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. *Immunome Res* 6:9. doi:[1745-7580-6-9](https://doi.org/10.1186/1745-7580-6-9) (pii) [10.1186/1745-7580-6-9](https://doi.org/10.1186/1745-7580-6-9)
22. Lin HH, Zhang GL, Tongchusak S, Reinherz EL, Brusic V (2008) Evaluation of MHC-II peptide binding prediction servers: applications for vaccine research. *BMC Bioinformatics* 9(Suppl 12):S22. doi:[10.1186/1471-2105-9-S12-S22](https://doi.org/10.1186/1471-2105-9-S12-S22)
23. Wang P, Sidney J, Dow C, Mothe B, Sette A, Peters B (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput Biol* 4(4):e1000048. doi:[10.1371/journal.pcbi.1000048](https://doi.org/10.1371/journal.pcbi.1000048)
24. Bordner AJ, Mittelmann HD (2010) MultiRTA: a simple yet reliable method for predicting peptide binding affinities for multiple class II MHC allotypes. *BMC Bioinformatics* 11:482. doi:[10.1186/1471-2105-11-482](https://doi.org/10.1186/1471-2105-11-482)
25. Zhang GL, Deluca DS, Keskin DB, Chitkushev L, Zlateva T, Lund O, Reinherz EL, Brusic V (2010) MULTIPRED2: a computational system for large-scale identification of peptides predicted to bind to HLA supertypes and alleles. *J Immunol Methods.* doi:[S0022-1759\(10\)00345-5](https://doi.org/10.1016/j.jim.2010.11.009) (pii) [10.1016/j.jim.2010.11.009](https://doi.org/10.1016/j.jim.2010.11.009)
26. Kullback S, Leibler RA (1951) On Information and Sufficiency. *Ann Math Statist* 22: 76–86
27. Rapin N, Hoof I, Lund O, Nielsen M. The MHC motif viewer: a visualization tool for MHC binding motifs. *Curr Protoc Immunol.* 2010; Chapter 18: Unit 18 17. doi:[10.1002/0471142735.im1817s88](https://doi.org/10.1002/0471142735.im1817s88).
28. Erup Larsen M, Kloverpris H, Stryhn A, Koohfethile CK, Sims S, Ndung'u T, Goulder P, Buus S, Nielsen M (2011) HLArestrictor—a tool for patient-specific predictions of HLA restriction elements and optimal epitopes within peptides. *Immunogenetics* 63(1):43–55. doi:[10.1007/s00251-010-0493-5](https://doi.org/10.1007/s00251-010-0493-5)
29. Rao X, Costa AI, van Baarle D, Kesmir C (2009) A comparative study of HLA binding affinity and ligand diversity: implications for generating immunodominant CD8+ T cell responses. *J Immunol* 182(3):1526–1532. doi:[10.1007/s00251-008-1526-3](https://doi.org/10.1007/s00251-008-1526-3) (pii)
30. Stranzl T, Larsen MV, Lundsgaard C, Nielsen M (2010) NetCTLpan: pan-specific MHC class I pathway epitope predictions. *Immunogenetics* 62(6):357–368. doi:[10.1007/s00251-010-0441-4](https://doi.org/10.1007/s00251-010-0441-4)
31. Hoof I, Perez CL, Buggert M, Gustafsson RK, Nielsen M, Lund O, Karlsson AC (2010) Interdisciplinary analysis of HIV-specific CD8+ T cell responses against variant epitopes reveals restricted TCR promiscuity. *J Immunol* 184(9):5383–5391. doi:[jimmunol.0903516](https://doi.org/10.4049/jimmunol.0903516) (pii) [10.4049/jimmunol.0903516](https://doi.org/10.4049/jimmunol.0903516)
32. Larsen MV, Lelic A, Parsons R, Nielsen M, Hoof I, Lamberth K, Loeb MB, Buus S, Bramson J, Lund O (2010) Identification of CD8+ T cell epitopes in the West Nile virus polyprotein by reverse-immunology using

- NetCTL. PLoS One 5(9):e12697. doi:[10.1371/journal.pone.0012697](https://doi.org/10.1371/journal.pone.0012697)
33. Nielsen M, Lundsgaard C, Lund O, Kesmir C (2005) The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 57(1–2):33–41. doi:[10.1007/s00251-005-0781-7](https://doi.org/10.1007/s00251-005-0781-7)
34. Larsen MV, Lundsgaard C, Lamberth K, Buus S, Brunak S, Lund O, Nielsen M (2005) An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. Eur J Immunol 35(8):2295–2303. doi:[10.1002/eji.200425811](https://doi.org/10.1002/eji.200425811)
35. Peters B, Bulik S, Tampe R, Van Endert PM, Holzhutter HG (2003) Identifying MHC class I epitopes by predicting the TAP transport efficiency of epitope precursors. J Immunol 171(4):1741–1749

Chapter 20

Evaluating CD8⁺ T Cell Responses In Vitro

François A. Lemonnier

Abstract

The ⁵¹Cr-release assay described in the 1960s has been for decades the gold standard cytolytic assay and remains in use in many laboratories. Whereas other radioactive tests were later on described, they never fully replaced the ⁵¹Cr-release assay. More thorough understanding of CTL biology and killing pathways has more recently resulted in the design of reliable nonradioactive tests to analyze CD8⁺ T cell responses which are likely to supplant in a close future the ⁵¹Cr-release assay.

Key words: Elispot, Interferon γ , CTL, Cytolytic assay

1. Introduction

(1, 2)

Naïve lymphocytes are functionally inactive. Following engagement, at the surface of a dendritic cell, of their clonotypic T Cell Receptors for antigen (TCR) with a specific nonself peptide/MHC class I molecule complexes (p/MHC), they start to divide and differentiate in effector cytolytic T lymphocytes (CTL).

Effector cytolytic CD8⁺ T lymphocytes release Interferon γ , produce and accumulate Perforin, Granzyme A and B in secretory vesicles of early-endosomal origin, and express at cell surface Fas-Ligand. Once differentiated, CTL can kill any type of cells as long as they express the same nonself p/MHC class I complexes as those seen on dendritic cells during the activation phase. CTLs are powerful sensors and, for some CTL, it has been shown that less than 10 complexes per target cell suffice for lysis.

Target cells following a CTL attack die by apoptosis with DNA fragmentation, plasma membrane blebbing with formation of apoptotic bodies, and leakage of cytosolic content. Once infection is cured, most CTL die, but a small fraction (CTL memory cells) survives. Cytolytic T cells can also get exhausted in case of intense

and sustained solicitation as observed in several chronic infectious diseases. Such exhausted CTLs still express their TCR and can be labelled with appropriate HLA class I multimers (see Chapter 10) but have lost the capacities to kill target cells and to produce Interferon γ .

The interaction of TCR with the p/MHC complex is of low affinity but is reenforced by the number of TCR simultaneously engaged, the interaction of the accessory CD8 molecules with the $\alpha 3$ domain of MHC Class I heavy chains, and a series of additional interactions LFA 1/ICAM1 and ICAM2, CD2/CD 58 ... that form all together the immunological synapse. Few minutes after CD8 $^{+}$ effector cells have contacted their target, the CTL secretory vesicles accumulate at the CTL-target interface and Perforin and Granzymes are released directionally in the enclosed intercellular space inducing exclusively target cell-apoptosis. Once the target is killed, the synapse, on the target side, breaks up liberating the CTL that can engage sequentially with other targets.

CTLs utilize 2 pathways for killing, the exocytosis Perforin–Granzyme pathway and the Fas ligand–Fas pathway. In most circumstances, the exocytosis pathway dominates. In both cases, the end point is target cell apoptosis through the activation of diverse aspartic acid-specific cysteine proteases named Caspases. The Fas pathway results in the activation of Caspase 8 that subsequently activates Caspase 3 that liberates CAD, a Caspase-activated DNase responsible for the fragmentation of the target cell DNA. Granzyme B, which is a serine protease, also activates Caspase 3 but additionally cleaves BID (for BH3-interacting domain death agonist protein) that permeabilizes the mitochondrial membrane, liberating additional apoptotic mediators in the target's cytosol.

2. Materials

Most of the material needed can be purchased from many companies.

2.1. Common Consumables and Lab Equipment

1. A fully equipped tissue culture room: Vertical laminar flow hoods, humidified 37°C CO₂ incubators, regular and inverted microscopes, refrigerated low-speed $\leq 10,000$ rpm centrifuge, autoclavable waste disposal containers.
2. Gamma or X-ray irradiator (alternatively Mitomycin C, see Note 1).
3. Basic sterile plastic-ware: 2, 5, 10, and 25 ml pipettes, 25, 75, and 150 cm² tissue culture flasks, 20, 200, 1,000 μ l disposable (preferentially cotton-plugged) tips, cell strainers, Petri dishes, sterile reservoirs, 96-well U-bottomed, flat-bottomed, and V-bottomed culture plates.

4. Multichannel 20–200 µl pipettes (8 or 12 channels).
5. Ficoll-Hypaque ($d=1.077$) to purify lymphocytes.

2.2. Mouse CD8⁺ T Cell Cultures

1. RPMI mouse complete medium: RPMI 1640 supplemented with 10% heat-inactivated (56°, 30 min) Fetal Calf Serum (FCS, preferentially tested for T cell growth support in a CTLL-2 proliferation assay, see Note 2), fresh (less than 1 month old) 2-mercaptoethanol (essential for the growth of mouse T cells in vitro, (3)), at a 5×10^{-5} M final concentration 100 U/ml Penicillin and 100 µg/ml Streptomycin.
2. TCGF (to be in-house prepared, see Note 3) or recombinant human IL-2 (several suppliers) to be both tested first in a CTLL-2 proliferation assay to determine the effective working concentration, usually 10% final concentration for TCGF and 50–100 IU or 10–15 BRMP U/ml for recombinant human IL-2 (see Note 3).

2.3. Human CD8⁺ T Cell Cultures

1. RPMI 1640 supplemented with 5–10% human AB serum (see Note 4), 100 U/ml Penicillin, and 100 µg/ml Streptomycin (RPMI human complete medium).
2. AIM-V Medium Research Grade (serum-free medium supporting in vitro human T cell growth).
3. Recombinant human IL-2 has to be used since the rat IL-2 does not activate efficiently human cells.
4. Phytohemagglutin (PHA) at 1 µg/ml that provides an antigen-independent potent stimulus for T cells through cross-linking of their TCR.
5. A set of HLA-typed EBV-transformed lymphoblastoid cell lines (see Note 5).
6. B95.8 cotton top marmoset cell line (ATCC.CRL 1612) for the production of infectious EBV particles and the transformation of human B lymphocytes in EBV-lymphoblastoid cell lines (see Subheading 3.2.4).

2.4. Radioactive Assays

1. Radioactive work must be done in approved and posted radioactive use areas and according to safety practice for the handling of radioactive compounds: protection of the working surface with a one-side absorbent paper, lead shielding to protect from the radioactive source, laboratory coat, gloves, dosimeter, Geiger counter for monitoring radiation exposure (see Note 6).
2. A cell harvester adapted to the handling of ³H-thymidine-labelled cells.
3. Luminescence counter for beta and gamma radiations and adapted 96-well plates (⁵¹Cr) and glass-fiber membranes (³H-thymidine).

4. $\text{Na}_2^{51}\text{CrO}_4$, 1 mCi/ml in isotonic sterile medium (half-life of 27.7 days), ^3H -thymidine, 1 mCi/ml (half-life of 12.32 years), ^{35}S -methionine, 1 mCi/ml (half-life 87.4 days) (see Note 6).
5. HCl or Triton X-100.

2.5. Nonradioactive Assays

1. ELISPOT reader.
2. ELISA reader.
3. Flow cytometer.
4. Many mouse and human Interferon γ ELISPOT kits are commercially available, but the various components can be independently purchased for much lower cost:
 - (a) PolyVinylDeneFluoride (PVDF) or nitrocellulose membrane-bottomed 96-well plates (similar performances).
 - (b) Anti-mouse Interferon γ and anti-human Interferon γ matched pairs of antibodies: Usually, pairs of mAb directed at 2 distinct epitopes of the Interferon γ molecule are used, one for capture and the other biotinylated for detection but in some cases biotinylated detection polyclonal antibodies are used (see Note 7).
 - (c) Streptavidin or Extravidin (a de-glycosylated form that exhibits less nonspecific binding) conjugated to alkaline phosphatase or horse radish peroxidase.
 - (d) NBT/BCIP substrate (Nitro-Blue Tetrazolium chloride 5-Bromo-4-Chloro-3'-Indolylphosphatep-Toluidine salt, commercially available) for detection with Alkaline Phosphatase-coupled to Streptavidin or Extravidin.
 - (e) AEC Substrate for Horse Radish Peroxidase-coupled Streptavidin/Extravidin, commercially available or to be prepared. Final substrate solution: Add 333.3 μl of AEC stock solution (100 mg AEC in 10 ml DMF (N,N-Dimethylformamide, see Note 8) to 10 ml 0.1 M acetate solution (148 ml of 0.2 M glacial acetic acid, 352 ml of 0.2 M sodium Acetate, adjust pH to 5.0 and volume to 1 L with water), filter through a 0.45 μm filter, add 5 μl of H_2O_2 (30%), and use immediately.
 - (f) Diamino Benzidine (DAB)/metal: Another substrate for horse radish peroxidase that is commercially available.
5. Reagents specific for the Lactodeshydrogenase (LDH)-release assay:
 - (a) Sodium L (+) Lactate solution at 36 mg/ml in 10 mM Tris buffer pH 8.5.
 - (b) Iodonitrotetrazolium chloride (tetrazolium dye INT) stock solution at 20 mg/ml in DMSO to be used diluted 1:10 in PBS 1 \times .

- (c) Nicotinamide Adenine Dinucleotide (NAD⁺)/diaphorase solution: Oxidized b-NAD⁺ phosphate oxidized, solution at 3 mg/ml and Diaphorase 13.5 U/ml, in PBS 1× supplemented with 0.03% Bovine Serum Albumin and 1.2% sucrose (to be kept aliquoted and frozen at -20°C).
- (d) Oxamic acid solution stock at 12 mg/ml in PBS 1×.

3. Methods

3.1. Interferon γ ELISPOTS

Principle: CD8⁺ T cells that have been in vivo primed (effector or memory CD8⁺ T cells) are peptide restimulated in vitro in the presence of antigen-presenting cells. Culture plates have been coated with a first anti-mouse or anti-human Interferon γ mAb that will capture the Interferon γ in the immediate vicinity of the stimulated CD8⁺ T cells. As a result, when a second (detection) anti-mouse or anti-human Interferon γ is used to reveal the captured molecules of Interferon, spots are observed on sites of Interferon γ-producing cells (4–6).

3.1.1. Mouse Interferon γ ELISPOT Procedure

1. Prepare PVDF or nitrocellulose membranes in the 96-well plates by addition of 50 µl/well of ethanol 70% for 30 s (see Note 9).
2. Wash (×3) with 100 µl sterile PBS 1×.
3. Coat 96-well ELISPOT plate with capture antibody (diluted 10 µg/ml in PBS 1×, 50 µl/well). Incubate at 4°C overnight or 2 h at 37°C. When sterile plates are used they can be stored at this stage at 4°C for a month.
4. Before use, empty the wells, tapping plates dry gently to preserve the membrane, and wash (×3) with 100 µl sterile PBS 1×.
5. Block the plates by adding 200 µl per well of RPMI 1640 mouse complete medium and incubate for 1 h at 37°C or 2 h at room temperature.
6. Wash wells in PBS 1× once. (If necessary, the plates can also be stored at 4°C at this stage in PBS 1× for a month.)
7. Add the relevant (see Note 10) peptide(s) in 100 µl diluted 10 µg/ml in RPMI complete medium, and prepare negative (100 µl of RPMI complete medium) and positive (100 µl of RPMI complete medium + 5 µg/ml Concanavalin A) control wells.
8. Add the relevant cells in a volume of 100 µl of RPMI mouse complete medium. Depending on the anticipated frequency of responder cells, from 10⁵ to 10⁶ cells will be distributed per well in triplicates.

9. Culture overnight at 37°C in a CO₂ incubator. Do not move the plates (see Note 11). During the overnight incubation cells will secrete Interferon γ , which will be bound by the primary antibody.
10. Wash cells and unbound cytokine away by incubating with PBS 1×, 0.1% Tween 20 for 10 min. Then wash the plates three times with PBS 1×, 0.1% Tween 20.
11. Detect by using the biotinylated secondary antibody (100 µl/well, diluted 1 µg/ml in PBS 1×, FCS 0.5%). Incubate for 1–2 h at room temperature.
12. Wash both sides of the membranes ($\times 3$) with PBS 1× (see Note 12, one can stop here in PBS 1×).
13. Add 100 µl/well of Streptavidin or Extravidin conjugated to either Alkaline Phosphatase or Horse Radish Peroxidase diluted according to supplier recommendations and incubate at room temperature for 1 h.
14. Enzyme substrate color development:
 - (a) Wash both sides of the membrane ($\times 3$) under running distilled water (see Note 13). Park plates face down without reattaching plastic under drain.
 - (b) Gently dry with a paper tissue the posterior face. Eliminate last water drops from the wells by vigorous tapping.
 - (c) Then add 100 µl of NBT-BCIP (Alkaline Phosphatase) or 100 µl of AEC (Horse Radish Peroxidase) per well. Cover the plate with aluminum foil (see Note 14).
15. Wash the plates with distilled water to stop the spot formation (take the base off the plates and wash both sides of the membrane).
16. Let membranes dry completely and count.

3.1.2. Human Interferon γ *ELISPOT Procedure*

The whole procedure is identical to the mouse one except:

1. That human Interferon γ -specific mAb are used for capture and detection of human Interferon γ .
2. That a serum-free culture medium AIM-V is used throughout the assay.
3. That human AB serum (10% final concentration in PBS 1×) is used for the saturation step (step 5).
4. That Phytohemagglutin A (1 µg final concentration) is added in positive control wells.

3.2. Cytolytic Assays

Mouse and human cytolytic assays are performed following similar procedures. Whereas some laboratories assayed human CTLs in media supplemented with human AB serum, others supplement cytolytic media with FCS (see Note 4).

Three radioactive compounds have been used to label target cells and evaluate the killing capacity of effector T lymphocytes: ⁵¹Chromium, ³H-thymidine, and ³⁵S-methionine. For ³⁵S-methionine-based assays see Note 15.

3.2.1. ⁵¹Chromium Release Assay

Routinely used in most immunological laboratories since the first descriptions (7–10).

Principle: At 37°C cells incorporate ⁵¹Cr O₄⁻ that is thought to bind to intracellular proteins. The incorporated ⁵¹Cr is not released into the culture supernatant unless cells have been killed. The cytolytic activity of effector cells incubated usually for 4 h with ⁵¹Cr-labelled targets is evaluated by measuring the amount of ⁵¹Cr released in the culture supernatant. However, a fraction of the ⁵¹Cr does not associate to large cellular molecules and consequently leaks out during the assay of cells that have not been lysed; this Spontaneous Release must be determined not to overestimate the lytic activity of the effector cells.

Procedure

1. Count exponentially growing target cells, check (Trypan Blue exclusion test) their viability (see Note 16), and spin down ($350 \times g$, 5 min, room temperature) the desired number of cells, considering that usually 5,000 target cells are used for each experimental point and that each condition will be tested in triplicate.
2. Eliminate the supernatant and gently resuspend the cell pellet in the few μl of medium that remain in the bottom of the tube.
3. Add 120 μl of pre-warmed (37°C) culture medium (see Note 17) and the needed amount of ⁵¹Cr (usually 100 μCi for 10^6 cells).
4. Place immediately in the CO₂ incubator for 1–2 h, shaking gently the tubes from time to time to resuspend the cells settled at the bottom of the tube.
5. Wash three times ($350 \times g$, 5 min, room temperature) with 5 ml of complete culture medium the targets, the third washing being done just before the final step of the assay assembling process. Resuspend at $5 \times 10^4/ml$ and distribute 100 μl per well of 96-well V-shaped plates (see Notes 18 and 19).
6. In addition to the experimental wells (in triplicates), prepare 6 wells for evaluating the Spontaneous Release and 6 to evaluate the Total Release (see infra).
7. Collect and count effector cell(s). Resuspend in complete culture medium. Add 100 μl of effectors in each experimental well. Effector cells are typically used at 30/, 10/, 3.3/, and 1.1/1 Effector-to-Target (E/T) ratios (see Note 20).

8. Add 100 μ l of complete culture medium in place of effectors in the spontaneous release wells.
9. Spin for 2 min at $50 \times g$ (optional) to force contact between effectors and targets and incubate for 4 h at 37°C in a CO_2 incubator.
10. Total release is obtained from wells in which targets are destroyed by various means: addition of HCl 10% in water, 10% bleached water, 1% triton X-100 in water (see Note 21).
11. Spin for 5 min at $350 \times g$ and collect a fraction of the cell supernatants to evaluate the release of ^{51}Cr (see Note 22).
12. Results are expressed in % of specific release:

$$E - S / T - S \times 100,$$

where E is experimental release, S spontaneous release, and T total release that are graphically represented in Fig. 1.

13. Treat all tips, pipettes, and other materials that contacted radiolabelled cells as radioactive and discard appropriately.
14. Waste: Solid and liquid wastes should be separately disposed of in containers for short-half-life isotopes.
15. Decontaminate the working area with appropriate anionic detergent at the end.

3.2.2. The JAM Assay (See Note 23)

Principle: In this assay, target cells are labelled with ^3H -thymidine and then incubated with effector cells. DNA-fragmentation is an early event during cell apoptosis. These fragments, initially held in the cytosol, and then released into the cell supernatant following plasma membrane disintegration, are too small to be retained by glass-fiber filters whereas undamaged chromosomal DNA is

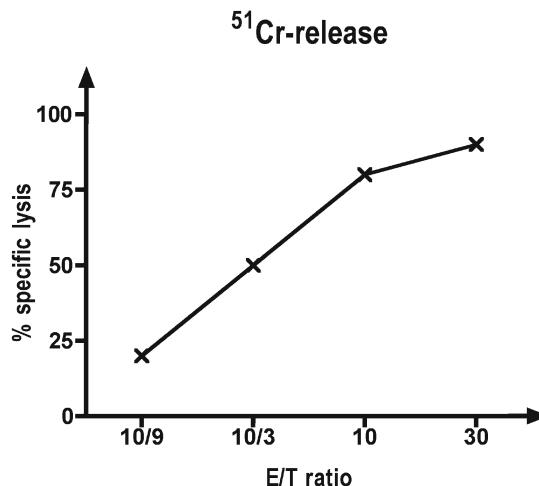


Fig. 1. A graphical representation of the equation $E - S / T - S \times 100$, where E is experimental release, S spontaneous release, T total release.

retained allowing a quantification of the living cells at the end of the assay (11).

JAM Assay Procedure

All culture incubations are done in a 37°C, 5% CO₂ incubator.

1. Label exponentially growing target cells (usually overnight but with rapidly growing cells labelling can be shorten to 4–6 h) by adding 5 µCi/ml of ³H-thymidine (5 × 10³ to 1 × 10⁴ cells are needed by experimental point, each condition being tested in triplicate).
2. Once incubation with ³H-thymidine is over, collect radiolabelled cells, wash ×3 (350×g, 5 min, room temperature), and count cells.
- 3–7. Steps 3–7 of the JAM assay are like steps 5–9 of the ⁵¹Cr-release assay, see Subheading 3.2.1.
8. At the end of the 4-h incubation with effector cells, collect wells (cells and supernatants) with a 96-well plate cell harvester on appropriate glass-fiber filters.
9. Dry the filters in a microwave oven (2 min, full power).
10. Soak the filter with scintillation fluid, place it in a sealed plastic bag, and count: By measuring the radioactivity retained by glass-fiber filters (which corresponds to the number of living target cells at the end of the assay) in the presence (*E*, Experimental values) and in the absence (*S*, maximal retained value) of effectors, the percentage of target cell lysis can be calculated:

$$S - E / S \times 100.$$

3.2.3. Lactate Dehydrogenase-Release Assay

Non radioactive cytolytic assays have been developed as alternatives to the radioactive ones (LDH-Release Assay, Metabolically active assays, Cellular DNA fragmentation ELISA, Granzyme B and Caspase 3 assays). Below the LDH-Release Assay protocol, for the other nonradioactive cytolytic assays, see Note 24.

Principle: Killed target cells released their cytoplasmic LDH into the culture supernatant. The LDH activity of the supernatant is measured coupling the transformation of lactate into pyruvate with the reduction of NAD⁺ in NADH⁺H⁺. NADH⁺H⁺ serves as a proton donor to transform Tetrazolium salt in red Formazan easily quantified by ELISA (12).

LDH-Release Assay Procedure

The general schemes of the LDH-release assay and ⁵¹Cr-release assay are very similar.

1. Prepare LDH-release assay medium, i.e., RPMI 1640, supplemented with either low concentrations of serum (2%) or preferably with 1% Bovine Serum Albumin (see Note 25).

2. Count exponentially growing target cells, check (Trypan Blue exclusion test) their viability (see Note 16), and wash $\times 3$ with LDH-release assay medium ($350 \times g$, 5 min, room temperature).
3. Resuspend in LDH-release assay medium the target cells, (usually 5×10^3 to 10^4 target cells are used for each experimental point) and distribute target cells in U- or V-bottomed 96-well plates, each experimental condition being tested in triplicate.
4. Include additional triplicated wells to evaluate (a) the background LDH activity of the LDH assay medium alone, (b) the spontaneous release of LDH enzyme by target cells in the absence of effectors, (c) the spontaneous release of LDH enzyme for each concentration of effectors used in the assay (see Note 26), and (d) the maximal total LDH release by the targets.
5. Collect and count effector cells. Resuspend in LDH-release assay medium. Add 100 μl of effectors in each experimental well. Effector cells are typically used at 30/, 10/, 3.3/, and 1.1/1 Effector-to-Target ratios (see Note 20).
6. Spin for 2 min at $50 \times g$ (optional) to force contact between effectors and targets and incubate for 4 h at $37^\circ C$ in a CO_2 incubator.
7. Evaluate background LDH activity of LDH assay medium from triplicated wells filled with 200 μl of LDH assay medium.
8. Evaluate the target cell spontaneous LDH enzyme release from triplicated wells with target cells alone in 200 μl of LDH assay medium.
9. Evaluate the effector cell spontaneous LDH enzyme release for each concentration of effector cells tested from triplicated wells with effector cells alone in 200 μl of LDH-release assay medium (see Note 26).
10. Evaluate the maximal target LDH release from triplicated wells in which target cells in 100 μl are destroyed by adding 100 μl of LDH-release assay medium supplemented with Triton X-100 for a final 1% concentration.
11. Incubate at $37^\circ C$, 5% CO_2 incubator for 4 h.
12. Spin ($350 \times g$, 5 min, room temperature) and transfer 100 μl to 96-well flat-bottomed plates adapted to ELISA test.
13. Add 20 μl of lactate solution and then 20 μl of tetrazolium solution (stock solution diluted 1:10 in PBS 1 \times) to each well.
14. Start the enzymatic reaction by adding 20 μl of the NAD $^+$ /diaphorase solution and let the reaction proceed protected from light for 15–20 min at room temperature.

15. Stop the reaction by adding 20 µl of oxamate solution per well.
16. Measure the absorbance of the samples at 490 or 492 nm (see Note 27).
17. Calculate the % specific lysis according to the formula:

$$E - S / T - S \times 100,$$

where E is the absorbance at 490 nm of target cells incubated with a given concentration of cytotoxic effector cells minus the spontaneous LDH release of effector cells alone at the same concentration (step 9), S is the absorbance at 490 nm of target cells alone (step 8), and T is the maximal target cell LDH release as determined in step 10 (see Note 28).

3.2.4. Epstein–Barr Virus Immortalization of B Lymphocytes (13)

1. Prepare supernatant containing Epstein–Barr Virus (EBV) by transferring exponentially growing B95.8 cells (1×10^6 /ml) in fresh RPMI 1640 medium supplemented with 10% FCS, 100 U/ml Penicillin, and 100 µg/ml Streptomycin (RPMI complete medium).
2. Collect 3 days later the culture EBV-containing supernatant by centrifugation ($350 \times g$, 5 min, room temperature), filter on a 0.45 µm pore-size filter, and store frozen (-80°C) as aliquots of 2.5 ml (virus titration of such supernatants can be determined in standard EBV-transformation assay (14), they contain usually 10^2 to 10^3 transforming units/ml, or, as more recently described, by quantitative PCR (15)).
3. Infect Ficoll Hypaque-purified PBMC (10^7 in 2.5 ml of RPMI complete medium) with 2.5 ml of EBV-containing supernatant in a 50 ml conical tube and incubate for 2 h in a 37°C water bath.
4. Transfer in a 25 cm² culture flask and add 5 ml of RPMI complete medium supplemented with Cyclosporine A, see Note 29 (to prevent the activation of EBV-reactive T lymphocytes that would kill the EBV-transformed B lymphocytes), for a final 500 ng/ml concentration.
5. Usually, the immortalized lymphoblastoid cells forming large round clusters appear after 3–4 weeks.
6. As soon as the medium turns light pink-yellow as becoming slightly acidic, collect the cells by centrifugation ($350 \times g$, 5 min, room temperature), discard the used medium, dissociate gently the cell pellet, resuspend cells in RPMI complete medium, and divide in 3 culture flasks.
7. About a week later, the cell line can be frozen when in log-phase growth (from 1×10^6 to 5×10^6 per ml of ice cold freezing mixture, see Note 30) and place the tubes in a cotton wool isolated box in a -80°C freezer for 24 h and transfer the tube in a liquid nitrogen container the following day.

4. Notes

1. Mitomycin C that cross-links irreversibly the complementary strands of the DNA double helix can be used to block cell division in the stimulating cell population. Cells are incubated for 1 h at 37°C with Mitomycin at a 50 µg final concentration and then washed four times to totally remove the drug.
2. The selection of an FCS batch that supports efficiently mouse T cell growth is achieved with the IL-2-dependant CTLL-2 cell line. CTLL-2 cells must be collected in active log-phase growth. After at least 2 washes in 50 ml of plain RPMI medium ($350 \times g$, 5 min, room temperature) to remove residual FCS and IL-2, cells are re-suspended at 4×10^4 cells/ml and 50 µl distributed per well of a 96-well flat-bottom culture plate. Having determined in preliminary experiments with an arbitrary batch of FCS the concentration of human recombinant IL-2 that corresponds on day 3 of a culture of 10^4 CTLL-2 cells per well to 50% of the maximal proliferation, the different batches of FCS are tested comparatively at a final 10% concentration under the same conditions (RPMI 1640 medium, 5×10^{-5} M 2-mercaptoethanol, Penicillin, Streptomycin and the selected IL-2 concentration, 200 µl final culture volume per well) and the proliferation of the CTLL-2 cells is comparatively evaluated on day 3.
3. Mouse IL-2 is expensive; as a rule rat TCGF or human IL-2 are used for the cloning of mouse CD8⁺T lymphocytes. We used to prepare each time a large batch of TCGF from 20 rats (LOU or Wistar) since TCGF can be conserved at -20°C without decline in activity. Spleens are removed aseptically and cell suspensions prepared from each spleen immediately after its excision. Spleens are crushed in 10 ml of RPMI complete medium using a 100 mm diameter tissue culture dish with the flat end of a 10 ml syringe. Clumps of cells are disrupted by repeated up and down pipetting and large debris finally eliminated by filtration in a 50 ml sterile conical tube through a cell strainer. Cells (2.5×10^6 /ml) are cultured for 36 h in RPMI 1640 FCS 10%, 2-mercaptoethanol 5×10^{-5} M, Penicillin, and Streptomycin supplemented with 5 µg of Concanavalin A (large 150 cm² culture flasks in horizontal position). Supernatants are collected following centrifugation (15 mn, $2,000 \times g$, 4–20°C) and Concanavalin A lectin activity competitively inhibited by the dissolution of 20 mg/ml of α-Methylmannoside. Forty ml aliquots are then frozen, each aliquot being again centrifuged before use (15 mn, $2,000 \times g$, 4–20°C) to eliminate clumps of debris that constantly formed during the freezing/thawing process and to facilitate the final sterilization

by filtration through a 0.45 µm disposable filter. Titration of the TCGF is performed in a CTLL-2 assay, as described in Note 2, and then used in cloning cultures at a final concentration (usually \approx 10%) that insures 80% of CTLL-2 maximal proliferation. Recombinant human IL-2 can be obtained from different suppliers. Biological activity is either expressed in International or Biological Response Modifier Program (BRMP) units. One BRMP unit equals 6 International units. The usually recommended working final concentration is 10–15 BRMP U/ml. However, since we observed important differences in the activity between human recombinant IL-2 of different origins, titration in a CTLL-2 assay is strongly recommended.

4. FCS proteins act as antigens for human T lymphocytes. Therefore, as a rule, AB human sera that are furthermore devoid of natural anti-blood group antibodies are used for human T lymphocyte cultures. However, when culturing cloned human T cells, FCS-supplemented media can be used since TCRs do not structurally change by somatic mutations. The use of FCS-supplemented media is also acceptable at the end-stage of the culture process when testing T lymphocytes functionally.
5. A panel of EBV-transformed cells representative of the major HLA class I alleles and/or HLA class I-transfected C1R cells ([16](#)) with essentially cell surface expression of a single species of HLA class I molecules are essential tools to determine the restricting HLA class I allele of the analyzed CTL responses.
6. Access to radioactive compounds is subordinated to licensing by the state competent authority. ^{51}Cr emits high-energetic gamma rays that are incompletely arrested by lead shields even of 4 cm thickness with, additionally, emission of X-rays; therefore, one should remain for as little time as possible in the vicinity of the main ^{51}Cr source. Handling of ^{35}S -methionine and deposit of ^{35}S -methionine-contaminated solids and liquids must be carried out according to the same safety instructions as for ^{51}Cr , knowing that shielding is not required for ^{35}S due to the low penetrating power of the emitted beta rays. Like ^{35}S , ^3H is a low-energy beta ray-emitting radiation; therefore no shielding is required but all other safety rules should be carefully followed since ^3H half-life exceeds 13 years.
7. The anti-mouse Interferon γ clone R4-6A2 is available from ATCC HB-170.
8. Dispense DMF in fume hood and store in glassware.
9. PVDF and nitrocellulose membrane-bottomed 96-well plates can be used indifferently. In their original forms, PVDF and nitrocellulose membranes have very hydrophobic properties

and poor binding capacities. Activation by alcohol makes the membranes more hydrophylic and increases their binding capacities.

10. Most MHC class I-restricted peptides are hydrophobic, and require 10% DMSO to dissolve. In case a single peptide is added and for a usual stock-solution peptide concentration of 2 mg/ml, the final concentration of DMSO (0.05%) is negligible. For peptide bank screening, in which case the initial tests are usually conducted with pools of peptides, it should be kept in mind that DMSO is toxic for cells especially when activated (more than 0.5% final concentration is toxic for lymphocytes in a 6-h assay).
11. Do not move the plates while the cells are culturing since the spots will have a snail trail shape and be ignored by the ELISPOT reader. Do not pile up plates since they will not warm homogeneously resulting in a peripheral warmer edging effect.
12. Plates can be kept at this stage overnight, even over weekend, at 4°C prior to final spot development.
13. The base of the plate should be removed after incubation with AP- or HRP-conjugated Streptavidin or Extravidin in order to wash both sides of the membranes; if not, a small leak would result in high staining background.
14. Monitor spots visually until they appear optimally developed, usually in about 10 min.
15. Target cells labelled with ^{35}S -methionine can be used to evaluate the killing capacity of effector cells (17). As being incorporated into target cell proteins, the spontaneous release of radiolabelled compounds is significantly reduced compared to chromium allowing to perform long-term (16 h) cytolytic assays. ^{35}S -methionine can also be used to end-label the target cells still alive at the end of the incubation with CTL; however, the incorporation of the label by the effector cells themselves has to be evaluated for each effector cell concentration used and has to be subtracted from the corresponding experimental values.
16. Target Cells should be in perfect condition to minimize the spontaneous release. The significance of a ^{51}Cr -release assay in which the spontaneous release is >25% of the total release is highly questionable. When tumor or virally transformed cells are used as targets, it is strongly recommended to perform regular in vitro passages the days prior to the assay so that the targets are in exponential growth phase when labelled. When lectine or lipopolysaccharide in vitro-activated lymphocytes are used as targets, viable cells need usually to be purified on a Ficoll Hypaque gradient.
17. In our hands, the highest the proportion of serum, the lowest the spontaneous released, especially for cells that are very susceptible to lysis.

18. Once the target cells have been distributed, the plates should be placed in the CO₂ incubator to maintain as much as possible the medium pH and temperature and reduce consequently the target cell spontaneous release. It is good practice to only have outside the CO₂ incubator the only plate on which one is working.
19. If peptides or antibodies have to be added in the assay, targets will first be distributed, subsequently peptides or antibodies, and finally effectors for a 200 µl final volume.
20. Under a regular microscope, activated CTL are relatively large white cells with a banana shape. Following bulk cell population in vitro primary restimulation, the specific effectors are still in a minority; hence a 30/1 as the highest E/T ratio is a safe start (beyond 100/1 nonspecific steric hindrance results in decreased cytolysis). By contrast, when working with cloned CTL, the E/T ratios should be lowered.
21. There are as many methods to determine the 100% of lysis as there are laboratories working with CTL. Be aware that some of these methods rely on volatile products (e.g., HCl) in which case the 100% wells should be assembled in a separate plate.
22. Collection of supernatants usually achieved with a multichannel pipette does not require tips to be changed between wells; inter-well contamination is minimal.
23. JAM is the acronym for Just Another Method. The JAM assay is more sensitive than the ⁵¹Cr-release assay since it measures DNA fragmentation, an apoptotic event that precedes cytosolic leakage. It also circumvents problems arising in case of high spontaneous Chromium release and therefore is well adapted for assays requesting long incubation times.
24. Additional nonradioactive cytolytic assays:
They are all commercially available.
 - (a) *Metabolically active assay:* Some Tetrazolium salts (e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) or nitro-5-sulfophenyl-5-[9phenyl-amino carbonyl]-2H-Tetrazolium hydroxide (XTT)) penetrate through intact plasma membrane, and are reduced intracellularly in viable cells in colored Formazan compounds that can be quantified by ELISA. Dead cells do not carry out such reaction. Therefore as for ³⁵S-End-Labeling assay, the cytolytic activity of an effector population of cells can be evaluated.
 - (b) *Cellular DNA fragmentation ELISA* is a nonradioactive alternative to the JAM-assay. Target cells having integrated in their DNA BromodeoxyUridine (BrdU) are then mixed with effector cells. At the end the incubation, target cells are lysed to liberate the apoptotic DNA fragments contained

into the cytosol of target cells that have not yet disrupted their plasma membranes and the amount of apoptotic DNA fragments is evaluated in an ELISA assay using an anti-DNA “capture” m.Ab., and a biotinylated “detection” anti-BrdU m.Ab.

(c) *Caspase 3 and Granzyme B assays:* The aminoacid recognition motif specific for Granzyme B (VGPD↓FGR) or for Caspase 3 (DEVD↓GIN) is inserted in a peptide backbone. At the N and C ends of this peptide have been coupled 2 fluorophores. As long as the peptide is intact, stacking interactions between the 2 fluorophores result in fluorescence quenching that is relieved following peptide cleavage (18).

25. LDH is present in biological liquids (serum, ascitis); cell culture media supplemented with either low concentrations of serum (2%) or preferably with 1% Bovine Serum Albumin should be used. Culture media supplemented with pyruvate (a competitive inhibitor of the LDH enzyme) should not be used.
26. LDH is a constitutively expressed ubiquitous protein; thus this assay is valid for any target cell-type. However, effector cells also expressed LDH; therefore the spontaneous release of LDH by target cells and by each effector cell concentration used in the assay must be evaluated. Ficoll-Hypaque purification of viable effector cells tested at high E/T ratios is strongly recommended.
27. Whereas supernatants collected at the end of the cytolytic assay can be kept frozen until assessing the LDH activity, once the LDH reaction is performed, absorbances must be evaluated at most in the following hour.
28. The LDH activity of the sole LDH-release assay medium should be lowered as much as possible and separately evaluated in each experiment.
29. Cyclosporine A prevents the activation of EBV-reactive T lymphocytes that would kill the EBV-transformed B lymphocytes.
30. To optimize the freezing-thawing process, we always used a pre-cooled (4°C) 90% of FCS 10% DMSO mixture and for thawing cell culture medium supplemented with 20% FCS.

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References

1. Murphy K, Travers P, Walport M (2008) Janeway's Immunobiology. Garland Science, New York
2. Berke G, Clark WR (2005) Killer lymphocytes. Springer, Dordrecht
3. Bevan M, Epstein R, Cohn M (1974) The effect of 2-mercaptoethanol on murine mixed lymphocyte cultures. *J Exp Med* 139:1025–1030
4. Sedgwick JD, Holt PG (1983) A solid-phase immunoenzymatic technique for enumeration of specific antibody-secreting cells. *J Immunol Methods* 57:301–309
5. Czerkinsky CC, Nilsson L-A, Nygren H, Ouchterlony Ö, Tarkowski A (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* 65: 109–121
6. Czerkinsky CC, Anderson G, Ekre H-P, Nilsson L-A, Klareskog L, Ouchterlony Ö (1988) Reverse ELISPOT assay for clonal analysis of cytokine production I. Enumeration of gamma-interferon-secreting cells. *J Immunol Methods* 110:29–36
7. Sanderson A (1965) Quantitative titration, kinetic behaviour, and inhibition of cytotoxic mouse isoantisera. *Immunology* 9:287–300
8. Wigzell H (1965) Quantitative titration of mouse H-2 antibodies using Cr51-labeled target cells. *Transplantation* 85:423–431
9. Holm G, Perlman P (1967) Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. *Immunology* 12:525–536
10. Brunner KT, Mauel J, Cerottini J-C, Chapuis B (1968) Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 14:181–196
11. Matzinger P (1991) The JAM test A simple assay for DNA fragmentation and cell death. *J Immunol Methods* 145:185–192
12. Korzeniewski C, Callewaert DM (1983) An enzyme-release assay for natural cytotoxicity. *J Immunol Methods* 64:313–320
13. Miller G, Shope T, Lisco H, Stitt D, Lipman M (1972) Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. *Proc Natl Acad Sci U S A* 69:383–387
14. Miller G, Lipman M (1973) Release of infectious Epstein-Barr virus by transformed Marmoset leukocytes. *Proc Natl Acad Sci U S A* 70:190–194
15. Fecenko-Tacka KL, Schina L, Beiswanger CB (2008) Quantitative analysis of Epstein-Barr virus supernatant by real-time PCR assay. *Cell Preserv Technol* 6:73–81
16. Zemmour J, Little AM, Schendel DJ, Parham P (1992) The HLA-A, B “negative” mutant cell line C1R expresses a novel HLA-B35 allele, which has a point mutation in the translation initiation codon. *J Immunol* 148: 1941–1948
17. Katz A, Feldman M, Eisenbach L (1992) The use of [³⁵S] methionine as a target cell label in long term cytotoxic assays. *J Immunol Methods* 149:255–260
18. Packard BZ, Telford WG, Komoriya A, Henkart PA (2007) Granzyme B activity in target cells detects attack by cytotoxic lymphocytes. *J Immunol* 179:3812–3820

Chapter 21

Cloning CD8⁺ Cytolytic T Lymphocytes

François A. Lemonnier

Abstract

CD8⁺ T lymphocyte cloning has resulted in many fundamental advances: structural elucidation of peptide-MHC recognition, spatiotemporal dissection of the thymic positive and negative selection processes and is further expected, TCRs being the key molecules controlling T cell activation, to provide us with molecular tools of immuno-therapeutic interest for infectious, tumor, and autoimmune diseases. However, cloning CD8⁺ T lymphocytes remains a relatively difficult enterprise. Cloning mouse CD8⁺ T lymphocytes that will be first considered is to some extent facilitated by our complete control of the *in vivo* priming process and the unlimited access we usually have to perfectly suited (syngeneic) antigen presenting cells. Cloning human CD8⁺ T lymphocytes is more difficult largely but not exclusively for ethical reasons.

Key words: T cell clone, Limiting dilution, CTL

1. Introduction (1)

CD8⁺ T lymphocytes recognize through their receptor for antigen (TCR) peptides commonly called epitopes (usually 8–10 amino acid long) inserted in the peptide-binding groove of the $\alpha 1\alpha 2$ domains of the Major Histocompatibility Complex (MHC) class I molecule heavy chain. TCR interaction is of much lower affinity (roughly 100-fold) than that of antibody but is reinforced by the interaction of the CD8 accessory molecules with the MHC heavy chain $\alpha 3$ domain as well as by additional interactions (CD2 and CD58, LFA-1 and ICAM1 and ICAM2,...) shared with other cells of the immune system.

CD8⁺ T lymphocytes are essential effector cells for host defense against pathogens that multiply in the cytoplasm of infected cells. Conversely, pathogenic auto-reactive CD8⁺ T lymphocytes are responsible for various autoimmune disorders. In these two opposite situations, CD8⁺ T cell cloning is of interest, either to identify the protective or pathogenic epitopes or to analyze phenotypically

and functionally (cytokines produced, cell surface molecules expressed, structure of their TCR) the mobilized CD8⁺ T cells.

The tremendous structural diversity (in the 10¹⁸ theoretical range) of the TCR α and β chains is both genetically determined (random association of β chain VDJ and α chain VJ segments) and genetically not determined (random excision and addition of nucleotides at the DNA 3' ends during the segment joining process). As a consequence, the peripheral CD8⁺ T cells are extremely diverse in terms of TCR they express and it is estimated that in the absence of encounter with antigen, in the whole body, the total number of CD8⁺ T cell expressing the same TCR (i.e., a naïve T cell clone) is at most around 100. It should finally be noted that TCR diversity is entirely acquired in the thymus: unlike antibodies, TCRs do not structurally change by somatic hypermutations.

Following antigen encounter, naïve CD8⁺ T cells are activated, start to transcribe the IL-2 receptor α chain and upregulate the expression of high-affinity IL-2 receptors. Whereas these activated CD8⁺ T cells differentiate in Interferon γ, TNF α, Granzymes A and B, and Perforin high producing cells, they produce relatively low amount of IL-2 and are therefore heavily dependant upon IL-2 high producer CD4⁺ T helper lymphocytes for their proliferation and survival.

The discovery in the mid-1970s of the IL-2 cytokine (initially named T Cell Growth Factor, TCGF, (2)) paved the way to the long term in vitro maintenance of CD8⁺ T cell lines and to the isolation and in vitro propagation of CD8⁺ T cell clones. The amplification of a homogeneous population of lymphocytes has enabled the fine dissection of CD8⁺ T cell responses against antigens (identification of the peptide/epitope recognized and cDNA cloning of the TCR responsible for such recognition) that have resulted in considerable breakthroughs regarding the structure of TCRs, the thymic positive and negative selection processes, the requirements for the peripheral survival and peripheral activation of naïve T cells. Today, we start to make use of this gained knowledge for a rational design of vaccines (3) and, more recently, adoptive immunotherapies (4–6).

2. Materials

Most of the material needed can be purchased from any company.

2.1. Common Consumables and Lab Equipment

1. A fully equipped tissue culture room: vertical laminar flow hoods, humidified 37°C CO₂ incubators, regular and inverted microscopes, refrigerated low speed ≤10,000 rpm centrifuge and autoclavable waste disposal containers.
2. Gamma or X-ray irradiator (alternatively Mitomycine C, see Note 1).

3. Sterile plastic-ware: 2, 5, 10, 25 ml pipettes, 25, 75, and 150 cm² tissue culture flasks, 20, 200, 1,000 µl disposable (preferentially cotton-plugged) tips, cell strainers, Petri dishes, sterile reservoirs, 96-well U-bottomed, 96-well flat-bottomed, and 24-well flat-bottomed culture plates.
4. Multi-channel 20–200 µl pipettes (8 or 12 channels).
5. CTLL-2 cells (ATCC TIB-214TM).
6. Tritiated ³H-thymidine, ⁵¹Chromium O₄⁻ (both radioactive products have to be handled and discarded under specific and tightly controlled procedures and require access to a Beta/Gamma counter. Alternatively, nonradioactive assays can be selected to evaluate cell proliferation (BrdU incorporation assay) and CD8⁺ T cell functional activity (i.e., LDH release cytolytic assay, mouse or human Interferon γELISPOT, see Chapter 20)).

2.2. Mouse CD8⁺ T Cell Cloning

1. Responder mice and syngeneic mice. As a rule 6–8 weeks old mice are used.
2. Stimulating antigens:
 - Synthetic peptides (purity >70%) dissolved in PBS 1× usually supplemented with 10–20% dimethylsulfoxide (DMSO), to be kept aliquoted at -20°C.
 - Cesium-gradient- or column-purified recombinant mammalian expression plasmid vector.
3. Incomplete (IFA) and Complete (CFA) Freud Adjuvant.
4. Poly-(I:C) and CpG oligonucleotides.
5. Cardiotoxin from cobra snake (at least 2 cardiotoxins from Naja nigricolis and Naja mossambica which are commercially available have been used for DNA vaccination purposes).
6. Syringes 1 and 5 ml and 27-gauge needles.
7. Dissecting instruments (scissors and forceps).
8. RPMI mouse complete medium: RPMI 1640 supplemented with 10% heat inactivated (56°C, 30 min) Fetal Calf Serum (FCS, ideally tested for T cell growth support in a CTLL-2 proliferation assay (see Note 2)), fresh (less than 1 month old) 2-mercaptoethanol (essential for the growth of mouse T cells in vitro, (7)), at a 5×10^{-5} M final concentration 100 U/ml Penicillin and 100 µg/ml Streptomycin (RPMI mouse complete medium);
9. TCGF (to be in house prepared, see Note 3) or recombinant human IL-2 (several suppliers) to be both tested first in a CTLL-2 proliferation assay to determine the effective working concentration, usually 10% final concentration for TCGF and 50–100 IU or 10–15 BRMP U/ml for recombinant human IL-2 (see Note 3).
10. Freezing mixture: 90% FCS, 10% (DMSO).

2.3. Human CD8⁺ T Cell Cloning

Important: work with human material must comply with legal and safety rules.

1. Tissue-procurement regulations: protocol validation by an ad hoc committee, full information and written consent of the patient.
2. Safety considerations: when working with human biological specimen (whether blood, serum, or biopsies) information must be gained regarding the health status (Hepatitis B, C, Human immunodeficiency virus infection,...) of the donor, and cultures should be handled and discarded according to the appropriate biohazard procedures.
 - RPMI human complete medium: RPMI 1640 supplemented with 5–10% human AB serum (supplied by several companies), 100 U/ml Penicillin, and 100 µg/ml Streptomycin.
 - Recombinant human IL-2 has to be used exclusively since rodent IL-2 does not activate efficiently human T cells. Each new batch has to be tested first in a CTLL-2 assay to define the optimal working concentration, usually 50–100 IU or 10–15 BRMP U/ml (see Note 3).
 - Phytohemagglutinin (PHA) and anti-CD3 mAb (i.e., 12F6, Quartett Immunodiagnostika und Biotechnologie Vertriebs GmbH) that provides both an antigen-independent potent stimulus for T cells through cross-linking of their TCR; Phorbol 12-Myristate 13-Acetate (PMA), and Cyclosporine A.
 - Conditioned medium from human PBMC to be in house prepared (see Note 4 and Subheading 3.2.5).
 - Ficoll-Hypaque to purify blood lymphocytes and antigen presenting cells (APC).
 - B95.8 cotton top marmoset cell line (ATCC.CRL 1612) for the production of infectious EBV particles (8) and the transformation of human B lymphocytes in EBV- lymphoblastoid cell lines (see Subheading 3.2.4).
 - Freezing mixture: 90% FCS, 10% (DMSO).

3. Methods

Cloning of mouse and human CD8⁺ T cells will be considered separately.

3.1. Cloning of Mouse CD8⁺T Cells

For clarity reasons, the cloning of mouse CTL specific of a known epitope will be described as a prototypic procedure. Other more

prospective experimental conditions aiming at either the identification of the CTL-targeted antigenic molecules or at inventorying the CTL epitopes they contain will be mentioned in Note 5.

Except for CD8⁺ T cell responses directed at allo-specific MHC Class I and II antigen differences (in which cases the frequency of reactive naïve CD8⁺ T cells is usually very high), in vitro CD8⁺ T cell cytolytic responses against other less potent stimuli (minor histocompatibility antigens, viruses, tumor antigens,...) need in vivo priming for the expansion of the activated T cell clones prior to in vitro culture and analysis. In vivo priming can be achieved in various ways mentioned in Note 5. Two of them, injection of synthetic peptides and intra-muscular DNA injection of mammalian expressing plasmid vectors are detailed below.

3.1.1. Conditioning the Responder Mice by Peptide Immunization

1. Emulsified peptides in IFA (v/v) by vigorous vortexing resulting in a milk-white viscous suspension (as a rule 100 µg/mouse of the MHC class I-restricted peptide mixed with an equimolar amount of a MHC class II “helper peptide” see Note 6 and in some cases with additional adjuvant substances see Note 7, dissolved in PBS 1× with or without DMSO (most MHC class I-restricted peptides require DMSO to dissolve).
2. Inject mice subcutaneously at the base of the tail with a maximal total volume of 200 µl/mouse.
3. Depending upon the immunogenicity of the MHC class I-restricted peptide, in vivo priming may have to be similarly repeated a week later.
4. Seven days after the last injection, spleen, and inguinal lymph nodes will be aseptically removed.

3.1.2. Conditioning the Responder Mice by DNA Immunization

1. Inject anesthetized mice in tibialis anterior muscle of the two hind legs with 100 µl of a 10 µM solution of Cardiotoxin in PBS 1× (see Note 8).
2. Five to nine days later, inject anesthetized mice in each tibialis anterior muscle with 50 µg of purified DNA (see Note 9).
3. Twelve to fourteen days later DNA re-boost mice similarly.
4. Twelve to fourteen days after the last re-boost the spleen will be aseptically removed.

3.1.3. In Vitro Restimulation: Primary T Cell Culture

1. Dilacerate spleen and, in case of peptide immunization, draining lymph nodes, in a 5 cm diameter Petri dish containing 5 ml of RPMI mouse complete medium. Dissociate cell clumps by pipetting (5 ml pipette) up and down the cell suspension see Note 10, then filter through a cell strainer to eliminate spleen and lymph node capsules.
2. Once counted, put responder cells in culture as soon as possible: about 40×10^6 cells in 20 ml RPMI mouse complete

medium per 25 cm² culture flask maintained in a vertical position in the CO₂ incubator (see Note 11).

3. Add solely the MHC class I restricted peptide usually at a 10⁻⁵ M (about 10 µg/ml) final concentration.
4. On day 5 of in vitro restimulation, collect approximately half of the non-adherent cells, wash thrice in RPMI complete medium (350×g, 5 min, room temperature), count viable cells and test for function in a cytolytic or Interferon γ ELISPOT assay (see Chapter 20).

3.1.4. In Vitro Secondary/Tertiary T Cell Culture

1. If the expected CD8⁺ T cell response has been obtained and is of significant magnitude (see Note 12), the following day, collect by centrifugation (350×g, 5 min, room temperature) the fraction of cells remaining in the original flask (at this stage there is usually no need to split these cells that are not as many as they were at the initiation of the culture) and restimulate with peptide as detailed for primary cultures in Subheading 3.1.3 with however two important differences.
 - First, supply the new culture with about 20×10⁶ per 25 cm² flask irradiated (20 Gy) splenocytes from a syngeneic mouse as source of APC/feeder cells;
 - Second, 2 days post-restimulation, supplement the whole culture with appropriate amounts of either TCGF or recombinant human IL-2 (see Note 3) for the limiting dilution cloning to be performed with effector cells growing exponentially.
2. If the expected response after the first in vitro restimulation is weak, secondary even tertiary restimulations on a weekly basis have to be performed until the response becomes robust enough for cloning.

3.1.5. Limiting Dilution Cloning of Mouse CD8⁺ Cells

The principle of limiting dilution cloning is detailed in Note 13. As soon as lymphocyte activation and proliferation become obvious in the secondary T cell culture (see Note 14) the cells should be cloned by limiting dilution.

All cell preparations and dilutions have to be performed in RPMI mouse complete medium supplemented with appropriate concentration of either TCGF or IL-2 (see Note 3).

Since the cloning efficiency (i.e., the number of cells growing versus the number (based on Trypan Blue exclusion) of viable cells seeded) for CD8⁺ T lymphocytes is as a rule much lower than 1, plates should be seeded with relatively high number of cells per well.

1. Prepare a 2×10⁶ cells/ml suspension of irradiated (20 Gy), unfractionated splenocytes (see Note 10) from mice syngeneic to responder cells as source of feeder/APC cells, add the MHC class I-restricted peptide so as to reach a 10⁻⁵ M final concentration

at the end of the culture-well assembly and distribute the stimulating cells and peptide in a final 100 µl volume per well in six U-bottomed 96-well plates. As soon as the stimulating population is distributed, place the culture plates in the CO₂ incubator (see Note 15).

2. Collect the effector cells, pellet them by centrifugation (350×g, 5 min, room temperature), decant the supernatant, shake by hand the tube to completely dissociate the pellet, resuspend in few ml of TCGF- or IL-2-supplemented RPMI complete medium and count viable cells.
3. Prepare 20 ml of a suspension of effector cells to be cloned at a concentration of 100 cells/ml. The suspension should be devoid of aggregates as judged by examination with a microscope (see Note 16).
4. Prepare serial dilutions of the effector cells, making sure to change tips or pipettes and to resuspend cells carefully at each step of the dilution process, which is described in the following steps (see Note 17).
5. Using a multichannel pipette, 100 µl per well of the effector cell suspension is distributed in the first plate in which appropriate amounts of feeder/APC irradiated cells and antigen have been distributed. This plate will contain 10 effector cells/well.
6. Add 20 ml of complete TCGF- or IL-2-supplemented mouse RPMI medium to the remaining 10 ml of the effector cell suspension, mix and distribute 100 µl per well of the diluted cell suspension into two 96-well plates previously supplied with 100 µl feeder cell suspension. The effector cell concentration in these plates will be 3.3 cells per well.
7. Add 20 ml of medium to the remaining 10 ml of effector cells, mix gently and distribute the solution into the three remaining plates already containing feeder cells. These plates will contain 1.1 effector cells per well.
8. Some laboratories recommend to feed on a weekly basis the wells (partial change—half the volume—of RPMI mouse complete medium supplemented with TCGF or IL-2). In our experience, this is unnecessary. By contrast, wells should be examined regularly to identify as early as possible the growing wells, and the level of water in the incubator should be often checked (see Note 18).
9. In 2–3 weeks the clones should appear (see Note 19).
10. When the pellets of the growing wells become visible by naked eyes, transfer them in 24-well plates. Transfer the whole content of a growing well of the 96-well plates in one well of a 24-well plate filled with 2 ml of TCGF- or IL-2-supplemented RPMI mouse complete medium, with about 1 × 10⁶ irradiated

(20 Gy) syngeneic feeder/APC cells/well and peptide at a final 10^{-5} M concentration (see Note 20).

11. Wells of the 24-well plates should be examined daily and can be functionally tested (cytolytic or ELISPOT assay) when half-confluent. Generally, cells grow on one side of the well with a characteristic quarter lunar shape (see Note 21).
12. Clones exhibiting the expected function will be expanded in 25 cm² culture flasks containing 20 ml of TCGF- or IL-2-supplemented RPMI mouse complete medium, about 20×10^6 irradiated (20 Gy) syngeneic feeder/APC cells/well and peptide at a final 10^{-5} M concentration.
13. As soon as possible the selected clones should be frozen when in log-phase growth (from 1×10^6 to 5×10^6 per ml of ice cold freezing mixture, see Note 22) and place the tubes in a cotton wool isolated box in a -80°C freezer for 24 h and transfer the tube in a liquid nitrogen container the following day.

3.2. Cloning of Human CD8⁺ T Cells

The general principles and limiting dilution cloning strategy are common to human and mouse CD8⁺ T cell cloning: in vitro primary and secondary restimulation to expand the T cell clones of interest prior to limiting dilution cloning, availability of an appropriate functional assay to identify among all growing clones those with the desired reactivity. But human CD8⁺ T cell cloning has a number of specificities. For clarity reasons, the cloning of viral-specific human CD8⁺ T cell will be described as a prototypic procedure, the differences linked to other experimental conditions (antitumoral, autoimmune responses) are briefly described in Note 23.

3.2.1. Primary In Vitro T Cell Culture

FCS is replaced at least until clones have been obtained by human AB serum at a final concentration of 5–10%, in order to avoid the stimulation of FCS-reactive T cells (see Note 24).

1. Separate peripheral blood mononuclear cells (PBMC) the usual source of both T lymphocytes and Antigen Presenting Cells (APC) from red blood cells by centrifugation of donor heparinized peripheral blood in a 50 ml conical tube over a 10 ml layer of Ficoll-Hypaque ($1,200 \times g$, 30 min, room temperature, without brake) (see Note 25). Resuspend cells in 10 ml of RPMI complete human medium and pellet cells ($400 \times g$, 10 min, room temperature).
2. Put cells back in suspension in RPMI complete human medium, count cells (a 20 ml sample should provide about 4×10^7 T lymphocytes and APC cells) and proceed to the primary T cell culture.
3. Resuspend responder cells at 1×10^6 per ml in RPMI complete human medium and put in culture as soon as possible $15-20 \times 10^6$ responder cells per 25 cm² culture flask maintained in a vertical position in the CO₂ incubator.

4. Addition of the stimulating antigens

Depending on distinct parameters, virus pathogenicity, cell type targeted, ease of production, either wild-type viruses (i.e., influenza virus), recombinant or pseudo-typed viruses are used.

- Pellet by centrifugation ($350 \times g$, 5 min, room temperature) a fraction of the donor Ficoll-Hypaque-purified donor PBMC for a final stimulating/responder cell ratio of 1 to 2:10 and resuspend these cells in 1 ml serum-free RPMI medium (see Note 26).
 - Incubate these cells at 37°C for 1.5 h with appropriate amounts (see Note 27) of wild type or pseudotyped virus preparations. Then depending on viruses either add directly the stimulating cells to the responder culture flask (i.e., influenza viruses or pseudotyped viruses) or proceed first to virus inactivation (i.e., recombinant vaccinia viruses see Note 28).
5. Freeze as in Subheading 3.1.5, step 13 the unemployed donor PBMC (10×10^6 cells per ml of ice cold freezing mixture and per freezing tube).

3.2.2. Secondary In Vitro T Cell Culture

1. On day 7 thaw frozen autologous PBMC, wash them twice in 5 ml of RPMI complete human medium pelleting them down after each wash ($350 \times g$, 5 min, 4°C) and resuspend them in 1 ml of serum-free RPMI medium.
2. Incubate with viruses or pseudotyped viruses as in Subheading 3.2.1, step 4, inactivate when required (i.e., vaccinia virus) and in all cases irradiate at 20 Gy.
3. Collect cells from the primary culture, spin them down, resuspend in fresh RPMI complete human medium, count the living cells (Trypan Blue exclusion), add stimulator cells as in Subheading 3.2.1, step 4 at a stimulating/responder ratio of 1:10 and supplement culture medium with human IL-2 (see Note 3).
4. Seven days later evaluate whether the expected CD8⁺ T cell response has been obtained and is of significant magnitude (see Note 12), and accordingly the following day proceed to either a third restimulation of the bulk culture or clone by limiting dilution.

3.2.3. Limiting Dilution Cloning of Human CD8⁺ T Cells

The principle of limiting dilution cloning, as detailed for mouse CD8⁺ in Note 13, applies to human CD8⁺ T cells.

Similarly, cloning efficiency for CD8⁺ T lymphocytes (i.e., the number of cells growing versus the number, based on Trypan Blue exclusion, of viable cells seeded) is as a rule much lower than 1, therefore plates should be seeded with relatively high number of

cells per well and care should be taken to clone cells in active exponential growth (see Note 14).

All cell resuspensions and dilutions have to be performed in RPMI human complete medium supplemented with appropriate concentration of human IL-2 (see Note 3).

1. Prepare the stimulating cell population: Thaw, infect and irradiate autologous PBMC as in Subheading 3.2.1, step 4 and distribute 10^5 cells in 100 µl per well in six U-bottomed 96-well plates. As soon as the stimulating population is distributed, place the culture plates in the CO₂ incubator (see Note 15).
2. Collect now the responder cells by centrifugation (350 ×g, 5 min, room temperature), resuspend in few ml of IL-2-supplemented RPMI complete human medium and count.
3. Prepare an initial 20 ml suspension of responder cells at a concentration of 100 cells/ml, check by microscope examination the absence of cell aggregates and proceed to the distribution in U-bottomed 96-well plates and serial dilutions as detailed in Subheading 3.1.5. Section, steps 4–7 of the mouse CD8⁺ cloning protocol.
- 4–13. Steps 4–13 of the human CD8⁺ cloning protocol are similar to steps 4–13 of the mouse CD8⁺ cloning protocol as detailed in Subheading 3.1.5 with the four following differences.
 - (a) The RPMI complete human medium must be supplemented with human IL-2, and until clones have been isolated with human AB serum see Note 24.
 - (b) Ideally autologous or MHC compatible PBMC should be used as source of feeder/APC cells during the whole cloning process, however this is often a problem. To circumvent this problem, irradiated (100 Gy) autologous (see Subheading 3.2.4) or MHC-compatible EBV-transformed lymphoblastoid cells can be used (4×10^4 /well of a 96 well-plate) with additionally 10^5 /well allogeneic irradiated (20 Gy) PBMC see Note 29. Alternatively, several laboratories start to use artificial APC (9).
 - (c) Most laboratories further supplement the culture medium with mitogenic compounds or conditioned medium see Note 4 and Subheading 3.2.5.
 - (d) Most laboratories feed bi-weekly the wells exchanging half the volume of RPMI human complete medium supplemented with human IL-2 with on day 14 addition of 10^5 irradiated (20 Gy) ideally autologous PBMC.

3.2.4. Epstein-Barr Virus (EBV) Immortalization of B Lymphocytes (8)

1. Prepare supernatant containing EBV by transferring exponentially growing B95.8 cells (1×10^6 /ml) in fresh RPMI 1640 medium supplemented with 10% FCS 100 U/ml Penicillin, 100 µg/ml Streptomycin (RPMI complete medium).

2. Collect 3 days later the culture EBV-containing supernatant by centrifugation ($350 \times g$, 5 min, room temperature), filter on 0.45 μm pore-size filter and stored frozen ($-80^{\circ}C$) as aliquots of 2.5 ml (virus titration of such supernatant can be determined in standard EBV-transformation assay (10), they contain usually 10^2 to 10^3 transforming units/ml, or, as more recently described, by quantitative PCR (11)).
3. Infect Ficoll Hypaque-purified PBMC (10^7 in 2.5 ml of RPMI complete medium) with 2.5 ml of EBV-containing supernatant in a 50 ml conical tube and incubate for 2 h in a $37^{\circ}C$ water bath.
4. Transfer in a 25 cm^2 culture flask and add 5 ml of RPMI complete medium supplemented with Cyclosporine A see Note 30 (to prevent the activation of EBV-reactive T lymphocytes that would kill the EBV-transformed B lymphocytes) for a final 500 ng/ml concentration.
5. Usually, the immortalized lymphoblastoid cells forming large round clusters appear after 3–4 weeks.
6. As soon as the medium turns light pink-yellow as becoming slightly acidic, collect the cells by centrifugation ($350 \times g$, 5 min, room temperature), discard the used medium, dissociate gently the cell pellet, resuspend cells in RPMI complete medium and divide in three culture flasks.
7. About a week later, the cell line can be frozen as in Subheading 3.1.5, step 13, $1-5 \times 10^6$ /ml of ice-cold freezing mixture.

3.2.5. Preparation of Conditioned Medium from Human PBMC

1. Ficoll-Hypaque-purify PBMC from a blood bag (≈ 400 ml).
2. Wash twice in RPMI human complete medium ($400 \times g$, 10 min, room temperature).
3. Resuspend cells (333×10^6) in 170 ml of RPMI 1640 human complete medium are incubate overnight in horizontally maintained 150 cm^2 culture flasks.
4. Add the following day, 0.5×10^6 /ml irradiated (100 Gy) LAZ 509 EBV-transformed cells (12), 50 ng/ml of PMA, and 1 μg /ml of PHA in the culture flasks and replace the flasks in the CO_2 incubator for 3 h.
5. Then remove the un-adherent cells and supernatants, adherent cells are washed $\times 3$ with PBS 1 \times , and incubated in 170 ml of fresh RPMI 1640 human complete medium for 36 h.
6. Spin conditioned supernatants are then spun ($2,000 \times g$, 15 min, room temperature), filtered (0.22 μm), aliquot and freeze.
7. Titrate the conditioned medium in a PHA lymphoblast or CTLL-2 proliferation assay and compare to a reference batch of human IL-2. As a rule, this conditioned medium is used at a 1% final concentration to supplement the cloning medium.

4. Notes

1. Mitomycin C that cross-links irreversibly the complementary strands of the DNA double helix can be used to block cell division in the stimulating cell population. Cells are incubated 1 h at 37°C with Mitomycin at a 50 µg final concentration and then washed four times to totally remove the drug.
2. The selection of a FCS batch that support efficiently mouse T cell growth is achieved with the IL-2-dependant CTLL-2 cell line. CTLL-2 cells must be collected in active log-phase growth. After at least two washes in 50 ml of plain RPMI medium ($350 \times g$, 5 min, room temperature) to remove residual FCS and IL-2, cells are resuspended at 4×10^4 cells/ml and 50 µl distributed per well of a 96-well flat-bottom culture plate. Having determined in preliminary experiments with an arbitrary batch of FCS the concentration of human recombinant IL-2 that corresponds on day 3 of a culture of 2×10^3 CTLL-2 cells per well to 50% of the maximal proliferation, the different batches of FCS are tested comparatively at a final 10% concentration under the same conditions (RPMI 1640 medium, 5×10^{-5} M 2-mercaptoethanol, Penicillin, Streptomycin and the selected IL-2 concentration, 200 µl final culture volume per well) and the proliferation of the CTLL-2 cells is comparatively evaluated on day 3.
3. Mouse IL-2 is expensive, as a rule rat TCGF or human IL-2 are used for the cloning of mouse CD8⁺T lymphocytes. We used to prepare each time a large batch of TCGF from 20 rats (LOU or Wistar) since TCGF can be conserved at -20°C without decline in activity. Spleens are removed aseptically and cell suspensions prepared from each spleen immediately after its excision. Spleens are crushed in 10 ml of RPMI complete medium using a 100 mm diameter tissue culture dish with the flat end of a 10 ml syringe. Clumps of cells are disrupted by repeated up and down pipetting and large debris finally eliminated by filtration in a 50 ml sterile conical tube through a cell strainer. Cells (2.5×10^6 /ml) are cultured for 36 h in RPMI 1640 FCS 10%, 2-Mercaptoethanol 5×10^{-5} M, Penicillin, Streptomycin supplemented with 5 µg of Concanavalin A (large 150 cm² culture flasks in horizontal position). Supernatants are collected following centrifugation (15 min, $2,000 \times g$, 4–20°C) and Concanavalin A lectin activity competitively inhibited by the dissolution of 20 mg/ml of α-Methylmannoside. Forty ml aliquots are then frozen, each aliquot being again centrifuged before use (15 min, $2,000 \times g$, 4–20°C) to eliminate clumps of debris that constantly formed during the freezing/thawing process and to facilitate the final sterilization by filtration

through a 0.45 µm disposable filter. Titration of the TCGF is performed in a CTLL-2 assay, as described in see Note 2 and then used in cloning cultures at a final concentration (usually $\approx 10\%$) that insures 80% of CTLL-2 maximal proliferation. Recombinant human IL-2 can be obtained from different suppliers. Biological activity is either expressed in International or BRMP (Biological Response Modifier Program) units. One BRMP unit equals 6 International units. The usually recommended working final concentration is 10–15 BRMP U/ml. However, since we observed important differences in activity between human recombinant IL-2 of different origins, titration in a CTLL-2 assay is strongly recommended.

4. Once clones have been obtained, since their TCR cannot change by hypermutation, IL-2 supplemented RPMI complete human medium can be further supplemented with nonspecific mitogenic compounds such as PHA (1 µg/ml) or anti-CD3 mAb (i.e., 12 F6 mAb, 0.05 µg/ml). Conditioned medium from human PBMC, produced as detailed in Subheading 3.2.5, is further added by some laboratories to IL-2 supplemented RPMI complete human medium, as a source of cytokines produced by activated dendritic cells and macrophages.
5. In vivo priming of donor mice can be achieved in many ways. Injection of synthetic peptides is a simple and usually effective priming strategy but, since it by-passes all intracellular antigen processing steps, the induction of a CD8⁺ T cell response against a synthetic peptide does not prove that this peptide is effectively naturally processed. Effective processing can by contrast be established following DNA immunization whatever the route (muscle, skin) of DNA administration. Furthermore, in combination with peptide libraries, DNA immunization enables the identification of unknown epitopes in any candidate molecule. Mice can also be in vivo primed by viral infection, injection of pseudo-typed viruses, injection of transfected syngeneic tumor cells expressing the gene(s) of interest, primary in vitro restimulation and CD8⁺ T cell cloning being performed in a manner similar to what is detailed for human CD8⁺ T cell cloning in Subheading 3.2 and see Note 23. Finally, mouse in vivo priming can be achieved by injection of lipopeptides, injection of apoptotic bodies, and injection of in vitro matured peptide- or antigen-loaded dendritic cells from sex-matched syngeneic third party mouse, approaches which have all been validated in the literature.
6. The in vivo induction of CTL responses in the absence of CD4⁺ T cell help is usually unsuccessful due to IL-2 shortage (13). A helper peptide, known to be efficiently presented by the MHC class II molecules of the immunized mice, has in most cases to be co-injected to insure CD4⁺ T cells concomitant activation.

7. The quasi-general usage of IFA as adjuvant is more based on historical and ethical (14) than on scientifically grounded reasons: IFA induces Th2-dominated responses, whereas Th1-dominated responses are induced by CFA. Although the adjuvant effect of CFA for the induction of CTL responses has been shown, for ethical reasons it is advisable for CD8⁺ epitopes of limited immunogenicity to rather supplement the peptide/IFA emulsion with Poly-(I:C) (100 µg/mouse) or CpG oligodeoxynucleotides (30–100 µg/mouse) that bind Toll-like.
8. Cardiotoxin from cobra snake venom induces extensive muscle necrotic injury resulting in local inflammation and 2–3 days later intense myocyte regeneration creating conditions that favor the cellular uptake of the injected mammalian expression DNA vectors. To insure successful injection in the tibialis muscle that results in visible muscle swelling, it is convenient to place a tubing on the 27-gauge needle (i.e., the end of a 10 µl tip) covering all the needle except its very end (about 2 mm) in order to limit the penetration in the muscle.
9. DNA mammalian expression vectors can be obtained from different companies, make sure that expression of the gene of interest is driven by a strong ubiquitous promotor such as the Cytomegalovirus early promotor. N-terminal ubiquitination signals may be added in order to enhance proteasomal degradation. It may in some circumstances be wised to use a vector deprived of mammalian cell selecting cassette to ascertain that the induced responses are related to the gene of interest.
10. This cell suspension, beside T cells, contains B lymphocytes, dendritic cells, macrophages, and also contains red blood cells that there is no need to eliminate.
11. During the first in vitro restimulation, addition of TCGF or IL-2 is not recommended, in order to favor the selection of the cells specific for the antigen of interest that should be the only cells receiving through their TCRs the appropriate activation signal.
12. The stronger the CD8⁺ T cell response, the higher the probability of successful cloning. Whereas one can evaluate precisely by Interferon γ ELISPOT the frequency among the responder population of the functional effector cells and adapt accordingly the cloning conditions (number of cells/well, number of cloning plates), such evaluation is not possible with cytolytic assays, in our experience with mouse CD8⁺ T cells, below 50% of specific lysis at a 10/1 effector to target ratio, the chances to obtain clones are low.
13. The principle of Limiting dilution cloning is as follow: by seeding in a 96-well U-bottomed plate statistically 1 cell per well, other parameters (amount of antigen, amount of irradiated

syngeneic feeder/accessory cells, supplementation of the complete RPMI with either TCGF or IL-2) being optimal, according to the Poisson law, the percentage of wells having received no seeding cell should be 37%, that of wells having received 1 cell should be 37%, that of wells having received 2 cells should be 18 and 8% of the wells should have received 3 or more cells. One easily sees that the probability that a growing well corresponds to a true clone increases when the number of cells statistically seeded per well decreases. For a 0.3 cell/well seeding, the Poisson law predicts that 74% of wells will receive no cell, that 22% will receive 1 cell and that 4% will receive 2 or more cells. However, based on Trypan Blue exclusion evaluation of cell viability, the cloning efficiency (i.e., the number of cells growing versus the number of viable cells seeded) for CD8⁺ T lymphocytes is as a rule much lower than 1. Therefore it is highly advisable to seed plates with relatively high number of cells per well.

We routinely seed 1 96-well U-bottomed plate with 10 cells per well, 2 96-well U-bottomed plates with 10/3 cells per wells, and 3 96-well U-bottomed plates with 10/9 cells per well. Plates in which less than 30% of wells exhibit cell growth are likely to correspond to real T cell clones.

14. An active culture contains numerous large isolated lymphoblasts visible with an inverted microscope in the areas where the layer of feeder cells is less dense. An active culture contains also several clusters of lymphoblasts with a blackberry shape that disrupt the flatness of the layer of feeder cells. Another sign of activity is a change of color of the culture medium that turns light pink-yellow as becoming slightly acidic.
15. It is essential not to allow the medium to become red-purple (basic), a change in pH that occurs very rapidly with 96-well plates (small volume of medium per well, large exchange surface with the surrounding atmosphere) and it is strongly advised all plates to be in the incubator except the one which is worked on.
16. Care must first be taken to prepare a true unicellular suspension of the effector cells to be cloned. Cells sediment rapidly, therefore it is essential to shake by hand and homogenize cell suspension by inverting tubes before any pipetting.
17. To limit the risks of error, serial tenfold dilutions are recommended, favoring relatively large volumes (i.e., 500 µl in 4,500 µl).
18. Care should also be taken to check regularly the level of sterile water in the water recipient of the incubator, the small volume of medium in each well, the large semi-open surface of the 96-well plates will result in substantial loss of medium and death of the cloned cells in case of insufficient humidification.

19. Wells in which the cloned CD8⁺ T lymphocytes grow can easily be identified: first the feeder cell pellet which was lightly red initially due to the presence of red cells will lose this red color when proliferating T lymphocytes out-numbered the feeder cells; second under inverted microscopic examination, despite the U-bottomed shape of the wells, a dense ring of lymphoblasts with a typical banana shape can be seen at the periphery of a central mass of stacked cells (robust clones will form a continuous and wide disk of cells surrounding the central mass).
20. The 24-well plates can be stored in the CO₂ incubator and then used 2–3 days in a row since clones growing at different speeds cannot usually be all transferred the same day.
21. In our experience this stage is critical possibly as a consequence of the fact that the bottom of these 24-well plates is flat, and often 50% of clones are lost at this stage.
22. Activated T lymphocytes withstand less efficiently than most other cells to be frozen. To optimize the freezing-thawing process, we always used a pre-cooled (4°C) 90% of FCS 10% DMSO mixture and for thawing, a TCGF- or IL-2- supplemented RPMI mouse complete medium supplemented with 20% FCS.
23. For cloning anti-tumor CD8⁺ T cells, Tumor Infiltrating Lymphocytes or TIL can efficiently be recovered by mechanical fine mincing and collagenase treatment of a fragment of the tumor. Adaptation to *in vitro* growth of patient autologous tumor cells is sometimes facilitated by temporary subcutaneous engraftment of immunodeficient, ideally RAG/ γ c double KO mice.

For cloning autoimmune CD8⁺ T cells, CD8⁺ T cells can sometimes be directly obtained from the inflammatory site (e.g., joint liquid in rheumatoid arthritis) but if the organ is not accessible such as the pancreas, auto-reactive CD8⁺ T cells have to be isolated from the blood and they will be more abundant at early stages of the disease, when the auto-antigenic charge is still significant.

If ones aims at cloning human CD8⁺ T cells of known peptide and MHC class I molecule reactivity, whatever the nature of their target, (viral, tumor, or auto-antigen) *in vitro* restimulation with synthetic peptide in the presence of appropriate APC (as detailed for mouse CD8⁺ T cells but adapted to human, AB serum, human IL-2,...) should work. In fact, in such situations, pre-enrichment in specific CD8⁺ T cells with Class I MHC-peptide multimers (see Chapter 23) might even enable to proceed directly to cloning (15).

24. FCS proteins act as antigens for human T lymphocytes that might outgrow the T cells of interest. AB human sera are used since they are devoid of natural anti-blood group antibodies.
25. Whereas classically the heparinized 20 ml of blood are drop after drop laid on top of the Ficoll-Hypaque cushion, we found more convenient to inverse the process by introducing through a sterile Pasteur pipette the Ficoll-Hypaque at the bottom of the tube under the blood layer.
26. Since virus adsorption to cells may be inhibited by proteins of the FCS, the stimulating cells are incubated at 37°C with viruses or pseudotyped viruses in FCS-free RPMI medium.
27. Knowing the titer of a viral preparation and selecting Multiplicities of Infection (MOI, ratio of infectious agents to target cells) of 3 or 5 results theoretically in the infection of 95% or 99.3%, respectively, of the target cells. Titration of pseudotyped preparations leading to the integration of non-replicating genes are based on the dosage of selected structural protein of the pseudotyped particule (i.e., p24 in the case of HIV 1-based lentiviral vectors) and efficiency of transduction have to be empirically defined.
28. Recombinant vaccinia viruses following infection of stimulating cells will propagate to and kill responder cells in in vitro cultures unless made replicative-incompetent by Psoralen and UV treatment that cross-links the viral DNA strands.
29. Since the T cell receptor cannot change by somatic hypermutation, allogeneic feeder cells can be used in conjunction with PHA for efficient activation and proliferation of the clones in the respect of their antigenic specificity. Once clones have been obtained they can be maintained on allogeneic peripheral blood lymphocytes or allogeneic EBV-transformed lymphoblastoid cells in conjunction with PHA (1 µg/ml) and human IL-2 (100 IU/ml) for efficient activation and proliferation in the respect of their antigenic specificity (see Note 19).
30. Cyclosporine A prevents the activation of EBV-reactive T lymphocytes that would kill the EBV-transformed B lymphocytes.

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References

1. Murphy K, Travers P, Walport M (2008) Janeway's Immunobiology. Garland Science, New York
2. Morgan DA, Ruscetti FW, Gallo R (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* 193:1007–1008
3. Sette A, Fikes J (2003) Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Curr Opin Immunol* 15:461–470
4. June CH (2007) Adoptive T cell therapy for cancer in clinic. *J Clin Invest* 117:1466–1476
5. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA, Nishimura MI (1999) Efficient transfer of a tumor antigen reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol* 163:507–513
6. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314:126–129
7. Bevan M, Epstein R, Cohn M (1974) The effect of 2-mercaptoethanol on murine mixed lymphocyte cultures. *J Exp Med* 139:1025–1030
8. Miller G, Shope T, Lisco H, Stitt D, Lipman M (1972) Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. *Proc Natl Acad Sci U S A* 69:383–387
9. Ye Q, Loisiou M, Evine BL, Suhoski MM, Riley JL, June CH, Coukos G, Powell DJ Jr (2011) Engineered artificial antigen presenting cells facilitate direct and efficient expansion of tumor infiltrating lymphocytes. *J Transl Med* 9:131–143
10. Miller G, Lipman M (1973) Release of infectious Epstein-Barr virus by transformed Marmoset leukocytes. *Proc Natl Acad Sci U S A* 70:190–194
11. Fecenko-Tacka KL, Schina L, Beiswanger CB (2008) Quantitative analysis of Epstein-Barr virus supernatant by real-time PCR assay. *Cell Preserv Technol* 6:73–81
12. Meuer SC, Schlossman SF, Reinhertz EL (1982) Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc Natl Acad Sci U S A* 79:4395–4399
13. Fayolle C, Deriaud E, Leclerc C (1991) In vivo induction of cytotoxic T cell response by a free synthetic peptide requires CD4+ T cell help. *J Immunol* 147:4069–4073
14. Aichele P, Hengartner H, Zinkernagel RM, Schulz M (1990) Antiviral cytotoxic T cell response induced by in vivo priming with a free synthetic peptide. *J Exp Med* 171:1815–1820
15. Dunbar PR, Ogg GS, Chen J, Rust N, van der Bruggen P, Cerundolo V (1998) Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. *Curr Biol* 8:413–416

Chapter 22

Production of CD4⁺ and CD8⁺ T Cell Hybridomas

David H. Canaday

Abstract

T cell hybridomas are very useful tools to investigate antigen presenting cell (APC) function. They were developed based on the fusion technology that led to monoclonal antibody section. Antigen-specific primary T cells are generated and fused to an immortal thymoma line. Unfused thymoma cells are eliminated by engineered metabolic selection. Antigen-specific hybridomas are identified and may be characterized in detail. Primary T cells are preferable for studies of the regulatory mechanisms intrinsic to T cells, but for study of antigen presentation T cell hybridomas have advantages over primary T cell clones, including their relative uniformity, stability over time, and ready availability in large numbers for extensive antigen presentation experiments.

Key words: Antigen presentation, T cell hybridoma, Antigen processing, Macrophage, Dendritic cell

1. Introduction

Fusion of primary B cells with an immortal fusion partner was developed initially to generate B-cell hybridomas that lead to the development of monoclonal antibody technology (1). A similar process was applied to T cells to produce T cell hybridomas that become activated after T cell receptor (TCR) signaling and have some measurable function most commonly IL-2 production (2, 3). Since very few antibodies exist that recognize specific major histocompatibility (MHC)-peptide complexes on APC, determining levels of antigen presentation by APC necessarily must be done with a bioassay involving T cells (4). T cell hybridomas provide a quantitative measurement of specific MHC-peptide complexes on APC. Most hybridomas are relatively costimulation (CD80 and CD86) independent making them a tool to study pure antigen processing and presentation less affected by costimulatory signals that influence primary T cells.

T cell hybridomas have a number of advantages over the primary T cell lines that they were generated from. Most importantly, their specificity can be very carefully defined. The result is a highly specific, reliable, reproducible, and convenient T cell reagent that can be grown to large numbers for extensive antigen presentation studies. In contrast to primary T cells, T cell hybridomas function well recognizing antigen presented by fixed APC. Fixation creates essentially a total separation of antigen uptake, processing, and presentation pre-fixation and the post-fixation readout of antigen presentation by T cell hybridoma. Fixation of APC allows detailed kinetic studies, use of inhibitors of antigen processing that will not come in contact with the T cell hybridoma readout, and the ability to study antigen presentation of live pathogens such as bacteria and virus that will be sterilized with fixation. T cell hybridomas are not appropriate to study aspects of T cell differentiation or regulation. Also, they are not suitable for in vivo experiments because they produce tumors in mice.

The process of making T cell hybridomas involves generating an activated antigen-specific T cell line that is fused to a fusion partner that can immortalize the primary T cell. A number of technical advances over the years have been applied to ease production of T cell hybridoma clones and maximize success in generating an antigen-specific hybridoma. The widely used version of fusion partner (BW5147) is a T thymoma line, which cannot survive in hypoxanthine/aminopterin/thymidine (HAT) selection medium unless it has successfully fused with a primary T cell. Additionally optimized fusion partners do not express endogenous TCR so there is no chance for the fusion partner's TCR α and β chains to combine with the primary T cell's TCR (5). The most effective fusion partner to generate CD8+ T cell hybridomas has been transfected with the CD8 molecule to maintain high CD8 levels even after fusion (3, 6, 7).

2. Materials

1. Mice of appropriate MHC type for in vivo priming with desired antigen (see Note 1).
2. Adjuvant for in vivo immunization and priming. Complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) are used most commonly.
3. Antigen or peptide for immunization and in vitro stimulation (see Note 2).
4. Glass syringes and Popper micro-emulsifying needle with reinforcement or a 3-way stopcock to create emulsion.

5. 50% (w/v) Polyethylene glycol (PEG): PEG 1500 in 75 mM HEPES 50% (w/v) from Roche is recommended.
6. 6/24/96-Well plates.
7. T25 Flasks.
8. 1× PBS sterile.
9. Dissecting instruments (scissors and forceps).
10. Culture dishes.
11. 1 ml Syringe.
12. DMEM unsupplemented.
13. Culture medium: DMEM, 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercapto-ethanol, 1% nonessential amino acids, 10 mM HEPES, 100 U/ml Penicillin/Streptomycin, 10 mg/ml Ciprofloxacin (see Note 3).
14. Murine or human IL-2. Both work well.
15. HAT and hypoxanthine/thymidine (HT) (see Note 4).
16. BW5147 fusion partner called BW5147.G.1.4 (ATCC, TIB-48). Other derivative lines such as BW1100 or BW5147-CD8 transfected can also be used but are not commercially available (see Notes 5 and 6).
17. APC in the same MHC restriction as the mouse at immunization. Primary splenocytes or bone marrow-derived macrophages (BMM) used most commonly.
18. Murine IL-2 detection system, either ELISA or CTLL-2 Bioassay (see Note 7).
19. Freeze medium: 90% FCS, 10% DMSO.
20. Freezing container (Nalgene Mr. Frosty) or vermiculite containing box.
21. Water bath that can fit in the hood or flask with water at 37°C.

3. Methods

3.1. Generate Antigen-Specific T Cells

1. Adjuvant antigen mixture preparation. For peptides use 5–20 µg/mouse in IFA. For soluble antigen use 10–25 mg/mouse in CFA. We use a 16 or 20 G Popper micro-emulsifying needle with reinforcement with glass syringes on each side to prepare the adjuvant. Make a 50/50 mixture of CFA or IFA and antigen or peptide in PBS. Plan on immunizing each mouse 50 µl of the adjuvant/antigen mixture. Draw up equal volumes of CFA or IFA in one syringe and antigen in PBS in the other. Screw each syringe onto the metal tube and inject it back and forth for 2–20 min to generate a firm emulsion (see Note 8).

2. Immunize mice in an area with a clear lymphatic drainage. Footpad or base of tail are the most common sites. Immunize with about 50 μ l of adjuvant/antigen per site (see Note 9).
3. At day 7, harvest the draining lymph nodes. For footpad harvest the popliteal lymph nodes. For base of tail injection harvest the inguinal lymph nodes.
4. Add the lymph nodes to 3–4 ml of DMEM in a small tissue culture dish. The lymph nodes are crushed against the bottom of the dish to free the cells. We use the back flat part of a disposable sterile 1 ml syringe that is normally where the thumb presses down to inject. Make sure each chunk of lymph node gets crushed against the bottom of the dish two or three times.
5. Harvest the cell containing supernatant and leave any tissue chunks behind.
6. Spin the cells at $200 \times g$ for 10 min and resuspend them in complete medium.
7. Culture the cells in 24-well plates at 5–8 million cells per well with 1–10 μ g/ml of antigen or peptide (see Notes 10 and 11).
8. On the day of harvest of the lymph nodes, thaw the fusion partner and begin to expand this up so it is ready for fusion in 5 days. If you will have a massive amount lymph node cells ($>10^7$ cells from immunization of over four mice) then thaw the cells prior to the lymph node harvest day to grow them to sufficient quantity.
9. At day 4, add 10–20 U/ml of IL-2 to each restimulation well.
10. At day 5, harvest the cells, spin them at $200 \times g$ for 10 min, and resuspend them in DMEM for counting.

3.2. Fusion (Day 5)

1. On the day of the fusion, warm 1 ml of PEG, 20 ml of DMEM, and 30 ml of complete medium at 37°C. Take several serologic pipettes (1 and 5 ml sizes) and put them in the incubator. Have a small water bath at 37°C or an Erlenmeyer flask of water at 37°C that can fit inside of the hood. After all of these components are at 37°C begin to prepare the fusion partner.
2. Spin down and count the fusion partner that was being expanded for at least the last 5 days.
3. Add lymph node cells from above and fusion partner in a 1:1 ratio to a 50 ml conical tube and spin the cells.
4. Aspirate the supernatant carefully to have the pellet be as dry as possible without aspirating the cells.
5. Prepare a timer to count 2, 5, and 2 min.
6. Add 0.4 ml of warm PEG to the cell pellet very slowly drop wise over 2 min. Have the bottom of the tube in the warm water bath or flask so that the whole fusion takes place at 37°C.

Gently mix the cells by stirring stir between drops with the PEG containing pipette.

7. Bring up 2 ml of warm DMEM in a warm pipette and add it very slowly drop wise to the suspension over 5 min with stirring between drops.
8. Next draw up 5 ml of DMEM and add it drop wise over 2 min while stirring.
9. Spin the cells gently at 200×*g* for 10 min.
10. After aspiration of supernatant, draw up 10 ml of complete medium in a pipette and tap the tube at the tip while adding 1–2 ml at a time of medium initially to loosen the pellet and then resuspend it. If there are still chunks of cells present after adding the first 10 ml then slowly pipette up and down once to help resuspend the cells. Finish by adding 20 ml more of complete medium.
11. Put the cells in a 37°C water bath to recover.
12. After 1 h perform serial fivefold dilution of the cells by taking 6 ml of the preceding dilution into 24 ml of complete medium to create the 1:5, 1:25, and 1:125 dilutions.
13. Label 8 *round bottom* 96-well plates with two plates for each dilution (neat, 1:5, 1:25, and 1:125).
14. Pipette 100 µl per well to fill the plates for each dilution.
15. The next day add 100 µl of 2× HAT solution in complete medium to every well.

3.3. Harvest the T Cell Hybridomas

1. Starting at day 7 begin to check the hybridoma plates. Hold the 96-well plates above your head to observe the pellet size. The cells were plated in round wells to allow a very quick visual screening to determine which wells are ready for harvesting and which wells need more time or have no growth. Wells that have a pellet size of 2–3 mm or that the medium is beginning to change color should be marked on the bottom of the well with a dark marker (see Notes 12 and 13).
2. Pipette up and down the marked wells and transfer them into 24-well plates. Most importantly mark on the plate lid what dilution the hybridomas came from.
3. Add 1 ml per well of 1× HT in complete medium to the 24-well plates.
4. Continue to harvest the 96-well plates daily for the next 2 days.

3.4. Screening

1. After 2 or 3 days the 24-well plates are ready for screening. The wells will be 30–80% confluent which is often at or near the time when the medium begins to change color.

2. Prepare APC for the screening. Splenocytes (2×10^5 /well) can be used fresh or cultured in complete medium in 96-well plates for 1–2 days in advance to have them ready for the successive days of screening. If BMM (5×10^5 /well) are used, they need to be treated with IFN-gamma at least 1 day prior to screening to upregulate MHC-II levels. Irradiation is not needed for any APC in this 1-day assay.
3. Open both your plates with APC and hybridomas place them side by side. Pipette up and down twice with a 1 ml pipette one row at a time from the 24-well plate. Use a new tip with every well to not cross-contaminate hybridomas.
4. Add 100 μ l of hybridomas to up and down rows in your APC plate from the 6 wells that were just resuspended. For example, the first wells are A1 and B1, second A2, B2, etc. The top row will be the no antigen row and the row beneath will be the antigen row. You can therefore screen 2 full 24-well hybridomas containing plates with a single 96-well plate of APC.
5. Add complete medium to the top row and antigen to the second row to have 200 μ l volume in all wells for the overnight antigen presentation assay.
6. Take the 24-well plates with the hybridomas in them and aspirate the cells and media to near empty then refill them with HT containing medium to keep them alive while you await the screening results to determine which wells have promising hybridomas in them.
7. After 20–24 h harvest 100 μ l of supernatant from each screening plate to 96-well plates.
8. Assay for IL-2 production as soon as possible. If IL-2 ELISAs will be performed, do them that day or the next. If CTL-L2 are used then start this right away. The urgency is that you have to keep the hybridomas in the 24-well plates alive while you await the screening results. The 24-well plates have to be examined daily and media and cells aspirated and refed with HT containing complete medium to keep them from getting too dense. T cell hybridomas apoptose fairly readily when they overgrow. One day of overgrowth may kill the lines (see Note 14).
9. The screening is not an exact process as precise numbers of hybridomas per well were not determined and it was only a singlet assay. All wells with IL-2 signal over background are then expanded up to 6-well plates or T25 flasks for a secondary screening to confirm antigen specificity and determine the sensitivity of the lines.

3.5. Secondary Screening

1. Monitor harvested hybridomas in 6-well plates or T25 flasks until they are 50–80% confluent then spin them down and count.
2. Perform antigen presentation assays as described above except set up the wells in duplicate or triplicate (1×10^5 hybridoma/well)

and use a series of concentrations of antigen to determine which lines are most sensitive.

3. If the most sensitive lines came from 96-well plates that had over 10 wells that grew then perform subcloning.
 4. Freeze down several vials of the most promising lines at the time of subcloning to have backup (see Subheading 3.7).
-
1. Spin down cells and count. Dilute the cells and make two plates of 1, 0.3, and 0.1 hybridoma/well in round bottom 96-well plates.
 2. Monitor the plates as growth takes 5–10 days at this point. Select subclones from plates that have ten hybridomas growing or less. Expand them up in T25 flasks when the pellet button is in the 2–3 mm range.
 3. Screen the clones as above and cryopreserve the antigen-specific subclones (see Note 15).

3.7. Cryopreservation of T Hybridomas

1. Spin the cells and resuspend in 1 ml freeze medium per vial to have 1–10 million cells per vial (see Note 16).
2. Place in Cryo-baby or box of vermiculite.
3. Transfer to liquid nitrogen within 2 weeks.

4. Notes

1. Standard commercially available mouse strains such as C57BL/6 or BALB are used most commonly to generate T cells that recognize antigens presented by murine APC. For human studies we and others have used HLA-transgenic mice to generate HLA-restricted T cell hybridomas that readily recognize human APC. The key to this is obtaining the HLA-transgenic mice. Several HLA-A and HLA-B expressing transgenic mice are available from Taconic and Jackson Laboratories. An HLA-DR4 transgenic mouse is available from Taconic. Transgenic mice expressing HLA-DRI, DR15, and DR3 have also been generated by other laboratories (8–10). We have used these mice to generate murine T cell hybridomas that respond readily to a range of human APC from monocytes, B cells, and dendritic cells (11–13). The methods to generate these HLA-restricted T cell hybridomas are identical to those described above with only one significant change. We use human APC for the screening after the fusion. This is important because hybridomas not capable of recognizing human APC presented antigen or that have a significant allogenic response are not useful. We use HLA-matched EBV-transformed B cells as the screening APC.

2. Plan on 5–25 µg per animal for the immunization depending on the robustness of the antigen and immune response expected. Some antigen/adjuvant is lost in preparation of the emulsion so extra must be made.
3. Ciprofloxacin is added to minimize mycoplasma contamination that can be a significant risk factor in the HAT and HT selection process.
4. We use fresh HAT every time as it is not that expensive and it comes in single vials (Sigma) that are resuspended at time of use. The HAT is very important to successful generation of hybridomas because the unfused fusion partner must die before the harvest process begins at days 7–10.
5. Although we suggest the ATCC BW-5147 basic line, it is not the optimal line to use for hybridoma generation. It does not have all of the TCR genes deleted so it is possible to have some recombinations that do not produce antigen-specific T cells. We use a derivative of BW5147 that has the TCR genes eliminated called BW1100. If this is not available or cannot be obtained collaboratively, the basic ATCC BW-5147 generally works reasonably well however.
6. For MHC-I hybridomas it has been shown that having a CD8-transfected BW5147 line increases the frequency of MHC-I-restricted lines generated (3). At least three groups have developed CD8-transfected BW5147 to optimally generate CD8 expressing T cell hybridomas (3, 6, 7). The standard BW5147 does not support continued expression of CD8 after fusion.
7. For the IL-2 measurement there are several techniques possible. We prefer CTLL-2 cells during the development of the hybridomas. We use a colorimetric readout rather than H³ thymidine for this assay as well (11, 14, 15). CTLL-2 cells can be obtained from ATCC (TIB-214). Some care and time have to be used to keep them successfully in passage as they are an IL-2-dependent cell line. The advantage is that they are inexpensive to use and require a few short bits of time to spin and add them to your supernatants rather than more expensive and time intensive ELISA assays. The amount of IL-2 generated from hybridomas is fairly significant in the 300–4,000 pg/ml range so a highly sensitive IL-2 ELISA is not necessary. For that reason we use a “homebrew” IL-2 ELISA using the recommended IL-2 antibody pair from EBiosciences. Many suppliers sell such antibody pairs. Standard commercial IL-2 ELISA kits are also widely available.
8. Be careful to make sure the syringe is tight onto the metal lock. If a stiff emulsion is very slow to develop then put the syringes at 4°C for 20–60 min and continue mixing. This cooling step

can facilitate development of a proper emulsion. When the emulsion is ready, it will be much harder to push through the metal tube. This condition often develops quite suddenly. Plastic syringes should be avoided as the black rubber breaks down and turns the emulsion gray, and also they are prone to leak. To test the emulsion, squeeze a tiny amount onto water in a Petri dish. There should be minimal or no oil leakage from the white emulsion. If there is a lot of oil leakage then keep squirting back and forth until it solidifies.

9. We use the popliteal lymph node as our preferred site of immunization. This means immunization generally with CFA into the footpad. We do not boost after this type of immunization. We have never seen a foot ulcer after hundreds of immunizations over the years. If your IACUC is concerned over footpad injections with CFA, a few options can be considered. A commonly used option is base of the tail immunization. Another possible site if you still want to harvest popliteal lymph node is to employ hock (lower leg below the knee) immunization. This has been shown to be the same as footpad injection (16).
10. It is important to have an activated antigen-specific cell line for fusion. The key reason for failure to generate antigen-specific hybridomas once you master the physical techniques is fusion with a poor primary T cell line. For very low precursor frequency T cells it may be necessary to immunize and boost *in vivo*. Another option we have used for MHC-I restricted lines is to do two rounds of *in vitro* restimulation. If this is planned, an enormous amount of cell death after the second round of restimulation should be expected. In spite of this, we have had success with fusion of less than a million cells in the past.
11. It is possible to screen the lines before fusion if there is concern that there will be very few antigen-specific cells. We have used intracytoplasmic staining most often in the past to perform this task (11). If we had on the order of 0.5–1% antigen-specific cells, generally this still lead to a successful fusion.
12. The investigator has to decide how many wells they plan to harvest for screening. Ideally hybridomas from plates that have 10 wells or less that grow on that dilution are more likely to be clonal. If it is uncertain how good of an immunization and T cell line were generated, it is better to err on the side of screening more wells. In a typical fusion we will harvest 100–200 wells total over 3 days for screening.
13. The harvesting and screenings of the T cell hybridomas requires nearly 2 weeks of daily work.
14. It is important not to allow the T cell hybridomas to overgrow as they apoptose and will visually develop a lot of vacuolization

and blebbing. Sometimes they can be passed and salvaged, but often lines are lost if they grow too thick.

15. The vast majority of T cell hybridoma clones we have generated are able to maintain their antigen specificity and sensitivity for several months in continuous culture.
16. If experiments with T cell hybridomas are planned intermittently, then large vials of 8–15 million cells per vial can be cryopreserved. The hybridoma vials can be thawed at the time of use and added in the typical 1×10^5 cells per well for antigen presentation assays. In preliminary work for several published manuscripts, we determined that the sensitivity of the freshly thawed hybridomas compared to hybridomas in continuous culture was similar (12, 17).

Acknowledgment

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References

1. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256(5517):495–497
2. Kappler JW, Skidmore B, White J, Marrack P (1981) Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med* 153(5):1198–1214
3. Rock KL, Rothstein L, Gamble S (1990) Generation of class I MHC-restricted T-T hybridomas. *J Immunol* 145(3):804–811
4. Zhong G, Reis e Sousa C, Germain RN (1997) Production, specificity, and functionality of monoclonal antibodies to specific peptide-major histocompatibility complex class II complexes formed by processing of exogenous protein. *Proc Natl Acad Sci USA* 94(25):13856–13861
5. Born W, White J, O'Brien R, Kubo R (1988) Development of T cell receptor expression: studies using T cell hybridomas. *Immunol Res* 7(4):279–291
6. Burgert HG, White J, Weltzien HU, Marrack P, Kappler JW (1989) Reactivity of V beta 17a+CD8+ T cell hybrids. Analysis using a new CD8+ T cell fusion partner. *J Exp Med* 170(6):1887–1904
7. Kanagawa O, Maki R (1989) Inhibition of MHC class II-restricted T cell response by Lyt-2 alloantigen. *J Exp Med* 170(3):901–912
8. Rosloniec EF, Brand DD, Myers LK, Whittington KB, Gumanovskaya M, Zaller DM, Woods A, Altmann DM, Stuart JM, Kang AH (1997) An HLA-DRI transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. *J Exp Med* 185(6):1113–1122
9. Kong YC, Lomo LC, Motte RW, Giraldo AA, Baisch J, Strauss G, Hammerling GJ, David CS (1996) HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene. *J Exp Med* 184(3):1167–1172
10. Finn TP, Jones RE, Rich C, Dahan R, Link J, David CS, Chou YK, Offner H, Vandenbergk AA (2004) HLA-DRB1*1501 risk association in multiple sclerosis may not be related to presentation of myelin epitopes. *J Neurosci Res* 78(1):100–114. doi:[10.1002/jnr.20227](https://doi.org/10.1002/jnr.20227)
11. Canaday DH, Gehring A, Leonard EG, Eilertson B, Schreiber JR, Harding CV, Boom WH (2003) T-cell hybridomas from HLA-transgenic mice as tools for analysis of human antigen processing. *J Immunol Methods* 281(1–2):129–142

12. Woc-Colburn L, Smulcea L, Ramachandra L, Canaday DH (2010) Preserved MHC class II antigen processing in monocytes from HIV-infected individuals. *PLoS One* 5(3):e9491. doi:[10.1371/journal.pone.0009491](https://doi.org/10.1371/journal.pone.0009491)
13. Jones L, McDonald D, Canaday DH (2007) Rapid MHC-II antigen presentation of HIV type 1 by human dendritic cells. *AIDS Res Hum Retroviruses* 23(6):812–816
14. Ahmed SA, Gogal RM Jr, Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [³H]thymidine incorporation assay. *J Immunol Methods* 170(2):211–224
15. Kwack K, Lynch RG (2000) A new non-radioactive method for IL-2 bioassay. *Mol Cells* 10(5):575–578
16. Kamala T (2007) Hock immunization: a humane alternative to mouse footpad injections. *J Immunol Methods* 328(1–2):204–214. doi:[S0022-1759\(07\)00248-7](https://doi.org/S0022-1759(07)00248-7) [pii] [10.1016/j.jim.2007.08.004](https://doi.org/10.1016/j.jim.2007.08.004)
17. Canaday DH, Burant CJ, Jones L, Aung H, Woc-Colburn L, Anthony DD (2011) Preserved MHC-II antigen processing and presentation function in chronic HCV infection. *Cell Immunol* 266(2):187–191. doi:[S0008-8749\(10\)00259-5](https://doi.org/S0008-8749(10)00259-5) [pii] [10.1016/j.cellimm.2010.10.003](https://doi.org/10.1016/j.cellimm.2010.10.003)

Chapter 23

Tracking Antigen-Specific CD8⁺ T Cells Using MHC Class I Multimers

**Cécile Alanio, Isabelle Bouvier, Hélène Jusforgues-Saklani,
and Matthew L. Albert**

Abstract

The tracking of epitope-specific T cells is a useful approach for the study of adaptive immune responses. This protocol describes how Major Histocompatibility Complex Class I (MHC-I) multimers can be used to stain, enrich, and enumerate (rare) populations of CD8⁺ T cells specific for a given antigen. It provides the detailed steps for multimer labeling, magnetic enrichment, and cytometric analysis. Additionally, it provides informations for multiplexing experiments in order to achieve simultaneous detection of multiple antigenic specificities, and strategies for coupling the protocol with functional assays (e.g., intracellular cytokine staining). Future developments in cytometric systems (e.g., mass spectroscopy-based cytometry) and gene expression studies (e.g., single cell PCR) will extend these approaches and provide an unprecedented assessment of the immune repertoire.

Key words: CD8⁺ T cells, MHC Class I multimers, Antigen-specific T cells, T-cell receptor

1. Introduction

Mature CD8⁺ T lymphocytes bear αβ T cell receptors (TCR) that are specific for a major histocompatibility complex (MHC) class I molecule bound to a unique peptide. A major goal in the study of adaptive immune responses is to understand the developmental progression of antigen-specific T cells from naive precursors to activated effector cells and long-lived memory cells (1, 2). Prior to 1996, limiting dilution analysis was the standard method for estimating the frequency of antigen-specific T cells. The major limitation of this approach is the requirement for exogenous stimulation and expansion, introducing potential bias and significant inter-assay variability. Notably, cloning efficiency is typically <40%, suggesting

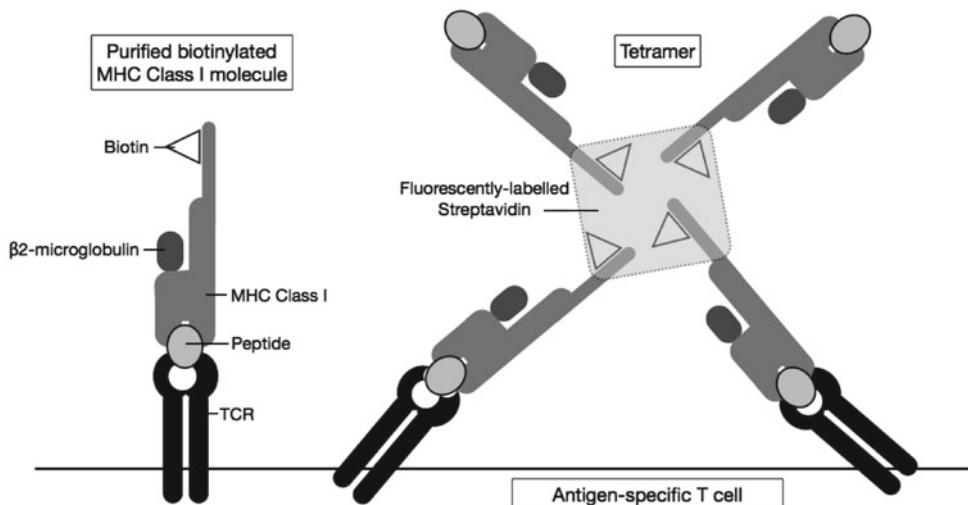


Fig. 1. Schematic representation of purified biotinylated MHC Class I molecule (*left*) and multimer (*right*) (adapted from Klenerman et al. [9]).

that the assay necessarily underestimates precursor frequencies (3). Other techniques such as ELISpot or intracellular cytokine staining (ICS) are based on the ability of antigen-specific T cells to secrete cytokines upon short *in vitro* restimulation with the cognate peptide (4, 5). Such approaches identify lymphocytes possessing the capacity to secrete a given cytokine at the time of the assay, however this represents only a fraction of the antigen-specific population(s).

The generation of MHC class I tetrameric or multimeric complexes (referred to herein as MHC multimers), originally described by Altman and Davis (6, 7), represents a major technical advance for the study of T cell repertoires. MHC multimers are reagents that carry multiple MHC Class I/peptide (MHC-I/pep) complexes, and thus have the ability to interact with multiple TCRs on a single CD8⁺ T cell (Fig. 1). Fluorescent-labeling of MHC multimers permits identification of antigen-specific T lymphocytes based on the avidity of their TCR, independent of their functional or differentiation state. This technology has been recently reviewed by Davis, Altman, and Newell (8). Using MHC multimers, it is now possible to directly track and quantitate antigen-specific T cells during the course of immunization (9). And by co-staining with antibodies directed against phenotypic cell surface proteins, one can define subsets of cells of interest based on their activation or differentiation state, or chemokine receptors expression (10). MHC multimer technology has also been successfully coupled to conventional functional assays (e.g., CFSE dilution, ICS), and specific T cells can be sorted for ELISpot, cytotoxicity, gene expression studies or for generating long-term cultures (11). Multimers are also widely used in the immune monitoring of T cell responses

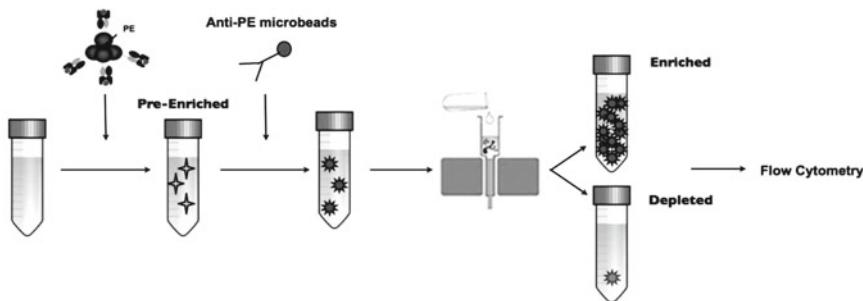


Fig. 2. Schematic representation of the enrichment procedure. Starting from cellular suspension, cells are stained with PE-labeled multimer, then incubated with antiPE-microbeads before loading on a MACS column. Flow through is the Depleted fraction. By removing the column, you then have access to your Enriched fraction, containing increased numbers of multimer-positive cells.

following therapeutic or prophylactic vaccination. Finally, the recent availability of GMP-grade multimers is enabling the ex vivo expansion of T cells for immunotherapy (12–15).

Several key improvements have been reported since the initial description of multimer technology (8, 16, 17). First, as multimerization is the key to overcoming the relatively low intrinsic affinity of TCR/MHC interaction, MHC-I multimers now exist as tetramers, pentamers, and dextramers (with the latter containing >10 MHC I/pep complexes). Monomer production has also been substantially optimized. Most notable is the work of Schumacher and colleagues, who demonstrated the possibility to generate high-throughput production of MHC I/pep complexes using a photo-destructible peptide that permits an exchange reaction with peptides of interest (18). Additionally, the implementation of a dump channel, dual tetramer labeling and multiplexing have all helped establish a robust foundation for translating this technology into the clinics (18–21). Nonetheless, there exist remaining technical limitations: staining methods, analysis protocols, validation and data sharing have to be standardized (22); and the limit of detection for standard multimer assays is 10^{-4} , which does not allow for direct detection of rare antigen-specific populations such as naive ones (23).

In order to improve the limit of detection, MHC multimer staining has recently been combined with magnetic bead enrichment (24), a concept initially developed in mice for assessment of CD4⁺ T and CD8⁺ T cells (25–27). Following from these studies, our lab, as well as others, developed a similar enrichment protocol for human CD8⁺ T cells (Fig. 2) (23, 28). Efforts have been made to standardize the procedure—herein described in details—and to optimize any details in order to achieve sufficient sensitivity to allow detection of naive antigen-specific T cells from human peripheral blood. This protocol permits up to 100-fold increased detection of antigen-specific populations, allowing assessment of populations with frequencies as low as 10^{-6} . As such, it is now

possible to characterize the naive T cell repertoire, opening up new opportunities for defining how T cells are selected, as well as to investigate aspects of their homeostasis (29). These approaches may also serve as powerful strategies for tracking rare antigen-experienced self-, tumor-, transplant- or microbe-specific T cells, either in mice or in humans, in turn providing insight into parameters that shape immune T-cell responses. This unit describes our method for labeling antigen-specific CD8⁺ T cells obtained from mice or human peripheral blood with MHC class I multimers, for enriching and enumerating them, and eventually multiplexing the assay and/or coupling it to ICS procedure. Future developments in cytometric systems (e.g., mass spectroscopy-based cytometry) and gene expression studies (e.g., single cell PCR) will further extend these approaches and provide an unprecedented look at the immune repertoire (8, 11).

2. Materials

2.1. Common Reagents

1. Fresh or frozen sample (for mice, see Subheading 3.1; for humans, prepare PBMCs according to standard procedures (see Note 1)).
2. For humans studies only: fluorescently labeled mAb specific for MHC class I molecules of interest, suitable for flow cytometry (e.g., anti-human HLA A2 antibody, BD Biosciences), and corresponding isotype (see Note 2).
3. 60-mm Petri dish.
4. Falcon 15 mL tube.
5. 5 mL FACS tubes.
6. FcR blocking reagents.
7. Anti-PE microbeads (see Note 3).
8. MACS separation columns, magnets, stands (see Note 4).
9. BD Falcon Cell Strainer 70 µm.
10. More than five-color flow cytometer, ideally with possibility to cell sorting.

2.2. Buffers

1. PBS.
2. PBS–2% FCS.
3. Human Pulldown Buffer (HPB; ~50 mL for one enrichment sample): PBS 1×, 5% of Human Serum Albumin 20% (final concentration 1%), 5% Citrate Dextrose Anticoagulant (see Note 5).
4. Mice Pulldown Buffer (MPB): PBS 1×, 2% FCS, 0.001% Sodium Azide.

5. Mice Pulldown Buffer (MPB) without azide.
6. Mice R-10 buffer: RMPI, 10% fetal calf serum, 10 mM HEPES, 1× nonessential amino acids, 1 mM sodium pyruvate, 60 nM 2-mercaptoethanol, 20 ng/mL gentamycin.

2.3. Flow Cytometry

1. PE- and/or APC-labeled MHC class I multimers (see Notes 6 and 7).
2. For multiplexing experiments (determination of multiple specificities in one single tube), biotinylated monomers (see Note 8) and streptavidin coupled to fluorochrome or reporter of interest (PE-, APC-, PE-Cy7-, APC-Cy7-, Qdots-streptavidin, 1 mg/mL).
3. Cocktail of fluorescently labeled mAb that are known to be expressed on cells you wish to exclude from analysis (e.g., monocytes, B cells, and NK cells) (see Note 9). These mAb should be coupled to a common fluorochrome, for example Pacific Blue, thus giving a positive signal in one fluorescent channel, which will be referred to as the “dump channel” in our gating strategy (see Subheading 3.5 and Fig. 5a).
4. Viability marker that will specifically stain dead cells (e.g., DAPI Nucleic Acid Stain, Invitrogen) (see Note 10).
5. Fluorescently labeled mAbs including at least an anti-CD8 antibody. Others will be chosen depending on the desired phenotypic characterization of target T cells (see Note 11).
6. For ICS in mice: CpG formulated with DOTAP, and specific peptide for in vivo restimulation.
7. For ICS, BD Cytofix/Cytoperm Fixation/Permeabilization solution kit with BD GolgiPlug containing Brefeldin A (BD biosciences).
8. For ICS, LIVE/DEAD fixable dead cell stain kit such as Aqua (Invitrogen) (see Note 12).

3. Methods

Please note that we describe in this section both mice and human protocols, and the reader should be careful to utilize the appropriate reagents and buffers.

For human experimentation, you will start with HLA typing (Subheading 3.2), then stain with multimer(s) (Subheading 3.3), with the option to perform enrichment (Subheading 3.4), and finally acquire your samples on flow cytometer (Subheading 3.5) and evaluate precursor frequency (Subheading 3.6).

For mice studies, you will start with mice dissection (Subheading 3.1), then stain with multimer(s) (Subheading 3.3),

and optionally continue by enrichment (Subheading 3.4), and/or ICS (Subheading 3.7). In all cases you will acquire your samples on a flow cytometer (Subheading 3.5), and evaluate precursor frequency (Subheading 3.6).

3.1. Mice Dissection

1. Harvest 15 lymph nodes (2 inguinal, 2 axillary, 2 brachial, 4 cervical-deep and superficial, 2 peri-aortic, and the mesenteric chain) and the spleen in a 60 mm-Petri dish containing 2 mL of Mice R-10 buffer.
2. Mash the organs and transfer the cells into a Falcon 15 mL tube after filtering the cell suspension with a 70 μm cell strainer.
3. Wash the well with 3×1 mL Mice R-10 to recover the maximum of cells.
4. Add 10 mL of Mouse Pulldown Buffer (MPB), count them, spin down at $300 \times g$ for 5 min at 4°C , and go to Subheading 3.3.

3.2. HLA Typing (Human)

1. Generic haplotyping of the sample can be easily achieved by flow cytometry, and is sufficient for most multimer uses.
2. Count PBMCs and resuspend in PBS at 10^7 cells/mL.
3. Dispense 2×50 μL of this solution into 5 mL FACS tubes. The remaining cells will be spun down ($300 \times g$, 5 min, 4°) and used for multimer staining (Subheading 3.3).
4. Add either isotype or anti-HLA antibody titrated to the optimal concentration to each FACS tube (optimal $C_f = 1/400$ in our hands, meaning that you put 1 μL of a solution diluted 1/8 in 50 μL staining volume).
5. Incubate for 15 min at 4°C in the dark.
6. Wash cells once at $300 \times g$ for 5 min at 4°C and resuspend in 100 μL of PBS.
7. Acquire these 100 μL in flow cytometry (Fig. 3).

3.3. Multimer Staining

1. Use cells prepared as detailed above, Subheadings 3.1 (mice) or 3.2 (human). Resuspend cells in cold Pulldown Buffer (MPB for mice or HBP for human, hereafter referred as PB), and aliquot defined numbers of cells in Falcon 15 mL tubes (one for each specificity) (see Note 13).
2. Wash once in PB ($300 \times g$, 5 min, 4°) and resuspend each sample in 90 μL cold PB.
3. Add 10 μL of FcR Blocking Reagent to each tube. Vortex.
4. Incubate 10 min at 4°C .
5. Add PE MHCI multimer and APC MHCI multimer of the same specificity at the appropriate concentration (see Notes 14 and 15, and Fig. 4).

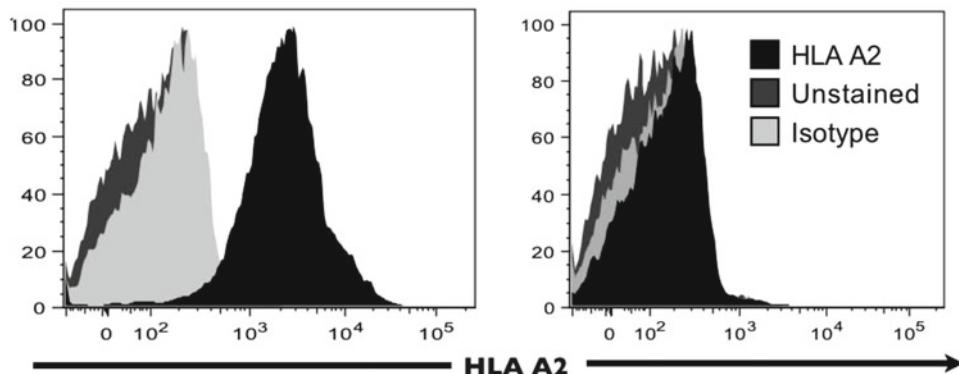


Fig. 3. Flow cytometry based-HLA typing (Human). PBMCs are prepared, then incubated with either isotype or anti-HLA antibody of interest titrated to the optimal concentration. Histograms represent data obtained from one HLA-A2 positive (*left*) and one HLA-A2 negative (*right*) blood donors.

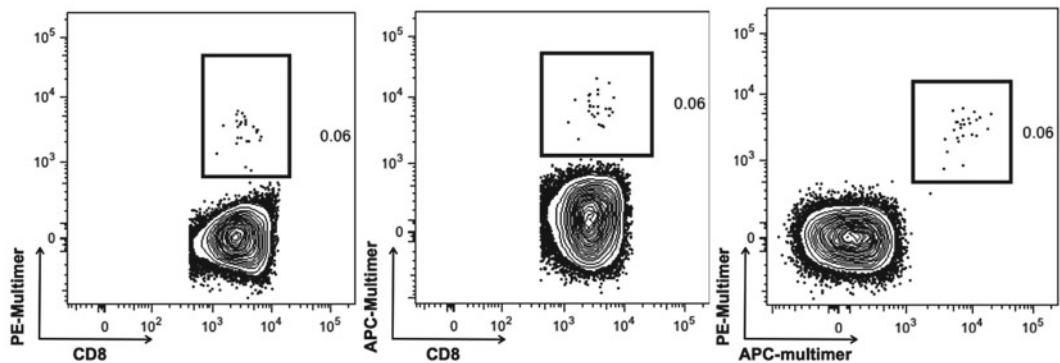


Fig. 4. Representative example of single and double multimer staining. PBMCs from one healthy donor were stained with Influenza A-Matrix1₅₈₋₆₆ MHC-I multimer labeled either in PE (*left*), APC (*middle*), or both (*right*). Plots are gated into global CD8⁺ population using the gating strategy described in Subheading 3.5 and Fig. 5a.

6. If needed, it is possible to multiplex the experiment (i.e., determine multiple specificities—up to 25—within one single tube) by preparing each specific multimer with a unique combination of two different colors (21). In the case you want to simultaneously enrich your target populations with antiPE microbeads, one of these two colors will have to be PE (see Note 16 and Fig. 6a).
7. Vortex gently and incubate 30 min at 4°C (see Note 17).
8. Wash once in 2 mL PB, spinning at 300×*g* for 5 min at 4°C.
9. If you stop here, transfer your cells into 5 mL FACS tubes, spin, resuspend in 90 µL of PBS–2% FCS, and proceed directly to flow cytometry analysis on Subheading 3.5. Otherwise, you can follow the procedure by enrichment (Subheading 3.4).

3.4. Enrichment

1. To start the enrichment protocol, resuspend labeled cells obtained in Subheading 3.3 (step 8) in 400 μ L PB.
2. Take a 10 μ L aliquot of labeled cells, and place it into 5 mL FACS tubes. Complete with 90 μ L with PBS–2% FCS. This gives you your “Pre-enriched” fraction.
3. To the cells used for enrichment, add 100 μ L of anti-PE microbeads (see Note 18).
4. Vortex and incubate for 20 min at 4°C in the dark.
5. Wash twice in 2 mL cold PB, spinning cells at $300 \times g$ for 5 min at 4°C.
6. During the washing step, prepare MACS columns (one per Falcon 15 mL tube) on a magnet support (see Note 4). Rinse each column with PB (discard elution). Label Falcon 15 mL tubes for collecting the flow through fraction.
7. Resuspend each sample in 1 mL PB, and load the column.
It is important to filter cells just prior to loading on the column in order to remove any clumped cells.
8. Wait until the sample has completely passed through the column bed.
9. Add 1 mL of PB to the initial Falcon 15 mL tube (wash step to get every last cell).
10. Load column with this fraction.
11. Wait until the sample has completely passed through.
12. Collect first flow-through fraction and load it on the same column a second time (again, an effort to capture all multimer-labeled cells).
13. Again, wait until the sample has completely passed through the column bed.
14. Wash the column with 3×1 or 2×3 mL of PB (for MS and LS columns, respectively).
15. Wait until the sample has completely passed through: the collective liquid in the collection tube (flow through fraction) is your “Depleted fraction.”
16. Remove one column at a time. Place it in a corresponding labeled Falcon 15 mL tube. Add 2–5 mL (for MS and LS columns, respectively) of PB to the upper fraction of the column. Push the plunger using steady pressure.
17. Gently remove the plunger.
18. Add again 2–5 mL of PB to the upper fraction of the column.
19. Push the plunger. The collective liquid (total volume is 4–10 mL) is considered the “Enriched fraction.”
20. Spin Depleted and Enriched fractions at $300 \times g$ for 5 min at 4°C.

21. For the Depleted fraction (see Note 19):

- (a) Resuspend in 1 mL of PBS–2% FCS.
- (b) Aliquot 90 µL in one 5 mL FACS tube and add Ab mix.
- (c) Incubate 20 min at 4°C in the dark.
- (d) Wash in 3 mL PBS–2% FCS at 300×g for 5 min at 4°C.
- (e) Resuspend in 300 µL PBS–2% FCS.

22. For the Enriched fraction, you can either continue with ICS (proceed to Subheading 3.7) or prepare your samples for flow cytometry analysis on Subheading 3.5:

- (a) Resuspend in 90 µL PBS–2% FCS.
- (b) Add your Ab mix directly into the Falcon 15 mL tube.
- (c) Incubate 20 min at 4°C in the dark.
- (d) Add 1 mL of PBS–2% FCS; transfer to 5 mL FACS tubes.
- (e) Add 1 mL of PBS–2% FCS to the initial Falcon 15 mL tube.
- (f) Transfer the 1 mL to the same 5 mL FACS tubes.
- (g) Spin at 300×g for 5 min at 4°C.
- (h) Resuspend in 300 µL PBS–2% FCS.

(Examples for mice and human data are provided in Fig. 5b.)

3.5. Flow Cytometry

1. If you came directly from Subheading 3.3, add your mAb mix and incubate 20 min at 4°C in the dark (see Note 20).
Wash in 3 mL PBS–2% FCS at 300×g for 5 min at 4°C.
Resuspend in 300 µL PBS–2% FCS.
2. If you have pursued with enrichment on Subheading 3.4, your Depleted and Enriched fractions are now ready to be analyzed.
3. Add DAPI to each sample just prior to acquisition ($C_f = 1/5,000$; 3 µL of solution 1/50 in 300 µL of cells).
4. Set stopping gate at 2,000,000 events on Single cells (SSC-A^{low}/SSC-W^{low}).
5. Importantly, acquire all samples for Enriched fraction (add PBS twice) (see Note 21).
6. Gating strategy: SSC-A vs. SSC-W to exclude doublets; Dump vs. CD3 to isolate viable pure CD3; CD3 vs. CD8 to gate on CD3⁺CD8⁺; Multimer-PE vs. CD8 gated on CD3 to have background evaluation; Multimer-PE vs. CD8 gated on CD8 to have percentages; any further phenotypic analysis on Multimer-PE⁺ cells (Fig. 5a).
7. If you enriched multiple specificities, you will gate on CD8⁺PE⁺ multimer positive cells, then discriminate antigen specificity

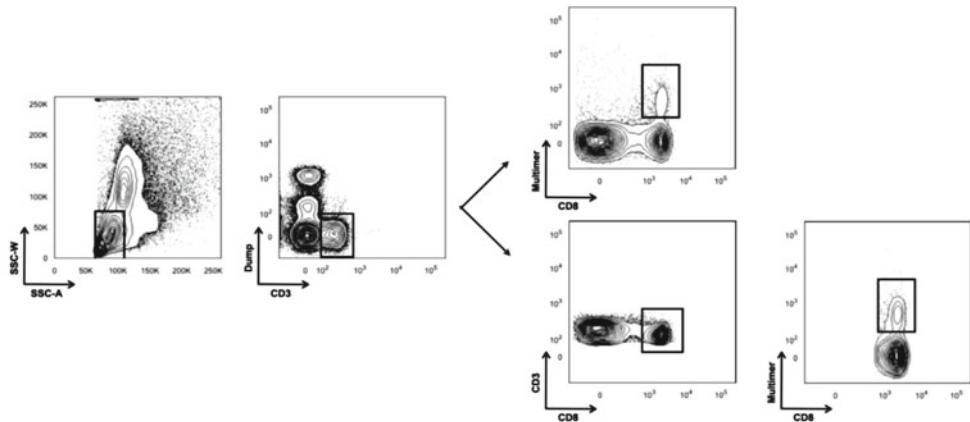
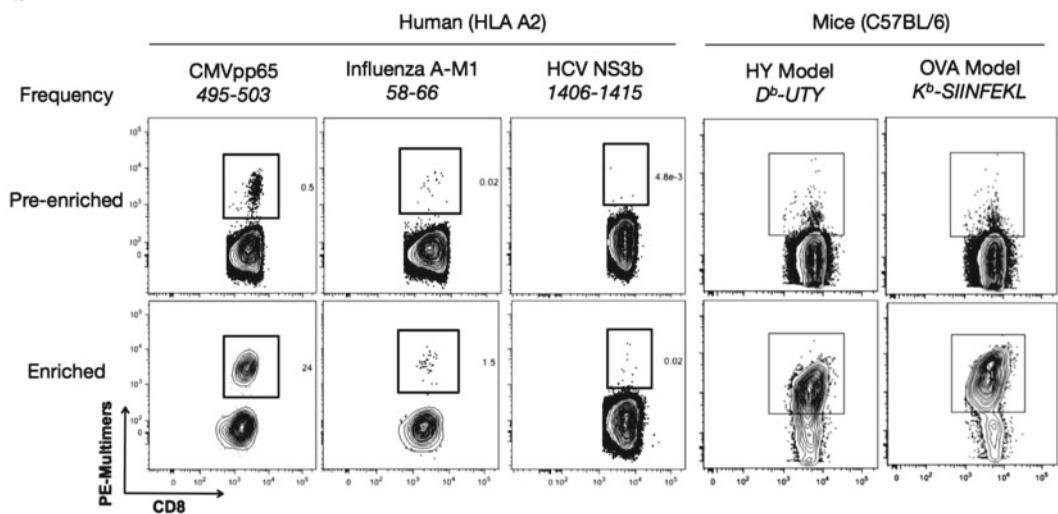
a**b**

Fig. 5. Gating strategy and Enrichment. (a) Example of the gating strategy applied to an Influenza A-Matrix 1₅₈₋₆₆ enriched human sample. SSC-A^{low}/SSC-W^{low} initial gating permit to exclude doublets, then Dump vs. CD3 contour plot permits to isolate viable pure CD3 for further analysis. *Upper line* shows evaluation of background on a Multimer-PE vs. CD8 contour plot still gated on the total CD3 population. *Bottom line* illustrates CD3⁺CD8⁺ selection, then finally evaluation of Multimer-PE⁺ cells percentages within CD8⁺ T cells. (b) *Left plots*: PBMCs from a healthy donor have been incubated with CMV, Flu or HCV MHC I multimers, then enriched as described in the protocol. *Right plots*: C57BL/6 mice were immunized intradermally, either with male (HY model) or K^bmOva (Ova model) splenocytes. On day 11, the spleen and lymph nodes were harvested and enrichment was performed as described in the protocol, using D^b-UTY (HY model) or K^b-SIINFEKL (Ova model) multimers.

from another by gating on double positive T cells: PE⁺color-A⁺ will be T cells with specificity A, PE⁺color-B⁺ will be T cells with specificity B,... (see Note 22 and Fig. 6b).

3.6. Precursor Frequency

1. To determine the size of the epitope-specific populations within each sample, we recommend a precise calculation, initially proposed by Moon et al. (26).
2. The absolute number of total CD8⁺T cells within any sample is determined using the following equation: absolute number of

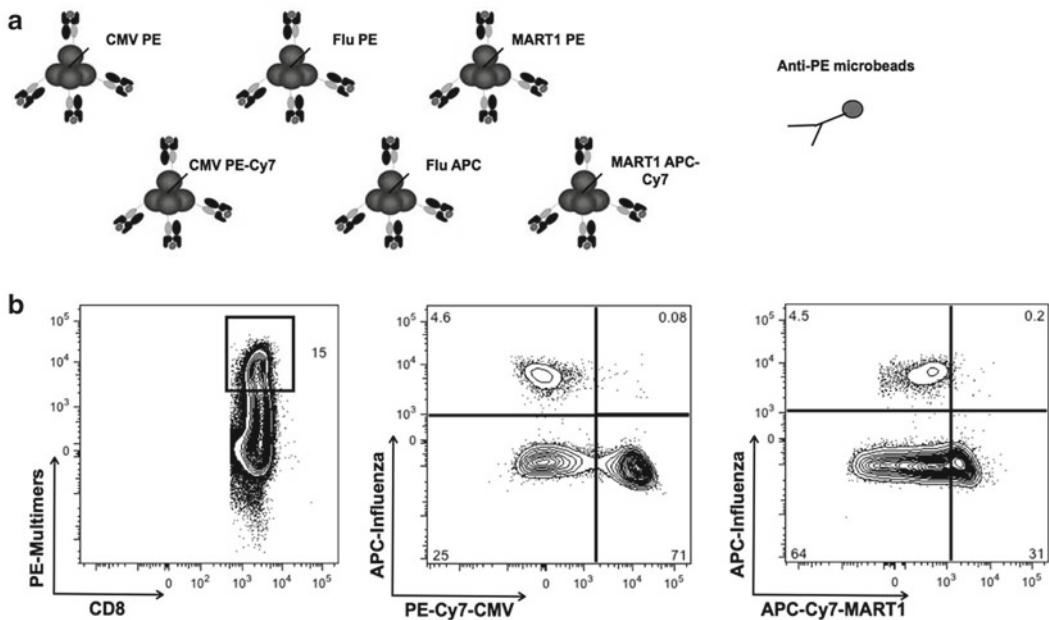


Fig. 6. Multi-enrichment. **(a)** PBMCs from a healthy donor are incubated with a cocktail of MHC I multimers. Each specificity (CMV, Flu, MART1) is labeled with PE and with another color (PE-Cy7, APC, APC-Cy7, respectively). Enrichment is performed with anti-PE microbeads as described in Subheading 3.4. **(b)** After applying the gating strategy described in Subheading 3.5 and Fig. 5a, CD8⁺PE⁺ cells are gated (*left plot*). Each specificity is then identified within CD8⁺PE⁺ population using the second color readout (*middle and right plots*).

CD8⁺T cells = (number of CD8⁺ T cells acquired in the pre-enriched sample) × [(total number of PBMCs in the pre-enriched sample)/(total number of cells acquired in the single cell gate in the pre-enriched sample)].

3. The absolute number of multimer-positive T cells is the number of multimer-positive cells within the “single, live, non-dump CD3⁺CD8⁺” T-cell gate present in the enriched fraction. (see Note 23).
4. The frequency of circulating multimer-positive cells is defined as the absolute number of multimer-positive T cells/absolute number of CD8⁺ T cells.

3.7. Intracellular Cytokine Staining (Optimized for Mice)

1. Restimulation of cells is performed *in vivo*. Three hours prior to leukocyte harvest, inject mice intravenously with 5 µg of CpG/DOTAP formulated as a mixture with 1 µg specific peptide (e.g., SIINFEKL peptide in the Ovalbumin model).
2. Perform the staining and enrichment as described in Subheadings 3.3 and 3.4 with the addition of BD GolgiPlug containing Brefeldin A during multimer and beads incubation steps (final concentration 1/1,000).
3. After elution from the column, resuspend enriched cells in MPB without azide, add Aqua fluorescent reactive dye (final

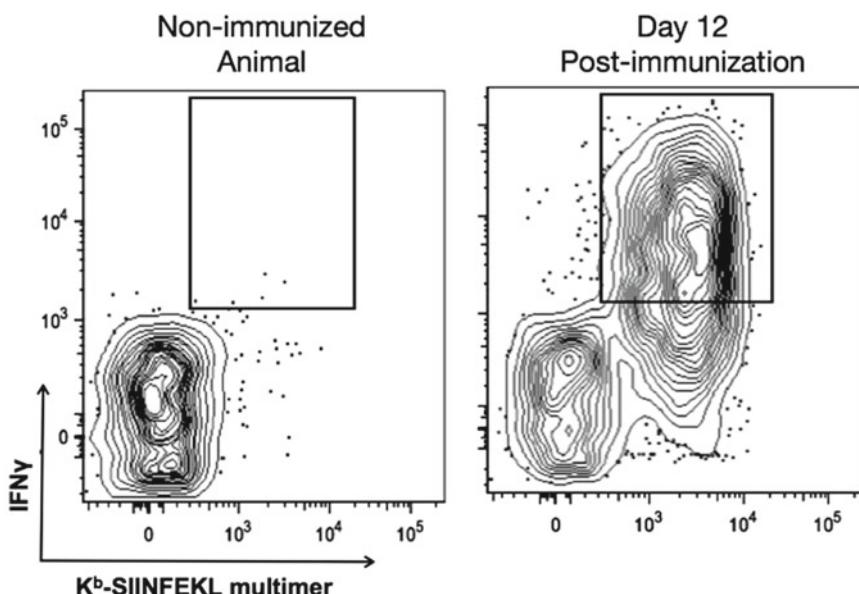


Fig. 7. Intracellular cytokine staining on mouse samples. C57BL/6 mice were immunized intradermally with K^bmOva splenocytes. On day 12, mice were injected intravenously with CpG/DOTAP and SIINFEKL peptide. 3 h later, the spleen and lymph nodes were harvested and Ova-specific T cells were enriched with K^b-SIINFEKL multimer, fixed and stained intracellularly for IFN γ as described in the protocol.

concentration 1/1,000) to stain dead cells (see Note 12), and incubate 30 min at 4°C in the dark.

4. Spin 5 min at 300 $\times g$ at 4°C in 3 mL MPB without azide.
5. Resuspend cells in 100 μ L MPB without azide, add the mix of antibodies for surface staining, and incubate 20 min at 4°C in the dark.
6. Wash with 3 mL of MPB without azide and resuspend thoroughly cells with 250 μ L of Cytofix/Cytoperm reagent. Vortex and incubate 20 min at 4°C.
7. Wash with 1 mL of 1× Perm/wash buffer.
8. Incubate cells for 30 min at 4°C with anti-IFN γ antibody diluted in Perm/Wash buffer.
9. Wash cells once with Perm/Wash buffer and once with MPB without azide.
10. Resuspend in 300 μ L PBS–2% FCS and acquire sample in flow cytometry (Fig. 7).

4. Notes

1. Although the protocol described here focuses on antigen-specific T cells harvested from human peripheral blood and from mice, similar procedures can be applied to other nonhuman

samples (25–27) and to other tissues (e.g., tumor infiltrating lymphocytes, TILs). For human peripheral blood, prepare PBMCs using Ficoll separation. For tissue-based applications, we recommend including a CD45 staining in one of the channels in order to segregate CD45-positive hematopoietic cells, and decrease noise in the assay.

2. Although the protocol described here focuses on HLA-A2 individuals as example, it can be applied to any HLA specificity without modification. Moreover, enrichment protocols would also be applicable for CD4⁺ T cell, NK-T, and $\gamma\delta$ T cell populations, using respective multimer reagents.
3. Although the protocol described here is based on the combination of PE-labeled multimers and anti-PE microbeads, you can similarly stain with APC-labeled multimers and enrich with anti-APC microbeads.
4. When establishing the assay on human samples, we found a better recovery of rare specific T cells when using MS columns, regardless of the number of loaded PBMC used (1×10^7 – 4×10^8 starting cell populations tested). Exceptions concern TILs, for which you need to use LS columns in order to avoid clumps and blockage of the column. Similarly, for mouse experiments, LS columns are recommended due to potential of stromal tissue from lymph nodes and spleen to clog the columns.
5. This recipe was chosen based on our experience in the lab. Other conventional sorting buffers can be used, but may result in slightly different background signals. Note that sodium azide should be omitted if planning to cultivate the cells or perform functional assays.
6. Concerning MHC multimers, there are two options. Commercial vendors exist and will sell off-the-shelf reagents as well as generate custom materials. Providers include Beckman Coulter, ProImmune and Immudex. The alternative and recommended option is to prepare your own monomers, thus facilitating multimerization with streptavidin coupled to your desired fluorescent tag. This approach allows you to work with the same multimer labeled in different colors, thus improving specificity of the assay (see Note 14) and permits multiplexing different specificities in the same tube (see Notes 16 and 22). Note that regardless of the source, high-quality monomers are important, with monomer purity impacting multimerization. Moreover, the choice of streptavidin reagent is critical, and it is recommended to purchase high-quality streptavidin conjugated to bright fluorochromes.
7. When using MHC multimers, it is recommended to choose an appropriate method to validate the specificity of tetramer-stained cells. Positive controls will be multimers targeting abundant populations of CD8 T cells. For human, EBV

BMLF1₂₈₀₋₂₈₈ or Influenza A-M1₅₈₋₆₆ can be used as a positive control. For mice, the strategy will be to stain splenocytes from a TCR-transgenic mouse with the corresponding multimer (OT-I CD8⁺ T cells that are specific for H2-K^b-SIINFEKL complexes stained with H2-K^b-SIINFEKL multimer for instance). It will help you to establish the assay and ensure that enrichment is sufficient for detection of rare cells. Concerning naïve cells in humans, MART1_{26-35(Leu27)} is a good choice, as it will also be a useful reference for establishing gating parameters for naïve vs. memory populations (30). Negative control tetramers can be employed to help establish the assay, although there is the potential to observe CD8⁺ T cells with the capacity to bind any MHC I, including self-antigens. An alternative option is the evaluation of background staining based on the nonspecific labeling of CD4⁺ T cells. That said, recent work has suggested that even this interaction might be of physiologic relevance (28). More definitive controls are also important, such as assessment of TCR CDR3-variable region usage skewing, peptide-induced TCR downregulation and, after cell sorting, TCR sequences analysis, or TCR genes transfer into immortalized cell lines to show that the specificity can be reconstituted (8).

8. Biotinylated monomers may be stored for months at -80°C. Stability testing is recommended. In contrast, multimers are less stable, should be stored at 4°C and ideally should be used within 4 weeks. Best is even to multimerize the amount you will need for each experiment 1 day before.
9. The use of a “Dump channel” is essential as it excludes cells that bind nonselectively to the MHC multimer reagents. Its composition has to be reviewed in the context of the experimental aims. For example, CD56 is useful for exclusion of NK cells in human samples when evaluating naive cell repertoires, but should be used with caution when studying human memory or activated T cell populations as some cells express CD56 and would thus be lost in the gating strategy. Similarly, it can be useful to add anti-CD33 and anti-CD34 antibodies when studying T cell populations present in bone-marrow populations. As indicated above, we deliberately do not include CD4 as we use the staining of this population as an assessment of background, but this can be added when multiple-free channels are needed for complex multicolor experiments. In the same way, the Dump channel must be chosen carefully for mouse experiments: some markers, such as CD11c, may in fact upregulate on activated T cells.
10. Addition of a viability marker is necessary in order to avoid nonspecific background staining on dead cells. While this may be omitted in some instances in which fresh blood is utilized,

it should be noted that the enrichment columns have an affinity for dead cells. Ideally, select a viability dye in the same channel as the Dump channel—thus keeping the maximum number of channels free for phenotypic characterization.

11. At least four fluorescent channels are necessary for careful assessment of enriched T cells: (a) a Dump channel; (b) CD3 staining for gating on T cells; (c) CD8 staining for gating of CD8⁺ T cells; and (d) the multimer-conjugated label for the specificity of interest. This can be extended when differently labeled multimers are included in the same experiment (for reducing nonspecific binding in the assay and/or for multiplexing enrichment). Anti-CD3 can eventually be omitted if you really need a maximum of free channels for phenotypic characterization. Additional channels that are available will depend upon the technical specifications of the cytometer and antibodies will be chosen depending on the experimental questions being evaluated. Note that it is crucial to stain with multimers prior to washing and staining with other Abs, especially for CD8 and CD4, as some clones have been shown to influence multimer staining (16).
12. As cells will be fixed to perform intracellular staining, DAPI cannot be used as a viability marker. We therefore recommend the use of a fixable live/dead cell marker such as Aqua. Note that Aqua labeling has to be done in azide-free buffer.
13. The starting number of cells is a critical point. It is required in order to calculate precursor frequency; and in most instances it is the determinant of the limit of sensitivity for the assay. For example, if you suppose your population to be around 10⁻⁶ (meaning 1 cell into 10⁶ CD8), you will need to start from at least 10⁷ cells to maximize the possibility of achieving a well-defined multimer-positive population. Of note, during the enrichment procedure, cell loss is in the range of 10–30%.
14. Staining cells with two multimers sharing the same specificity but labeled in different colors permits a further decrease in the nonspecific binders (21). We and others strongly recommend to include dual labeling, especially if your aim is to detect ultra-rare populations of cells of variable avidity. Of course, if you want to pursue with enrichment with anti-PE microbeads, one of the two colors need to be PE. Otherwise, you can use any fluorochrome combination (up to 25) (21), as soon as you titrate both streptavidin and multimers before use, and be cautious with settings and compensations.
15. Concentration of multimers is another important parameter to consider. Optimal concentrations must be defined for each multimer by titrating on specific cell lines. In general, we recommend working at high concentrations, i.e., 10–20 nM=3–10 µg/mL

final concentration (NB: PE Tetramers \sim 500 kDa; APC Tetramers \sim 350 kDa). Of note, the receptor density of TCR on responding T cells is also critical. While not thoroughly validated, it may be of interest to evaluate exposure to the protein tyrosine kinase inhibitor dasatinib prior to staining and/or enrichment as a means of enhancing tetramer binding (16).

16. For example, if you aim to stain and enrich T cells specific for CMV, Influenza A, and MART1-specific populations at the same time, you will put in the same tube, at the same incubation step, the following multimers: PE-CMV, PE-Cy7-CMV, PE-Flu, APC-Flu, PE-MART1, APC-Cy7-MART1 (see Note 22 and Fig. 6a).
17. Temperature is a critical parameter in multimer staining. It is advisable to assess the effects of temperature (and time) for each individual system. In our assays, MHC I staining is performed at 4° for 30 min. For staining at room temperature, be cautious with respect to internalization of multimers—which might interfere with the enrichment procedure.
18. Please note that the number of beads used here is not adjusted to the number of targeted cells but rather fixed at a high level, sufficient for enrichment of rare or common populations.
19. We recommend to systematically analyze the Depleted fraction, at least during assay optimization in order to evaluate cell loss during the procedure (usually 10–30% in our hands).
20. All reagents should be titrated before use.
21. To ensure you can detect rare specific T-cell populations, it is important to start with sufficient number of cells (see Note 13), and acquire sufficient number of events (31).
22. In the example provided above (see Note 16 and Fig. 6), after gating on CD8 $^{+}$ PE-multimer-positive T cells (which will contain the four T cell specificities), CMV-specific T cells may be segregated based on PE-Cy7 positivity; Flu-specific cells will be stained with APC; and MART1-specific T cells will be APC-Cy7 labeled. With the increased number of available fluorescent channels (e.g., 18-parameter cytometer), one can theoretically combine up to ten enriched specificities at the same time, the difficulty being the optimization of compensation settings. The quality of the enrichment procedure, and capacity of detecting rare events will in fact also depend on ones experience using the cytometer.
23. This calculation is based on the hypothesis that you recover all epitope-specific T cells while acquiring your Enriched sample and should therefore be considered as the lower limit of precursor frequency. However, this requires rigorous adherence to the protocol, and in particular the standardization of wash

steps, and consistent acquisition of the Enriched sample. Mixing studies with known input numbers of monoclonal TCR transgenic cells in wild-type congenic mix, or T cell clones into HLA-mismatched PBMCs may be used to evaluate efficiency and establish in-house criteria (23, 25).

References

- Arens R, Schoenberger SP (2010) Plasticity in programming of effector and memory CD8 T-cell formation. *Immunol Rev* 235(1):190–205. doi:[10.1111/j.0105-2896.2010.00899.x](https://doi.org/10.1111/j.0105-2896.2010.00899.x)
- Schumacher TNM, Gerlach C, Heijst JW (2010) Mapping the life histories of T cells. *Nat Pub Group* 10(9):621–631. doi:[10.1038/nri2822](https://doi.org/10.1038/nri2822)
- Coulier PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, Boon T (2002) Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen. *Immunol Rev* 188:33–42
- Lamoreaux L, Roederer M, Koup R (2006) Intracellular cytokine optimization and standard operating procedure. *Nat Protoc* 1(3):1507–1516. doi:[10.1038/nprot.2006.268](https://doi.org/10.1038/nprot.2006.268)
- Sun Y, Iglesias E, Samri A, Kamkamidze G, Decoville T, Carcelain G, Autran B (2003) A systematic comparison of methods to measure HIV-1 specific CD8 T cells. *J Immunol Methods* 272(1–2):23–34
- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274(5284):94–96
- Altman JD, Davis MM (2003) MHC-peptide tetramers to visualize antigen-specific T cells. *Curr Protoc Immunol Chapter* 17:Unit 17.13. doi:[10.1002/0471142735.iml1703s53](https://doi.org/10.1002/0471142735.iml1703s53)
- Davis MM, Altman JD, Newell EW (2011) Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. *Nat Rev Immunol* 11(8):551–558. doi:[10.1038/nri3020](https://doi.org/10.1038/nri3020)
- Klenerman P, Cerundolo V, Dunbar PR (2002) Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol* 2(4):263–272. doi:[10.1038/nri777](https://doi.org/10.1038/nri777)
- Romero P, Valmori D, Pittet MJ, Zippelius A, Rimoldi D, Lévy F, Dutoit V, Ayyoub M, Rubio-Godoy V, Michielin O, Guillaume P, Batard P, Luescher IF, Lejeune F, Liénard D, Rufer N, Dietrich P-Y, Speiser DE, Cerottini J-C (2002) Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol Rev* 188:81–96
- Baitsch L, Baumgaertner P, Devevre E, Raghav SK, Legat A, Barba L, Wieckowski S, Bouzourene H, Deplancke B, Romero P, Rufer N, Speiser DE (2011) Exhaustion of tumor-specific CD8⁺ T cells in metastases from melanoma patients. *J Clin Invest* 121(6):2350–2360. doi:[10.1172/JCI46102](https://doi.org/10.1172/JCI46102)
- Kanodia S, Wieder E, Lu S, Talpaz M, Alatrash G, Clise-Dwyer K, Molldrem JJ (2010) PR1-specific T cells are associated with unmaintained cytogenetic remission of chronic myelogenous leukemia after interferon withdrawal. *PLoS One* 5(7):e11770. doi:[10.1371/journal.pone.0011770](https://doi.org/10.1371/journal.pone.0011770)
- Rosenberg SA, Dudley ME, Restifo NP (2008) Cancer immunotherapy. *N Engl J Med* 359(10):1072. doi:[10.1056/NEJM081511](https://doi.org/10.1056/NEJM081511)
- Appay V, Douek DC, Price DA (2008) CD8⁺ T cell efficacy in vaccination and disease. *Nat Med* 14(6):623–628. doi:[10.1038/nm.f.1774](https://doi.org/10.1038/nm.f.1774)
- Ma Q, Wang C, Jones D, Quintanilla KE, Li D, Wang Y, Wieder ED, Clise-Dwyer K, Alatrash G, Mj Y, Munsell MF, Lu S, Qazilbash MH, Molldrem JJ (2010) Adoptive transfer of PR1 cytotoxic T lymphocytes associated with reduced leukemia burden in a mouse acute myeloid leukemia xenograft model. *Cytotherapy* 12(8):1056–1062. doi:[10.3109/14653249.2010.506506](https://doi.org/10.3109/14653249.2010.506506)
- Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK (2009) Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology* 126(2):147–164. doi:[10.1111/j.1365-2567.2008.02848.x](https://doi.org/10.1111/j.1365-2567.2008.02848.x)
- Bakker AH, Schumacher TNM (2005) MHC multimer technology: current status and future prospects. *Curr Opin Immunol* 17(4):428–433. doi:[10.1016/j.coim.2005.06.008](https://doi.org/10.1016/j.coim.2005.06.008)
- Toebes M, Coccini M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ, Van De Kasteele W, Rimmelzwaan GF, Haanen JBAG, Ova H, Schumacher TNM (2006) Design and use of conditional MHC class I ligands. *Nat Med* 12(2):246–251. doi:[10.1038/nm1360](https://doi.org/10.1038/nm1360)
- Hadrup SR, Toebes M, Rodenko B, Bakker AH, Egan DA, Ova H, Schumacher TNM

- (2009) High-throughput T-cell epitope discovery through MHC peptide exchange. *Methods Mol Biol* 524:383–405. doi:[10.1007/978-1-59745-450-6_28](https://doi.org/10.1007/978-1-59745-450-6_28)
20. Newell EW, Klein LO, Yu W, Davis MM (2009) Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. *Nat Pub Group* 6(7):497–499. doi:[10.1038/nmeth.1344](https://doi.org/10.1038/nmeth.1344)
 21. Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, Castermans E, Thor Straten P, Blank C, Haanen JB, Heemskerk MH, Schumacher TN (2009) Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* 6(7):520–526. doi:[10.1038/nmeth.1345](https://doi.org/10.1038/nmeth.1345)
 22. Chattopadhyay PK, Melenhorst JJ, Ladell K, Gostick E, Scheinberg P, Barrett AJ, Wooldridge L, Roederer M, Sewell AK, Price DA (2008) Techniques to improve the direct ex vivo detection of low frequency antigen-specific CD8+ T cells with peptide-major histocompatibility complex class I tetramers. *Cytometry A* 73(11):1001–1009. doi:[10.1002/cyto.a.20642](https://doi.org/10.1002/cyto.a.20642)
 23. Alanio C, Lemaitre F, Law HKW, Hasan M, Albert ML (2010) Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies. *Blood* 115(18):3718–3725. doi:[10.1182/blood-2009-10-251124](https://doi.org/10.1182/blood-2009-10-251124)
 24. Day CL (2003) Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* 112(6):831–842. doi:[10.1172/JCI200318509](https://doi.org/10.1172/JCI200318509)
 25. Moon J, Chu H, Pepper M, McSorley S, Jameson S, Kedl R, Jenkins M (2007) Naive CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27(2):203–213. doi:[10.1016/j.immuni.2007.07.007](https://doi.org/10.1016/j.immuni.2007.07.007)
 26. Moon JJ, Chu HH, Hataye J, Pagán AJ, Pepper M, McLachlan JB, Zell T, Jenkins MK (2009) Tracking epitope-specific T cells. *Nat Protoc* 4(4):565–581. doi:[10.1038/nprot.2009.9](https://doi.org/10.1038/nprot.2009.9)
 27. Obar J, Khanna K, Lefrancois L (2008) Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity* 28(6):859–869. doi:[10.1016/j.immuni.2008.04.010](https://doi.org/10.1016/j.immuni.2008.04.010)
 28. Legoux F, Debeaupuis E, Echasserieau K, De La Salle H, Saulquin X, Bonneville M (2010) Impact of TCR reactivity and HLA phenotype on naive CD8 T cell frequency in humans. *J Immunol* 184(12):6731–6738. doi:[10.4049/jimmunol.1000295](https://doi.org/10.4049/jimmunol.1000295)
 29. Jenkins MK, Chu HH, McLachlan JB, Moon JJ (2010) On the composition of the preimmune repertoire of T cells specific for peptide-major histocompatibility complex ligands. *Annu Rev Immunol* 28(1):275–294. doi:[10.1146/annurev-immunol-030409-101253](https://doi.org/10.1146/annurev-immunol-030409-101253)
 30. Pittet MJ, Valmori D, Dunbar PR, Speiser DE, Liénard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini JC, Romero P (1999) High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J Exp Med* 190(5):705–715
 31. Roederer M (2008) How many events is enough? Are you positive? *Cytometry* 73A(5):384–385. doi:[10.1002/cyto.a.20549](https://doi.org/10.1002/cyto.a.20549)

Chapter 24

The Purification of Large Numbers of Antigen Presenting Dendritic Cells from Mouse Spleen

David Vremec and Elodie Segura

Abstract

Dendritic cells (DC) are found at low frequency in lymphoid and non-lymphoid tissues. Different DC subsets are adept at different roles in immunity in diverse scenarios of attack by infectious agents, as well as in the maintenance of self-tolerance. A key element in the ability of DC to initiate adaptive immune responses is their capacity to capture and process antigen, whether from pathogens, vaccines or self-components, and present it to T cells. Our typical procedure for isolation of the different DC types from murine spleen involves their digestion from the tissue using collagenase, selection of cells of light density, and negative selection for DC. DC may then be separated into their functionally distinct subpopulations using immunofluorescent labeling and flow cytometric cell sorting. If the availability of mice is limiting, our protocol can cater for DC numbers boosted by the administration of fms-like tyrosine kinase 3 ligand (Flt3L), directly via subcutaneous injection or via the introduction of a Flt3L secreting melanoma cell line. Large numbers of in vitro equivalents of the spleen DC subsets may also be produced by culturing bone marrow with Flt3L. If flow cytometric sorting time is a limitation splenic DC subpopulations may instead be separated using a combination of fluorescent antibody labeling and anti-fluorochrome magnetic beads. Careful segregation of these functionally distinct subpopulations of DC will enable a thorough examination of their antigen processing and presenting capabilities.

Key words: Dendritic cell, DC isolation, DC expansion, DC subpopulations, Conventional DCs, Plasmacytoid DCs

1. Introduction

Dendritic cells (DC) play an indispensable role in the immune system. Contact between immature DC and antigen results in antigen uptake, processing and presentation and finally T cell priming (1). Mice lacking DC are unable to mount an effective immune response upon attack by pathogens (2–4). In the absence of pathogens or inflammation, the DC system is crucial in maintaining self-tolerance by presenting self-antigen and inducing either unresponsiveness or deletion of T cells (5, 6) and inducing, or expanding,

regulatory T cells (7). The majority of steady state, spleen DC in the mouse are in an immature state. They express relatively low levels of MHC II and co-stimulatory molecules and can efficiently endocytose and process antigen, which is loaded onto MHC molecules, and transported to the cell surface where it can be presented (8).

Antigen processing and presentation can follow several pathways and different DC subsets possess different abilities in this area. All mature DC efficiently present endogenous proteins on their MHC class I and II molecules. Presentation of exogenous antigen is largely restricted to MHC class II molecules (9). However, a small population of DC in the spleen has the unique capacity to present exogenous antigen via MHC class I in a process termed “cross-presentation” (10). Cross-presentation is crucial for generating antiviral and antitumor cytotoxic T cell responses and in the induction and maintenance of tolerance (11).

As the DC are activated they undergo a cessation of antigen uptake and processing, an upregulation of MHC and co-stimulatory molecules such as CD40, CD80, and CD86, and they produce many cytokines and chemokines (8).

Early DC isolation procedures separating DC from macrophages and other non-adherent cells after overnight culture utilizing the transient adherence of the DC may have inadvertently commenced this maturation process thus altering both the phenotype and function of the DC (12). Even very short culture periods at 37°C induce DC maturation, particularly in conditions of high DC concentration (13). Any study of spleen DC phenotype or function, including antigen capture, processing and presentation, must therefore ensure that the DC isolated are equivalent to the immature DC found *in vivo* in the steady state and have not been induced to undergo maturation by the methods used in their isolation. Our procedure has been designed with this limitation in mind. Our short collagenase digestion at room temperature does not appear to activate DC, based on the observed surface expression of MHC class II and costimulatory molecules which remain at levels seen on the much lower yield of DC which can be isolated without collagenase using mechanical disruption at 4°C.

It was also important to consider the heterogeneity of the DC network when designing our protocol, so as to include all DC subtypes in our analysis. Our DC isolation procedure efficiently extracts all DC subpopulations based on differences in their expression of selected cell surface molecules, without evident bias. This is important as although all DC share the classical role of antigen uptake and processing, they differ in function due to differences in their location, their array of cell surface molecules, including pathogen receptors such as Toll-like receptors and C-type lectins, and the cytokines they secrete (14, 15).

DC constitute less than 1% of total splenocytes, so any effective study of them necessitates an initial isolation procedure comprised of extraction by digestion with collagenase at room temperature, followed by a short treatment with EDTA to dissociate multicellular complexes between DC and T cells. Dead cells and erythrocytes are removed and DC are substantially enriched by performing a density cut and selecting the ~5% lightest density cells. At this point DC account for 10–15% of light density cells and it is possible to isolate them directly using either flow cytometric cell sorting or positive selection using immunomagnetic beads. However this is still a lengthy and/or expensive task, so further enrichment is advised via negative selection. Non-DC lineage cells are coated with a cocktail of monoclonal antibodies and depleted with anti-immunoglobulin-coated immunomagnetic beads. Care must be taken when selecting the monoclonal antibodies to include in the cocktail, in order to avoid losing DC subtypes that bear molecules found more commonly on T cells, B cells, and macrophages. Residual contaminating autofluorescent macrophages can be a problem, but these can be removed during fluorescence activated cell sorting or analysis by gating out autofluorescent cells (16, 17).

Mouse spleen contains both migratory plasmacytoid DC (pDC) and lymphoid tissue resident conventional dendritic cells (cDC). Multicolor immunofluorescent labeling and flow cytometry can be used to identify and separate these different DC subtypes using their different arrays of surface molecules (18).

pDC are migratory DC produced in the bone marrow that migrate to all lymphoid organs via the blood stream. Upon activation pDC acquire characteristic DC morphology, upregulate MHC class II and rapidly produce high levels of type I interferon (18). All pDC have the phenotype CD11c^{int}CD317⁺ (19) and are enriched during DC isolation if the appropriate monoclonal antibody depletion cocktail is used in the procedure. Conversely they may be depleted if desired by adding anti-CD45R monoclonal antibody to the depletion cocktail (18).

All cDC in the spleen are tissue resident DC that have developed in the spleen from a blood-borne precursor and then exhibit typical DC morphology and function in the steady state (20). They are readily identified by CD11c which is expressed at high levels. CD11c can also be used in conjunction with CD317 to separate cDC from pDC, as cDC have higher levels of CD11c and do not express CD317 (19) (see Fig. 1a).

Traditionally cDC in the mouse spleen have been divided using CD4 and CD8 α into three distinct subtypes: CD4⁻CD8 α ⁻, CD4⁺CD8 α ⁻, and CD4⁻CD8 α ⁺ (16) (see Fig. 1b). The CD8 α ⁺ cDC are found in the T cell areas of the spleen and are the major producers of the pro-inflammatory cytokine IL-12p70, causing polarization of T cells to a Th1 response (21). A subset of the CD8 α ⁺ DC is the most efficient at cross presenting antigens (22, 23).

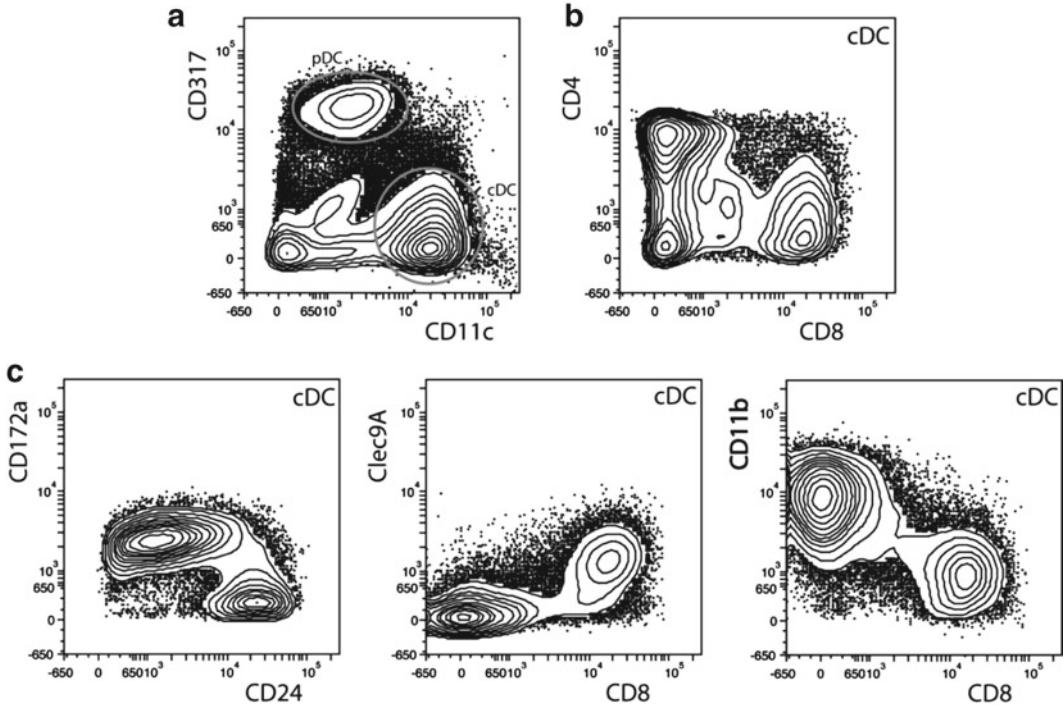


Fig. 1. Separation of mouse spleen DC into discrete subpopulations. (a) DC are separated into CD11c^{int}CD317⁺ plasmacytoid DC (pDC) and CD11c⁺CD317⁻ conventional DC (cDC). (b) Traditionally cDC have been separated using a combination of CD4 and CD8 into three populations: CD4⁺CD8⁻, CD4⁻CD8⁻, and CD4⁻CD8⁺. (c) CD172a, CD24, CD11b, and Clec9A can all be used to distinguish between the CD8⁺ and CD8⁻ subsets. The CD172a^{low}CD24^{high}CD11b⁺Clec9A⁺ and CD172a^{high}CD24^{low}CD11b⁺Clec9A⁻ populations correspond to the CD8⁺ and CD8⁻ populations, respectively.

The CD4⁺ DC population is found in non-T cell areas of the spleen and is the population that produces the highest levels of inflammatory chemokines (24). Together with the CD4⁻CD8^{α-} subtype the CD4⁺ DC are the most potent presenters of MHC II-antigen complexes to CD4⁺ T cells (23).

The introduction of new markers provides a more complete characterization of the two DC populations. These two populations may then be defined as CD8^{α+}CD24^{high}CD205⁺CD11b⁻CD172a^{low}Clec9A⁺ and CD8^{α-}CD24^{low}CD205⁻CD11b⁺CD172a^{high}Clec9A⁻ subtypes (25). Clec9A is an especially useful marker as it allows us to align the CD8⁺ DC subtype in mouse with its functional human equivalent, which does not express CD8 (26) (see Fig. 1c).

A deterrent facing those studying DC is the relatively small number that can be efficiently isolated from spleen and the difficulties in studying those cells *in vivo*. Precursors of all DC subtypes are found in the Flt3⁺ (27) fraction of bone marrow and it is possible to produce large numbers of both pDC and cDC by exposing these precursors to fms-like tyrosine kinase 3 ligand (Flt-3L)

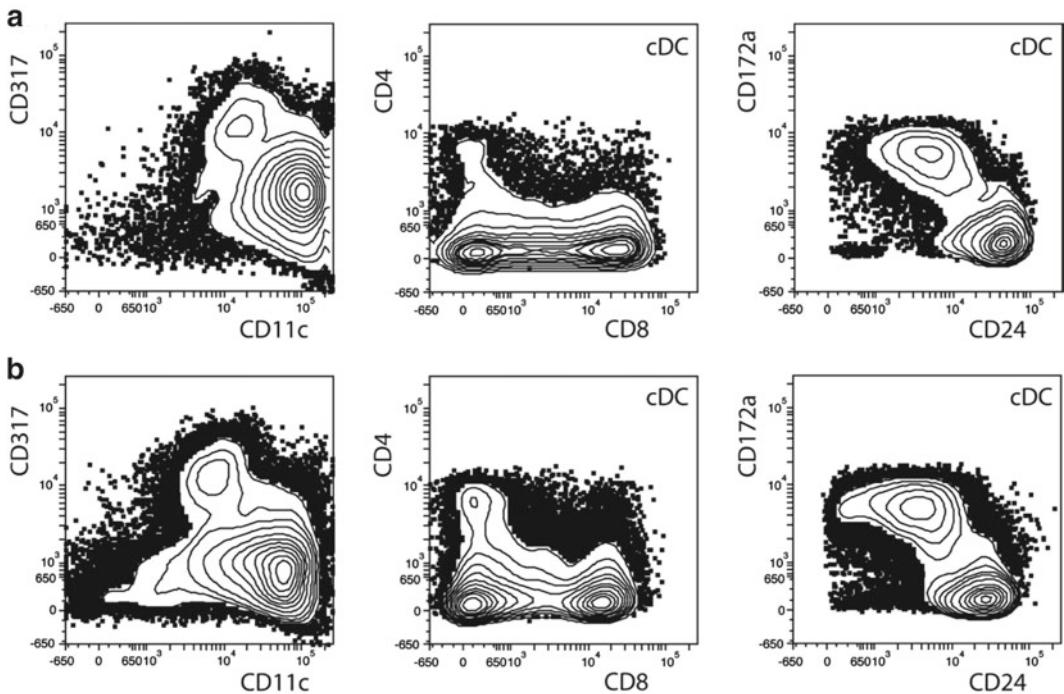


Fig. 2. Separation of DC from the spleen of mice treated with Flt3L *in vivo* into discrete subpopulations. pDC and cDC can be identified on the basis of differential expression of CD317 and CD11c and the cDC readily separated into CD8⁺ and CD8⁻ subpopulations using the same markers as used for untreated mice, regardless of whether the Flt3L was (a) administered via a Flt3L secreting melanoma or (b) administered directly via subcutaneous injection. In both cases both populations are expanded but the CD8⁺ fraction is expanded to a much greater extent.

either *in vivo* or *in vitro*. Administration of Flt-3L via daily intravenous injections for a period of 10 days can result in up to a 17-fold increase in numbers of DC in the spleen (28) and subcutaneous injection of the Flt-3L secreting melanoma B16FLT3L, can increase numbers up to 20-fold in the spleen (29). In both cases a two- to threefold increase in spleen cellularity also results and the DC subtypes can be identified and separated using the same markers used for untreated spleen. Expansion of both cDC subsets occurs although the CD8⁺ fraction is expanded to a greater extent (see Fig. 2).

Large numbers of pDC and cDC can also be produced from cultures of whole bone marrow treated with Flt-3L (30, 31). CD4 and CD8 α are not expressed by DC generated in culture with Flt3L and so these markers are inadequate for aligning the cDC subtypes. The cDC produced can nevertheless be divided into the functional equivalents of CD8⁻ and CD8⁺ DC using the markers CD11b, CD24, and CD172a (25) (see Fig. 3). Recently we have demonstrated that DC numbers recovered from these cultures can be expanded even further with initial addition of small amounts of

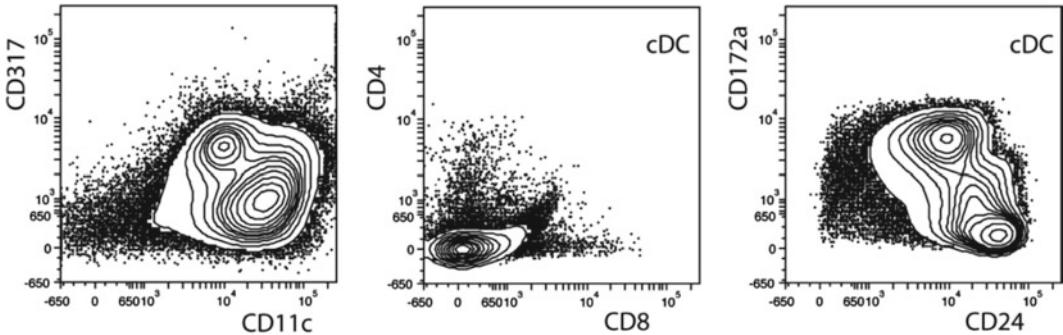


Fig. 3. Separation of DC generated in Flt3L bone marrow cultures into discrete subpopulations. pDC can be separated from cDC using a combination of CD11c and CD317. Although cDC generated in these cultures do not express CD4 or CD8 they are still separated into CD8⁺ and CD8⁻ equivalents using a combination of other markers, most commonly CD24 and CD11b or CD172a.

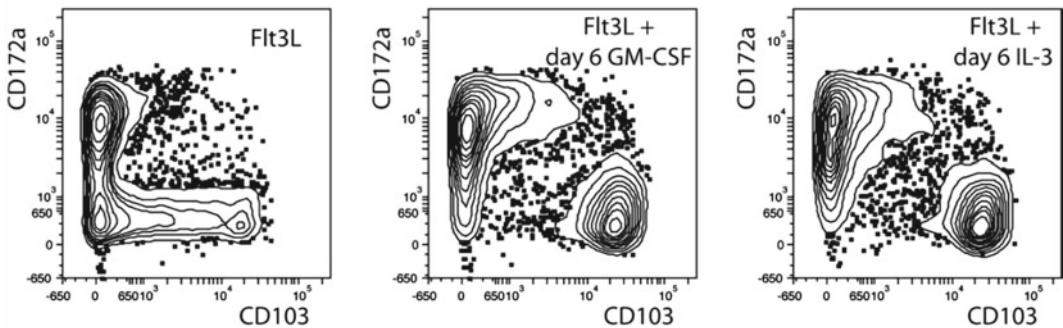


Fig. 4. Modification of cDC subpopulations in Flt3L bone marrow cultures. Addition of GM-CSF or IL-3 on day 6 of culture produces an increase in the proportion of CD103⁺ DC.

GM-CSF, and how addition of GM-CSF, or IL-3, after 6 days of culture will preferentially expand the CD103⁺ cDC which represents the DC capable of cross-presentation in these cultures (32) (see Fig. 4). Thus large numbers of the equivalents of the steady-state mouse spleen DC populations can now be produced *in vivo* and *in vitro*.

Sorting of cDC subpopulations is expensive and time consuming. An alternative method of isolating large numbers of highly purified DC subtypes without flow cytometric sorting is via positive selection using anti-fluorochrome-conjugated magnetic beads. Cells of one subtype are stained with a fluorochrome-conjugated antibody specific for that population, allowed to bind to anti-fluorochrome magnetic beads and then selected with a magnet. If desired, staining the negative cells with an antibody specific for the other subtype but conjugated to another fluorochrome and repeating the positive selection step allows the sequential selection of each of the two populations (33, 34) (see Fig. 5).

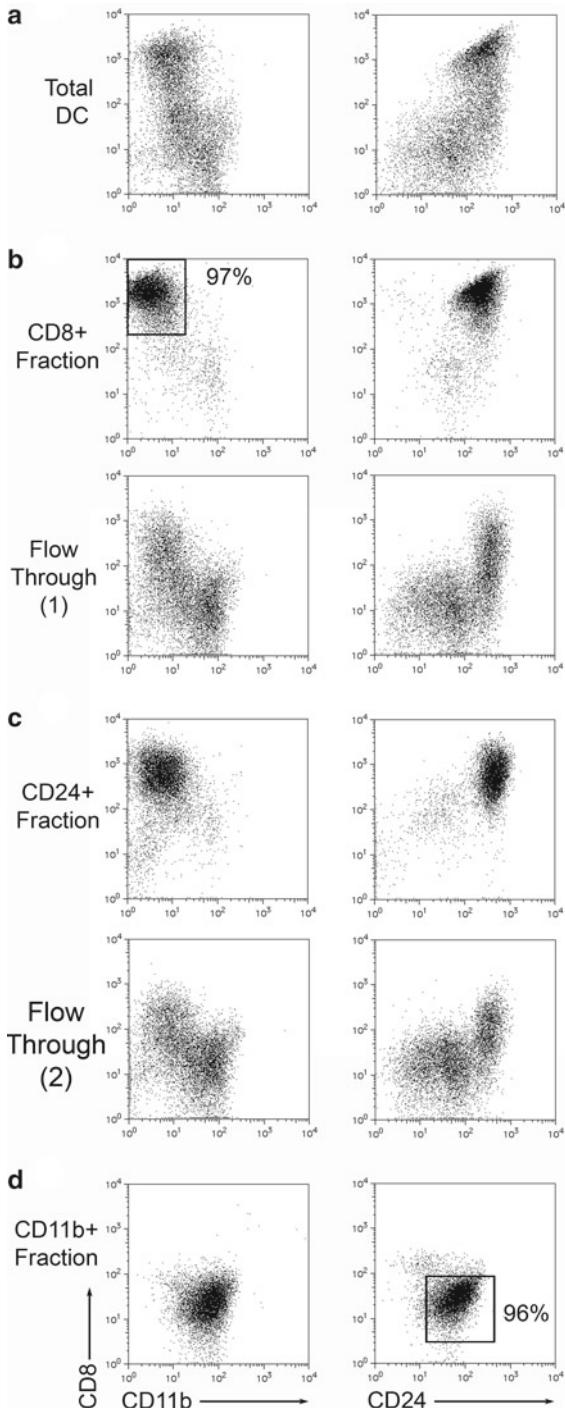


Fig. 5. The sequential separation of CD8⁺ and CD8⁻ cDC using immunomagnetic beads. **(a)** Two major subsets of spleen DC can be visualized using the markers CD8, CD24 and CD11b: CD8⁺CD24^{high}CD11b⁻ and CD8⁻CD24^{low}CD11b⁺. **(b)** The first round of selection for CD8⁺ DC results in a highly purified CD8⁺CD24^{high}CD11b⁻ population and a flow through with a greatly reduced proportion of CD8⁺ cells. **(c)** A second round of depletion targeting CD24 binds many of the remaining CD8⁺CD24^{high}CD11b⁻ and also CD24^{high}CD8⁻ precursors of the CD8⁺ cDC lineage. The second flow through contains a greatly enriched CD8⁻CD24^{low}CD11b⁺ population. **(d)** The final round of depletion utilizes CD11b and results in a very pure CD8⁻CD24^{low}CD11b⁺ population.

Working with large numbers of highly purified DC populations in an immature state, unaffected by the isolation procedure is crucial in studying antigen processing, presentation and functional specialization. The ability to expand DC numbers using Flt3L either *in vivo* or *in vitro* and using an economical procedure is essential in these endeavors.

2. Materials

2.1. In Vivo

Administration of Flt3L

1. Donor mice.
2. 27 G needle.
3. MTPBS: Mouse tonicity (308 mOsm/kg) phosphate-buffered saline. Filter to sterilize using a 0.2 µM filter unit and store at 4°C.
4. Flt3L: recombinant murine fms-like tyrosine kinase 3 ligand purified from the tissue culture supernatant produced by a CHO cell line transfected with a soluble murine Flt3L-FLAG peptide construct in the mammalian expression plasmid pEF-BOS (N. Nicola, WEHI, Australia) (see Note 1).
5. MSA: Mouse serum albumin (Sigma Aldrich). Prepare a 10 µg/ml stock solution in MTPBS. Filter using a 0.2 µM filter unit to sterilize and store at 4°C.
6. Flt3L/MSA: MTPBS containing 100 µg/ml Flt3L and 1 µg/ml MSA. Store at 4°C for up to 10 days.
7. B16FLT3L melanoma: Retroviral-mediated gene transfer generated B16-F10 melanoma line secreting murine Flt3L (J. Villadangos, WEHI, Australia) (see Note 2).

2.2. Organ Removal

1. FCS: Fetal calf serum. Aliquot and store at -20°C or for short periods of time at 4°C.
2. RPMI-FCS: modify RPMI-1640 to mouse osmolarity (308 mOsm/kg) and add pH 7.2 HEPES buffering to reduce dependence on CO₂ concentration. After adjusting to ~pH 7 with CO₂, filter through a 0.2 µM filter unit to sterilize and store at 4°C. Add FCS to a final concentration of 2% before use.
3. Dissecting instruments (scissors and forceps).

2.3. Digestion of Spleen and Release of DC

1. Enzyme digestion mix: dissolve collagenase TypeIII (Worthington Biochemicals) at 7 mg/ml and DNase I (Boehringer Mannheim) at 140 mg/ml in RPMI-FCS. Ensure the collagenase used is free of trypsin and other trypsin-like proteases (see Note 3). Divide into 1 ml aliquots and store as a 7× stock solution frozen at -20°C. Dilute each 1 ml aliquot

with 6 ml RPMI-FCS immediately preceding use. Pass through a 0.2 μM filter unit if sterility is required.

2. EDTA solution: 0.1 M ethylenediamine tetra-acetic acid disodium salt adjusted to pH 7.2. Pass through a 0.2 μM filter to sterilize and store at 4°C.
 3. EDTA-FCS: Add 1 ml of 0.1 M EDTA per 10 ml FCS preceding use.
1. BSS-EDTA: Modified salt solution containing 150 mM NaCl and 3.75 mM KCl (no Ca^{2+} or Mg^{2+}) and 5 mM EDTA. Adjust to pH 7.2 and mouse osmolarity (308 mOsm/kg). Filter to sterilize using a 0.2 μM filter unit and store at 4°C.
 2. BSS-EDTA-FCS: BSS-EDTA containing 2% EDTA-FCS.
 3. Nycodenz-EDTA: Nycodenz AG powder (Nycomed Pharma AS) is dissolved in water to produce a 0.372 M stock solution, then diluted and adjusted to the desired density of 1.077 g/cm³ at 4°C and osmolarity of 308 mOsm/kg using BSS-EDTA (see Note 4). Filter to sterilize using a 0.2 μM filter unit and store in 10 ml aliquots at -20°C. Thaw at room temperature, mix thoroughly and cool to 4°C prior to use (see Note 5).
 4. Polypropylene tubes: 14 ml polypropylene round bottom tubes (Becton Dickinson Labware).

2.5. Depletion of Non-DC Lineages

1. Monoclonal antibody depletion cocktail (see Note 6): Combine pre-titrated amounts of rat monoclonal antibodies specific for non-DC lineage cells.
 - (a) For purification of cDC only: KT3-1.1 (anti-CD3 ϵ), T24/31.7 (anti-CD90), TER119 (anti-erythroid lineage), 1A8 (anti-Ly6G), and RA3-6B2 (anti-CD45R).
 - (b) For purification of both cDC and pDC: KT3-1.1 (anti-CD3 ϵ), T24/31.7 (anti-CD90), TER119 (anti-erythroid lineage), 1A8 (anti-Ly6G), and 1D3 (anti-CD19)Dilute to the appropriate volume with BSS-EDTA-FCS. Saturating levels of all monoclonal antibodies are used (see Note 7). Pass through a 0.2 μM filter to sterilize, aliquot, and store at -20°C.
2. Immunomagnetic beads: BioMag goat anti-rat IgG coated beads (Qiagen) or Dynabead sheep anti-rat IgG coated beads (Dynal).
3. Spiral rotator: Spiramix 10 (Denley).

2.6. In Vitro Administration of Flt3L

1. Red Cell Lysis Medium (RCLM): 0.168 M NH_4Cl . Filter through a 0.2 μM filter unit to sterilize and store at 4°C.
2. Flt3L DC culture medium: Modified RPMI-1640, iso-osmotic with mouse serum, with additional HEPES-buffering at pH

7.2, supplemented with 10% FCS, 50 µM 2-mercaptoethanol, and 2 mM l-glutamine, filter sterilized using a 0.2 µM filter and stored frozen at -70°C. Add 200 ng/ml murine Flt3L immediately prior to use (see Note 8).

3. GM-CSF: recombinant murine granulocyte macrophage colony stimulating factor (PeproTech, Inc.).
4. IL-3: recombinant murine interleukin 3 (R&D Systems, Inc.).

2.7. Immuno-fluorescent Staining and Purification via Flow Cytometric Sorting

1. Monoclonal antibodies are purified from hybridoma culture supernatant using Protein G sepharose (Amersham Biosciences) and subsequently conjugated to fluorochromes in-house. All are titrated to determine saturating levels.
2. Fluorochrome-conjugated mAb conjugated following the manufacturer's instructions:
 - (a) Conjugate P84 to FITC (Molecular Probes, Inc.).
 - (b) GK1.5 and M2/90 to phycoerythrin ((PE) Prozyme).
 - (c) NLDC145 to allophycocyanin ((APC) Prozyme).
 - (d) YTS169.4 to PerCp.Cy5.5 (Innova Biosciences).
 - (e) M1/69 to Pacific Blue (Molecular Probes, Inc.).
 - (f) N418 to Quantum dots 655 (Invitrogen).
 - (g) 120G8 to Quantum dots 605 (Invitrogen).

Add FCS to 1% and NaN₃ to a final concentration of 10 mM (see Note 9). Titrate to determine saturating levels. Aliquot stocks of FITC and Pacific Blue conjugates and store at -70°C. Stocks of PE, APC, Quantum dots 655, Quantum dots 605, and PerCp.Cy5.5 conjugates (see Note 10) and working stocks of FITC and Pacific Blue conjugates are stored at 4°C, protected from light. Dilute to their final concentration immediately prior to use.

3. PI: prepare a 100 µg/ml propidium iodide (Calbiochem) stock solution in normal saline. Aliquot and store at 4°C protected from light (see Note 11).
4. BSS-EDTA-FCS-PI: dilute the PI stock in BSS-EDTA-FCS to a final working concentration of 500 ng/ml before addition to cells.
5. FACSAria (BD Biosciences).

2.8. Immuno-fluorescent Staining and Purification via Immunomagnetic Beads

1. Fluorochrome-conjugated mAb:
 - (a) Conjugate M1/70 to FITC (Molecular Probes, Inc.).
 - (b) Conjugate YTS169.4 and M1/69 to phycoerythrin ((PE) Prozyme).

2. Anti-fluorochrome beads (Miltenyi Biotec):
 - (a) Anti-FITC Microbeads.
 - (b) Anti-PE Microbeads.
3. BSS-EDTA-0.5%FCS: BSS-EDTA containing 0.5% EDTA-FCS.
4. MACS column (Miltenyi Biotec):
 - (a) MACS LS column.
 - (b) MACS LD column.
5. Magnet and stand (Miltenyi Biotec):
 - (a) Mini MACS magnet.
 - (b) MACS multistand.

3. Methods

3.1. In Vivo Administration of Flt3L

3.1.1. Soluble Flt3L

3.1.2. B16Flt3L

1. Inject 100 µl of Flt3L/MSA subcutaneously using a 27 G needle, into the nape of the neck of each mouse daily for 10 days (see Note 12).
1. Grow the B16Flt3L melanoma in culture RPMI.
2. Begin the harvest of the cells by removing the supernatant and washing with 5 ml of MTPBS.
3. Remove the MTPBS and treat with 2 ml of trypsin at 37°C for 2 min.
4. Shake flask to dislodge adherent cells, remove, and pool all cells.
5. Centrifuge, resuspend the pellet in MTPBS, and count.
6. Dilute cells to 25×10^6 /ml in MTPBS.
7. Inject 200 µl (5×10^6) cells per mouse subcutaneously into the nape of the neck using a 27 G needle (see Note 13).
8. Monitor the injected mice daily (see Note 14).
9. Allow melanoma to grow for 10 days (see Note 15).

3.2. Organ Removal

3.3. Digestion of Spleen and Release of DC

1. Remove eight spleens from untreated mice or two spleens from Flt-3L-treated mice (see Note 16) into cold RPMI-FCS. Take care to remove the organs with as little fat or connective tissue as possible.
1. Prepare the enzyme digestion mix slightly ahead of time and allow it to warm to room temperature before use.
2. Strip away any remaining residual fat (see Note 17) from the tissue using two 20 G needles and transfer the organs to a small

petri dish containing 7 ml of enzyme digestion mix. Use a sharp pair of scissors or a single-sided razor blade to cut the tissue into very small fragments (see Note 18). Transfer the mix containing the fragments to a 10 ml polypropylene tube using a wide bore pasteur pipette and digest with frequent mixing, with the same pipette, for 20–25 min at room temperature (~22°C) (see Note 19).

3. Add 600 µl of EDTA solution to the digestion mix and continue mixing for a further 5 min (see Note 20).
4. Remove and discard any remaining undigested tissue by running the digestion mix through a sieve. Dilute to 9 ml with RPMI-FCS, underlay with 1 ml of cold FCS-EDTA and recover the cells by centrifugation (see Note 21).
1. Thaw two 10 ml aliquots of Nycodenz-EDTA of appropriate density at room temperature. Once thawed ensure they are mixed well and kept at 4°C until required (see Note 22).
2. Prepare a cell suspension by resuspending the cell pellet in 10 ml of Nycodenz-EDTA (see Note 23).
3. Transfer 5 ml of the remaining Nycodenz-EDTA into the bottom of each of two polypropylene tubes.
4. Gently layer 5 ml of the cell suspension over the Nycodenz-EDTA in each of the two tubes (see Note 24). Add a 1–2 ml layer of EDTA-FCS over the cell suspension.
5. Gently mix the interface by inserting the tip of a pasteur pipette, swirling, and removing it (see Note 25).
6. Perform the density cut in a swing-out head, refrigerated centrifuge, set at 4°C, for 10 min at $1,700 \times g$ with the brake set on low.
7. Use a pasteur pipette to collect the light density fraction in the upper zones down to the 4 ml mark (see Note 26) and discard the bottom 4 ml and the cell pellet.
8. Transfer the light density fraction to a 50 ml tube, dilute to 50 ml with BSS-EDTA, mix thoroughly, and centrifuge to recover the cells (see Note 27).
9. Resuspend the cells in 5 ml BSS-EDTA-FCS and count (see Note 28).

3.5. Depletion of Non-DC Lineages

1. Calculate the volume of monoclonal antibody depletion cocktail required if 10 µl is needed per 10^6 cells.
2. Add the required volume of the appropriate monoclonal antibody depletion cocktail (see Note 29) to the cell pellet, resuspend, and incubate at 4°C for 30 min.
3. Calculate the required volume of immunomagnetic beads required (see Note 30) and transfer them to 5 ml polypropyl-

ene tube. Wash the beads with BSS-EDTA-FCS (see Note 31) by diluting, placing the tubes into the magnet, allowing the beads to move to the magnet, and removing the supernatant. Repeat the washing step three to four times. After the final wash, pellet the beads at the bottom of the tube in a small amount of BSS-EDTA-FCS and place the tube at 4°C until required.

4. Dilute the cells up to 9 ml with BSS-EDTA-FCS and underlay with 1 ml of FCS-EDTA. Centrifuge the cells and remove the supernatant from the top, leaving the FCS layer over the cells (see Note 32). Sit the tube at 4°C for a short time to allow any remaining supernatant to run down the wall of the tube. Then remove the supernatant and the FCS (see Note 33). Resuspend the cells in 400–500 µl of BSS-EDTA-FCS.
5. Remove the BSS-EDTA-FCS from the magnetic bead pellet and add the cells. Resuspend the beads and cells by vortexing the tube very briefly to produce a slurry (see Note 34). Seal the tube and mix the slurry continuously for 20 min at 4°C at an angle of 30° on a spiral mixer (see Note 35).
6. Dilute the bead-cell slurry with 3 ml of BSS-EDTA-FCS, mix very gently and attach the tube to the magnet for 2 min.
7. Recover the supernatant containing unbound DC with a pasteur pipette and transfer to a second 5 ml polypropylene tube. Discard the tube containing magnetic beads bound to non-DC (see Note 36). Place the tube containing the supernatant into the magnet for a further 2 min to remove any remaining beads. Transfer the supernatant to a 10 ml tube.
8. Layer 1 ml of FCS-EDTA under the cell suspension and centrifuge to recover the DC fraction. Resuspend the cells in 2 ml of BSS-EDTA-FCS and count.
9. Maintain the cells at 4°C until they are required for immunofluorescent labeling.

3.6. In Vitro Administration of Flt3L

1. Remove the femurs and tibiae from the desired number of mice, removing as much tissue and sinew as possible and place in RPMI-FCS at 4°C (see Note 37).
2. Use sharp scissors to remove the top of each bone. Hold the bones with a pair of forceps and flush the bone marrow from the bones, using a 23 G needle attached to a syringe filled with RPMI-FCS. Mix the suspension up and down in a syringe fitted with a 26 G needle to create a single cell suspension. Underlay with FCS and centrifuge.
3. Remove the supernatant and gently resuspend the cells in 2–3 ml of RCLM. Expose the cells to RCLM for a maximum of 30 s (see Note 38). Immediately dilute the cells with RPMI-FCS, pass them into a new tube through a sieve to remove

clumps, underlay with FCS and wash by centrifugation. Repeat the washing step twice. Resuspend the cells and count.

4. Centrifuge the cells and resuspend the pellet at $1.5 \times 10^6/\text{ml}$ in Flt3L culture medium (see Note 39). Culture the cells for 8–9 days at 37°C in 10% CO₂ in air (see Note 40).
5. If desired, add 5 ng/ml GM-CSF or 5 ng/ml IL-3 at day 6 of culture, to increase the proportion of CD103⁺ cells in the cells harvested at day 8 or 9.
6. Harvest the cells after culture by gently washing the flasks several times with cold BSS-EDTA-FCS (see Note 41) and centrifuge to pellet. Resuspend the cells in BSS-EDTA-FCS and count to determine recovery.
7. Maintain at 4°C until required for immunofluorescent labeling.

3.7. Immuno-fluorescent Staining and Purification

3.7.1. Via Flow Cytometric Sorting

1. Prepare a cocktail of pre-titrated fluorochrome-conjugated monoclonal antibodies at the appropriate concentration immediately prior to use.
2. Centrifuge the cells and remove the supernatant.
3. Add 10 μl of the fluorochrome-conjugated antibody cocktail per 10^6 cells, resuspend by flicking, and incubate at 4°C for 30 min (see Note 42).
4. Resuspend up to a larger volume with BSS-EDTA-FCS and underlay with FCS-EDTA. Centrifuge to wash the cells.
5. Remove the supernatant leaving the FCS-EDTA layer above the cells and centrifuge again for 30 s to allow any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS-EDTA layer.
6. Resuspend cells in BSS-EDTA-FCS-PI and maintain cells at 4°C until ready for flow cytometry (see Note 43).
7. Sort the DC using a FACSAria (see Note 44). The flow cytometer should be set up with standard lasers in place: a blue 488 nm emitting laser for detection of FITC, PE, PI, PerCp, Cy5.5, and PE.Cy7, a violet/near UV laser, emitting wavelengths of 375 and 405 nm, to detect Quantum Dot655, Quantum Dot605, and Pacific Blue and a red laser emitting a wavelength of 640 nm to detect APC, as well as the appropriate filters and dichroic mirrors. Select DC on the basis of high forward and side light scatter, excluding dead cells with high PI fluorescence. Remove contaminating macrophages by gating out autofluorescent cells using the PI channel in combination with another unused fluorescence channel (see Note 45). Identify CD11c^{int}120G8⁺ pDC and CD11c^{hi}120G8⁻ cDC (see Note 46). Use combinations of conjugated antibodies to identify and sort other dendritic cell subtypes (see Note 47).

3.7.2. Via Immuno-magnetic Beads

1. Centrifuge cells to a pellet.
2. Add YTS169.4-PE (anti-CD8) (see Note 48) at $10 \mu\text{l}/10^6$ cells (see Note 49), resuspend, and incubate at 4°C for 20 min (see Note 50).
3. Resuspend up to a larger volume with BSS-EDTA-FCS and underlay with FCS-EDTA. Centrifuge the cells.
4. Remove the supernatant leaving the FCS-EDTA layer above the cells and centrifuge again for 30 s to allow any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS-EDTA layer.
5. Add anti-PE microbeads (see Note 51) at $1 \mu\text{l}/4 \times 10^6$ cells in a final volume of $2.5 \mu\text{l}/10^6$ cells BSS-EDTA-0.5%FCS (see Note 52).
6. Resuspend gently and incubate for 15 min in a 4°C cold room (see Note 53).
7. Resuspend up to a larger volume with BSS-EDTA-FCS and underlay with FCS-EDTA. Centrifuge the cells to wash away unbound microbeads.
8. Equilibrate a MACS LS column (see Note 54) by placing into a cold miniMACS magnet suspended on a MACS multistand and washing with 3 ml BSS-EDTA-0.5%FCS (see Note 55).
9. Resuspend the cells in 3 ml BSS-EDTA-0.5%FCS and apply them to the column. Rinse the column with 3 ml of BSS-EDTA-0.5%FCS. Repeat the wash three more times and collect the flow through containing the unbound CD8⁻ fraction of cells.
10. Remove the column from the magnet and add 4 ml BSS-EDTA-0.5%FCS to wash the bound CD8⁺ cells from the column. Add a further 4 ml of BSS-EDTA-0.5%FCS. Use the supplied plunger to force all liquid from the column to ensure all cells are removed. Count the recovered CD8⁺ cells.
11. Count the CD8⁻ cells and centrifuge them.
12. Add M1/69-PE (anti-CD24) at $10 \mu\text{l}/10^6$ cells, resuspend, and incubate at 4°C for 20 min (see Note 56).
13. Resuspend up to a larger volume with BSS-EDTA-FCS and underlay with FCS-EDTA. Centrifuge the cells.
14. Remove the supernatant leaving the FCS-EDTA layer above the cells and centrifuge again for 30 s to allow any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS-EDTA layer.
15. Add anti-PE microbeads at $1 \mu\text{l}/4 \times 10^6$ cells in a final volume of $2.5 \mu\text{l}/10^6$ cells BSS-EDTA-0.5%FCS.
16. Repeat steps 6–7.

17. Equilibrate a MACS LD column (see Note 57) by placing into a cold miniMACS magnet suspended on a MACS multistand and washing with 2 ml BSS-EDTA-0.5%FCS (see Note 54).
18. Resuspend the cells in 1 ml BSS-EDTA-0.5%FCS and apply them to the column. Rinse the column with 1 ml of BSS-EDTA-0.5%FCS. Repeat the wash one more time and collect the flow through containing the unbound CD24⁻CD8⁻ fraction of cells. Discard the column (see Note 58).
19. Count the CD24⁻CD8⁻ cells and centrifuge them.
20. Add M1/70-FITC (anti-CD11b) at 10 µl/10⁶ cells, resuspend, and incubate at 4°C for 20 min.
21. Resuspend up to a larger volume with BSS-EDTA-FCS and underlay with FCS-EDTA. Centrifuge the cells.
22. Remove the supernatant leaving the FCS-EDTA layer above the cells and centrifuge again for 30 s to allow any remaining media to wash down the wall of the tube. Remove the remaining media and the FCS-EDTA layer.
23. Add anti-FITC microbeads at 1 µl/4 × 10⁶ cells in a final volume of 2.5 µl/10⁶ cells BSS-EDTA-0.5%FCS.
24. Repeat steps 6–8.
25. Resuspend the cells in 3 ml BSS-EDTA-0.5%FCS and apply them to the column. Rinse the column with 3 ml of BSS-EDTA-0.5%FCS. Repeat the wash three more times and discard the flow through containing the unbound CD24⁻CD8⁻CD11b⁻ fraction of cells.
26. Remove the column from the magnet and add 4 ml BSS-EDTA-0.5%FCS to wash the bound CD24⁻CD8⁻CD11b⁺ cells from the column. Add a further 4 ml of BSS-EDTA-0.5%FCS. Add a further 4 ml of BSS-EDTA-0.5%FCS. Use the supplied plunger to force all liquid from the column to ensure all cells are removed. Count the recovered CD24⁻CD8⁻CD11b⁺ cells (see Note 59).

4. Notes

1. Alternatively use recombinant human Flt3L.
2. The B16Flt3L melanoma was derived from male C57/BL6 mice. Injection into mice of other strains, or into female mice, may elicit an immune response and affect the DC recovered.
3. The level of contamination of collagenase with trypsin or trypsin-like proteases can vary between batches, so each should be tested prior to use. Proteases can strip cell surface molecules

and alter the surface phenotype of the DC. We test each new batch of collagenase for the presence of these proteases by using them to digest thymocytes for 30 min at 37°C and then screening for the loss of the trypsin-sensitive cell surface markers CD4 and CD8 by flow cytometry.

4. A pycnometer is used to determine the density of the Nycodenz accurately, by reference to water, using an analytical balance. The pycnometer is a glass flask with a close-fitting ground glass stopper with a capillary tube in it, which allows air bubbles or excess Nycodenz to escape from the vessel. The pycnometer is weighed empty, full of water, and full of Nycodenz, and the specific gravity of the Nycodenz calculated. A correction needs to be made as the density of water will not be 1 g/cm³ at 4°C.
5. Temperature, pH, and osmolarity all affect the buoyant density of cells, so Nycodenz of higher, or lower, than recommended density will affect the purity and the yield of recovered cells. We calculate the density of Nycodenz at pH 7.2, 308 mOsm/kg and 4°C. Temperature is of particular importance during the density cut so care must be taken to ensure the Nycodenz and the centrifuge to be used for the density cut are at 4°C.
6. The monoclonal antibodies we have included in the cocktail for depletion of non-DC lineage cells and those we have utilized to identify DC and DC subpopulations are available commercially.
7. Appropriate levels should be determined for each batch of antibody to be included in the cocktail. The rat anti-mouse monoclonal antibodies are individually titrated via flow cytometry using a fluorochrome-conjugated anti-rat Ig secondary reagent in order to determine their working dilution. Concentrations determined to result in cell surface saturation of the antigen are considered adequate for efficient depletion and are added to the cocktail.
8. Flt3L should be titrated prior to use by small-scale bone marrow culture. A range of 50–300 ng/ml should be tested. Suboptimal levels will vastly reduce DC yield. Optimal levels can result in DC recoveries of up to 130% of the starting number of bone marrow cells.
9. All proper precautions should be taken when using sodium azide, particularly when preparing the stock solution, where adequate protective clothing, including safety glasses, gloves, and face mask, should be worn. Sodium azide is extremely toxic if ingested.
10. Do not freeze phycoerythrin (PE), allophycocyanin (APC), PerCp.Cy5.5, or Quantum Dots or their conjugates. They are extremely sensitive to freezing and thawing and will lose activity.

11. All proper precautions including protective clothing, safety glasses, gloves, and face mask should be worn during preparation of the stock solution of propidium iodide. Propidium iodide is an irritant and potentially toxic.
12. MSA is used as a carrier protein to minimize loss of Flt3L sticking to tubes, syringes, etc. It may be possible to substitute with endotoxin-free BSA (bovine serum albumin) or even FCS (fetal calf serum) but care must be taken not to elicit an immune response against the carrier protein.
13. Aim to insert the needle and inject as centrally between the shoulder blades as possible. The melanoma will develop rapidly into a large growth and if not located centrally will make normal movement for the mouse very difficult.
14. Mice should be monitored regularly and any showing signs of distress or illness euthanized.
15. The rate of growth of the melanoma should not adversely affect the mice for at least 10 days after injection. After 10 days however, mice will begin to show signs of distress and/or illness. It is therefore recommended that the melanoma should not be allowed to develop for longer than 10 days.
16. Provision has been made to cater for the greatly increased size of spleens treated with Flt3L. A proportional increase or decrease of all listed amounts and volumes should also be made to cater for any change in the starting number of organs.
17. Residual fat will reduce cell viability and combined with undigested connective tissue will accumulate and cause clumping. It is therefore important to clean the organs as much as possible before commencement of the digestion. We use two 20 G needles to perform the cleaning but any suitable instrument may be used.
18. The organs are cut into very small fragments to increase the surface area available to the enzymes. This ensures adequate digestion and maximizes cell yield.
19. Inadequate digestion will result in a lower recovery and the preferential loss of certain DC populations that are more firmly entrenched in the tissue. A digestion time of 20 min should prove sufficient to digest all but the pulpy tissue from spleen, provided the tissue was cut up adequately prior to the digestion and adequate mixing occurred during the digestion. The digestion may be extended to 25 min if required.
20. EDTA inhibits collagenase and effectively ends the digestion. EDTA also chelates Ca^{2+} and Mg^{2+} ions and will dissociate lymphocytes complexed to DC. EDTA must be added to all media from this point onwards to stop the reformation of these multicellular complexes. Failure to do so will cause loss of DC

during purification and possible contamination of the recovered DC with lymphocytes.

21. All centrifugation steps are performed at $1,000 \times g$ for 7 min at 4°C unless otherwise stated. Underlaying the sample with FCS, thereby incorporating a zonal centrifugation step increases the efficiency of separation of cells from smaller particles and soluble material in the supernatant. It is therefore eliminates the need for repetitive “washing” of the cells.
22. Nycodenz has a tendency to settle over time so mix it thoroughly prior to aliquoting and again prior to use to ensure a solution of uniform density.
23. Efficiency of separation will be lost and yields reduced if the density separation is overloaded. Do not load more than four untreated organs or one Flt3L treated (5×10^8 – 10^9 cells) per 10 ml of Nycodenz.
24. A discrepancy between the density of the Nycodenz at the top of an aliquot and the bottom, or between different aliquots (most likely due to inadequate mixing), will affect the ability to layer Nycodenz containing cells over the Nycodenz at the bottom of the tube. Ensure Nycodenz has been adequately mixed and is of uniform density before use.
25. Disruption of the interface creates a density gradient rather than a sharp band and increases the efficiency of the density separation.
26. The light density fraction of cells will be found as a band at the interface zone between the FCS and Nycodenz, while dense cells will have formed a pellet. Cells of intermediate density will be found in the gradient between these zones, so collect all cells down to the 4 ml mark while concentrating on the light density band at the interface. The 4 ml mark is an arbitrary one and collecting a few ml either side of this will not alter recovery greatly. It is just important to be consistent.
27. Adequate dilution and mixing of the light density fraction of cells with EDTA-BSS is essential to recover them as a pellet during centrifugation.
28. This count is used to calculate the appropriate volume of mAb depletion cocktail and immunomagnetic beads required in subsequent steps. It is important that all cells, including any remaining erythrocytes, are included in the count. As a rough guide the light density fraction should represent 5–7% of the starting number of cells.
29. Anti-CD45R is included in the cocktail to deplete B cells but will also deplete pDC, which are all CD45R+. In order to include pDC, but continue to deplete B cells, replace anti-CD45R with anti-CD19.

30. For the isolation of spleen DC we recommend BioMag beads at a 10:1 bead to cell ratio. BioMag beads provide optimal economy and reasonably good efficiency. For optimal efficiency we recommend a two-stage depletion which will optimize efficiency but increase cost by using an initial 5:1 BioMag to cell ratio followed by Dynabeads at a 3:1 bead to cell ratio on the reduced number of cells.
31. Immunomagnetic beads must be washed prior to use to remove preservative.
32. Centrifuging through a layer of FCS separates cells from unbound (excess) mAb.
33. It is important to carefully remove all the supernatant after washing as any remaining unbound mAb will compete for binding to the beads thus decreasing the efficiency of the depletion.
34. The efficiency of the depletion is greatly increased by maximizing contact between beads and cells in the concentrated slurry.
35. Fitting a wide ring around the top of the tube increases the angle of rotation to 30° from the horizontal and keeps the bead-cell slurry concentrated at the bottom of the tube. The wide ring also serves to slow the rate of rotation.
36. If numbers are critical, more DC can be recovered by washing the beads and attaching the tube to the magnet a second time, however this will result in reduced purity.
37. We routinely harvest $4-7 \times 10^7$ cells per mouse.
38. RCLM is toxic so it should be added and mixed gently and then washed away quickly. An exposure of 15–30 s is sufficient to lyse erythrocytes.
39. The optional addition of 300 pg/ml GM-CSF to the Flt3L culture medium will improve DC yield.
40. Maximum yield of the DC subpopulations can also vary with culture time, so an optimal culture time should be determined for each batch of Flt3L. Production of pDC peaks earlier than that of cDC.
41. This gentle washing step should be sufficient to remove any slightly adherent DC from the plastic but leave behind any more adherent macrophages.
42. All immunostaining steps should be performed at 4°C to promote cell viability and to prevent capping of monoclonal antibody from the cell surface.
43. Propidium iodide is used for dead cell exclusion during flow cytometric analysis.
44. Any flow cytometer with sorting capabilities and appropriate lasers and optical set-up can be used.

45. Remove autofluorescent cells (mainly macrophages) during fluorescence-activated cell sorting or analysis by gating out cells that have low levels of fluorescence in two or more fluorescent channels. Ideally use a combination of the PI and an unused channel. During multicolor sorting or analysis, it may be necessary to combine autofluorescence in the PI channel with low fluorescence in a channel that is being used. Choose a parameter where all DC will fluoresce brightly (i.e., CD11c) and gate out cells of low fluorescence.
46. CD45RA and CD45R (B220) are alternative markers to 120G8 and are often used to separate pDC from cDC.
47. If functional studies are to be undertaken the cells should be washed to remove propidium iodide post-sorting.
48. If using DC expanded by Flt3L administration it is advisable to purify the more abundant CD8⁺ fraction first in order to avoid contamination of the CD11b⁺ fraction.
49. Too high a concentration of antibody added at this stage tends to increase the nonspecific loss of DC and lower the yield. We routinely use antibodies at a quarter to a half their saturating levels, but would recommend that each user carefully titrate their antibody for optimal performance.
50. It is not important what fluorochrome the anti-CD8 monoclonal antibody is conjugated to provided an appropriate anti-fluorochrome bead is available.
51. Although directly coupled anti-CD8 beads are available, we find that efficiency of separation is lowered when they are used.
52. This is a lower bead:cell ratio than recommended by the manufacturer. We have compensated for this by reducing the volume in order to increase the final concentration and maximize bead to cell contact. This has resulted in an economical and more efficient process.
53. Incubation may also be completed in a refrigerator, however ice should be avoided as temperatures below 4°C will decrease binding.
54. Do not overload the column. This will result in a lower recovery and reduced purity. An LS column can accommodate up to 5×10^8 cells with a maximum of 10^8 positive cells and an LD column up to 10^9 cells with a maximum of 5×10^8 positive cells. Ensure accurate counts of cells are made and appropriate numbers of columns used.
55. Cell viability is increased at 4°C so cooling the magnet before use ensures that the column and cell suspension passing through it are kept cold during the separation. Placing the magnet at -20°C and the columns at 4°C for 15 min before use is recommended.

56. A second depletion for CD8 bearing cells using anti-CD24 conjugated to PE is necessary to improve purity of the CD11b⁺ cells. This depletion also removes the CD8-CD24⁺ precursors of the CD8⁺ DC.
57. LS columns are designed for selection and LD columns for depletion. So when positively selecting a population (CD8⁺ or CD11b⁺) we use an LS column and when trying to select a population we wish to discard (CD24⁺) we use an LD column.
58. As the bound fraction of cells in this case will be a mix of CD8⁺CD24⁺ and CD8-CD24⁺ cells it is typically discarded. If this fraction is required it may be recovered using the typical washing procedure used to recover the CD8⁺ and CD11b⁺ fractions.
59. Purity of all collected populations may be tested. Fluorochromes on the surface of the cells are not affected by bound beads and can be readily detected using flow cytometry.

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References

1. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9:271-296
2. Jung S, Unutmaz D, Wong P, Sano G, De Los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA (2002) In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8⁺ T cells by exogenous cell-associated antigens. *Immunity* 17:211-220
3. Liu CH, Fan YT, Dias A, Esper L, Corn RA, Bafica A, Machado FS, Aliberti J (2006) Cutting Edge: Dendritic cells are essential for *in vivo* IL-12 production and development of resistance against Toxoplasma gondii infection in mice. *J Immunol* 177:31-35
4. Ciavarra RP, Stephens A, Nagy S, Sekellick M, Steel C (2006) Evolution of immunological paradigms in a virus model: are dendritic cells critical for antiviral immunity and viral clearance? *J Immunol* 177:492-500
5. Watanabe N, Wang YH, Lee HK, Ito T, Cao W, Liu YJ (2005) Hassall's corpuscles instruct dendritic cells to induce CD4⁺CD25⁺ regulatory T cells in human thymus. *Nature* 436:1181-1185
6. Luo X, Tarbell KV, Yang H, Pothoven K, Bailey SL, Dind R, Steinman RM, Suthanthiran M (2007) Dendritic cells with TGF-β1 differentiate naïve CD4⁺CD25⁻ T cells into islet-protective Foxp3⁺ regulatory T cells. *Proc Natl Acad Sci U S A* 104:2821-2826
7. Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, Steinman RM (2003) Direct expansion of functional CD25⁺CD4⁺ regulatory T cells by antigen presenting dendritic cells. *J Exp Med* 198:235-247
8. Wilson NS, El-Sukkari D, Belz GT, Smith CM, Steptoe RJ, Heath WR, Shortman K, Villadangos JA (2003) Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 102:2187-2194
9. Villadangos JA, Schnorrer P (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets *in vivo*. *Nat Rev Immunol* 7:543-555

10. Heath WR, Kurts C, Miller JF, Carbone FR (1998) Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. *J Exp Med* 187:1549–1553
11. Heath WR (2004) Cross presentation, dendritic cell subsets, and generation of immunity to cellular antigens. *Immunol Rev* 199:9–26
12. Vremec D, Shortman K (1997) Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes on incubation and differences between thymus, spleen and lymph nodes. *J Immunol* 159:565–573
13. Vremec D, O'Keeffe M, Wilson A, Ferrero I, Koch U, Radtke F, Scott B, Hertzog P, Villadangos J, And Shortman K (2011) Factors determining the spontaneous activation of splenic dendritic cells in culture. *Innate Immun* 17:338–352
14. Shortman K, Caux C (1997) Dendritic cell development: multiple pathways to nature's adjuvant. *Stem Cells* 15:409–419
15. Shortman K, Liu YJ (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151–161
16. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K (2000) CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 164:2978–2986
17. Vremec D (2010) The isolation of mouse dendritic cells from lymphoid tissues and the identification of dendritic cell subtypes by multi-parameter flow cytometry. In: Naik SH (ed) *Dendritic cell protocols*, vol 595, Methods in molecular biology. Humana, Totowa, NJ, pp 205–229
18. O'Keeffe M, Hochrein H, Vremec D, Caminschi I, Miller JL, Anders EM, Wu L, Lahoud MH, Henri S, Scott B, Hertzog P, Tatarczuch L, Shortman K (2002) Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8⁺ dendritic cells only after microbial stimulus. *J Exp Med* 196:1307–1319
19. Asselin-Paturel C, Brizard G, Pin J-J, Briere F, Trinchieri G (2003) Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* 171:6466–6477
20. Naik SH, Shortman K (2007) Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7:19–30
21. Hochrein H, Shortman K, Vremec D, Scott B, Hertzog P, O'Keeffe M (2001) Differential production of IL-12, IFN- α and IFN- γ by mouse dendritic cell subsets. *J Immunol* 166:5448–5455
22. Den Haan JM, Lehar SM, Bevan MJ (2000) CD8⁺ but not CD8⁻ dendritic cells cross-prime cytotoxic T cells *in vivo*. *J Exp Med* 192:1685–1696
23. Pooley JL, Heath WR, Shortman K (2001) Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8⁻ dendritic cells, but cross-presented to CD8 T cells by CD8⁺ dendritic cells. *J Immunol* 166:5327–5330
24. Proietto AI, O'Keeffe M, Gartlan K, Wright MD, Shortman K, Wu L, Lahoud MH (2004) Differential production of inflammatory chemokines by murine dendritic cell subsets. *Immunobiology* 209:163–172
25. Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, Lahoud MH, O'Keeffe M, Shao QX, Chen WF, Villadangos JA, Shortman K, Wu L (2005) Cutting edge: generation of splenic CD8⁺ and CD8⁻ dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 174:6592–6597
26. Caminschi I, Proietto AI, Ahmet F, Kitsoulis S, Teh JS, Lo JCY, Rizzitelli A, Wu L, Vremec D, van Dommelen SLH, Campbell IK, Maraskovsky E, Braley B, Davey GM, Mottram P, van de Velde N, Jensen K, Lew AM, Wright MD, Heath WR, Shortman K, Lahoud MH (2008) The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* 112:3264–3273
27. D'Amico A, Wu L (2003) The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. *J Exp Med* 198:293–303
28. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* 184:1953–1962
29. Mach N, Gillessen B, Wilson SB, Sheehan C, Mihm M, Dranoff G (2000) Differences in dendritic cells stimulated *in vivo* by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60:3239–3246
30. Brasel K, De Smedt T, Smith JL, Maliszewski CR (2000) Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 96:3029–3039
31. Gilliet M, Boonstra A, Paturel C, Antonenko S, Xu XL, Trinchieri G, O'Garra A, Liu YJ (2002) The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT-3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med* 195: 953–958

32. Sathe P, Pooley J, Vremec D, Mintern J, Jin JO, Wu L, Kwak JY, Villadangos JA, Shortman K (2011) The acquisition of antigen cross-presentation function by newly formed dendritic cells. *J Immunol* 186:5184–5192
33. Bedoui S, Prato S, Mintern J, Gebhardt T, Zhan Y, Lew AM, Heath WR, Villadangos JA, Segura E (2009) Characterization of an immediate splenic precursor of CD8⁺ dendritic cells capable of inducing antiviral T cell responses. *J Immunol* 182:4200–4207
34. Segura E, Kapp E, Gupta N, Wong J, Lim J, Ji H, Heath WR, Simpson R, Villadangos JA (2010) Differential expression of pathogen-recognition molecules between dendritic cell subsets revealed by plasma membrane proteomic analysis. *Mol Immunol* 47: 1765–1773

Chapter 25

Preparation of Dendritic Cells by In Vitro Cultures

Mirjana Weimershaus and Peter van Endert

Abstract

In vitro cultures of bone marrow-derived precursors are a convenient method for generating dendritic cells (DC). This method additionally overcomes the problem of low availability of certain DC types, DC heterogeneity, and laborious procedures encountered using ex vivo isolation protocols.

Here we describe two standard protocols for in vitro differentiation of steady-state DC equivalents with Fms-like tyrosine kinase 3 ligand (Flt3L) and inflammatory-like DC using granulocyte-macrophages-colony-stimulating factor (GM-CSF). These protocols allow for obtaining up to 2×10^8 CD11c^{high} inflammatory-like DC and up to 5×10^6 equivalents of each CD8+ and CD8- conventional DC and plasmacytoid DC.

Key words: Dendritic cells, GMCSF, Flt3L, Bone marrow-culture

1. Introduction

Dendritic cells (DC) are the most potent cells in antigen presentation and T cell priming. Many of their specific features and their crucial role in mediating immune responses and maintaining tolerance can and have been studied in in vivo systems. However, ex vivo analyses of DC by biochemical and cell biological methods that often require large numbers of cells can be hampered by the fact that DC constitute a relatively rare population in vivo (1), accounting for only 1–2% of total cells in most tissues. The isolation and use of ex vivo DC are further complicated because DC are functionally and phenotypically heterogeneous. For instance, at least three distinct DC subpopulations can be distinguished in the spleen, the major site of T cell priming against blood-borne antigens: CD8+ and CD8- conventional dendritic cells (cDC) and plasmacytoid DC (pDC) (2). CD8+ DC excel in antigen processing and presentation via the MHC class I pathway, by both direct presentation and cross-presentation (3, 4), and are thought to be the main DC subset

responsible for the in vivo induction of CD8+ T cell responses against pathogens, especially viruses (5). In contrast, CD8- DCs are the most efficient subset in presenting internalized antigen to CD4+ T cells via the MHC class II pathway (6, 7). The salient property of pDCs is their capacity to rapidly secrete high amounts of type I interferons (IFN) upon viral stimulation (8), which is triggered via signaling through the highly expressed TLR7 and 9 (9).

The development of these steady-state DC subsets critically depends on signaling through the cytokine receptor Fms-like tyrosine kinase 3 and its ligand (Flt3L), and Flt3-deficient mice show strongly reduced DC numbers under steady-state conditions (10).

Additionally, a distinct subtype of DC differentiates from monocytes in vivo under inflammatory conditions in the presence of the cytokine granulocyte/macrophage-colony stimulating factor (GM-CSF) and in response to specific microbial components (11).

In vitro cultures of bone-marrow precursors supplemented with Flt3L or GM-CSF allow for generating steady-state and inflammatory DC, respectively, in a controlled and technically simple fashion, overcoming also the important limitation of cell numbers of certain subsets, such as CD8+ DC. While generally no more than 5×10^5 cells of this subtype can be isolated from a single mouse spleen, culturing bone-marrow precursors from femurs and tibiae of one mouse in Flt3L-containing medium will yield up to 5×10^6 equivalents of this rare DC type.

2. Materials

2.1. Common Consumables and Lab Equipment

1. Donor mice.
2. Dissecting instruments (scissors and forceps).
3. Petri dishes, diameter 100 and 150 mm.
4. 50 ml conical centrifugation tube.
5. 5 ml or 10 ml syringes and 25 gauge needles.
6. Flow cytometer with possibility of cell sorting.

2.2. Generation of Dendritic Cells in GMCSF-Stimulated Bone-Marrow Cultures

1. Sterile Phosphate-buffered saline (PBS).
2. Conditioned complete Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol, and 20 ng/ml granulocyte-macrophage colony-stimulating factor (see Note 1).
3. Monoclonal antibody to CD11c (clone N418) and access to cell sorting facility or CD11c microbeads (Miltenyi) for MACS sorting via manual columns (Miltenyi) or AutoMACS®.

4. Optional: Red cell lysis buffer, commercial or made as 10× solution using 0.15 M ammonium chloride (8.26 g NH₄Cl/L), 10 mM potassium bicarbonate (1 g KHCO₃/L) and 0.1 mM EDTA (0.037 g/L) in H₂O (see Note 2).
1. Sterile Phosphate-buffered saline (PBS).
2. Conditioned complete Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol, and 200 ng/ml Fms-like tyrosine kinase 3-ligand (Flt3L) (see Note 3) and 300 pg/ml granulocyte-macrophage colony-stimulating factor.
3. Optional: Red cell lysis buffer, commercial or made as 10× solution from 0.15 M ammonium chloride (8.26 g NH₄Cl), 10 mM potassium bicarbonate (1 g KHCO₃), and 0.1 mM EDTA (0.037 g) in H₂O (see Note 2).
4. Monoclonal antibodies anti-CD11c (clone N418), anti-CD11b (M1/70), anti-CD24 (M1/69), anti-CD45R/B220 (RA3-6B2), and anti-SIRPα (p84).

3. Methods

3.1. Generation of Dendritic Cells in GM-CSF-Stimulated Bone-Marrow Cultures

1. Euthanize mice. Turn mice on the back and wet the hind leg region with 70% ethanol (see Note 4).
2. To remove the skin, first perform a longitudinal cut on the inner side of the leg from the hip downwards to the foot. Pull away the skin to both sides and cut off the foot at the ankle without opening the cavity of the tibia. Separate the bone from the skin starting from the distal end by cutting as close as possible along the bone in order to remove the bulk of muscle material. Sever femur from hip without opening the bone cavity. Remove all muscle as well as the fibula, disjoint the knee ligaments and carefully separate femur and tibia at the knee.
3. Place dissected femurs and tibiae into 100 mm sterile PBS-filled Petri dishes and repeat step 2 for all hind legs.
4. The following steps have to be performed under the cell culture hood. Sterilize scissors and forceps with 96% ethanol. Transfer the bones into a new 100 mm Petri dish containing 70% ethanol and submerge for no more than 30 s.
5. Transfer bones into a new 100 mm Petri dish containing sterile PBS. Attach a 25 g needle to a 5 ml or 10 ml syringe and fill with PBS.
6. Using scissors and forceps, cut off the distal and proximal end of each bone.

7. Insert needle into the cavity and flush the bone with PBS until bone turns white. Collect bone-marrow-containing PBS in a sterile 50 ml conical centrifugation tube.
8. Fill up tube with serum-free IMDM and centrifuge for 5–10 min at $500 \times g$.
9. Discard supernatant.
10. Resuspend bone-marrow cells at a concentration of 5×10^5 cells/ml in conditioned complete IMDM containing 20 ng/ml GMCSF and transfer into 150 mm Petri dishes (see Notes 2 and 5).
11. Culture at 37°C , 5% CO_2 .
12. After 4 or 5 days of culture, collect non-adhering cells by transferring culture supernatant into 50 ml conical centrifugation tubes and loosely adhering cells by washing plates twice with cold PBS. Add wash fluid to 50 ml conical centrifugation tubes.
13. Centrifuge 5–10 min at $500 \times g$, and discard supernatant.
14. Resuspend cells at 5×10^5 cells/ml in conditioned complete IMDM containing 20 ng/ml GMCSF and transfer into new 150 mm Petri dishes.
15. Re-culture at 37°C , 5% CO_2 for 2–5 days, depending on application (see Note 6). Collect cells from cultures cells as in step 12.
16. Fully differentiated DC can be distinguished and/or separated from precursor cells by staining with anti-CD11c antibodies and subsequent FACS or MACS according to reagent supplier's information.

**3.2. Generation
of Dendritic Cells
In Flt3L-Stimulated
Bone-Marrow Cultures**

1. Follow Subheading 3.1, steps 1–9.
2. Resuspend bone marrow cells at a concentration of 1×10^6 cells/ml in conditioned complete IMDM containing 300 ng/ml Flt3L and 300 pg/ml GM-CSF (see Notes 2, 5 and 7) and distribute in 100 mm Petri dishes or smaller cell culture plates as desired.
3. Culture at 37°C , 5% CO_2 , for 5–7 days (see Note 8).
4. 48 h before the planned harvest date of the DC, add GM-CSF to a final concentration of 1 ng/ml (see Note 9).
5. Continue culture for 2 days at 37°C , 5% CO_2 .
6. Collect non-adhering cells by transferring culture supernatant into 50 ml conical centrifugation tubes, and loosely adhering cells by washing plates twice with cold PBS and also transfer wash medium into 50 ml conical centrifugation tubes.
7. Equivalents of the in vivo subsets of pDC, CD8+ DC, and CD8- DC can be discriminated and/or sorted by staining with

anti-CD11c, anti-CD11b, anti-CD24, anti-B220/CD45RA, and anti-SIRP α (see Note 10) followed by flow-cytometry-based analysis or sorting according to reagent supplier's information.

4. Notes

1. Purified recombinant murine GMCSF is commercially available from many companies. Another widely used source of GM-CSF are cell lines, e.g. J558 cells, stably transfected with murine GM-CSF. The concentration of GM-CSF secreted into the culture supernatant by these transfectants can be quantified by ELISA (available from different companies, e.g. eBiosciences, Cat.: 88-7334-22). The J558 culture supernatant will then be added to the DC medium at a final concentration of 20 ng/ml. However, we find it also convenient to functionally determine the appropriate J558 supernatant concentration by performing small-scale BM cultures in DC medium containing different concentrations of J558 supernatant (for instance 0, 1, 2, 5, 10 and 20%) and assay the DC differentiation and maturation on day 6 by staining for CD11c, MHC class II, CD86, and CD80.
2. The bone marrow is highly vasculated and therefore rich in red blood cells. These cells should not be taken into account when counting cells for setting up the correct per-well concentration of hematopoietic precursors. Red blood cells can be distinguished from leukocytes under the light microscope. However, it is also possible to eliminate red cells prior to counting by short exposure (maximum 5 min) of the isolated bone-marrow to red cell lysis buffer (commercially available or prepared in the lab).
3. Purified recombinant human or murine Flt3L is commercially available, e.g. from R&D Systems (human; Cat.: 308-FK) or eBiosciences (murine; Cat.:14-8001-62). Flt3L has cross-species activity in mouse and human. Flt3L can also be affinity-purified from culture supernatant of Chinese Hamster Ovary (CHO) cell lines transfected with Flt3L-FLAG available in different laboratories ([2](#), [4](#), [12](#)).
4. Subheading [3.1](#), steps 1–3 can be performed outside the cell culture hood, while Subheading [3.1](#), steps 4–14 must be performed under sterile conditions.
5. The use of Petri dishes is preferable to cell culture-treated surfaces because the DC adhere less to the untreated plastic surface and largely conserve their immature phenotype, allowing for controlled induction of maturation by TLR ligands or other agents, as well as for other experiments requiring immature DC properties, such as high phagocytic activity.

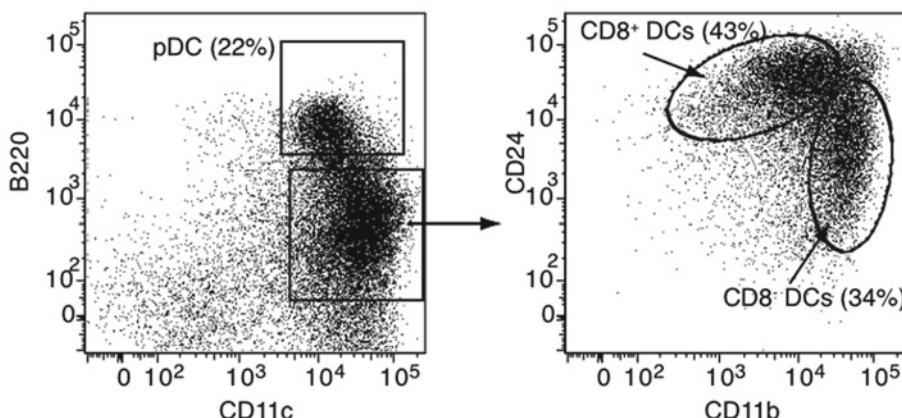


Fig. 1. Generation of steady-state DC equivalents in vitro. Typical FACS profile showing gating allowing for DC subset sorting from an Flt3L culture on day 7.

6. When first setting up the culture, staining and FACS analysis for CD11c and maturation markers, including CD80 and CD86, should be performed on day 5, 6, and 7 (and later time points if desired) in order to determine the optimal culture duration to obtain a maximal number of immature ($CD86^{\text{low}}$ $CD80^{\text{low}}$ $CD11c^{\text{high}}$) DC (also see Note 5).
7. Low levels of GM-CSF in the culture are required in order to confer to the CD8+ equivalent subset the capacity to cross-present antigen (12).
8. It should be noted that the numbers of the different DC subsets peak at different time points. For instance, for obtaining the highest portion of pDC equivalents, 5 days of culture are optimal, whereas the cDC subsets numbers increase until day 10. If all three subsets are desired, 7–8 days of culture are suitable.
9. The addition of 1 ng/ml GM-CSF during the last 48 h of culture increases the portion of fully differentiated, cross-presentation-competent CD103+ CD8+ DC equivalents (12).
10. PDC equivalents are B220+ CD11c+, while CD8+ DC equivalents are B220- CD11c+ CD24+ CD11b- SIRP α -, and CD8- DC equivalents B220- CD11c+ CD24 $^{\text{low}}$ CD11b+ SIRP α + (Fig. 1).

References

1. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811
2. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7(1):19–30
3. den Haan JM, Lehar SM, Bevan MJ (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192(12):1685–1696
4. Kamphorst AO, Guermonprez P, Dudziak D, Nussenzweig MC (2010) Route of antigen uptake differentially impacts presentation by

- dendritic cells and activated monocytes. *J Immunol* 185(6):3426–3435
5. Lopez-Bravo M, Ardavin C (2008) In vivo induction of immune responses to pathogens by conventional dendritic cells. *Immunity* 29(3):343–351
 6. Carter RW, Thompson C, Reid DM, Wong SY, Tough DF (2006) Preferential induction of CD4+ T cell responses through in vivo targeting of antigen to dendritic cell-associated C-type lectin-1. *J Immunol* 177(4):2276–2284
 7. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumppheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG, Steinman RM, Nussenzweig MC (2007) Differential antigen processing by dendritic cell subsets in vivo. *Science* 315(5808):107–111
 8. Siegal FP, Kadokami N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ (1999) The nature of the principal type 1 interferon-producing cells in human blood. *Science* 284(5421):1835–1837
 9. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* 98(16):9237–9242
 10. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Teepe M, Lyman SD, Peschon JJ (2000) Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 95(11):3489–3497
 11. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, Jeffrey KL, Anthony RM, Kluger C, Nchinda G, Koh H, Rodriguez A, Idoyaga J, Pack M, Velinzon K, Park CG, Steinman RM (2010) Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143(3):416–429
 12. Sathe P, Pooley J, Vremec D, Mintern J, Jin JO, Wu L, Kwak JY, Villadangos JA, Shortman K (2011) The acquisition of antigen cross-presentation function by newly formed dendritic cells. *J Immunol* 186(9):5184–5192

Chapter 26

Monitoring Dendritic Cell Activation and Maturation

Ben Fancke and Meredith O'Keeffe

Abstract

Since the 1997 discovery that the first identified human homologue of *Drosophila* Toll could activate the innate immune system, the innate arm of immunity has rapidly taken on a new light as an important player in the recognition of pathogens and damaged self. The recognition of danger by dendritic cells (DC) is a crucial step in activating the adaptive immune system. Different DC express varied subsets of pattern recognition receptors (PRR), enabling both overlap and exclusivity in the recognition of danger signals by DC. PRR-mediated DC maturation and activation can be measured by changes in the surface expression of costimulatory molecules and changes in size and shape of the DC and by their production of different cytokines.

Key words: Dendritic cell, Dendritic cell activation, Dendritic cell maturation, Pattern recognition receptor, Innate immune responses

1. Introduction

Dendritic cells (DC) are immune sentinels vital for the initiation and regulation of immune responses. They represent a highly heterogeneous population within the body that differ in phenotype and function depending on where they are found and the immunological roles they play. In the “steady state,” nonactivated/immature DC constantly sample their local environment in search of foreign antigen (Ag) to present to T-cells. These immature DC typically express high levels of CD11c and intermediate levels of MHCII and have low levels of costimulatory molecules such as CD25, CD40, CD69, CD80, CD83, and CD86 on their surface. DC that capture Ag in the periphery during this steady state can move to draining lymph nodes to present captured Ag to T-cells. These migratory DC may not be “activated” as such but are often classified as “mature” due to their increased expression

of MHCII and costimulatory markers and their migratory capacity. The activation of a DC relies on its recognition of “danger signals” via PRR.

DC can therefore generally be categorized into three distinct subsets based on their maturation/activation status:

- (a) Immature, nonactivated DC such as the conventional DC found in the “steady-state” spleen expressing high levels of CD11c and low-to-intermediate levels of MHCII and costimulatory markers. Without prior activation these DC do not produce inflammatory cytokines but are able to stimulate naïve T cells and are potentially tolerogenic in a nonactivated state. The plasmacytoid (p) DC express lower levels of CD11c and MHCII, are poor stimulators of naive T cells, and do not produce pro-inflammatory cytokines in the steady state.
- (b) Mature but nonactivated, and potentially tolerogenic, migratory DC that show intermediate CD11c expression and higher levels of MHCII and costimulatory markers on their surface but do not produce inflammatory cytokines.
- (c) Mature, activated DC that have encountered “danger signals” in response to an invading pathogen or damaged self. These DC produce large amounts of inflammatory cytokines that differ depending on the DC subset and express very high levels of MHCII and costimulatory molecules on their surface.

Pathogen-associated “danger signals” are recognized by DC via Pattern Recognition Receptors (PRR) located within the DC or on its surface. Since the discovery of the first mammalian Toll-like Receptor (TLR) that activated the innate system (1), the innate immune system has been shown to possess a previously unrecognized plasticity in its ability to respond to invading pathogens. These activation-inducing “danger signals” recognized by DC include the following:

- (a) Pathogen-Associated Molecular Patterns (PAMPs). Evolutionarily conserved molecules associated with pathogens such as bacterial cell wall components or viral/bacterial DNA and RNA segments that are not normally found within eukaryotic organisms.
- (b) Damage-Associated Molecular Patterns (DAMPs) such as nuclear or cytosolic proteins released by necrotic cells of self-origin.
- (c) Inflammatory cytokines released by proximal DC or other cells.

Activation in response to danger signals causes changes in the DC phenotype and morphology. These changes can include; increased distribution of MHC to the cell surface; increased surface expression of costimulatory markers, adhesion molecules, and chemokine

receptors; secretion of cytokines, chemokines, and proteases; and architectural transformation to a stellate morphology showing the characteristic dendrites after which the cells are named. All of these maturation- or activation-induced changes are detectable by microscopy, FACS analysis, or soluble protein excretion assays such as ELISA or Multiplexed Bead-based Immunoassays.

Due mostly to a lack of availability of lymphoid organs for research most of what we know of human DC derives from those found in blood. While mouse blood DC show an immature phenotype and poor T-cell stimulatory capacity without prior maturation (2), DC found in human blood express higher levels of costimulatory molecules and can stimulate division of naïve T-cells with an efficiency similar to that of mouse spleen DC without further maturation. Whether this difference is due to the clean versus unclean development of laboratory mice and humans respectively, or an inherent species difference, is unknown. Mature or activated human DC subsets regulate surface expression of activation markers and produce proinflammatory cytokines in a similar manner to mouse DC. The DC subsets present in human blood have now with reasonable confidence been correlated to DC found in mouse lymphoid organs through comparative functional studies (3–6) and genome profiling (7). These correlations are vital if DC are to be targeted to promote or inhibit immune responses by vaccination. Several recent reviews succinctly summarize the types of PRR present in DC subsets, the components of their signaling pathways, the PRR localization within the cell, and the PAMPs they recognize (8–16).

2. Materials

2.1. *In Vitro DC Culture*

Unless otherwise specified all media components available from GIBCO.

1. Human or mouse purified DC.
2. Culture Media: RPMI-1640 containing GlutaMAX™ (see Note 1), with 100 U/mL Penicillin/100 µg/mL Streptomycin, 0.1 mM 2-Mercaptoethanol, 10% heat-inactivated (see Note 2) FCS (see Note 3). Culture Media can be aliquoted and frozen for later use.
3. Tissue culture plates, 96-Well U-Bottom (see Note 4) with low evaporation lid. Sterile, polystyrene, nonpyrogenic (BD FALCON).
4. Culture stimulants (see Note 5).
5. Light microscope.

2.2. FACS Analysis of Cultured DC

1. 0.1 M Ethylenediaminetetraacetic Acid (EDTA) pH 7.2, sterile filtered through a 0.2 µm filter pack. Stored at 4°C.
2. FACS Buffer: PBS adjusted to mouse or human tonicity (see Note 6) containing 2 mM EDTA and 2% FCS.
3. Blocking reagent: Anti-CD16/32 mAb.
4. Fluorescent antibodies for labelling of surface markers (see Note 13)
5. Cell viability indicator: Propidium iodide.
6. Flow cytometer and appropriate software analysis program such as FlowJo (Tree Star Inc) or Weasel (WEHI).

3. Methods

DC should be kept at 4°C to reduce DC metabolism while in media. Ensure that media and cells are stored on ice and centrifugations are refrigerated.

3.1. In Vitro Activation of Purified DC Populations

1. DC should be purified using established protocols previously outlined for mouse DC (3) or human DC (4).
2. Wash the purified DC populations once in Culture Media and pellet by centrifugation, $400 \times g$, 7 min, 4°C.
3. Taking into account the cell number you have and require/well (see Note 4) and the activation conditions you wish to test (in duplicate, see Note 5), resuspend cells in Culture Media at $2 \times$ final concentration and keep on ice.
4. To avoid adding concentrated stimulants directly to cells, which may cause inconsistent results and cell death, prepare culture stimulants at $2 \times$ final concentration (see Note 5) in Culture Media.
5. Transfer the required number of cells at $2 \times$ concentration to labeled culture plate (see Note 4). Add equal volume of $2 \times$ concentrated stimulants to duplicate wells containing DC, thus bringing DC/stimulants to end concentrations (see Note 5).
6. Be sure to include duplicate *Culture Media-only* control wells of each DC population (see Note 7, Fig. 1) and to fill the surrounding wells with Culture Media to reduce evaporation from outer wells. Culture in humid 37°C incubator, ≥ 18 h (see Note 8).
7. Cells should be monitored by light microscope for visual confirmation of activation (Fig. 2, see Note 9).

Dotted Black Line: 37 degrees 20h Media without Ab staining

Thick Black Line: 4 degrees 20h Media

Grey Filled: 37 degrees 20h Media

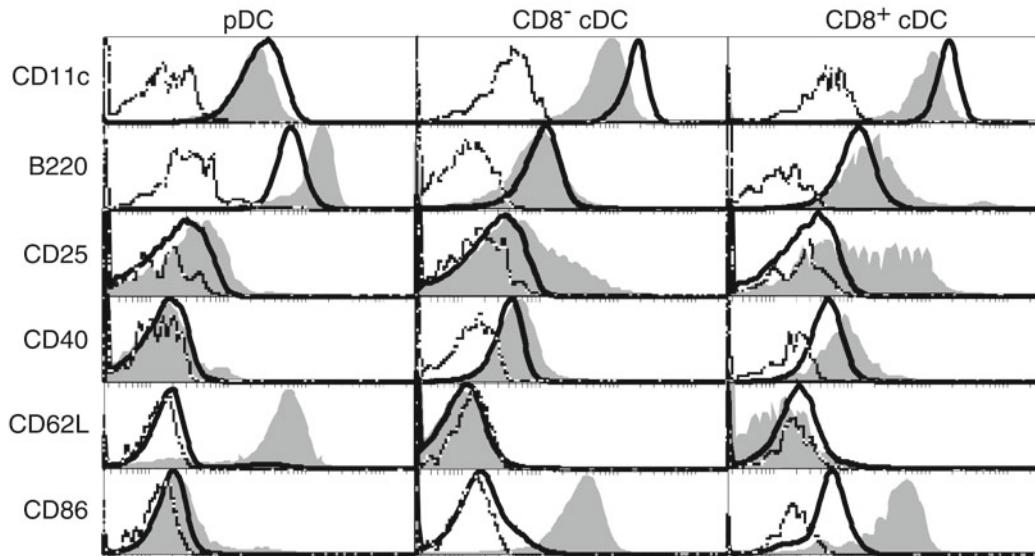


Fig. 1. C57BL/6 splenic DC populations sorted by FACS into three DC populations: pDC ($CD11c^{int}$, $B220^+$) and $2 \times$ cDC ($CD11c^{hi}$, $B220^-$) populations based on CD8 expression. Sorted DC were then either kept on ice (*black line*) or cultured at 37°C in 96-well U-bottom culture plates at 1×10^6 cells/mL (*grey filled*) for 20 h. Cell populations were then restained for CD11c and B220 using the same antibody fluorochromes as for sorting and tested for expression of various activation markers. An unstained control is included to show baseline fluorescence (*dotted black line*) after overnight culture at 37°C.

3.2. Surface Staining of Activated DC

1. Individually resuspend each well after culture and transfer to Eppendorf tube. Spin cell in minifuge at $6,000 \times g$, 5 min, 4°C (see Note 10).
2. Return supernatants to respective wells for later cytokine analysis (see Note 11). Plates can be sealed with parafilm and frozen at this stage. Resuspend DC pellets in FACS Buffer. The cells from duplicate wells can be pooled at this stage. Spin cells in minifuge at $6,000 \times g$, 5 min, 4°C.
3. Cell pellets are incubated with 1 mg/mL purified anti-CD16/32 mAb (or similar blocking reagent) for 20 min on ice (10 µL per 1×10^6 cells, minimum 15 µL) (see Note 12).
4. An equal volume of 2× concentrated antibody mix (see Note 13) is added to the cell suspension and incubated for a further 20 min. Cells are washed 1× in FACS buffer and resuspended in 200–300 mL of FACS Buffer containing 1 µg/mL propidium iodide (PI) or similar cell viability indicator (see Note 14) for FACS analysis (see Note 15).

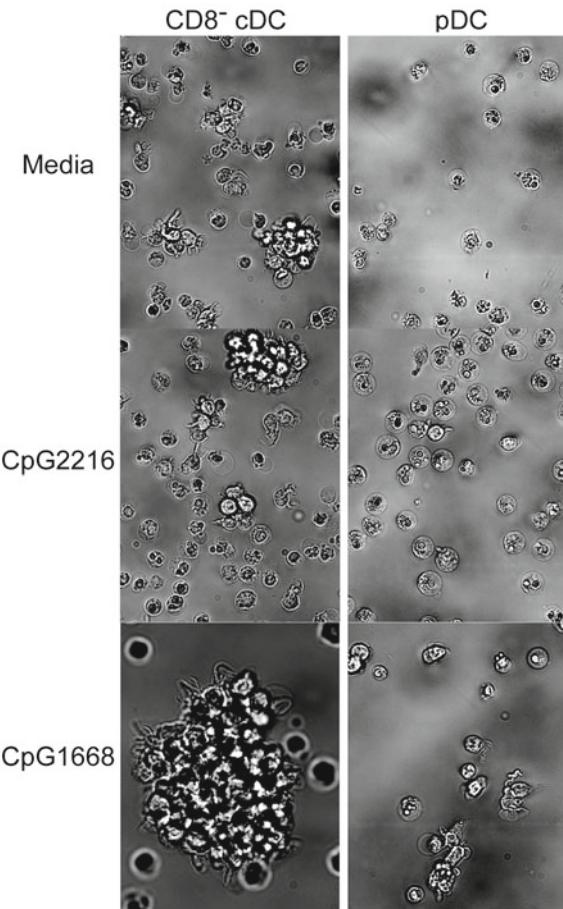


Fig. 2. 1×10^5 pDC or CD8⁻ cDC were cultured at 1×10^6 cells/mL in μ -Slide 8 well ibiTreat slide (Ibidi) in media or stimuli for 20 h at 37°C in a humidified atmosphere containing 5% CO₂. Images collected by Olympus IX51 light microscope ($\times 40$ images shown). The use of flat-bottom plates required for imaging reduces clustering of activated DC.

4. Notes

1. GlutaMAX™ is a commercial substitute for l-glutamine that is more stable in long-term culture, preventing buildup of ammonia within the cultures that may hinder cell development.
2. FCS batches need to be heat inactivated (HI) before use in culture to denature complement that may be present in serum. Place thawed bottle of FCS in 56°C water bath for 40 min. Be sure to mix contents manually by swirling regularly to ensure even temperature distribution and reduce precipitation of the FCS. FCS being added to RPMI should be passed through a 0.2 μ m filter to ensure sterility and reduce precipitates within Culture Media.

3. Large variation is seen between manufacturers and batches of FCS. We have found that the best way to test the suitability of FCS for DC culture is to test batches before purchasing for their ability to induce in vitro DC development in Flt3-ligand cultures of mouse bone marrow (17).
4. In culture, DC prefer and are more likely to survive when in proximity to each other. This is true to a point as overcrowding can also be problematic. Do not exceed 2×10^6 cells/mL for 96-well U-bottom plates. Wells typically hold no more than 250 μ L (=Max 5×10^5 cells/well). To maintain proximity between DC $\leq 25 \times 10^3$ cells/well should be cultured in 96-well V-bottom cell culture microplates (Costar®, Corning Inc.). Flat-bottom wells can be used but the U- and V-bottom wells are favorable.
5. As understanding of DC activation increases so does the list of ligands such as inflammatory cytokines, PAMPs, and DAMPs that can induce these responses. The known PRR that are expressed by heterogeneous DC populations are available in the literature (8, 9, 13–16). The concentrations of PRR ligands and other stimulants to add to DC cultures really need to be determined empirically, titrating out the stimulant to establish the concentration that gives maximal activation with minimal death. This is particularly important when working with viruses. Different cytokines can be added to stimulants to skew cytokine responses. For example, peak IL-12p70 production is obtained in the presence of GM-CSF and IFN- γ , and IL-4. However, whilst in the presence of GM-CSF, IFN- γ without IL-4, peak IL-12 p40, and p(40) 2 responses are elicited from DC (18). Working concentrations of stimulants and cytokines that we commonly use are; CpG-containing oligonucleotides (TLR9 ligands) 0.5 μ M; Poly I:C (TLR-3 and Rig-like helicase ligand) 100 μ g/mL; LPS (TLR4 ligand) 1 μ g/mL; R848 (TLR7 ligand) 10 μ g/mL; Loxoribine (TLR7 ligand) 100 μ M; Pam3-Cys (TLR2 ligand) 1 μ g/mL; GM-CSF 20 ng/mL; IFN- γ 20 ng/mL; and IL-4 20 ng/mL. Many more stimulants are available. Invivogen is a good source of PRR ligands and further information.
6. The culture and manipulation of DC in media that is as close as possible to their “natural” environment are beneficial to maintain optimal function. The osmolarity of media can be tested by reading with an Osmometer. We prepare all mouse media at 308 mOsm and human media at 290 mOsm. Media can be adjusted to the correct osmolarity by the addition of concentrated NaCl (increase Osm) or distilled water (decrease Osm).
7. DC in Culture Media only, particularly cDC, will mature and increase activation marker expression without addition of any stimulus. This is believed to be a result of as-yet uncharacterized

soluble factors produced by DC as a normal physiological response to the changed conditions and cross talk between associating DC facilitated by culture (19). Due to this when measuring DC activation by FACS the true negative control must be cells incubated in Culture Media alone (Figs. 1 and 3). Not all cDC preparations are noticeably activated upon culture in media only. cDC from Flt3-ligand bone marrow cultures do not show the same upregulation of costimulation markers as ex vivo DC when cultured in media only.

8. As culture periods increase so does cell death which decreases the FACS data obtainable from these cultures. We recommend 18 h culture as optimal for ample inflammatory cytokine production by DC with minimal cell death.
9. Stimulation of DC can be confirmed before subsequent FACS or cytokine analyses by microscopic visualization. pDC in humans and mouse are the most extreme example of simple activation confirmation by visualization. In media alone pDC remain as round, non-dendritic-like cells. With activation by, for example, TLR9 ligands, the shape of pDC changes. The extent of the morphological changes visualized depends on the activation stimulus and the type of culture well. As shown in Fig. 2 pDC rapidly die in media alone and do not show any resemblance to a classical “dendritic” cell. A-type oligonucleotides (CpG2216) induce some changes in the shape of pDC but

Filled Grey: Media only
 Thick Black Line: CpG 2216
 Grey Line: CpG 1668
 Dotted Black Line: Poly IC

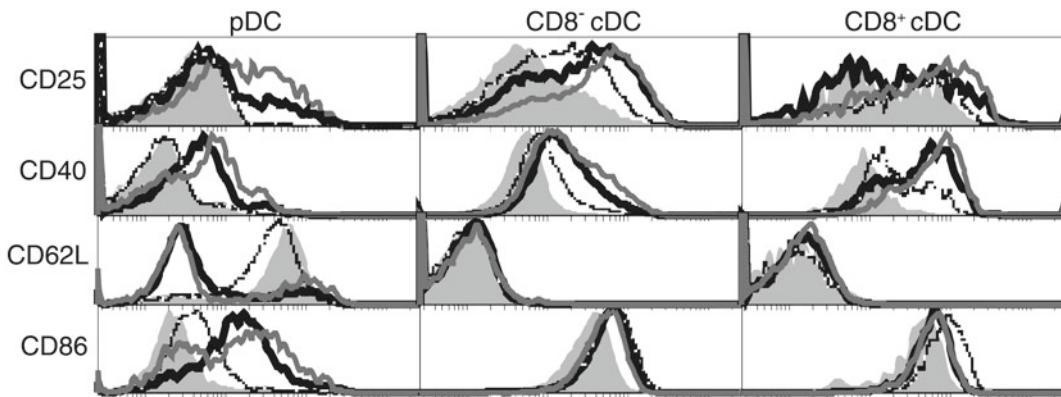


Fig. 3. C57BL/6 splenic DC populations sorted by FACS into three DC populations: pDC ($CD11c^{int}$, $B220^+$) and $2 \times$ cDC ($CD11c^{hi}$, $B220^-$) populations based on CD8 expression. Sorted DC cultured at 37°C in 96-well U-bottom culture plate at 1×10^6 cells/mL for 20 h in Culture Media alone (filled grey), $0.5 \mu\text{M}$ Type-A CpG2216 (thick black line), $0.5 \mu\text{M}$ Type-B CpG1668 (grey line), or $100 \mu\text{g}/\text{mL}$ polycytidylic acid (Poly IC, dotted black line). Cells were then stained for CD25, CD40, CD62L, and CD86 expression.

little to no clustering of cells. The images shown are from a flat well microscopic slide to optimize the quality of the images, but small clusters are sometimes seen in round-bottom plates. With B-type oligonucleotides (CpG1668) pDC become more dendritic-like, tend to cluster, and the survival improves somewhat. The clusters of pDC in a round-bottom plate are much larger and resemble more those seen for the CD8⁻ cDC (Fig. 2). We have not visualized morphological activation of mouse pDC in response to cytokines, however, IL-3 does induce morphological activation of human pDC and it has recently been shown that GM-CSF behaves similarly on human pDC (20). For ex-vivo cDC the assessment of activation by microscopy is more difficult since they cluster and appear dendritic in media alone. Some stimuli (for example CpG1668, Fig. 2) induce very large clusters of cDC which enable immediate confirmation that activation has been successful. It is important to make a habit of visualizing DC after every culture, before removing cells for cytokine analyses for example, and particularly important if FACS is not being carried out. A negative result for cytokine production may not necessarily mean that the DC were not activated but rather could be indicative of rapid cell death perhaps due to toxicity of the stimuli or additives used. For example, high concentrations of DMSO are extremely toxic to DC.

10. Pelleting at higher speeds in a minifuge creates tight pellets on the outer side of the tube. This allows total supernatant to be collected leaving cell pellet behind.
11. DC culture supernatants can be tested for the production of proinflammatory cytokines by ELISA or Cytometric Bead Array (CBA, BD) or FlowCytomix™ (eBioscience). ELISAs can usually only be used to measure the expression of one cytokine per ELISA. CBA and Flowcytomix multiplex systems require smaller supernatant volumes and can measure several cytokines simultaneously.
12. The incubation with anti-CD16/32 blocks nonspecific Fc-receptor binding of the subsequently applied antibodies. Commercially available reagents to block Fc-receptor binding include Fc-Block (BD) or FcR Blocking Reagent (Miltenyi).
13. The different DC subsets vary by which costimulatory markers they upregulate. All antibodies used to detect surface expression of upregulated markers should always be pre-titrated on activated DC to ensure that expression levels stay on-scale during FACS analysis. This is particularly important for CD80 and MHCII expression on activated cDC. CD8, CD69, CD80, CD86, and MHCII are all nicely upregulated during pDC activation while CD62L is downregulated (see Fig. 3).

MFI of activation markers on DC subsets in varying stimuli

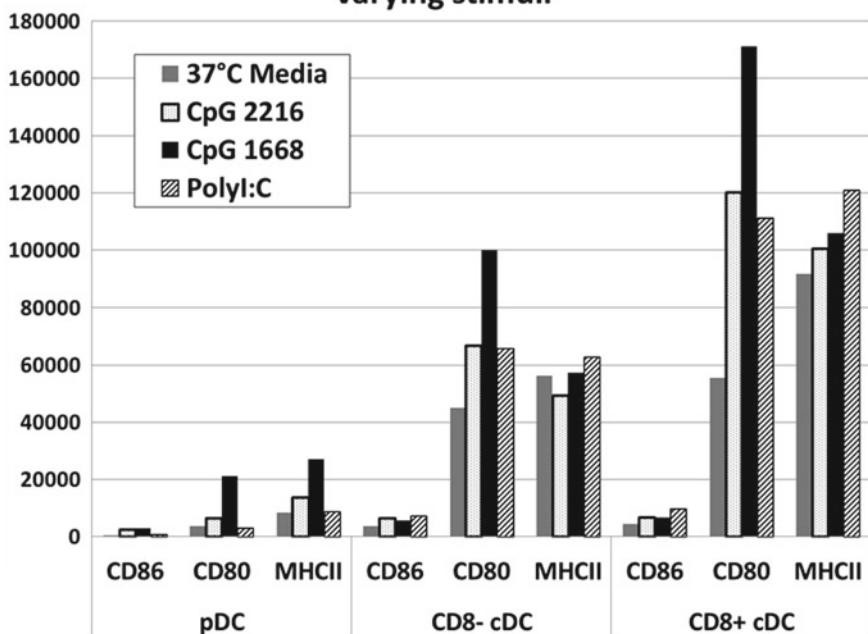


Fig. 4. Sorted mouse spleen DC cultured at 37°C in 96-well U-bottom culture plate at 1×10^6 cells/mL for 20 h in Culture Media alone, 0.5 μ M Type-A CpG2216, 0.5 μ M Type-B CpG1668, or 100 μ g/mL Poly IC. Histograms were plotted as in Fig. 3 using Weasel FACS analysis to calculate the mean fluorescence intensity of the cultured DC subsets after staining for CD80, CD86, and MHCII expression.

CD25, CD40, CD80, and MHCII are highly upregulated on activated cDC.

As mentioned above to prevent overstaining of highly expressed activation markers all antibodies should be pre-titrated on activated DC to determine an optimal dilution. MHCII is commonly upregulated to such an extent, particularly by cDC, that activated cells appear off scale. It is recommended that fluorescent MHCII antibodies be “spiked” with unconjugated MHCII antibody to allow marker saturation. All antibodies are available from BD Biosciences.

When DC are sorted by FACS if possible avoid reusing fluorochromes that may still be present on the cell after culture. While some surface markers are lost during culture period others may still be present with fluorescent antibodies still attached. The activation of the cell may also alter the uptake or turnover of the stained surface proteins. Thus it is important to include an unstained control in each of the stimulated samples to ascertain any “carryover” fluorescence on the surface of cells under different stimulatory conditions.

14. PI is a membrane-impermeable dye that intercalates between the bases of nucleic acids and is thus used to stain the nuclei of dead and dying cells as an indicator of cell death. Other commercial alternatives now available emit light in differing wavelengths to PI which may increase FACS flexibility. One example is the Invitrogen Live/Dead® Fixable Aqua Dead Cell Stain Kit excited by the 405 nm laser with an emission spectra similar to that of Pacific-Orange.
15. Data from FACS analysis can be represented in various ways. Overlaying histograms as in Fig. 3 allows direct comparison of activation marker expression on mouse DC after culture in Culture Media alone and in various stimulants. Figure 4 shows the mean fluorescence intensity of selected activation markers. As this is a simplified graphical means of displaying the data, information regarding the heterogeneity of marker expression on activated DC is lost.

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References

1. Medzhhitov R, Preston-Hurlburt P, Janeway CA (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388(6640):394–397
2. O'Keefe M, Hochrein H, Vremec D, Scott B, Hertzog P, Tatarczuch L, Shortman K (2003) Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood* 101(4):1453–1459
3. Lauterbach H, Bathke B, Gilles S, Traidl-Hoffmann C, Luber CA, Fejer G, Freudenberg MA, Davey GM, Vremec D, Kallies A, Wu L, Shortman K, Chaplin P, Suter M, O'Keefe M, Hochrein H (2010) Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J Exp Med* 207(12):2703–2717
4. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, Chen CJ, Dunbar PR, Wadley RB, Jeet V, Vulink AJ, Hart DN, Radford KJ (2010) Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med* 207(6):1247–1260
5. Piccioli D, Tavarini S, Borgogni E, Steri V, Nuti S, Sammicheli C, Bardelli M, Montagna D, Locatelli F, Wack A (2007) Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood* 109(12):5371–5379
6. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN (2002) Characterization of human blood dendritic cell subsets. *Blood* 100(13):4512–4520
7. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, Xu H, Vivier E, Sellars M, Pierre P, Sharp FR, Chan S, Kastner P, Dalod M (2008) Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* 9(1):R17. doi:[gb-2008-9-1-r17 \[pii\]](https://doi.org/10.1186/gb-2008-9-1-r17)
8. Duthie MS, Windish HP, Fox CB, Reed SG (2011) Use of defined TLR ligands as adju-

- vants within human vaccines. *Immunol Rev* 239(1):178–196
9. Kumagai Y, Akira S (2010) Identification and functions of pattern-recognition receptors. *J Allergy Clin Immunol* 125(5):985–992
 10. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11(5):373–384
 11. Barton GM, Kagan JC (2009) A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol* 9(8):535–542
 12. Benko S, Magyarics Z, Szabo A, Rajnavolgyi E (2008) Dendritic cell subtypes as primary targets of vaccines: the emerging role and cross-talk of pattern recognition receptors. *Biol Chem* 389(5):469–485
 13. Takeuchi O, Akira S (2009) Innate immunity to virus infection. *Immunol Rev* 227(1):75–86. doi:[IMR737 \[pii\]](#)
 14. Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7(3):179–190
 15. Hochrein H, O'Keeffe M (2008) Dendritic cell subsets and toll-like receptors. *Handb Exp Pharmacol* 183:153–179
 16. Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, Akira S, Wiegand M, Hochrein H, O'Keeffe M, Mann M (2010) Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32(2):279–289
 17. Naik SH, O'Keeffe M, Proietto A, Shortman HH, Wu L (2010) CD8+, CD8-, and plasmacytoid dendritic cell generation in vitro using flt3 ligand. *Methods Mol Biol* 595: 167–176
 18. Hochrein H, O'Keeffe M, Luft T, Vandenabeele S, Grumont RJ, Maraskovsky E, Shortman K (2000) Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med* 192(6):823–833
 19. Vremec D, O'Keeffe M, Wilson A, Ferrero I, Koch U, Radtke F, Scott B, Hertzog P, Villadangos J, Shortman K (2011) Factors determining the spontaneous activation of splenic dendritic cells in culture. *Innate Immun* 17(3):338–352
 20. Ghirelli C, Zollinger R, Soumelis V (2010) Systematic cytokine receptor profiling reveals GM-CSF as a novel TLR-independent activator of human plasmacytoid predendritic cells. *Blood* 115(24):5037–5040

Chapter 27

Monitoring the Intracellular Routing of Internalized Antigens by Immunofluorescence Microscopy

Lars Franken, Christian Kurts, and Sven Burgdorf

Abstract

Professional antigen-presenting cells such as dendritic cells (DCs) and macrophages internalize extracellular antigens, process them intracellularly, and present the resulting antigen-derived peptides in the context of MHC I or MHC II molecules. Since the intracellular routing of the antigen determines whether antigens are presented on MHC I or MHC II molecules, a profound analysis of the intracellular distribution of the internalized antigens is of high interest. Here, we describe an immunofluorescence protocol to monitor the intracellular routing of the model-antigen Ovalbumin in bone marrow-derived dendritic cells (BM-DCs). This protocol describes a procedure to stain such cells with antibodies against different endosomal markers, such as EEA1 and LAMP1, and can be easily adopted to other antigen-presenting cells or antigens.

Key words: Immunofluorescence microscopy, Antigen routing, Dendritic cell, Endosomes, Antibody staining

1. Introduction

To investigate the intracellular location of internalized antigens, DCs are usually challenged with fluorochrome-labeled antigens for a defined period of time, before they are fixed and stained with markers for subcellular compartments. Antigen co-localization with the respective markers can then be determined using an immunofluorescence microscope.

As an example of such experiments, this protocol demonstrates how to determine the intracellular distribution of the soluble model antigen ovalbumin (OVA), which is taken up in bone marrow-derived dendritic cells (BM-DCs) by mannose receptor (MR)-mediated endocytosis (1). BM-DCs are commonly used as model system for dendritic cells and antigen uptake in these cells can be studied easily in vitro.

To monitor uptake and intracellular routing of OVA in these cells, we used OVA, which was directly labeled with Alexa Fluor 647. This fluorochrome is very stable and not susceptible to differences in endosomal pH or to photo-bleaching due to light-exposure, which inevitably occurs during the experimental procedure.

In this chapter, we used for the analysis of the subcellular location of OVA LAMP-1 as a lysosomal and EEA-1 as an early-endosomal marker.

Note that this protocol is aimed at analyzing antigens that are internalized in high amounts by receptor-mediated endocytosis.

2. Materials

Prepare all solutions using sterile PBS and analytical grade reagents. All reagents and buffers are prepared and stored at room temperature (unless indicated differently).

2.1. Consumables and Lab Equipment

1. Ø12mm coverslips (autoclave before use).
2. Object slides (do not need to be sterile).
3. 24-well tissue culture plate.
4. 10 cm Petri dishes.
5. LS-MACS Columns (Miltenyi).
6. QuadroMACS Separator (Miltenyi).
7. Fluorescence microscope (Olympus IX71) and ImageJ software (<http://rsbweb.nih.gov/ij/>).

2.2. Buffers

1. Sterile PBS.
2. Fixation Buffer: 4% paraformaldehyde (PFA) in sterile PBS. 3 mL Aliquots of 20% PFA can be stored at -20°C. Dilute with 12 mL PBS before usage. If the PFA does not dissolve, incubate shortly at 50°C. Cool buffer to room temperature before use. Store 4% PFA no longer than 1 week at 4°C.
3. Permeabilization/blocking buffer: 5% sugar-free Milk Powder, 0.1% Triton X-100, 0.5% BSA in PBS. Add 1% serum from the same host species as the secondary antibody. This will reduce unspecific binding. Store no longer than 1 week at 4°C.
4. Washing buffer: 1% BSA in sterile PBS.
5. BMDC-Medium: 70% fresh IMDM (10% FCS, 1% Penicillin/Streptomycin, 100 µM 2-Mercaptoethanol) mixed with 30% of the supernatant of the GM-CSF-producing R1 cell line.
6. 2 mM EDTA: Prepare a sterile stock of 0.5 M EDTA in PBS. Add a few drops of NaOH to the EDTA to facilitate dissolution.

Afterwards adjust pH to 8. Add 2 mL 0.5 M EDTA to 500 mL sterile PBS to obtain a 2 mM EDTA working solution.

7. MACS-Buffer: Sterile PBS with 0.5% BSA and 2 mM EDTA.

2.3. Antibodies and Reagents

1. Ovalbumin, Alexa Fluor647 conjugate (Invitrogen) dissolved in PBS.
2. Rat anti-mouse Lamp1 (CD107a) antibody (BD Bioscience, clone 1D4B).
3. Rabbit anti-mouse EEA-1 antibody (Dianova, clone PA1-063).
4. Chicken anti-rat Alexa Fluor 488 conjugated secondary antibody (Invitrogen).
5. Donkey anti-rabbit Alexa Fluor 586 conjugated secondary antibody (Invitrogen).
6. ImmuMount (Thermo Fisher Scientific).
7. CD11c microbeads (Miltenyi).

3. Methods

3.1. Preparation of BM-DCs

BM-DCs were prepared as described extensively before (2, 3). All steps are carried out in a sterile workbench.

1. Open both sides of femur and tibia from both rear legs of an at least 8-week-old mouse with a pair of scissors at the end of the bone in close proximity to the joints. Subsequently, the bone marrow is flushed out of the bone with PBS using a small syringe. The bone marrow is collected in 10 cm petri dishes.
2. Separate the cells by pipetting up and down and transfer the cell solution through a sterile 50 µm nylon mesh into a collection tube.
3. Collect cells by centrifuging for 5 min at $300 \times g$ at 4°C.
4. Plate cells on three different 10 cm untreated petri dishes in BM-DC Medium (17 mL medium per plate) (see Note 1) and incubate at 37°C with 5% CO₂.
5. After 3–4 days, all cells are collected and replated in fresh medium. To this end, collect supernatant from each plate in a 50 mL falcon tube and cover the adherent cells with 10 mL 2 mM EDTA. Incubate for 5 min at room temperature and wash cells from the petri dish. Pool the cell suspension and supernatant and collect cells by centrifuging for 5 min at $300 \times g$ at 4°C. Resuspend each pellet in BM-DC medium and plate on six fresh 10 cm petri dishes (17 mL per plate).
6. Culture for another 3 days.

3.2. MACS-Based Cell Separation of CD11c⁺ BM-DCs

To isolate DCs from the bone marrow culture, CD11c-positive cells can be enriched by magnetic cell separation using the MACS technology.

1. Harvest cells with 2 mM EDTA as described above (Subheading 3.1, step 5). Wash the cells one time with 20 mL MACS-Buffer.
2. Collect cells by centrifuging for 5 min at $300 \times g$ at 4°C.
3. Resuspend the cells in MACS buffer and add to all cells from one mouse 60 µL micro beads directed against mouse CD11c.
4. Incubate for 15 min at 4°C.
5. Wash cells with 20 mL MACS-Buffer. Collect cells by centrifugation at $300 \times g$ for 5 min at 4°C. In the meantime, attach an LS-MACS column to the MACS-magnet and equilibrate with 5 mL MACS-Buffer.
6. Resuspend the cells in 3 mL MACS-Buffer and pass them through a 50 µm filter on the MACS-column.
7. Wash the MACS-column three times with 2 mL MACS-buffer.
8. Remove the MACS-Column from the magnet and elute the CD11c-positive cells with 5 mL MACS-Buffer.
9. Collect the cells by centrifugation at $300 \times g$ for 5 min at 4°C.
10. Wash the cells once with PBS and collect the cells by centrifugation at $300 \times g$ at 4°C.
11. Put sterile coverslips on the bottom of a 24-well plate and put 300,000 cells in 400 µL IMDM per Well. Let cells adhere for 45–60 min in the incubator at 37°C before starting antigen uptake.

3.3. Antigen Uptake

Antigen concentrations described in this protocol are intended to monitor uptake of high amounts of antigen by receptor-mediated endocytosis (see Note 2).

1. Prepare IMDM with 500 ng/mL fluorochrome-labeled OVA. Replace the medium on the cells with OVA-containing medium and place in the incubator at 37°C. To monitor background fluorescence, it is advisable to include cells that were not treated with OVA (see Note 3).
2. After 15 min, OVA-containing medium can be replaced and fresh medium can be added for different time points. This chase step enables analyzing antigen routing at different time points after internalization.

3.4. Immuno-cytochemistry

All steps should be carried out on a shaker plate in the dark. Therefore, the 24-well plate can be covered with tinfoil. To monitor background staining of the antibody, it is advisable to include isotype control antibodies and samples without primary antibody.

1. Remove medium and wash cells two times carefully with PBS.
2. Remove PBS and add 250 µL Fixation buffer to the cells. Incubate for 15 min at room temperature.
3. Remove fixation buffer and wash cells twice with PBS for 5 min.
4. Add 500 µL permeabilization/blocking buffer and incubate for 1 h at room temperature.
5. Remove permeabilization/blocking buffer, and wash cells twice with PBS for 5 min.
6. Add the primary antibodies (in this example α -EEA1 and α -LAMP1) in 1% BSA-containing PBS in a concentration of 2.5 ng/µL. Incubate for 1 h at room temperature (see Note 4).
7. Remove the staining buffer and wash the cells three times for 5 min with PBS at room temperature.
8. Add the secondary antibodies in 1% BSA-containing PBS in a concentration of 1 ng/µL. Incubate for 30 min at room temperature.
9. Remove the staining buffer and wash the cells three times for 5 min with PBS at room temperature. For staining of the nucleus, include 0.5 µg/mL DAPI or Hoechst 33258 in the second washing step.
10. Prepare an object slide with one drop of ImmuMount.
11. Recover coverslips with a forceps from the 24-well plate (see Note 5) and move it with the forceps shortly through distilled water. This removes traces of PBS and avoids crystal formation after mounting the coverslips. Carefully move the edge of the coverslip over a paper tissue. This removes the remaining fluid.
12. Lay the coverslip on the drop of ImmuMount on the object slide. In this step, the fixed cells should be on the bottom side of the coverslip, making direct contact with the mounting medium (see Note 6). The ImmuMount will seal the coverslip. Let the ImmuMount dry in a dark, dry place overnight before analysis.

3.5. Data Analysis

Our slides were analyzed with an Olympus IX71 fluorescence microscope (see Note 7). For analyzing co-localization, we used a 60 \times oil objective and took single color grey scale pictures. The pictures were analyzed using the freely available ImageJ software (<http://rsbweb.nih.gov/ij/>). Overlay pictures of the individual fluorescence channels can be generated with the RGB merge tool. Co-localization can be quantified and plotted as depicted in Fig. 1 using the Manders coefficients tool of the co-localization analysis plug-in of ImageJ.

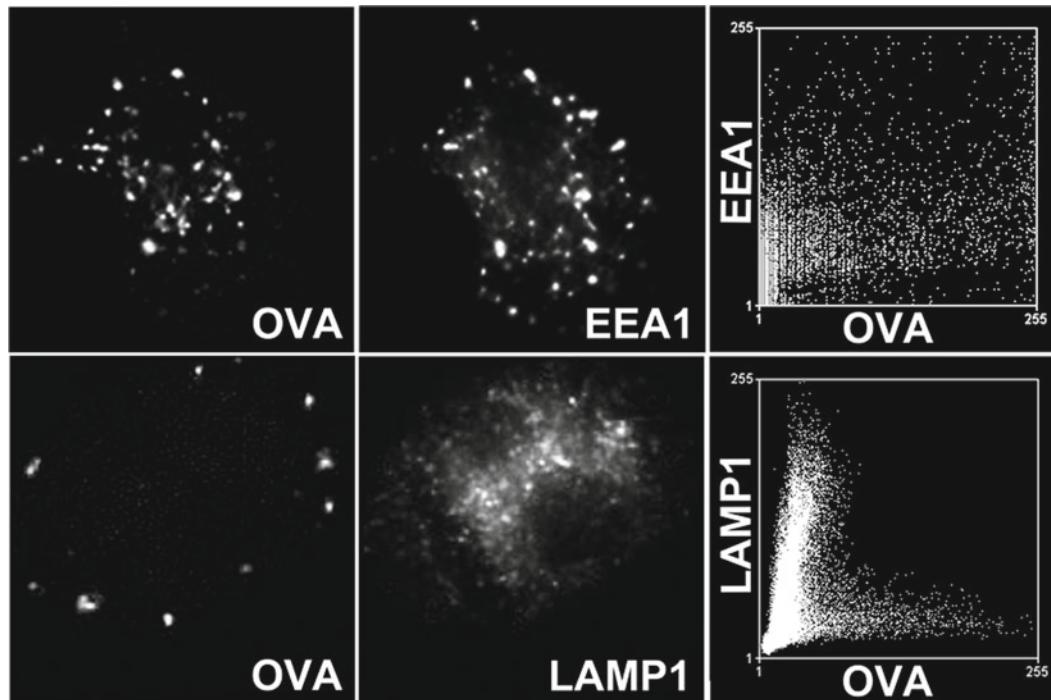


Fig. 1. Example of a staining performed on BM-DCs with the protocol described here. Cells were incubated for 15 min with fluorochrome-labeled OVA before staining. The *upper panel* shows a staining against EEA1. Most of the Ovalbumin co-localizes with the early endosomal marker EEA-1. The *lower panel* shows a staining against LAMP-1. No co-localization between OVA and LAMP-1 is observed. Plots were generated using the colocalization analysis plug-in of imageJ.

4. Notes

1. Do not culture the BMDCs on coated tissue culture plates, since the treated plastic might induce DC maturation. Additionally, the cells adhere strongly to those plates and it is nearly impossible to remove them from the plate by using 2 mM EDTA in PBS. The cells can be collected by using Trypsin, but this will stress the cells further and will result in the unspecific digestion of surface proteins by the trypsin, which may alter the antigen uptake by the BMDCs.
2. To monitor pinocytosis, antigen concentrations might be increased or Lucifer Yellow (LY) can be used as a surrogate pinocytosis marker. LY can be stained using specific antibodies with the same protocol as described here.
3. Certain treatments (e.g., some fixation procedures) might result in an increased background fluorescence signal, which can be misinterpreted as antigen internalization signal. To monitor such background fluorescence, it is advisable to prepare controls that did not receive fluorochrome-labeled antigen.

4. To verify the specificity of the antibody staining, it is highly recommended to include specificity controls. These can be DCs from mice lacking the protein of interest. In these cells, no signal should be observed. Alternatively, cells expressing a GFP fusion protein of the protein of interest can be used as specificity control. If the staining is specific, the GFP signal should co-localize with the antibody signal.
5. To remove the coverslip from the 24-well plate, use a thin forceps and a 200 µL pipette tip. The coverslip will not cover the whole surface of the well. Enter the well with the pipette tip and place it on the surface of the well that is not covered by the coverslip. Then gently press the coverslip against the opposite site of the 24-well. Now you can slowly lift the pipette tip and the coverslip will also be lifted on one end. This end can then be grabbed using the forceps. This procedure works very well with some practice. Be careful not to apply too much pressure to the coverslip as it will easily break. When using the forceps, grab the coverslip firm but not too hard, as it is likely to break.
6. Make sure that no air bubbles are trapped in the mounting medium, since this will result in a very high background signal. Air bubbles can be removed by gently pressing the coverslips into the mounting medium.
7. Co-localization between the internalized antigens and the stained endosomal markers can be analyzed by regular immunofluorescence microscopy. Nevertheless, analysis by confocal microscopy is much more reliable, since it avoids false-positive events, which can result from the limited in-depth resolution of a standard-microscope.

Acknowledgments

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References

1. Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C (2007) Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316:612–616
2. Burgdorf S, Lukacs-Kornek V, Kurts C (2006) The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J Immunol* 176:6770–6776
3. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77–92

Chapter 28

Isolation of a Specialized, Antigen-Loaded Early Endosomal Subpopulation by Flow Cytometry

Achmet Imam Chasan, Marc Beyer, Christian Kurts, and Sven Burgdorf

Abstract

Isolation and characterization of antigen-containing endosomes remains difficult utilizing standard purification techniques. Here, we describe a method, which allows isolation of antigen-loaded endosomes, that is based on flow cytometrical analysis and sorting. We specifically isolated antigen-containing endosomes from cells that had taken up fluorochrome-labeled ovalbumin via mannose receptor-mediated endocytosis. The protocol described here allows for the isolation of pure fractions of ovalbumin-containing endosomes and the extraction of proteins from these endosomes for analysis by western blot. Importantly, this protocol can be easily extended to other fluorochrome-labeled antigens and to primary antigen-presenting cells like bone marrow-derived dendritic cells.

Key words: Antigen uptake, Mannose receptor, Endosome, FACS, FAOS, Organelle purification

1. Introduction

Isolation, purification, and analysis of distinct organelle types is a widely used approach in cell biology and biochemistry to determine their components (1). Whereas the isolation and characterization of phagosomes containing particulate antigens (2) has been established extensively, the isolation of endosomes containing soluble antigens remains much more difficult. Classical approaches to isolate pure fractions of such endosomes frequently include density gradient ultracentrifugation (1, 3). However, since it is impossible to separate organelles with the same or similar density using this technique, the purity of the obtained fractions is not always satisfactory. Since it is of great interest to determine proteomes of distinct organelles or endocytic vesicles, several novel approaches have been developed, including ferromagnetic isolation of endocytic compartments (4) the flow-cytometrical sorting of fluorescently labeled organelles, termed FAOS (fluorescence assisted organelle sorting) (5).

In this chapter, we describe an approach for flow-cytometrical analysis and sorting of distinct antigen-loaded endosomes. We used HEK293T cells that were stably transduced with the murine mannose receptor (HEK-MR), an endocytic receptor that is able to internalize high amounts of the model antigen ovalbumin (OVA) (6). After treating these cells with fluorochrome-labeled OVA, we mechanically disrupted the cells and isolated specifically the antigen-containing endosomes based on their fluorescence. We provide a detailed protocol to isolate an endosomal subpopulation by FAOS, which can easily be extended to other fluorochrome-labeled antigens or other cell types like primary antigen-presenting cells.

2. Materials

All reagents were purchased in the highest available (analytical) purity. Solutions were prepared with double distilled H₂O and stored at 4°C unless otherwise stated. Buffers and stock solutions for organelle preparation were filtered through 0.2 µm filter membranes prior to use to remove possible containing particles that may interfere with organelle detection.

2.1. Tissue Culture

1. 10 cm tissue culture dishes.
2. MR-expressing HEK293T cells were maintained in DMEM, 10% FCS, 1% sodium pyruvate, and Penicillin (10 U/ml)/Streptomycin (1 mg/ml), and were split before confluence was reached (see Note 1).
3. Sterile PBS.
4. 2 mM EDTA/PBS was prepared by diluting a stock of 0.5 M EDTA in PBS.
5. Dissolve 2 mg of Ovalbumin-Alexa Fluor 647 conjugate (Molecular Probes) in 400 µl sterile PBS. Aliquots of 40 µl are stored at -20°C in the dark. Prepare working solution by addition of 760 µl sterile PBS to one aliquot (endconcentration: 250 µg/ml) and store at 4°C in the dark. Use 1:1,000 (250 ng/ml) in complete medium for internalization experiments.
6. To enable analysis of antigen-bearing endosomes, it is important to include a proper control (in our case OVA-treated control HEK293T cells) (see Note 2).

2.2. Organelle Preparation

1. Homogenization Buffer: 25 mM MES (2-(*N*-morpholino)-ethanesulfonic acid), 150 mM NaCl, 1× protease inhibitor mix (Complete, Roche) pH 6.5 (see Note 3).
2. Stainless steel tissue grinder (Greiner) (see Note 3).

2.3. Flow Cytometry

1. High resolution FACS-Analyzer/Sorter. For this study, we used a LSRII analyzer and an AriaIII sorter, both from BD Biosciences.
2. Sorting sheath fluid: 25 mM MES, 150 mM NaCl pH 6.5 (0.2 μ m filtered!). Depending on sort duration, 3–6 L is needed (see Note 4).
3. 20 \times protease inhibitor mix (Complete, Roche), dissolved in 25 mM MES, 150 mM NaCl, pH 6.5.
4. Fluorescent beads (TetraSpeck microspheres 0.5 μ m, Invitrogen).
5. 15 ml collection tubes.
6. Aqueous solution containing 5% paraformaldehyde and 0.1% glutaraldehyde.

2.4. Downstream Analysis

1. Methanol–Chloroform ratio 2:1 (see Note 5).
2. Methanol p.A.
3. Pasteur pipettes.
4. 50 ml tubes and 1.5 ml reaction tubes.
5. Sonification device.
6. Lysis buffer/SDS sample buffer: Mix phosphate lysis buffer: 20 mM NaPO₄ pH 8, 140 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, with 2 \times SDS sample buffer: 250 mM Tris–HCl pH 6.8, 6.6% SDS in a ratio of 1:1.
7. 3 \times NEM Solution: 25 mg *N*-ethylmaleimide, 660 μ l ddH₂O, 330 μ l glycerol saturated with bromophenol blue.
8. Standard SDS-PAGE and western blot reagents and buffers.
9. Silver staining kit (Silversnap II, Pierce).

3. Methods**3.1. Antigen Loading**

1. Harvest cells by treatment with 2 mM EDTA for 5 min. Pellet cells by centrifugation at 300 \times g for 5 min, resuspend in fresh medium and plate out on 10 cm tissue culture dishes (6×10^6 cells/dish). Let the cells adhere for 1 h in the incubator at 37°C. Use at least 30×10^6 cells to obtain a sufficient amount of organelles for sorting experiment (see Note 6).
2. Aspirate medium carefully and add 5 ml of medium containing 250 ng/ml fluorochrome-labeled OVA per dish for 45 min at 37°C.
3. Discard medium by aspiration and harvest cells by treatment with 2 mM EDTA. Centrifuge at 300 \times g for 5 min (see Note 6).

4. Wash cells twice with PBS. After each washing step, pellet cells by centrifugation at $300 \times g$ for 5 min, always discarding the supernatant (see Note 7).
5. Hold back a small aliquot of cells (about 500 k) for flow-cytometrical analysis of overall antigen internalization.

3.2. Homogenization of Cells

All steps are performed on ice with cooled buffer and equipment.

1. Resuspend cell pellet in 1 ml homogenization buffer and transfer cell suspension into a stainless tissue grinder. Disrupt cells with 5–10 strokes (see Note 8).
2. Transfer homogenate to a 1.5 ml reaction tube. Rinse grinder with 500 μ l homogenization buffer to collect remaining debris and organelles. Add suspension to the same reaction tube.
3. Centrifuge at $2,000 \times g$ for 10 min to pellet cellular debris and nuclei. Take off the post nuclear supernatant (PNS) (see Note 9).
4. Transfer PNS to a new reaction tube and repeat centrifugation at $2,000 \times g$ (see Note 9).
5. Transfer cleared PNS to a new reaction tube (see Note 9) and perform flow-cytometrical analysis (see Note 10).

3.3. Flow Cytometrical Analysis and Sorting

Set FSC threshold to a minimum of 200 before starting. To avoid the detection of multiple organelles simultaneously, samples are analyzed at lowest possible flow speed with a maximum of 1,000 events/second. Dilute samples with homogenization buffer if necessary.

1. Adjust cytometer settings with the sample of intact cells. Voltages for FSC and SSC photomultipliers are altered until the cell population is placed in the top right corner of the FSC-A/SSC-A plot (Fig. 1a). Gate on cells and check antigen internalization (Fig. 1b).
2. With these settings, organelles are displayed further to the bottom left part of the FSC-A/SSC-A plot (Fig. 1c). These events have been shown before to be intact antigen-containing endosomes (7). For analysis of the fluorescence signal of these organelles, it is recommended to display the scales as bi-exponential and eventually to adjust photomultiplier voltages for analyzed fluorescence channels in such a way, that the center of control organelle population (=nonfluorescent) is placed on the axes origins (Fig. 1d). Organelles derived from cells capable of antigen internalization (HEK-MR) should contain a subpopulation of endosomes with increased fluorescence (Fig. 1d) (see Note 11).
3. For sorting of OVA-positive endosomes, install a 70 μ m nozzle and replace sheath fluid by sorting sheath fluid. Add 500 μ l of 20 \times protease inhibitor solution into two 15 ml collection tubes each and place them into the sorter. According to the properties

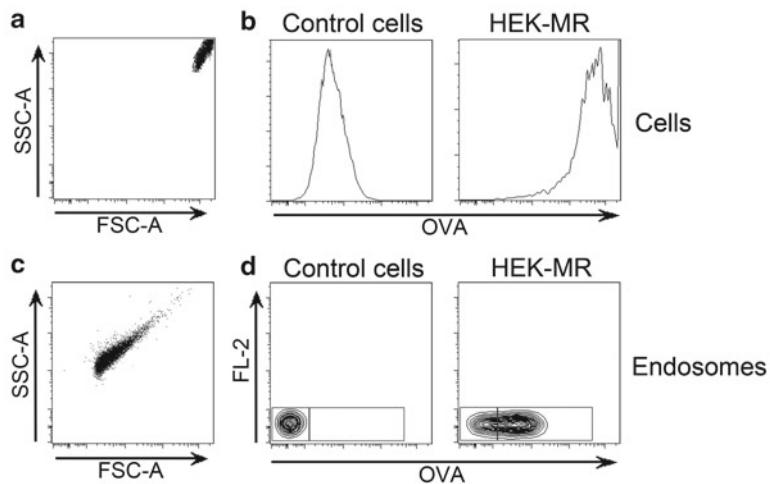


Fig. 1. Flow cytometrical analysis of cells and prepared organelles. **(a)** FSC-A/SSC-A plot of intact cells. **(b)** Internalization of OVA in HEK-MR and control HEK293T cells. **(c)** FSC-A/SSC-A plot of enriched endosomes using the same cytometer settings as in **(a)**. **(d)** Detection of OVA in isolated endosomes.

of the sorting machine, it might be possible to sort 20,000 events/second. However, best results are obtained with sorting a maximum amount of 5,000 events/second. Determine the control organelle population as OVA-negative organelles and sort OVA-negative and OVA-positive populations (Fig. 2). About 6×10^6 individual organelles can be sorted into one tube in a final volume of approx. 10 ml. For sufficient protein detection in downstream applications, sort at least 12×10^6 organelles for each sample (see Note 12).

4. Hold back an aliquot of unsorted organelles (5–10% of initial volume) as control sample for SDS-PAGE/western blot (see Note 13).
5. In the reanalysis of the sorted material, OVA-negative events might appear in the OVA-positive fraction (Fig. 2). These events are generally not due to the contamination of the sample with OVA-negative endosomes, but are due to the disruption of some of the sorted endosomes during sorting, which leads to the loss of soluble OVA.

This can be verified by two independent experiments.

First, samples can be mixed with fluorescent beads, which should have a similar size compared to endosomes. These beads can be sorted into the OVA-negative fraction (Fig. 3a). Reanalysis of the sorted samples should show that all beads are indeed sorted into the correct fraction, demonstrating that no false positive particles are sorted into the positive fraction and confirming the reliability of the sorting procedure.

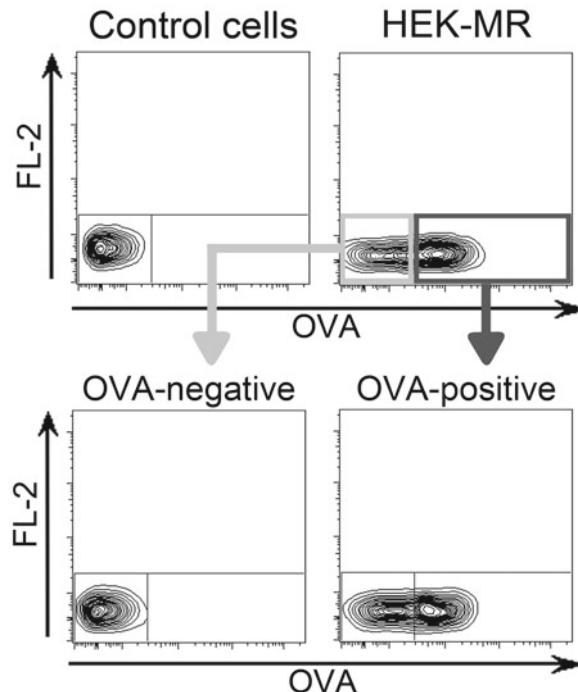


Fig. 2. Sorting of OVA-positive endosomes. HEK-MR or HEK293T control cells were treated with fluorochrome-labeled OVA. Subsequently, the endosomal fraction was isolated and sorted for OVA-positive endosomes.

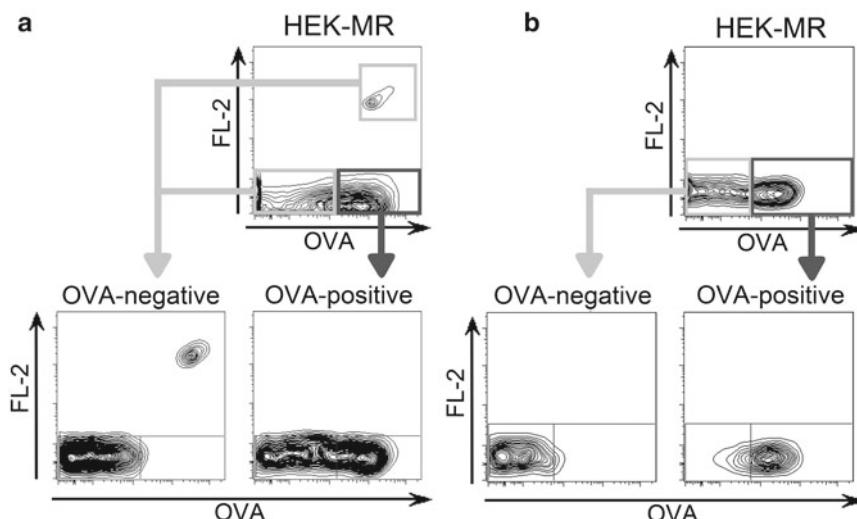


Fig. 3. Determination of sorting purity. (a) Endosomes from OVA-treated HEK-MR or control HEK293T cells were mixed with fluorescent 0.5 μm microspheres (TetraSpeck) and sorted as indicated. Beads were only detected in the OVA-negative fraction. (b) Endosomes from OVA-treated HEK-MR or control HEK293T cells were isolated, fixed by adding 5% paraformaldehyde and 0.1% glutaraldehyde and sorted as indicated.

Second, endosomes can be fixed by adding paraformaldehyde (end concentration 5%) and glutaraldehyde (end concentration 0.1%) for 15 min prior sorting (Fig. 3b). After fixation, no OVA-negative events should be present in the positive fraction anymore, demonstrating that a putative loss of fluorescence in the unfixed samples is due to the disruption of the endosomes during sorting.

3.4. Protein Extraction for Downstream Applications

Perform precipitation at room temperature.

1. Prepare a mixture of methanol and chloroform in a ratio of 2:1 and mix well until the solution is clear (see Note 5).
2. Transfer sorted organelles into 50 ml tubes (10 ml/tube). Add 36 ml of the prepared methanol/chloroform mixture. Close the cap and mix well by inverting the tube. Lipids are extracted by chloroform, leading to the destruction of organelle structure and the release of endosomal material. The solution should be homogenous, since the amount of methanol is sufficient to act as intermediary between the hydrophobic chloroform component and the hydrophilic aqueous component.
3. Addition of 5 ml H₂O and mixing by inversion leads to phase separation, which is accelerated by centrifugation at 3,200×*g* for 10 min at RT. Proteins can be found as small precipitates in the interphase.
4. Carefully aspirate and discard the lower chloroform-containing phase with a Pasteur pipette from the very bottom of the tube leaving as less chloroform behind as possible (but without aspirating protein precipitates). Thereafter, use a new Pasteur pipette to generously collect the interphase including proteins and transfer it into a 1.5 ml reaction tube (see Note 14).
5. In order to extract remaining traces of chloroform, fill up the tubes with methanol and mix by inverting. Protein precipitates are not dissolved and can be pelleted by centrifugation at 20,000×*g* for 10 min.
6. Aspirate and discard supernatant and air-dry protein precipitates overnight (see Note 15).
7. Add 30 µl of a 1:1 mixture of lysis buffer and SDS sample buffer. Make sure that all precipitates are covered with buffer. Dissolve proteins by sonification for 15 min.
8. Boil samples for 3 min at 100°C, spin putative condensates down, and add 15 µl NEM solution.
9. Separate an aliquot (4 µl) of the samples to determine the overall protein content of the individual samples (see Note 16).
10. Separate remaining samples by SDS-PAGE to perform western blot analysis. A typical western blot analysis of sorted samples is depicted in Fig. 4a, which shows a strong enrichment of the

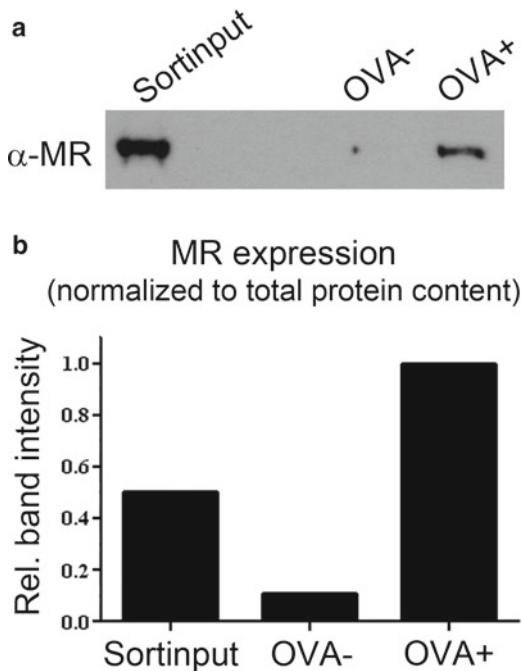


Fig. 4. Analysis of recovered proteins from sorted organelles. (a) Endosomes from OVA-treated HEK-MR cells were sorted. MR expression in the sorted samples was detected by western blot. (b) Quantified MR expression in sorted samples, normalized to the total protein content of the according sample.

MR in OVA-positive sorted endosomes compared to the control preparations (Fig. 4b). Alternatively, endosome preparations can be separated by SDS-PAGE and, after the excision of small gel bands, analyzed by mass spectrometry.

4. Notes

1. This protocol can be easily adapted for primary bone marrow-derived dendritic cells (7).
2. Including a proper control is crucial, since organelles derived from control cells are defined as “fluorescent negative”.
3. A stainless steel homogenizer can easily be cooled to 4°C by placing it on ice for a few minutes. The homogenizer maintains its temperature over a longer period of time.
4. The use of a suited homogenization buffer is of crucial importance. The addition of sucrose is not suitable for FACS, because it delivers many background events that could be misinterpreted as organelles. The buffer used here additionally has

been described to allow peripheral proteins to remain attached on the prepared organelles (8), which enables analysis/detection also of proteins attached to the endosomes.

5. Check resistance of plastics against chloroform before using it in the experiment.
6. Do not harvest cells with trypsin. The number of cells plated strongly depends on the cell lines used.
7. It is important to remove any excess fluid before adding homogenization buffer. We therefore place the tubes upside down on a paper towel. Cells remain sticky at the bottom of the tube.
8. Do not be too harsh with homogenization. In any case, avoid the generation of air bubbles since it will result in bad organelle preparations.
9. Carefully collect PNS without disturbing debris. It is better to leave a small volume behind rather than having debris in the samples. Use a 1,000 µl pipette.
10. Do not be too harsh. Avoid generation of air bubbles.
11. We found that displaying of an “empty” fluorescence channel on the y -axis was best to visualize organelle populations, since it allows optimal characterization of antigen loaded organelles (9).
12. Before sorting is performed, set the device on the “cool sorting” parameter. Depending on the quality of the preparation and the amount of organelles to be sorted, the sorting procedure may take several hours. The more organelles are sorted the better downstream results will be. We observed that 10^7 sorted organelles seem to be the minimum to achieve a sufficient detection by SDS-PAGE/western blot.
13. A sample of the sort input is needed to detect overall protein content. After normalization to overall protein content loaded on the SDS-PAGE, sorting should lead to a clear enrichment of the engaged marker for detection.
14. Protein precipitates usually gather at the center. Therefore it is best to dip the Pasteur pipette at the border of the tube. At this point, precipitates initially derived from the same sort samples can be pooled.
15. Protein precipitates usually are attached only weakly to the side of the tube. Therefore, it is important to aspirate the supernatant very carefully. If precipitates loosen, do not aspirate further but leave supernatant in the tube. In that case, a second round of centrifugation should be performed.
16. This step is recommended to adjust protein contents of the different samples. For analysis, we used ImageJ software to measure intensities of silver stained bands and of bands detected by western blot.

Acknowledgments

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References

- Yates JR III et al (2005) Proteomics of organelles and large cellular structures. *Nat Rev Mol Cell Biol* 6:702–714
- Ackerman AL et al (2003) Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc Natl Acad Sci U S A* 100: 12889–12894
- Harford JB, Bonifacino JS (2001) Subcellular fractionation and isolation of organelles, in current protocols in cell biology. Wiley, Inc
- Bauer A, Richter H-P (1990) Ferromagnetic isolation of endosomes: a novel method for subcellular fractionation of *Xenopus* oocytes. *Biol Cell* 70:61–72
- Murphy RF (1985) Analysis and isolation of endocytic vesicles by flow cytometry and sorting: demonstration of three kinetically distinct compartments involved in fluid-phase endocytosis. *Proc Natl Acad Sci U S A* 82: 8523–8526
- Burgdorf S, Lukacs-Kornek V, Kurts C (2006) The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J Immunol* 176:6770–6776
- Zehner M et al (2012) Intraendosomal flow cytometry: A novel approach to analyze the protein composition of antigen-loaded endosomes. *Eur J Immunol* 42:2187–2190
- German CL, Howe CL (2009) Preparation of biologically active subcellular fractions using the Balch homogenizer. *Anal Biochem* 394: 117–124
- Zehner M et al (2011) Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation. *Proc Natl Acad Sci U S A* 108:9933–9938

Chapter 29

Preparing Antigens Suitable for Cross-presentation Assays In Vitro and In Vivo

Loredana Saveanu and Peter van Endert

Abstract

Cross-presentation is defined as the ability of certain professional antigen-presenting cells to take up, process and present extracellular antigens on major histocompatibility class I (MHC-I) molecules to CD8+ T cells. The stimulation of naive cytotoxic CD8+ T cells by this process, termed cross-priming, is involved in many different responses, including those to tumors, pathogens, graft tissues, and self-antigens. Dendritic cells (DCs), a heterogeneous cell population, are endowed with the highest cross-priming capacity. Investigation of their cross-presentation capacities, important both for vaccination and for the induction of immune tolerance can be performed by in vivo and in vitro assays. In this chapter we describe the preparation of antigens that can be used to test cross-presentation via pinocytosis, receptor-mediated endocytosis, and phagocytosis.

Key words: Cross-presentation, Dendritic cells, Antigen processing, Antigen presentation, MHC-I, Endocytosis, Phagocytosis

1. Introduction

MHC-I molecules bind short (8–10 amino acids) antigenic peptides and present them to CD8+ T cells. In the classical (or endogenous) pathway of antigen presentation by MHC-I the source of the antigenic peptides is the proteins synthesized in the cell (1). Alternatively antigenic peptides from exogenous proteins can be presented on MHC-I molecules and this phenomenon is called cross-presentation (2).

Unlike the endogenous pathway of MHC-I antigen presentation, which is functional in all nucleated cells of the body, cross-presentation is only observed for professional antigen presenting cells (pAPCs). Among the various pAPCs populations able to cross-present exogenous antigens, the most relevant and extensively studied are DCs that are able to prime naïve T cells.

According to their origin, phenotype and immune role, DCs themselves can be divided in several populations (3). In steady-state conditions, the main subsets among splenic DCs are the conventional CD8+ and CD8– DCs and the plasmacytoid DCs. During infection and inflammation, an abundant number of activated monocytes differentiate into inflammatory DCs, often called monocyte-derived DCs (moDCs) (4). All DC subsets can be obtained *in vitro* from bone marrow precursors with different cocktails of cytokines (see Chapter 25) and used for *in vitro* cross-presentation assays.

DCs acquire exogenous antigens by pinocytosis, receptor-mediated endocytosis, and phagocytosis. Antigen uptake often depends on the specific cell surface receptors that are expressed by a specific DC subset. Therefore, when performing cross-presentation assays, one must take into account that different DC subsets have different uptake abilities for various forms of antigens. For example, apoptotic cells are phagocytized preferentially by CD8+ DCs (5), while yeast cells are preferentially engulfed by CD8– DCs (Weimershaus et al., manuscript submitted).

In this chapter we describe how different forms of exogenous antigens can be prepared and used to test cross-presentation via pinocytosis, receptor-mediated endocytosis, and phagocytosis. Cross-presentation of exogenous antigens that are endocytosed via a receptor is generally much more efficient than cross-presentation of soluble antigens. All DC subsets express the receptor for the Fc (Fragment, crystallizable) region of antibodies, the Fc Receptor (FcR). Nevertheless, DC subsets express at the cell surface a large panel of receptors that can be involved in the uptake of the antigen (see Note 1). Therefore, we discuss two strategies for the study of cross-presentation via receptor-mediated endocytosis. The first one allows for the analysis of cross-presentation of antigens that are endocytosed via the FcR, while the second one is more versatile and allows the antigen targeting to any DC receptor for which a specific antibody is available. Next to receptor targeted endocytosis the antigen uptake via phagocytosis leads to efficient cross-presentation. We describe the preparation of a frequently used particulate model antigen, latex beads, but also the preparation of two other forms of phagocytized antigens: yeast cells and apoptotic cells. It is useful to analyze different forms of phagocytosis such as apoptotic cells and yeasts since the adaptive immune responses that follow these two forms of phagocytosis are very different (see Note 2).

2. Materials

Since a convenient readout of cross-presentation is the activation of transgenic CD8+ T cells specific for ovalbumin (OT1 T cells), we take the ovalbumin antigen as an example. Nevertheless, other

antigens can be analyzed by the same methods if specific and sensitive CD8+ T cells are available.

Use ultrapure reagents and ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C).

2.1. Soluble Antigens

1. Ovalbumin (OVA) (Worthington Biochemical Corporation) or EndoGrade® OVA (Hyglos) (see Note 3).
2. Bio-Spin® 6 Column from Bio-Rad (see Notes 4 and 5).

2.2. FcR Mediated Endocytosis

1. OVA (Worthington Biochemical Corporation).
2. Anti-ovalbumin rabbit polyclonal IgG antibody (SIGMA) (see Note 6).
3. Proteine G Sepharose 3 ml column (see Note 6).
4. Bio-Spin® 6 Column from Bio-RAD (see Note 4).
5. 96-well round bottom plates.

2.3. Cell Culture

1. Complete DC medium: IMDM (SIGMA) supplemented with Glutamine, 50 μM β-mercaptoethanol, 10% fetal calf serum.
2. DC isolated from mouse spleen or produced in vitro from bone marrow precursors (see Chapter 25).
3. Antigen specific CD8+ T cells: OT1 cells isolated from the lymph nodes of transgenic OT1 mice.

2.4. Versatile Targeting of Cell Surface Receptors Using Fusion Proteins

1. OVA-Fusion proteins: stock in PBS—10% glycerol (see Note 7).
2. Targeting antibodies; e.g., antibodies that we used successfully for fusion protein cross-presentation:
 - (a) Rat anti-mouse DEC-205 (clone NLDC-145).
 - (b) Hamster anti-mouse CD11c (clone N418-American Type Culture Collection).
 - (c) Rat anti-mouse TLR2 (AbD Serotec).
 - (d) Rat anti-mouse CD206 (Mannose Receptor, AbD Serotec).

2.5. Latex Beads

1. Latex beads (Polysciences, Inc): Polybead® Polystyrene microspheres 1 μm or 3 μm.
2. OVA (Worthington Biochemical Corporation).
3. BSA.
4. Fetal Calf Serum (FCS) heat-inactivated.
5. PBS (Phosphate Buffered Saline).
6. Refrigerated centrifuge for 15 ml tubes.
7. 0.004% Glutaraldehyde in 1× PBS.
8. 0.2 M Glycine pH 7 in 1× PBS, sterilized by 0.22 μm filtration. Store sterile at 4°C.

2.6. Yeast Cells

1. EBY100—control yeasts.
2. EBY100-OVA—yeasts expressing full length OVA (see Note 8).
3. Stratalinker® UV Crosslinker (Stratagene).

2.7. Apoptotic Cells

1. β_2 -microglobulin (β_2 m) ko splenocytes.
2. OVA (Worthington Biochemical Corporation).
3. Biorad—Gene Pulser Xcell™ Electroporation System or other equivalent electroporation system.
4. Electroporation buffer: 0.15 mM CaCl₂, 2 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 130 mM KCl, 25 mM Hepes. Sterilize the buffer before utilization by one passage through 0.22 μ m syringe filter.

3. Methods**3.1. Soluble Protein**

1. Prepare a solution of OVA in sterile PBS at 10 mg/ml. Vortex until the solution is clear. Centrifuge at 20,000 $\times g$ for 20 min to remove insoluble aggregates.
2. Prepare the Bio-Spin® 6 column according to the manufacturer instructions. Before sample addition, wash the column three times with 500 μ l sterile 1× PBS in order to change the column buffer that contains traces of sodium azide. Each wash should be followed by a 1 min centrifugation at 1,000 $\times g$.
3. Put the column in a new 2 ml collection tube. Add 75 μ l of OVA solution at 10 mg/ml and centrifuge for 4 min at 1,000 $\times g$. Usually, more than 90% of OVA is recovered. The column retains small contaminating peptides.
4. For in vitro cross-presentation assays, make serial dilutions of OVA recovered from the column in complete medium to obtain final concentrations of OVA between 1 and 0.03 mg/ml.
5. Add 100 μ l of each OVA dilution to 100,000 DCs in 96-well plates and incubate at 37°C. Use duplicates for each OVA concentration. A kinetic of antigen presentation is recommended at different time points between 2 and 16 h. Antigen processing can be stopped by DC fixation.
6. To fix the DCs:
 - (a) Centrifuge the plate at 550 $\times g$ and aspirate the medium
 - (b) Wash the cells once in 1× PBS
 - (c) Fix 30 s with 50 μ l 0.004% glutaraldehyde in 1× PBS at room temperature
 - (d) Neutralize the glutaraldehyde with 150 μ l 0.2 M Glycine pH 7 in 1× PBS

- (e) Wash once in 200 µl Glycine 0.2 M pH 7 in 1× PBS
 - (f) Add 100 µl complete medium
7. Add specific T cells and incubate for the desired period of time. This is usually between 16 and 24 h if the readout of T cell activation is IL-2 secretion or 3 days if the readout of T cell activation is IFN- γ secretion or T cell division measurement by a CFSE dilution assay.
 8. For in vivo cross-presentation assays dilute the OVA recovered from the Bio-Spin 6 column in sterile PBS at a final concentration of 200 µg/ml. Inject s.c. or i.v. 10–30 µg/mouse. The readout of in vivo cross-presentation after 3 days can be the activation of naïve OT1 T cells adoptively transferred in the mice 1 day before OVA injection. Alternatively, the endogenous polyclonal CD8+ T cell response can be analyzed on day 10 using H2-K^b-SINFEKL pentamers.

3.2. Fc Receptor-Mediated Endocytosis

1. Dialyze the anti-OVA polyclonal rabbit IgG previously purified on Protein G column against complete DCs medium. Make solutions of 1, 0.2, 0.04, and 0.008 mg/ml anti-ovalbumin IgG in complete DCs medium.
2. Prepare a solution of 1 mg/ml OVA and remove the small peptides using the Bio-Spin® 6 columns as described in Subheading 3.1, steps 2 and 3.
3. Dilute in complete DCs media the purified OVA at 20 µg/ml final concentration.
4. Distribute in 96-well round bottom plates 50 µl of each anti-OVA IgG dilution and 50 µl of OVA solution at 20 µg/ml. Incubate at 4°C for 1 h for the immune complexes formation.
5. Remove the plate from the refrigerator and warm it in a cell culture incubator at 37°C for 15 min. Add to each well 100 µl of medium containing the DCs. For most of DCs subsets that we analyzed we used 100,000 cells/well. Incubate at 37°C in CO₂ supplemented incubator overnight.
6. In the next morning, centrifuge the plate at 550× g , aspirate the supernatant, and wash the cells once with complete medium using a multichannel pipette.
7. Add specific T cells as mentioned in Subheading 3.1, step 7.

3.3. Versatile Targeting of Cell Surface Receptors Using Fusion Proteins

1. Mix in PBS the fusion protein with the targeting antibody in a molecular ratio of 1:1. Incubate the tube in ice for 30 min for the protein complexes formation (see Note 9).
2. At the end of the incubation prepare in complete medium dilutions of immune complexes with a range between 10 and 1 µg/ml fusion protein.

3. Seed 100,000 DCs per well, in 50 µl complete medium, in a 96-well round bottom plate.
4. Add 50 µl immune complexes dilutions. Incubate overnight at 37°C in a CO₂ supplemented incubator.
5. Next morning, centrifuge the plate at 550×g, aspirate the medium containing the immune complexes, wash once in complete medium and add the specific T cells as previously mentioned in Subheading 3.1, step 7.

3.4. Latex Beads

3.4.1. Latex Beads

Preparation

1. Prepare the following solutions in 1× PBS:
 - (a) 50 mg/ml OVA
 - (b) 25 mg/ml OVA and 25 mg/ml BSA
 - (c) 10 mg/ml OVA and 40 mg/ml BSA
 - (d) 5 mg/ml OVA and 45 mg/ml BSA
 - (e) 50 mg/ml BSA.
2. Prepare five sterile 1.5 ml tubes. Put in each tube 100 µl of latex beads and centrifuge them at 15,000×g for 5 min. Discard the supernatant. Wash the bead pellets twice in sterile 1× PBS.
3. Mix 100 µl of each OVA-BSA solutions (solutions prepared in step 1) with a bead pellet and shake overnight at 4°C.
4. In the next morning, centrifuge the tubes at 15,000×g for 5 min, discard the supernatant and wash three times the beads in 1.5 ml of cold 1× PBS.
5. Suspend the beads in 100 µl sterile PBS and use them immediately for phagocytosis (see Note 10). Do not store the beads, the proteins are passively adsorbed to the beads and therefore can readily detach from the latex.
6. Latex beads phagocytosis can be realized in 15 ml conical tubes (see Subheading 3.4.2) or in 96-well round bottom plates (see Subheading 3.4.3) according to the DC number available (see Note 10).

3.4.2. Latex Beads

Phagocytosis in Conical Tubes for Cross-presentation

1. Prepare five separate 15 ml conical tubes containing five million DCs in medium without FCS and add the five populations of latex beads containing various amounts of OVA.
2. Vortex well the tubes for mixing the cells and the beads.
3. Centrifuge the tubes (short spin at 550×g) to attach the beads to the cells.
4. Incubate in a water bath at 37°C for 20 min.
5. Centrifuge again at 550×g for 2 min at 4°C, discard the supernatant, and suspend the DC pellet in 4 ml cold 1× PBS.

6. Remove the beads by a FCS-flotation gradient as following:
 - (a) Put 2 ml of FCS in a 15 ml conical tube and add slowly, without mixing the two layers, the 4 ml of PBS containing the DCs.
 - (b) Centrifuge the tube with the gradient at $350 \times g$ and 4°C for 5 min, without acceleration and without brake.
 - (c) Recover the pellet and discard the supernatant, which contained the free latex beads.
7. Repeat step 6 once. Resuspend the cell pellet in 1 ml complete medium, count the cells, and use them as cross-presenting DCs.
8. Incubate in 96-well round bottom plates 100,000 DCs for several period of times, e.g., 2, 4, 8, 16 h. Make duplicate for each point.
9. At the end of the incubation fix the DCs as described in Subheading 3.1, step 6.
10. Add Specific T cells as mentioned in Subheading 3.1, step 7.

3.4.3. Latex Beads

Phagocytosis in 96-Well Plates for In Vitro Cross-Presentation

1. Dilute each type of OVA-BSA latex beads in complete DCs medium at 1/100, 1/300, and 1/900. Distribute 100 μ l of each dilution per well in duplicate in 96-well round bottom plates.
2. Add 100,000 DCs in each well. When the antigen is incubated in the wells and might not be removed before T cells addition, a control with prefixed DCs is strongly recommended (see Note 5). Incubate the plate at 37°C in a CO₂ supplemented incubator for the desired period of time. Kinetics of antigen processing between 2 and 16 h could be performed.
3. Before adding the specific T cells it is recommended to fix the DCs as in Subheading 3.1, step 6.
4. Add the specific T cells as previously mentioned in Subheading 3.1, step 7.

3.5. Yeast Cells

Cross-presentation

1. Take a frozen aliquot of 100 μ l yeast cells at 2×10^8 cells/ml and add 1 ml cold 1× PBS.
2. Centrifuge the tube at $20,000 \times g$ for 10 min. Discard the supernatant carefully. Suspend the yeasts pellet in 100 μ l complete DC medium.
3. Prepare serial dilutions 1/25, 1/50, 1/100, and 1/200 of the 100 μ l yeast aliquot in complete media and distribute in duplicate 100 μ l of each yeast dilution in 96-well round bottom plates. Do this step outside from the cell culture room to avoid cell culture facilities contamination by yeasts.
4. UV-Irradiate the plate (without cover) containing the yeasts in a Stratalinker® UV Crosslinker in energy mode at 200,000 μ J/cm². Put a sterile cover on the plate and continue to work in

cell culture facilities since irradiated yeasts are unviable and cannot contaminate the room.

5. Add 100,000 DCs per well and mix them with the irradiated yeasts. Since the antigen remains in the wells all during the assay, a control with prefixed DCS showing the background of exogenously generated SIINFEKL peptide, is absolutely required (see Note 5). Incubate at 37°C in a CO₂ supplemented incubator. A time series for antigen presentation can be done from 4 to 16 h.
6. Before the addition of specific T cells, the DCs can be fixed as described in Subheading 3.1, step 6.
7. Add the specific T cells as mentioned in Subheading 3.1, step 7.

3.6. Apoptotic Cells

1. Prepare a solution of 3 mg/ml OVA in electroporation buffer
2. Prepare single cell suspension of splenocytes from β2m ko spleens.
3. Centrifuge the splenocytes and resuspend them in electroporation buffer at final concentration of 40 million per ml.
4. Mix in the 4 mm gap electroporation cuvette 100 µl (four million cells) of splenocytes suspension with 100 µl OVA solution at 3 mg/ml.
5. Switch on the Gene Pulser machine and set the electroporation parameters at 300 V, 500 µF.
6. Place the cuvette in the Gene Pulser cuvette chamber and make the pulse.
7. Remove the cells from the cuvette, transfer them to a 15 ml conical tube, and wash them twice with 10 ml 1× PBS.
8. Resuspend the pellet in the desired volume of cell culture medium for in vitro assays or in sterile PBS for in vivo cross-presentation assay. Usually the injection of 0.5–2 million OVA electroporated splenocytes per mouse is sufficient for the detection of naïve OT1 T cells proliferation in an adoptive transfer experiment (6).

4. Notes

1. The immune response may depend on the nature of the receptor targeted. For example, routing into different intracellular compartments of ovalbumin internalized by two different receptors as, DEC 205 and 33D1 results in antigen presentation mainly by MHC class I or by MHC class II molecules (7). Different signaling molecules recruited by each receptor can explain these results.

2. The receptors involved in antigen recognition and its phagocytosis largely dictate the outcome of the immune response following antigen cross-presentation. While the mechanism of latex beads uptake is poorly characterized and the “latex beads receptor” not yet identified, the pathogens and apoptotic cells uptake mechanisms are better understood. The apoptotic cells express at their surface several “eat-me” molecules (8). Apoptotic cells phagocytosis triggers anti-inflammatory responses (9). Phagocytosis of pathogens, in contrast, triggers the inflammatory response, which indicates to the immune system the presence of infection. We use yeast cells engineered to express the antigens at the cell surface, as a model of pathogen phagocytosis. Dectin-1 and Mannose Receptor (MR) are the DCs receptors that mediate yeasts phagocytosis (10). These receptors, by their interaction with TLRs are involved in the induction of inflammatory responses.
3. The DC activation by TLR ligands may change the antigen presentation capacity of DCs. Traces of endotoxins are able to activate the DC via TLR-4, therefore the use of endotoxin free OVA is recommended when the antigen cross-presentation ability of immature DCs is analyzed. Moreover, if the antigen cross-presentation of activated DCs will be analyzed the use of endotoxin free OVA allows the tight control of DC activation by a unique, exogenously added TLR ligand.
4. Soluble ovalbumin might be contaminated by free H2-K_b ligand, the SIINFEKL peptide. Since the OT1 T cells are very sensitive, this contamination could interfere with the results of OVA cross-presentation assays. Separation of small size peptide from full-length ovalbumin using the Bio-Spin® 6 Columns is therefore strongly recommended.
5. Taking into account the high sensitivity of OT1 T cells receptor; all cross-presentation assays *in vitro* absolutely require a control for the presence of free SIINFEKL peptide in the antigen preparation. This control could be either DCs prefixed with 0.005% glutaraldehyde before the incubation with the antigen or DCs obtained from a MHC-I mismatched mouse strain (mice that do not bear H2-K_b allele, as for example BALB/c or CH3 strains). If the antigen cross-presentation assay background obtained with this control is high, the results of the assay may not be valid.
6. The anti-OVA antibody is supplied as a whole rabbit serum. FcR mediated cross-presentation assay require purified IgG. The isolation of IgG from the whole serum should be performed using the Protein G sepharose column.
7. Our laboratory has previously reported (11) the characterization of fusion proteins suitable for antigen targeting to an unlimited number of cell surface receptors. The general scheme



Fig. 1. Fusion protein composition. *White box* (1–26 aminoacids) represents the gp 64 insect protein signal peptide, *dark grey boxes* (26–238 aminoacids) illustrate three repeats of IgG binding domain of Protein G, *light grey box* indicates the ubiquitin and the *black box* designates the antigen, ovalbumin. Fusions with other antigens like the influenza protein matrix 1, neuraminidase (extracellular), nucleoprotein, and hemagglutinin are also available in Peter van Endert laboratory (11).

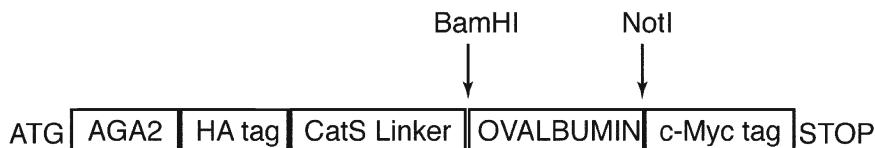


Fig. 2. Engineered yeast as a natural phagocytosis substrate. The antigens are expressed as a fusion protein between the yeast adhesion subunit of the α-agglutinin Aga2 and full length OVA. A linker containing a double repeat of the peptide EKARVLAEEAA that is cleaved by Cathepsin S precedes the ova sequence. The HA tag (YPYDVPDYA) and the c-Myc tag (EQKLISEEDL) can be used to monitor the yeast surface expression of the antigen by flow cytometry.

of the fusion proteins is represented in Fig. 1. The Protein G domain, which has broad Ig binding capacity covering several IgG isotypes from many animal species, allows for binding of the fusion proteins to the antibodies that are specific for DCs cell surface receptors. The Ub can enhance proteasomal degradation of antigenic proteins that are linked to the C terminal part of the Ub.

8. Based on the yeast surface display of various proteins strategy developed by the KD Wittrup laboratory (12), we designed and produced yeast plasmids expressing full-length OVA (Fig. 2). The antigen is fused to the yeast agglutinin protein Aga2, which attaches to the yeast cell wall through disulfide bonds to Aga1. Expression of the fusion protein Aga2-Antigen is under the control of a galactose-inducible promoter on the yeast display plasmid that is based on pCTCON-2 (12) backbone. The plasmid that expresses Aga2 and the antigen, also expresses an auxotrophy marker allowing its maintenance in yeast. The yeast strain used is EBY100, which expresses Aga1 from a galactose-inducible expression cassette integrated into the yeast genome. We have demonstrated that the yeast cells displaying full-length OVA are cross-presented via proteasome dependent cross-presentation (13).

9. The protein complexes might precipitate when the targeting antibody is the hamster anti mouse CD11c (clone N418). The precipitation can be avoided if the total protein concentration in the mixture is kept below 0.2 mg/ml.
10. According to the number of DCs available for the cross-presentation assay, the phagocytosis can be done in tubes or in 96-well round bottom plates. For the phagocytosis in separate tubes at least 25 million DCs are required. Phagocytosis in 96-well plates requires only two million DCs for each DC population that should be analyzed.

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References

1. Rock KL, Farfan-Arribas DJ, Shen L (2010) Proteases in MHC class I presentation and cross-presentation. *J Immunol* 184(1):9–15. doi:[10.4049/jimmunol.0903399](https://doi.org/10.4049/jimmunol.0903399)
2. Amigorena S, Savina A (2010) Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr Opin Immunol* 22(1):109–117. doi:[10.1016/j.co.2010.01.022](https://doi.org/10.1016/j.co.2010.01.022)
3. Shortman K, Naik SH (2007) Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7(1):19–30. doi:[10.1038/nri1996](https://doi.org/10.1038/nri1996)
4. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, Jeffrey KL, Anthony RM, Kluger C, Nchinda G, Koh H, Rodriguez A, Idoyaga J, Pack M, Velinzon K, Park CG, Steinman RM (2010) Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143(3):416–429. doi:[10.1016/j.cell.2010.09.039](https://doi.org/10.1016/j.cell.2010.09.039)
5. Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, Takahara K, Steinman RM, Inaba K (2002) The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med* 195(10):1289–1302
6. Saveanu L, Carroll O, Weimershaus M, Guermonprez P, Firat E, Lindo V, Greer F, Davoust J, Kratzer R, Keller SR, Niedermann G, van Endert P (2010) Fusion proteins for versatile
7. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumppheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG, Steinman RM, Nussenzweig MC (2007) Differential antigen processing by dendritic cell subsets in vivo. *Science* 315(5808):107–111. doi:[10.1126/science.1136080](https://doi.org/10.1126/science.1136080)
8. Jeannin P, Jaillon S, Delneste Y (2008) Pattern recognition receptors in the immune response against dying cells. *Curr Opin Immunol* 20(5):530–537. doi:[10.1016/j.co.2008.04.013](https://doi.org/10.1016/j.co.2008.04.013)
9. Henson PM, Hume DA (2006) Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol* 27(5):244–250. doi:[10.1016/j.it.2006.03.005](https://doi.org/10.1016/j.it.2006.03.005)
10. Bazan SB, Geginat G, Breinig T, Schmitt MJ, Breinig F (2011) Uptake of various yeast genera by antigen-presenting cells and influence of subcellular antigen localization on the activation of ovalbumin-specific CD8 T lymphocytes. *Vaccine* 29(45):8165–8173. doi:[10.1016/j.vaccine.2011.07.141](https://doi.org/10.1016/j.vaccine.2011.07.141)
11. Kratzer R, Mauvais FX, Burgevin A, Barilleau E, van Endert P (2010) Fusion proteins for versatile

- antigen targeting to cell surface receptors reveal differential capacity to prime immune responses. *J Immunol* 184(12):6855–6864. doi:[10.4049/jimmunol.0902555](https://doi.org/10.4049/jimmunol.0902555)
12. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD (2006) Isolating and engineering human antibodies using yeast surface display. *Nat Protoc* 1(2):755–768. doi:[10.1038/nprot.2006.94](https://doi.org/10.1038/nprot.2006.94)
13. Merzougui N, Kratzer R, Saveanu L, van Endert P (2011) A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep* 12(12):1257–1264. doi:[10.1038/embor.2011.203](https://doi.org/10.1038/embor.2011.203)

Chapter 30

Gene Transduction in Human Monocyte-Derived Dendritic Cells Using Lentiviral Vectors

Takeshi Satoh and Nicolas Manel

Abstract

Monocyte-derived dendritic cells (MDDCs) are widely used in the field of human immunology. Although a variety of gene delivery procedures have been used in MDDC, it has remained difficult to achieve robust gene transductions. In this chapter, we describe a procedure for high efficiency gene transduction in human MDDCs using lentiviral vectors. Gene transduction based on HIV-1-derived lentiviral vectors is restricted at the level of reverse transcription by the cellular protein SAMHD1 in MDDCs. Co-transduction of the MDDCs with helper particles derived from SIVmac that contain the viral protein Vpx removes this restriction, leading to a drastic increase in the rate of gene transduction. This procedure leads to nontoxic, efficient and stable transduction in MDDCs. It can be applied to any HIV-1-derived lentiviral vector, including shRNA lentiviral vectors for RNAi. Transduced MDDCs are not activated by the transduction and can be activated normally by TLR ligands.

Key words: Lentiviral vector, Dendritic cell, shRNA, RNAi, Vpx

1. Introduction

Lentiviral vectors have been developed to achieve stable gene transduction in dividing cells and nondividing cells. Most currently used lentiviral vectors derive from HIV-1 and have been rendered nonreplicating by removing genes coding for so-called “accessory” proteins (Vif, Vpr, Vpu, and Nef) and for the envelope glycoprotein and by dissociating expression of the viral proteins from expression of the viral nucleic acid. Instead of the natural envelope protein, the G protein of the Vesicular stomatitis virus (VSV-G) is used in the virus-production step. This envelope allows lentiviral vectors to broadly transduce most types of cells, as it is thought that the receptor for VSV-G is a ubiquitous lipid component of the cellular membrane. Furthermore, whereas the HIV-1 envelope is labile due to non-covalent interactions between gp120 and gp41,

VSV-G is stable and thus highly resistant to ultracentrifugation. However, lentiviral vector transduction in myeloid-type cells like macrophages and dendritic cells has been notoriously difficult. This phenomenon has been observed also with replication-competent HIV-1, while these cells are permissive to infection with HIV-2 and the macaque immunodeficiency virus (SIVmac). During HIV-1 infection of myeloid cells, SAMHD1 has been reported as the restriction factor that blocks infection (1, 2). On the other hand, HIV-2 and SIVmac express the Vpx protein. The Vpx protein is incorporated into viral particles through an interaction with HIV-2 or SIVmac Gag, and upon delivery into the target cell cytosol, it recruits a proteasome complex to SAMHD1 that includes CUL4, DDB1, and DCAF1 (3). The mechanism of restriction imposed by SAMHD1 is not completely understood, but it coincides with a blockade at the reverse transcription step. Through the action of Vpx, SAMHD1 is degraded and this restores efficient reverse transcription, allowing infection of myeloid cells by these viruses. Importantly, Vpx delivery by virus-like particles (VLPs) of SIVmac can alleviate the block to infection of MDDCs with HIV-1 (4). This indicates that the Vpx protein can act in *trans* and that co-transduction of myeloid with an HIV-1-derived vector and with SIVmac VLPs containing Vpx enables transduction.

Dendritic cells (DCs) constitute a key regulator of diverse immune responses, linking innate with acquired immunity. It had been possible to obtain stably transduced DCs by using CD34⁺ hematopoietic precursors (5), however this procedure is long and yields are not consistent. We took advantage of the principle of Vpx action to achieve efficient gene transduction in monocyte-derived dendritic cells (MDDCs) (6). In this chapter, we describe this procedure, which leads to nontoxic, efficient, and stable gene transduction (Fig. 1). Transduced MDDCs are not activated by the transduction and can be normally activated by TLR ligands.

The principle of the protocol can be summarized as follows. Two culture supernatants are generated by transient transfection of 293FT cells. The first supernatant contains the HIV-1-derived lentiviral vector particles for gene transduction (“lentiviral vector”), and the second supernatant contains the SIVmac-derived helper particles that encapsidate the Vpx protein but do not carry any genetic material (“SIV-VLP”). Next, fresh monocytes are isolated from blood and incubated at the day of isolation with a mixture of the two viral supernatants. This will lead to delivery of both the Vpx protein and the lentiviral vector in the monocytes. Vpx will remove the restriction, and the lentiviral vector, which consists essentially of a promoter and a gene of interest, will integrate in the monocyte genomic DNA. Monocytes are cultured in the presence of GM-CSF and IL-4 at all times, thus DC differentiation is induced. Expression of the lentiviral vector will start rapidly, typically by day 1 of the monocyte culture and will be maintained over

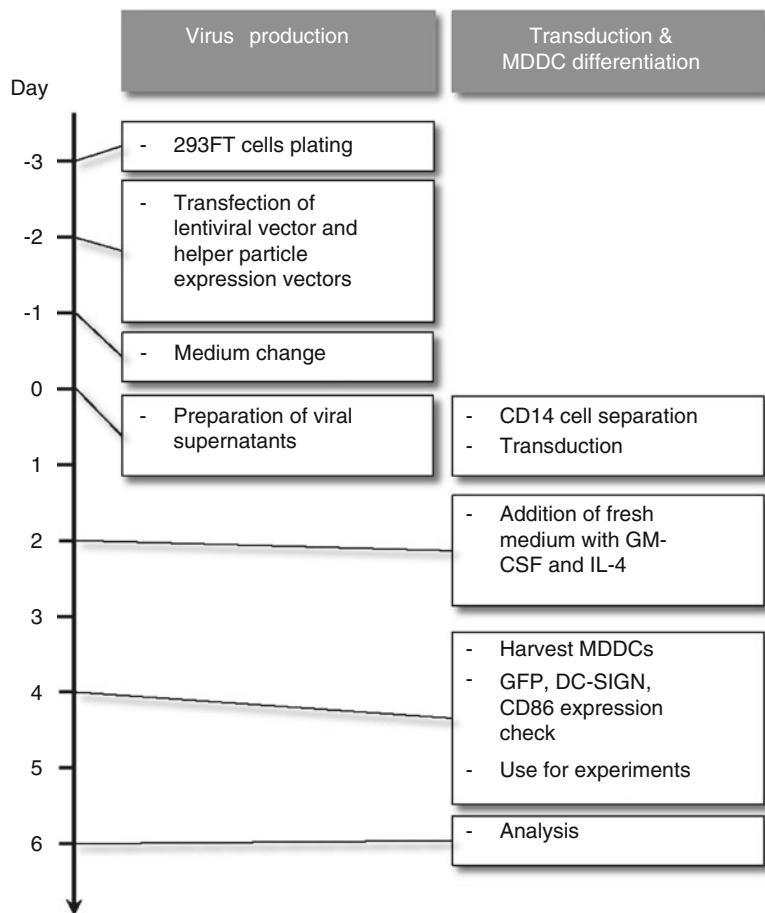


Fig. 1. Timetable of lentiviral vector transduction in human MDDCs.

the course of differentiation into DCs. In the case of a lentiviral vector encoding for a shRNA, such as pLKO.1, transduction of monocytes at day 0 allows for persistent inhibition of the target cellular mRNA by RNAi, which will lead to depletion of the target protein, according to its half-life (6). Alternatively, this protocol can be used to stably overexpress proteins of interest in MDDCs by using a different lentiviral vector.

2. Materials

All reagents must be prepared under endotoxin-free conditions. It is required to use disposable plastic ware and filter tips for reagent preparation and for all experiments.

- 2.1. Lentiviral Vector Production**
1. Purelink HiPure Plasmid Kit (Invitrogen).
 2. Plasmid pLKO.1gfp ([6](#)) (see Note 1).
 3. Plasmid psPAX2 (Addgene, see Note 1).
 4. Plasmid pSIV3+ ([7](#)) (see Note 1).
 5. Plasmid pCMV-VSVG (Addgene, see Note 1).
 6. 293FT cells (Invitrogen) (see Note 2).
 7. 293FT culture medium: DMEM supplemented with 10% fetal bovine serum (FBS), Penicillin/Streptomycin. Neomycin selection is recommended on an occasional basis.
 8. Virus production medium: DMEM supplemented with 10% DC FBS, Penicillin/Streptomycin, and Gentamicin (see Note 3).
 9. TransIT-293 Transfection Reagent (Mirus Bio).
 10. DMEM.
- 2.2. Monocyte Purification**
1. Human adult peripheral blood from anonymous, healthy, and consenting donor.
 2. Steriled phosphate buffered saline (PBS): 137.93 mM NaCl, 2.67 mM KCl, 1.47 mM KH₂PO₄, 8.06 mM Na₂HPO₄·7H₂O. Sterilized by Autoclave.
 3. Ficoll-Paque PLUS (GE Healthcare).
 4. Cell Strainer, 40 µm, blue.
 5. Human CD14 Microbeads (Miltenyi Biotec) (see Note 4).
 6. MACS buffer: PBS, 0.5% Bovine serum albumin, 2 mM EDTA. Sterilized by 0.2 µm filtration.
 7. MDDC culture medium: RPMI 1640 supplemented 10% DC FBS, Penicillin/Streptomycin, and Gentamicin.
 8. FITC-labeled anti-human CD14 antibody.
 9. Magnetic cell separator (Miltenyi Biotec).
 10. Flow cytometer.
- 2.3. Lentivirus Transduction and MDDC Differentiation**
1. Cellulose acetate syringe filter, pore size 0.45 µm.
 2. 1,000× protamine solution: 8 mg/ml protamine in distilled water. Sterilized by a 0.2 µm syringe filter.
 3. Recombinant human IL-4.
 4. Recombinant human GM-CSF.
 5. PE-labeled anti-human DC-SIGN.
 6. PE-labeled anti-human CD86.
 7. High molecular weight polyinosinic:polycytidylic acid (poly(I:C)).
 8. Ultrapure lipopolysaccharide (LPS).
 9. Flow cytometer.

Table 1
Ratios of DNAs for transfection of 293FT cells (for one well of a 6-well plate)

	Plasmid	Quantity
For lentiviral vector	pLKO.1gfp	1.6 µg
	psPAX2	1 µg
	PCMV-VSVG	0.4 µg
For SIV-VLP	PSIV3+ PCMV-VSVG	2.6 µg 0.4 µg

3. Methods

For experiments using human blood and lentiviral vectors, please follow specific regulations. It is typically needed to obtain approval of the experimental plan and procedure from the Biosafety and Ethics committee at your institution.

3.1. Lentiviral Vector and SIV-VLP Production

1. On day -3, plate 293FT cells to 6-well plates. 1×10^6 per well in 2 ml of 293FT culture medium (see Notes 5 and 6).
2. On day -2, aspirate the culture medium and add pre-warmed (37°C) 2 ml of 293FT culture medium (see Note 7).
3. Transfection of plasmid DNAs with TransIT-293 Transfection Reagent. Per well, use 8 µl TransIT-293 and 3 µg total DNA.
 - (a) DNA is purified with Invitrogen HiPure Plasmid Kit (see Note 8).
 - (b) Dispense 200 µl Plain DMEM to new microtube and suspend 8 µl TransIT-293 into Plain DMEM directly. Mix by vortex for 5 s. This is for 1 well transfection. If you transfet DNAs to a few wells, prepare this mixture for each. Two transfections must be performed independently for each transduction: one to generate the HIV-1-derived lentiviral vector particles for gene transduction, and one to generate the SIV-VLP helper particles that will provide Vpx.
 - (c) Incubate for 5 min on the bench.
 - (d) Add DNAs to TransIT-293 suspension and mix by tapping (Table 1).
 - (e) Incubate for 15 min at room temperature.
 - (f) Add DNA/TransIT-293 mixture to the well in a dropwise fashion.
 - (g) Incubate for 18 h in CO₂ incubator.

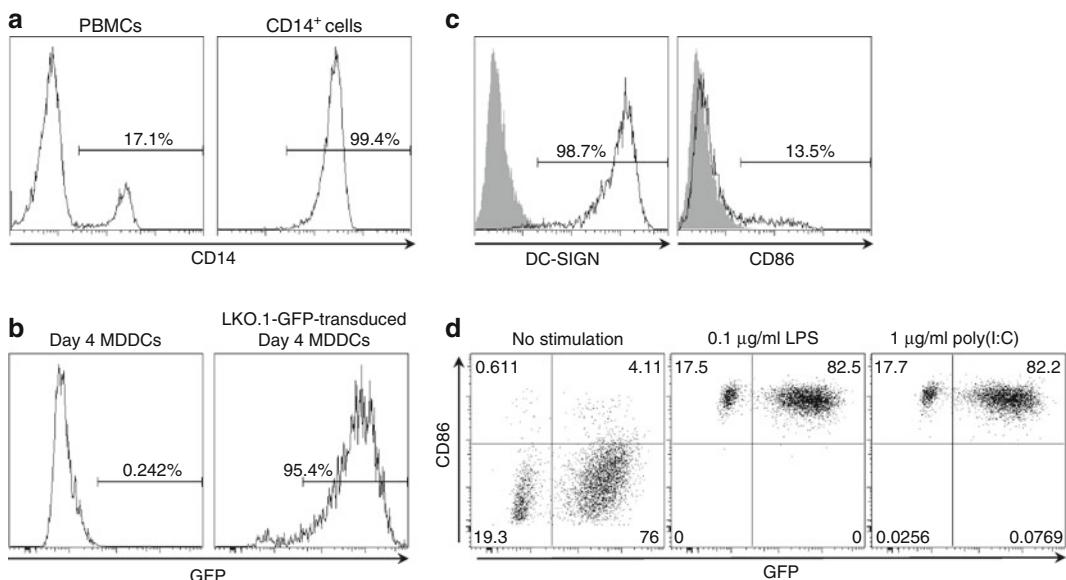


Fig. 2. Lentiviral transduction and MDDC differentiation. (a) Monocyte separation from PBMC. CD14⁺ cells were separated by MACS, stained with FITC-labeled anti-CD14 antibody, and analyzed by FACS. (b) GFP expression in lentivirus-transduced MDDCs at day 4. (c) DC-SIGN and CD86 expression in lentivirus-transduced MDDCs at day 4. (d) Lentivirus-transduced MDDCs are not activated and stimulated with TLR ligands similarly to regular MDDCs. Day 4 MDDCs were stimulated with 0.1 µg/ml LPS or 1 µg/ml Poly(I:C) for 48 h, stained with PE-labeled anti-CD86 antibody, and analyzed by FACS.

4. Day -1, aspirate the medium and add 3 ml pre-warmed (37°C) virus medium.
5. Incubate for 24 h in CO₂ incubator.

3.2. Monocyte Purification

1. On day 0, dilute peripheral blood with same volume of PBS.
2. Dispense 15 ml Ficoll-Paque PLUS to 50 ml tube and overlay the diluted blood slowly.
3. Centrifuge at 400×*g* for 30 min at room temperature without brakes.
4. Isolate PBMC phase with dropper and transfer to new 50 ml tube.
5. Fill up PBS to 50 ml.
6. To remove platelets, centrifuge the sample at low speed: 200×*g* at 4°C for 10 min.
7. Remove supernatant, suspend PBMC in MACS buffer, remove debris using a cell strainer, and count cell number.
8. Purify CD14⁺ monocyte with CD14 microbeads according to the manual.
9. Suspend monocyte in DC medium and count cell number.
10. Check the purity by Flow cytometer (Fig. 2a).

3.3. Lentiviral Transduction and MDDC Differentiation

1. Collect culture supernatant from transfected 293FT and remove debris with 0.45 µm syringe filter and keep at fridge or on ice (see Notes 9 and 10).
2. Suspend 1×10^6 per ml monocytes in MDDC medium and add recombinant human GM-CSF at 10 ng/ml and recombinant human IL-4 at 50 ng/ml.
3. Plate 3×10^6 per well monocytes to 6-well plates (see Notes 11 and 12).
4. Add 1.5 ml each of lentiviral vector and SIV-VLP supernatant.
5. Add 6 µl 1,000× Protamine solution (see Note 13).
6. Mix gently by pipetting (see Note 14).
7. Incubate in CO₂ incubator.
8. On day 2, remove 3 ml supernatant slowly and add 3 ml fresh MDDC culture medium with 10 ng/ml GM-CSF and 50 ng/ml IL-4 (see Note 15).
9. Incubate in CO₂ incubator.
10. On day 4, check transfection efficiency and MDDC differentiation by GFP and DC-SIGN expression (Fig. 2b, c, see Notes 16 and 17).
11. Stimulate MDDC with LPS or poly(I:C).
12. On day 6, analyze MDDC activation by measuring CD86 upregulation (Fig. 2d).

4. Notes

1. pLKO.1gfp is a shRNA-encoding lentiviral vector in which the GFP gene is substituted for the puromycin resistance gene, starting from pLKO.1puro (<http://www.addgene.org/8453/>) (6). Any pLKO.1 vector can be used, according to the experiment, such as pLKO.1puro (8). Alternatively, lentiviral vectors for gene expression such as pTRIP may also be used (9). We find that lentiviral vectors containing IRES sequences get less efficiently transduced in MDDCs. psPAX2 is a packaging plasmid expressing required HIV-1 viral proteins (<http://www.addgene.org/12260/>). pCMV-VSVG is an expression plasmid for the envelope glycoprotein G of the Vesicular Stomatitis Virus (<http://www.addgene.org/8454/>). pSIV3+ is a packaging vector derived from SIVmac251 that has been modified to express only viral proteins, including Vpx, but excluding Env and Nef (7).
2. 293T may be substituted for 293FT. It is very important to plate cells at optimal cell density for efficient transfection as well as for high titer of the lentiviral preparation. We recommend

titrating optimal cell density from 5×10^5 to 2×10^6 per well on 6-well plates and determine the best transfection condition in your hands.

3. It is required to identify a batch of FBS that is compatible with both efficient virus production and proper DC differentiation and viability. Refer to this batch as your “DC FBS.” Gentamicin is recommended to suppress environmental contaminations that may occur during monocyte preparation.
4. Monocytes can also be purified by negative selection using specific kits.
5. Culture may get contaminated with bacteria even if Gentamicine is added to culture medium because of the long-term culture with monocytes. To reduce the possibility of contamination, avoid using bacteria the day of the experiment.
6. For initial experiments, it is recommended to prepare one well of a single lentiviral vector and one well of SIV-VLP. Each will lead to approximately 2.5 ml of actual supernatant after filtration. This is sufficient to transduce a maximum of 5×10^6 monocytes.
7. When changing the medium, add new medium slowly to avoid for cells to detach from plates. Detachment of the cells reduce virus yield.
8. High purity endotoxin-free plasmids are required for this transfection. Contaminations of *E. coli* endotoxins and the other substances suppress transfection and may also be carried over, inducing activation of MDDCs. To check the contamination of endotoxin, add 1 µg of plasmid to a culture of 10^6 MDDCs for 24 h. Endotoxin-free plasmid should not induce DC activation (upregulation of CD86).
9. At this point, pLKO.1gfp-transfected 293FT cells must be almost all GFP positive. If this is not the case, check the condition of transfection or purity of plasmids.
10. Lentiviral particles are unstable at room temperature. If you don't use the virus immediately, you need to keep it on ice, in the refrigerator for short-term storage or at -80°C for long-term storage.
11. You may change the plate format. Check cell viability accordingly during the culture.
12. Because MDDC are nondividing cells, the concentration of intracellular dNTP for reverse transcription is thought to be limiting. If transduction efficiency is low, addition of deoxy-nucleosides (dNTP precursors, 1–5 mM) to culture medium may improve it (10).
13. Generally, polybrene is used to neutralize negative charges between the virus and cell surface phospholipidic membranes to facilitate binding of virus to cells. However, polybrene is

toxic especially for primary cells. Instead of polybrene, we found that protamine performs efficiently for lentiviral transduction to MDDCs. Protamine is not highly toxic for MDDCs and enhances the transduction to a similar extent, or more, than polybrene.

14. The cells must be mixed gently. Mixing vigorously induces MDDC activation.
15. You can change the day of fresh medium addition to day 1, 3, or 4 as needed. You must maintain cells in healthy conditions and limit cell death that could influence the remaining live cells.
16. At this point, 50–95% of the monocytes seeded at day 0 are recovered as viable live cells.
17. In this protocol, co-transduction of Vpx leads to degradation of the SAMHD1 protein at day 0 in monocytes. At day 4, it should be kept in mind that the expression level of SAMHD1 has not recovered and accordingly MDDCs are permissive to HIV-1 infection (6). Given the implication of SAMHD1 in Aicardi–Goutière Syndrome (11), absence of SAMHD1 may lead to an altered response to cytosolic DNA in MDDCs.

References

1. Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M, Srivastava S et al (2011) Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 474(7353):658–661
2. Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Ségéral E et al (2011) SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 474(7353):654–657
3. Srivastava S, Swanson SK, Manel N, Florens L, Washburn MP, Skowronski J (2008) Lentiviral Vpx accessory factor targets VprBP/DCAF1 substrate adaptor for cullin 4 E3 ubiquitin ligase to enable macrophage infection. *PLoS Pathog* 4(5):e1000059
4. Goujon C, Jarrosson-Wuillème L, Bernaud J, Rigal D, Darlix J-L, Cimarelli A (2006) With a little help from a friend: increasing HIV transduction of monocyte-derived dendritic cells with virion-like particles of SIV(MAC). *Gene Ther* 13(12):991–994
5. Boggiano C, Manel N, Littman DR (2007) Dendritic cell-mediated trans-enhancement of human immunodeficiency virus type 1 infectivity is independent of DC-SIGN. *J Virol* 81(5):2519–2523
6. Manel N, Hogstad B, Wang Y, Levy DE, Unutmaz D, Littman DR (2010) A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. *Nature* 467(7312): 214–217
7. Mangeot PE, Nègre D, Dubois B, Winter AJ, Leissner P, Mehtali M et al (2000) Development of minimal lentivirus vectors derived from simian immunodeficiency virus (SIVmac251) and their use for gene transfer into human dendritic cells. *J Virol* 74(18): 8307–8315
8. Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS et al (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9(4): 493–501
9. Zennou V, Petit C, Guétard D, Nerhbass U, Montagnier L, Charneau P (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101(2):173–185
10. Ravot E, Comolli G, Lori F, Lisziewicz J (2002) High efficiency lentiviral gene delivery in non-dividing cells by deoxynucleoside treatment. *J Gene Med* 4(2):161–169
11. Rice GI, Bond J, Asipu A, Brunette RL, Manfield IW, Carr IM et al (2009) Mutations involved in Aicardi-Goutières syndrome implicate SAMHD1 as regulator of the innate immune response. *Nat Genet* 41(7): 829–832

Chapter 31

Pulse–Chase Analysis for Studies of MHC Class II Biosynthesis, Maturation, and Peptide Loading

**Tieying Hou, Cornelia H. Rinderknecht, Andreas V. Hadjinicolaou,
Robert Busch*, and Elizabeth Mellins***

Abstract

Pulse–chase analysis is a commonly used technique for studying the synthesis, processing and transport of proteins. Cultured cells expressing proteins of interest are allowed to take up radioactively labeled amino acids for a brief interval (“pulse”), during which all newly synthesized proteins incorporate the label. The cells are then returned to nonradioactive culture medium for various times (“chase”), during which proteins may undergo conformational changes, trafficking, or degradation. Proteins of interest are isolated (usually by immunoprecipitation) and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the fate of radiolabeled molecules is examined by autoradiography. This chapter describes a pulse–chase protocol suitable for studies of major histocompatibility complex (MHC) class II biosynthesis and maturation. We discuss how results are affected by the recognition by certain anti-class II antibodies of distinct class II conformations associated with particular biosynthetic states. Our protocol can be adapted to follow the fate of many other endogenously synthesized proteins, including viral or transfected gene products, in cultured cells.

Key words: MHC class II, Biosynthesis, Maturation, Metabolic labeling, Immunoprecipitation

Abbreviations

MHC	Major histocompatibility complex
ER	Endoplasmic reticulum
Abs	Antibodies
Ii	Invariant chain
B-LCL	EBV-transformed B-lymphoblastoid cell lines
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MIIC	MHC class II compartments
LIP	Leupeptin-induced polypeptides
CLIP	Class-II-associated invariant chain peptides
Cys/Met	Cysteine/methionine

*These two authors have contributed equally to this chapter.

PAS	Protein A Sepharose
PGS	Protein G Sepharose
IP	Immunoprecipitation
APCs	Antigen presenting cells
SLB	Laemmli sample loading buffer
PMSF	Phenylmethylsulfonyl fluoride

1. Introduction

In the 1960s, Palade et al. devised pulse–chase labeling of secreted proteins in pancreatic tissue slices in order to investigate the role of subcellular compartments in protein trafficking (1, 2). These experiments showed that secretory proteins were sequentially transported from the rough endoplasmic reticulum (ER) to the Golgi complex and then to zymogen granules via small vesicles located in the periphery of the Golgi (1, 2). Since then, pulse–chase labeling has become a widely adopted tool for studying the posttranslational fate of proteins.

In a typical experiment, cells of interest are first cultured for a short period (the “pulse”) in the presence of radioactively labeled essential amino acids. This is often a mixture of ^{35}S -labeled cysteine and methionine (Cys/Met). Other essential amino acids may be labeled with ^3H or ^{14}C and used in a similar way; this may be useful for proteins with a low Cys/Met content. Nonessential amino acids are not suitable, as their endogenous synthesis would compete with labeling. During the pulse, all proteins synthesized de novo become radiolabeled in proportion to their rate of biosynthesis and their content of amino acids carrying the label. Competition with preexisting pools of unlabeled amino acids in the cells may be reduced, and labeling efficiency increased, by starving the cells of these amino acids prior to pulse-labeling. In order to track the fate of proteins labeled during the pulse, the cells are washed and recultured (“chased”) for varying amounts of time in the presence of normal media, containing excess unlabeled amino acids. This ensures that only those proteins that were made during the pulse are radiolabeled; their fate can then be traced during the chase.

The proteomes of mammalian cells are exceedingly complex, so for most purposes, proteins of interest must be enriched by, for example, selective extraction, subcellular fractionation, and/or chromatography. A versatile and specific method uses detergent extraction and small-scale affinity chromatography (i.e., immunoprecipitation (IP)) with antibodies (Abs) specific for the protein of interest. The Abs are coupled directly to sepharose beads or are recognized by Ab-binding proteins, such as staphylococcal protein A or G, coupled to sepharose. Monoclonal Abs are often selective for particular protein conformations, and this property must be considered during data interpretation. Such Abs can

provide information on the fate of conformationally distinct molecular subsets of the same protein. Ab-bound radiolabeled proteins may be quantified by scintillation counting or visualized after separation on SDS-PAGE gels by autoradiography. The latter method allows quantification by densitometric analysis of gel bands and improves the discrimination between specifically bound proteins of interest and background radioactivity from nonspecific binding. It also reveals intracellular processing steps that alter the molecular weight of proteins of interest (e.g., proteolytic cleavage, modification of carbohydrates, and oxidation of disulfide bonds) or their association with other proteins. These capabilities continue to justify the use of radioactivity in the pulse–chase approach, even though nonradioactive techniques for measuring protein synthesis and turnover have become available (for a recent application to MHC proteins, see ref. 3).

Our laboratories have had a long-standing interest in the analysis of MHC class II (MHC II) glycoproteins, which bind heterogeneous mixtures of peptides in endosomes and present them on the surface of antigen-presenting cells for inspection by CD4+ T helper lymphocytes. Pulse–chase analysis has been invaluable in tracing the complex processes of MHC II protein synthesis, maturation, trafficking, and peptide loading. Newly synthesized MHC II $\alpha\beta$ heterodimers are assembled with the invariant chain (Ii) polypeptide in the ER (4, 5). This association aids the correct folding of nascent MHC II molecules and prevents premature binding of misfolded polypeptides (6). The assembled $(\alpha\beta)_3Ii_3$ complexes travel through the Golgi apparatus and, targeted by sequence motifs in the cytoplasmic tail of Ii, are directed to late endosomal and pre-lysosomal compartments, which are called MHC class II compartments (MIIC) (7–11). In these compartments, Ii is progressively degraded by aspartyl and cysteine proteases, including cathepsin S (12–15), via intermediates (p21, p10/12) termed “LIP” (“leupeptin-induced polypeptides”) and “SLIP” (small LIP), respectively, until only small Ii fragments called CLIP (class-II-associated invariant chain peptide) remain bound in the peptide-binding groove of MHC II (16, 17). The subsequent release of CLIP from MHC II and exchange for antigenic peptide are both mediated by HLA-DM (18–20). The catalytic action of DM also selects for peptides that form kinetically stable complexes with MHC II, and may influence the conformation of MHC II molecules (21). One unusual biochemical consequence of loading with high-affinity peptides is that the resultant MHC α/β -peptide complexes become resistant to denaturation with SDS in vitro; this feature can be used to track peptide loading in pulse–chase experiments (22). Some variations in the migration of SDS-stable complexes have been noted, which relate to peptide occupancy and/or conformation (“compact” vs. more slowly migrating “floppy” dimers (23, 24)). Peptide-loaded MHC II molecules are transported to the cell surface.

MHC II molecules are encoded at several genetic loci (generating class II isotypes), most of which exhibit extensive allelic polymorphism. The details of the maturational steps described above depend to some extent on the MHC II variant being studied. For example, allelic and isotypic variation in the affinity of MHC II molecules for CLIP results in varying degrees of spontaneous CLIP release in DM-deficient cells, and in varying levels of SDS stability of MHC II complexes with CLIP; this, in turn, affects peptide loading and turnover of MHC II molecules (reviewed in ref. 25).

Here, we describe a protocol for pulse-chase analysis of MHC class II molecules in human Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL). We show experimental results for HLA-DR and -DQ molecules (two isotypes of human MHC II), after pulse-chase labeling of B-LCL with defined mutations in DM. These results illustrate what information may be obtained by tracking changes in the molecular associations and abundance of radiolabeled proteins, as well as by use of conformation-sensitive mAbs.

2. Materials

2.1. Cell Culture and Media

Cell culture plasticware and culture media suitable for cells of interest. B-LCL grow well in RPMI1640 medium supplemented with 2 mM L-glutamine and either 10% fetal bovine serum or 10% newborn calf serum.

Complete Cys/Met-free medium: Cell growth medium deficient in Cys/Met (e.g., Cys/Met-free RPMI1640, CellGro; or DMEM, Gibco) supplemented with 2 mM L-glutamine and 10% dialyzed FBS (Invitrogen). Sterile filter (unless prepared from sterile ingredients), store at 4°C, warm to 37°C before use.

Chase medium: Complete growth medium (from 10.2.1.1, as above) supplemented with 1 mM each nonradioactive Cys and Met (Sigma). Sterile filter, store at 4°C, warm to 37°C before use.

2.2. Radioactive Reagents

1. Radioactive labeling reagent: (^{35}S) Cys/Met labeling mix (Perkin Elmer) should be stored in a -80°C freezer. Thaw at room temperature before use. Ensure handling with appropriate safety precautions (see *Current Protocols in Molecular Biology*, Appendix, A.1F for safe use of radioisotopes and specific precautions with ^{35}S -labeled materials).
2. Radioactive markers: ^{14}C -labeled rainbow protein molecular weight markers (GE Healthcare).

2.3. Preparation and Analysis of Lysates

1. Lysis Buffer: 50 mM Tris-HCl, 150 mM NaCl, 1% v/v NP-40 (now commercially available as IGEPAL CA-630; see Note 1), pH 8.0. Add 1 mM phenylmethylsulfonyl fluoride (PMSF; from 100 mM stock in dry 100% ethanol, kept at -20°C; toxic)

and 1/50th volume of protease inhibitor cocktail (Roche) into lysis buffer immediately before use. To prepare a 50× stock of the inhibitor cocktail, dissolve a protease inhibitor tablet in 1 ml water. Aliquot and store at –20°C.

2. Beads for immunoprecipitation (IP) and preclearing:

- (a) Protein A (Sigma) or protein G (GE Healthcare) sepharose beads: If the beads are supplied as a lyophilized powder, incubate 100 mg of beads in 1 ml PBS at 4°C for at least 1 h to allow swelling. The rehydrated beads may then be washed three times with at least 10 volumes PBS on a sintered glass funnel; alternatively, the washes can be performed by diluting the bead suspension to 50 ml in a conical polypropylene tube, centrifuging for 30 min at 920–1,430 ×*g* in a refrigerated benchtop centrifuge, and carefully aspirating the supernatant. Thereafter, adjust the final volume with PBS to obtain a 50% slurry (i.e., 50% settled beads by volume) and add sodium azide (toxic) from 10% w/v stock to a final concentration of 0.02%. Store at 4°C. If beads are supplied as a slurry in preservative (e.g., 20% ethanol), the rehydration step may be omitted. However, the washes are recommended to minimize carryover of preservative into immunoprecipitations.
- (b) (Optional) Pansorbin cells (Calbiochem): protein A-bearing *Staphylococcus aureus* cells with IgG-binding capacity. Pansorbin cells may be used for preclearing. They are stored as a 10% v/v suspension in PBS with 0.02% sodium azide at 4°C. Transfer desired amount of Pansorbin cells to a microfuge tube. Centrifuge for 5 min in a benchtop microfuge at 3,000 ×*g* or at maximum speed for 1 min at 4°C. Aspirate the supernatant. Resuspend in 1.0 ml of cold lysis buffer and mix gently. Centrifuge again and remove the lysis buffer. Repeat wash.

3. Pre-immune normal serum with the same animal source as antiserum used for IP, or, for mAbs, a species- and isotype-matched control Ab.
4. Antibodies: The choice of mAbs for IP experiments will be dictated by the experimental goals. Owing to polymorphism and isotype of the MHC, one important consideration is the selectivity of the mAbs for particular MHC alleles and isotypes. Antibodies raised in mice against human MHC molecules are often, but not invariably, monomorphic (i.e., their binding is relatively unaffected by allelic polymorphism), whereas murine antibodies raised against murine MHC molecules from another strain generally bind to only a subset of alleles. Moreover, mAbs may exhibit conformational preferences, and these preferences often relate to biosynthetic states of class II. See Tables 1 and 2 below for examples of antibodies (anti-human

Table 1
Selected antibodies used to study biosynthesis and maturation of human MHC class II molecules

Antibody	Isotype (murine)	Specificity in immunoprecipitation	Reference (original and application)
PIN 1.1	IgG1	Anti-Ii: Recognizes a cytoplasmic, N-terminal epitope of human Ii and co-immunoprecipitates any Ii-associated HLA class II $\alpha\beta$ heterodimer. It also reacts with C-terminally truncated Ii fragments that retain the N terminus (Leupeptin-induced peptides, LIP, but not CLIP). Does not allow detection of Ii on the surface of intact cells by flow cytometry	(33)
BU45	IgG1	Anti-Ii: Recognizes the C-terminal luminal domain of Ii. Co-immunoprecipitates free and class II-associated Ii. Does not react with C-terminally truncated Ii fragments (LIP). Allows detection of Ii on the surface of intact antigen presenting cells (APCs) by flow cytometry	(34, 35)
CerCLIP.1	IgG1	Anti-CLIP: Recognizes the N-terminus of CLIP (Ii residues 81–105). Immunoprecipitates CLIP-loaded class II $\alpha\beta$ heterodimers. Also allows detection of MHC class II-CLIP complexes on intact APC by flow cytometry. Staining intensity, relative to that with anti-class II mAbs, is inversely correlated with DM-catalyzed CLIP release. CLIP levels also depend on the affinity of any given MHC allele(s) for CLIP	(36, 37)
6-39	IgG1	Recognizes DR3/CLIP complexes, but not free CLIP	(38)
I-5	IgG1	Recognizes DR3/CLIP complexes, but not free CLIP	(39)
DA6.147	IgG1	Anti-HLA-DR α chain: Recognizes a monomorphic (DR specific) determinant on the C-terminal cytoplasmic tail of DR α . The epitope is accessible for IP in Ii-associated DR but less so for mature DR molecules. It can also be used to immunoprecipitate DR α chain from denatured lysates. Also reacts with dissociated HLA-DR α chains on Western Blots. Not suitable for detection of DR on intact APCs by flow cytometry	(37, 40–42)
L243	IgG2a	Anti-HLA-DR: Recognizes a monomorphic, conformational determinant on the α chain of DR $\alpha\beta$ heterodimers. DR molecules associated with intact Ii are immunoprecipitated poorly (presumably due to steric masking of the epitope by the Ii C terminus), but DR molecules associated with LIP, CLIP, or endosomal peptides are immunoprecipitated well (see Fig. 1). The strength of the residual interaction with DR/Ii complexes may be dependent on the allele and the exact conditions for IP	

(continued)

Table 1
(continued)

Antibody	Isotype (murine)	Specificity in immunoprecipitation	Reference (original and application)
		L243 is poorly suited to the IP of SDS-stable DR/peptide complexes under non-denaturing conditions (without boiling), because these complexes are supershifted by L243, which fails to dissociate in SDS at room temperature. Such complexes (but not dissociated DR chains) are, however, detected by L243 on Western blots. Also detects DR molecules on the surface of intact APCs by flow cytometry and has been used for large-scale affinity chromatography to purify DR	(12, 41, 43, 44)
ISCR3	IgG2b	Anti-HLA-DR β chain: immunoprecipitates HLA-DR $\alpha\beta$ dimers and is relatively insensitive to conformational maturation. Allows visualization of immunoprecipitated SDS-stable peptide-loaded DR $\alpha\beta$ dimers, as it dissociates from antigen in reducing SDS-PAGE sample buffer at room temperature. Also, cross-reacts with MHC II molecules from other species	(6, 45, 46)
LB3.1	IgG2a	Anti-HLA-DR α chain: Recognizes both empty and peptide-loaded HLA-DR1 $\alpha\beta$ heterodimer (monomorphic). Has been used for large-scale affinity chromatography of DR	(45, 47, 48)
MEM-264	IgG2b	Anti-empty HLA-DR β chain: Recognizes an epitope on the β chain of empty HLA-DR1 $\alpha\beta$ heterodimers. Binding of the antibody is abolished by association with CLIP, antigenic peptides, or truncated model peptides	(26, 47, 48)
16.23	IgG3	Anti-HLA-DR3: Recognizes an allele-specific, DM-dependent epitope present on a subset of DR3 molecules. Empty DR3/DM complexes and DR3/peptide complexes have been reported to bind, but only after DM-mediated CLIP release and not after the alternative self-release exchange mechanism. CLIP-associated DR3 molecules are not recognized	(39, 49–53)
NFLD. D11	IgM	Anti-HLA-DR4: Recognizes an allele-specific, DM-dependent determinant on the HLA-DR0401 β chain.	(54)
SPVL3	IgG2a	Anti-HLA-DQ: Recognizes a monomorphic epitope on DQ $\alpha\beta$ dimers, but not the denatured individual α or β chains. The epitope is maturation- and DM-dependent (see Fig. 2)	(55–57)
2.12.E11	IgG1	Anti-HLA-DQ2: Recognizes a conformation-dependent, polymorphic epitope on DQ $\alpha\beta$ dimers, but not the denatured individual α or β chains. It is specific for DQB1*0201 or 0202 alleles	(55, 58, 59)

(continued)

Table 1
(continued)

Antibody	Isotype (murine)	Specificity in immunoprecipitation	Reference (original and application)
Ia3	IgG2a	Anti-HLA-DQ: Recognizes a monomorphic epitope on DQ $\alpha\beta$ dimers, but not the denatured individual α or β chains	(55, 60)
B7/21.2	IgG3	Anti-HLA-DP: Recognizes a monomorphic epitope on HLA-DP α and immunoprecipitates immature and mature DP heterodimers	(61)
XD5.A11	IgG1	Anti-MHC class II β chain: Recognizes both intact and denatured DR, DP and DQ β chains and therefore all MHC class II $\alpha\beta$ heterodimers	(62)

Note that the studies cited here may describe the original derivation or informative uses of these antibodies, or both

and murine MHC class II proteins, respectively) that have been used successfully and/or frequently in these types of experiments. The specificities of mAbs often become evident in the course of pulse-chase experiments (see Figs. 1 and 2). Antibodies to the cytoplasmic tails of MHC class II α or β chains provide conformation-independent reagents for denatured proteins, and sometimes, but not always, for native proteins (see Ab DA6.147 below).

Monoclonal Abs may be purchased commercially or purified from hybridoma culture supernatants by protein A- or G-sepharose affinity chromatography, using commercial kits. Stock solutions (≈ 1 mg/ml in PBS, quantified by UV absorption spectrophotometry, assuming $A_{280}=1.4$ for a 1 mg/ml solution) can be stored indefinitely at or below -20°C in aliquots. Thawed aliquots may be kept at 4°C with addition of sodium azide to 0.05% (from 10% w/v stock) for several weeks.

5. IP wash buffer: PBS supplemented with 1 mM PMSF (added fresh), 0.05% NP-40, and 0.05% sodium azide.

6. SDS Polyacrylamide Gel Components:

See *Current Protocols in Molecular Biology*, Chapter 10, Section II, for directions on making gels and required buffers, including Laemmli sample loading buffer (SLB), both reducing and nonreducing. Equipment and supplies for SDS-PAGE (gel plates, combs, spacers, e.g., Bio-Rad) are also provided in the above reference.

7. Amplify solution (GE Healthcare).

8. Heated vacuum gel dryer, e.g., Bio-Rad.

Table 2
Selected antibodies used to study biosynthesis and maturation of mouse MHC class II molecules

Antibody	Reactivity	Isotype (murine unless indicated)	Specificity in immunoprecipitation	Reference (Original and application)
In-1	Ii	Rat IgG2b	Recognizes cytoplasmic epitope near N terminus of Ii. Immunoprecipitates free Ii and Ii/class II complexes. Recognizes denatured or non-denatured, full-length and C-terminally truncated Ii fragments, but not CLIP	(23, 63)
P4H5	Ii	Armendariz Hamster	Recognizes residues 99–116 of Ii, a luminal epitope immediately C-terminal of CLIP	(64–66)
M5/114	I-A/I-E β I-A ^{b,d,q} , I-E ^{d,k} (Not reactive with I-A ^{k,s,f,g})	Rat IgG2b	Recognizes β chain epitope (β1 domain = β chain binding groove domain). Competes with MK-D6, but no detectable preference for immature/mature class II	(23, 64, 65, 67)
Y3P	I-A ^{b,f,p,q,r,s,u,v} , weakly with I-A ^k , also cross-reacts with all rat I-A-like molecules	IgG2a	Recognizes peptide binding domain of mature αβ dimers and αβ/Ii complexes	(23, 65, 68)
10-2.1.6	I-A ^{k,f,g} β but not reactive with I-A ^{b,d,p,q}	Mouse IgG2b	Recognizes β1 domain	(24, 65, 69, 70)
K24-199	I-Aα, including I-A ^{d,k,g}	IgG2a	Does not recognize newly synthesized class II in presence or absence of Ii, prefers a more mature conformation.	(65, 69, 71)
MKD6	I-A ^d β (not reactive with I-A ^{k,b,s,f,g})	IgG2a	Recognizes extracellular β chain epitope (β1 domain, competes with M5/114). Prefers more mature (e.g., DM-edited) conformations of I-A ^d (IP is poor at early time points, more efficient after ~1 h chase, and more efficient from DM-expressing than DM-negative cells)	(64, 69, 72, 73)
B21-2	I-A ^{b,d,g} β	Rat IgG2b	Recognizes β chain of properly folded αβ dimers. No detectable preference for mature/immature/DM-edited class II	(72, 74)
14-4-4S	Common I-Eα chain (N.B. not all haplotypes express I-E _c)	IgG2a	Recognizes α chain of properly folded αβ dimers	(65, 69, 75, 76)

9. Kodak Biomax MR Film (Scientific Imaging film, 20.3 × 25.4 cm, 8 × 10 in.).
10. Equipment for developing X-ray film.
11. (Optional) Equipment for film densitometry or phosphorimager system.

2.4. Laboratory Accommodations for Working with Radioactivity

1. Decontamination agent, e.g., Count-Off (PerkinElmer). Obtain appropriate training for dealing with a radioactive spill, in accordance with institutional regulations/standards.
2. Lab work area equipped with safety equipment for radioactive work (waste containers, disposable absorbent covering, secondary containment, hand-held Geiger counter). Equipment for disposal of radioactive waste should be in accordance with institutional regulations/standards.
3. Refrigerated benchtop centrifuge and microcentrifuge, dedicated for radioactive work.
4. Tissue culture incubator with dedicated space for radioactive work.
5. Tissue culture hood that can be reserved for radioactive work.

3. Methods

3.1. Prepare Cells for the Experiment

1. Cells should be highly viable and screened for microbial contamination, including mycoplasma. Proliferating cell lines should be in logarithmic growth.
2. The protocol used here is suitable for suspension cells, such as B-LCL; alternative procedures for adherent cells are in Note 2.
3. Cells and media are kept as close to 37°C as possible to minimize interference of the labeling protocol with normal cell metabolism.
4. Collect sufficient cells for the experiment (typically 10⁶–10⁷ cells per time point; see Note 3) in 50 ml conical polypropylene tubes by centrifuging for 5–10 min at 330–520 ×*g* in a benchtop centrifuge (e.g., a Beckman GS-6R centrifuge) kept at room temperature. Use the same centrifugation conditions in subsequent steps.

3.2. Wash and Starve

1. Wash cells once in at least 5 ml pre-warmed, unsupplemented Cys/Met-free media. The cell density after dilution should be <1 × 10⁷/ml.
2. Suspend cells in Cys/Met-free media plus glutamine and dialyzed FBS at a density of 5 × 10⁶ cells/ml (see Note 3) and incubate (starve) at 37°C in a 50 ml conical tube for 1 h to deplete

endogenous Cys/Met. During this time, place cells in a humidified CO₂ incubator and leave the cap partially unscrewed to allow gas exchange. Mix occasionally (see Note 3).

3.3. Pulse Labeling

1. Perform this and subsequent steps with safety precautions for radioactive work (see *Current Protocols in Molecular Biology*, Appendix, A.1F).
2. Add 100–150 µCi ³⁵S-Cys/Met per ml of starved cell suspension and mix well. Alternatively, cells may be centrifuged after step 3 and suspended in an equal volume of Cys/Met-free media supplemented with glutamine, 10% dialyzed FBS, and 100–150 µCi/ml ³⁵S-Cys/Met. Place the 50 ml conical tube, with the cap partially unscrewed, inside a secondary container inside the CO₂ incubator. Incubate at 37°C, mixing occasionally (see Note 3). The duration of pulse-labeling may be varied to suit specific experimental purposes (see Note 4).

3.4. Chase

1. Collect cells by centrifugation at room temperature. Remove radioactive culture supernatant and dispose using a liquid waste conversion kit or other disposal method, as indicated by institutional regulations. It is preferable to aspirate, rather than decant, radioactive supernatants, to avoid contaminating the outside surfaces or cap thread of the conical tube during decanting.
2. Wash cells once with 5 ml pre-warmed chase media. Record the time of adding chase media as the start time of the chase.
3. Resuspend cells in chase media at up to 5 × 10⁶ cells/ml. Distribute cells to individual 15 or 50 ml conical tubes. Place tubes into a secondary container with caps partially opened. Incubate in a tissue culture incubator at 37°C with occasional mixing (see Note 3). For longer chase times (≥8 h), cells should be diluted to 1 × 10⁶ cells/ml in chase media (or lower still, to allow for cell growth, if the chase is continued beyond 24 h), and appropriately sized tissue culture flasks should be used. Meanwhile, set the temperature of the benchtop centrifuge to 4°C.

3.5. Harvest

1. Harvest cells at the end of the pulse and after each time of chase. The timing of sampling should match the time scales of the processes to be investigated; intracellular trafficking, maturation, and peptide loading of MHC class II molecules can be sampled on time scales of hours (see Figs. 1 and 2 and Notes 4 and 5).
2. During collection, keep cells on ice and centrifuge at 4°C. Spin down cells and dispose of media as radioactive waste.
3. Wash cells once with ice-cold PBS containing 1 mM PMSF (added fresh). Record the time of PBS addition as the time of harvest. Remove the supernatant as completely as possible after spinning down, especially when samples will be lysed in a small volume.

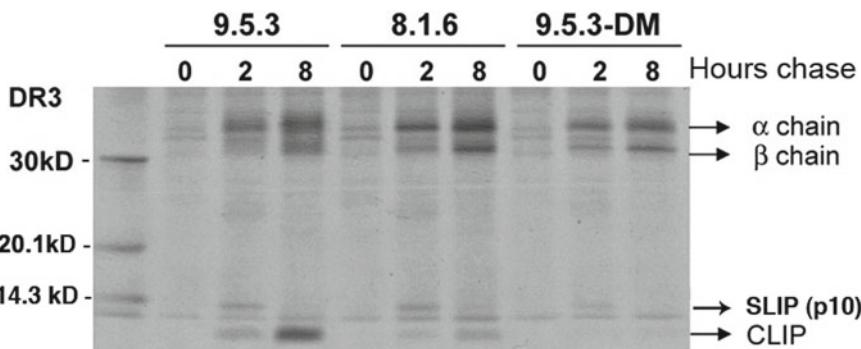


Fig. 1. Pulse–chase analysis of DR molecules in DM-expressing and non-expressing B-LCL. The DM-deficient cell line, 9.5.3, its wild-type progenitor, 8.1.6, and 9.5.3 cells transfected with DM (9.5.3-DM) were pulse-labeled for 1 h with (35 S)-Cys/Met and chased for 0, 2, or 8 h. NP-40 cell extracts (3×10^6 cell equivalents/lane) were immunoprecipitated with the anti-DR mAb L243 and analyzed by 12% SDS-PAGE. The positions of DR α and β chains and of a 10 kDa SLIP fragment of li are indicated. Levels of DR-associated CLIP are substantially diminished in DM-expressing 8.1.6 cells at 2 and 8 h of chase, and even further reduced when DM levels are increased by DM transfection of 9.5.3 cells (57). Also note inefficient IP of immature DR (mostly li-associated) in pulse-labeled cells with L243, which accounts for the weak bands at 0 h chase.

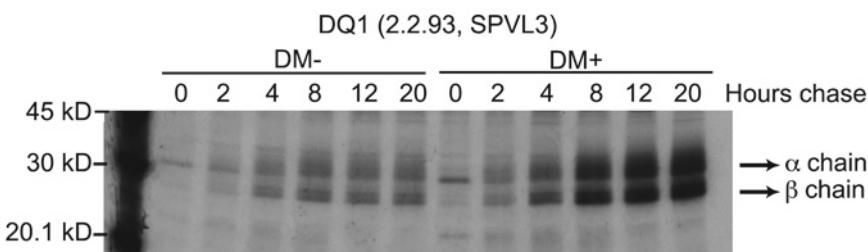


Fig. 2. Pulse–chase analysis of DQ1 molecules in DM-expressing and non-expressing B-LCL. 2.2.93 and 2.2.93-DM cells were pulsed for 1 h with (35 S)-Cys/Met and chased for the indicated time periods. Aliquots of cell lysates, normalized for counts, were immunoprecipitated with SPVL-3 for DQ1 and then analyzed by SDS-PAGE (26). This figure illustrates the preferential recognition of a mature confirmation of DQ1 by SPVL3, which is likely peptide-dependent and is significantly increased in the presence of DM.

4. Store the cell pellets from all time points in 15 ml (or 50 ml) tubes in designated boxes (with radioactive hazard warning label) at -20°C (short-term) or -80°C (long-term).

3.6. Detergent Extraction

1. Supplement ice-cold lysis buffer with 1 mM PMSF and Roche Complete protease inhibitors (toxic).
2. Add lysis buffer (1 ml for up to 10^8 cells per sample) to frozen cell pellets and mix until cell pellets are dispersed as a turbid but homogenous suspension. Avoid thawing frozen cell pellets before addition of lysis buffer, as this releases active proteases.
3. Continue lysis by rotating the microcentrifuge tubes at 4°C for 20–60 min or leaving them on ice with occasional vortexing.
4. Spin down non-solubilized material for 20 min at top speed in a microcentrifuge (13,000 rpm or $10,000 \times g$) at 4°C .

5. Transfer the cleared supernatant, which contains the extracted radiolabeled proteins, to a clean tube, or directly to the first preclear tube (below).

3.7. Preclearing

This step depletes any radiolabeled proteins that bind nonspecifically to the beads and/or antibody used for IP. In the case of B cell lines, preclearing also depletes endogenous Ig. Depending on Ab to be used for immunoprecipitation, preclearing can be done using Protein A (PAS) or protein G sepharose (PGS) beads (see Note 6). The preclearing step may be repeated several times (see Note 7).

1. For each sample, add to a fresh, labeled tube 30–60 µl 50% slurry of PAS or PGS and 3–5 µl pre-immune normal serum or 10 µg purified, isotype-matched Ig from the same species as the antibody to be used for IP.
2. Add radiolabeled cell extract (plus any additional lysis buffer) to a final volume of 750–1,000 µl and rotate tubes at 4°C for at least 1 h.
3. Spin down beads (full speed in a microfuge, 1–2 min, 4°C) and transfer supernatant to fresh 1.5 ml Eppendorf tubes.
4. In some experiments, it may be useful to quantify the amount of radioactivity incorporated into the precleared cell extract and to adjust volumes to match the amount of input radioactivity between samples (for details, see Note 8).

3.8. Immunoprecipitation

1. Add PAS or PGS beads (40–100 µl 50% v/v slurry) and desired antibody to each tube (see Note 9). A typical amount of mAb to use is 10 µg = 10 µl of a 1 mg/ml stock in PBS, or a few µl of a polyclonal antiserum; titrating these amounts may improve results.
2. Rotate tubes for at least 1 h at 4°C.
3. Spin down beads. Remove the supernatant (extract depleted of the protein of interest) and transfer to a fresh microfuge tube (see Note 10).

3.9. Washing Beads

Wash beads 3–5 times with 1 ml cold IP wash buffer. Each time, remove the supernatant by pipetting or aspiration, without disturbing the pellet (see Note 11). The number of washes may be optimized, and lysis buffer may be used instead of wash buffer. The last wash may be performed in PBS or water to reduce carryover of nonionic detergent into the elution step.

3.10. Preparation of SDS-PAGE Gels

1. Select the percentage acrylamide of the gel so as to maximize resolution of proteins of interest; gradient gels can be useful if a wide range of molecular weights needs to be analyzed. For MHC class II molecules, 12% gels generally are suitable.

2. Prepare SDS-PAGE acrylamide gels as described in detail in *Current Protocols in Molecular Biology*, Chapter 10, Section II, 10.2, or use commercially available pre-cast gels. The best molecular weight resolution is achieved on large gels ($14 \times 16 \times 0.15$ cm), but standard minigels may also be used.
3. Set up gels in an electrophoresis chamber as per manufacturer's instructions, and add 1× SDS-PAGE running buffer to the buffer compartments.

3.11. SDS-PAGE Sample Preparation and Separation

The conditions for elution of radiolabeled material from beads may be varied for specific purposes. Here, we describe alternative procedures for analysis of dissociated dimers (Subheading 3.11, step 1) and non-dissociated dimers (for assessment of SDS-stability, Subheading 3.11, step 2). Other alternatives not described here include elution in nonreducing conditions to visualize interchain disulfide bonding; denaturation followed by re-IP of individual chains, to identify co-precipitated polypeptides within a larger complex (26); or enzymatic digestion prior to SDS-PAGE separation, for example, using endoglycosaminidase H to remove high-mannose forms of N-linked glycans, which may be used to track intracellular transport through the Golgi apparatus (27).

1. Elution of dissociated dimers:
 - (a) Resuspend beads in an appropriate amount (limited by the size of gel wells, e.g., 30 μ l for a Bio-Rad minigel) of reducing SLB. If buffer volume is large compared to bead volume, 1× SLB may be used, but 1.5× or more concentrated SLB may be used for small elution volumes. This is to compensate for dilution by residual wash buffer in the bead pellet.
 - (b) Heat the samples for 10 min to 90–100°C in a heat block to elute protein from beads. Vortex and spin down beads.
 - (c) Proceed to step 3.
2. Alternative procedure for elution of non-dissociated dimers:
 - (a) For visualization of SDS-stable (compact or floppy, see Note 12) dimers, suspend beads in SLB and incubate at room temperature for 10–60 min (24). (Do not boil.) Vortex and spin down beads.
 - (b) Elution conditions are required that release immune complexes and dissociate the immunoprecipitating antibody from class II molecules, without also dissociating class II $\alpha\beta$ heterodimers associated with peptide. Conditions (SDS concentration, addition of reducing agent, elution time) may need to be varied to optimize this step.
 - (c) Note that some mAbs fail to dissociate from SDS-stable class II molecules under typical non-boiled elution conditions and cause super-shifting of bands (see Table 1).

3.12. Sample Loading and Electrophoresis

1. Using a Hamilton syringe (dedicated for radioactive work, and rinsed thoroughly between samples) or disposable gel loading tips, load the supernatants from each sample into wells of the SDS-PAGE gel. One well is set aside for radiolabeled molecular weight markers (Amersham/GE Healthcare ^{14}C -labeled rainbow markers), applied as per manufacturer's instructions (see Note 13).
2. Electrophorese samples until the loading dye front has reached the bottom of the gel (see Note 14).

3.13. Gel Fixation and Exposure to Scintillation Cocktail

Although gel fixation is recommended, we have found that it may be skipped. Transfer gel into enough Amplify solution to cover the gel. Incubate gel with gentle rocking for 15–30 min at room temp in the dark (covered with aluminum foil). We have found that the Amplify solution may be reused several times.

3.14. Gel Drying

1. Cut a piece of Whatman 3 M filter paper slightly larger than the surface area of the gels. Wet Whatman paper with water. Gently place the gel onto the filter paper and cover it with clear plastic wrap (Saran Wrap). Avoid introducing air bubbles between the layers of this sandwich.
2. Using a gel dryer according to the manufacturer's instructions, dry the gel for 1.5 h at 80°C with slow ramp and good vacuum seal. Breaking the seal early or heating the gel too fast will crack the gel.

3.15. Visualize Radioactive Bands

1. Remove plastic wrap from the dried gel.
2. For a high-resolution, publication-quality image, expose autoradiography film to the gel in a film cassette at –80°C, adjusting the exposure time between a few hours and several weeks, depending on the amount of radioactivity being visualized. The exposure time should be varied to achieve detection of radioactive bands of interest whilst minimizing nonspecific background and avoiding overexposure of abundant bands. Allow the frozen film cassettes to return to room temperature before opening them, or the gel and film may stick together and separating them may cause sparks that will leave black spots on the film when developed. Develop the film using an automatic film developer, according to manufacturer's instructions.
3. Semiquantitative analysis of band intensities may be achieved by film densitometry.
4. If a phosphorimager is available, a “snapshot” may be obtained by placing the gel on the light-blanked detector screen of a phosphorimager system overnight at room temperature. Phosphorimagers can be more sensitive than film and linear over a wider range of exposures. The data are immediately available for quantitation, but the images obtained are grainier than film and less suitable for publication.

4. Notes

1. In order to maintain protein-protein interactions that are sensitive to NP-40, or for specific class II alleles thought to have reduced stability (such as I-A^{g7}), digitonin (1%) or CHAPS (6 mM) can be used to replace NP-40. Alternative buffer systems may be required, for example, to maintain pH-dependent interactions, such as those between MHC II and DM (28); note, however, that interactions of antibodies with their antigens and with protein A/G-sepharose may also be pH-dependent.
2. Steps 1–6 apply to suspension cells. Adherent cells should be split 1 day before the experiment and allowed to grow in 10-cm or 60-mm tissue-culture dishes overnight. Depending on the abundance of proteins of interest, each chase time point may require one or more dishes of cells. The cells can be washed without centrifugation (by gentle aspiration and addition of media or buffers, as appropriate at each step) and harvested by scraping off cells using disposable cell scrapers, or by detaching with PBS/EDTA, with or without trypsin (unless the protein of interest is sensitive to trypsinization). Detached cells may then be centrifuged and further processed as described in the main protocol (step 6). Alternatively, adherent cells may be extracted without being detached, by washing in PBS followed by addition of ice-cold lysis buffer to culture dishes. After thorough dispersion of cells in lysis buffer, transfer the extract to 1.5 ml microcentrifuge tubes for further processing.
3. Cell density during starvation and subsequent pulse-labeling and chase may be adjusted according to the metabolism of the cell type under study and the particular experiment. The cell density during starvation and chase should be low enough to avoid nutrient depletion of media, but volumes of radiolabeled media should be minimized to limit disposal cost. Mixing of cells kept in conical tubes is important to avoid depletion of nutrients and oxygen after cells settle under gravity. Moreover, to allow oxygen diffusion and for effective CO₂ exchange for appropriate buffering of media, tubes should not be filled with media to a height of more than 2–3 cm. The total cell number per time point may be increased to isolate less abundant or poorly labeled proteins.
4. The pulse-labeling time varies depending on the experiment and should be adjusted based on the desired time resolution. To track subsequent intracellular trafficking of MHC II molecules on time scales of 2–24 h, a 30 min to 1 h pulse is suitable. Very brief pulses (on the order of a minute or even shorter) may be used to track early stages of MHC II assembly in the ER; however, this requires further modifications to this protocol to

allow precise timing of the pulse and chase (29, 30). Proteins synthesized at a low rate, or proteins with a low content of Cys/Met, may require longer labeling times to become detectable.

5. The spin/wash steps between the pulse and chase and the same steps at collection can each take up to 30 min. When using a long (≥ 30 min) pulse with chase time points at intervals of an hour or longer, the time for spinning/washing is not critical, because metabolism slows substantially once cell suspensions are handled in the cold. However, for experiments performed on shorter time scales, the time for manipulation can introduce error to estimated times for particular processing events. In order to ensure accurate timing in these experiments, the pulse may be terminated using protein synthesis inhibitors; labeled and chased cells may be rapidly diluted into a large excess of ice-cold PBS and kept rigorously in the cold thereafter. Centrifugation times may be shortened and $\times g$ forces increased to reduce processing time.
6. Protein A and G bind differentially to the different immunoglobulin isotypes, and the choice of protein A- or G-sepharose will be dictated by the mAb(s) used. For binding affinities of protein A and G for various IgG subclasses, see *Current Protocols in Molecular Biology*, Chapter 10, section VI, Table 10.16.1. A cheaper alternative is to use a 10% suspension of Pansorbin cells (fixed, heat-killed *S. aureus*, bearing protein A) for preclearing. The desired amount of Pansorbin cells (100 μ l for each time point) is washed twice before use (see Subheading 2). Add cell lysate and normal preclearing serum (plus any additional lysis buffer) directly to the Pansorbin cell pellet to a final volume of 750–1,000 μ l. Mix thoroughly by pipetting or vortexing. Otherwise proceed as described above.
7. Additional rounds of preclearing may further reduce nonspecific background radioactivity, but each step slightly dilutes the cell extracts and very unstable proteins may gradually denature or degrade, so this step may need to be optimized. When stable proteins are being isolated, one of the preclearing steps may be extended overnight. The final preclearing step may be performed with PAS or PGS alone, in order to remove any residual irrelevant antibody before the specific IP.
8. To quantify cell-associated radioactivity, mix a small aliquot of the extract (e.g., 5 μ l) with 1 ml scintillation fluid in a disposable scintillation vial, and count in a scintillation counter. Alternatively, protein-associated radioactivity may be quantified. Spot a small aliquot of the cell extract onto glass filter membranes, soak in 100% w/v aqueous trichloroacetic acid, wash, transfer to scintillation vials, add scintillant, and count. Before proceeding to IP, the volumes of cell extracts may be adjusted to match for equal counts of radioactivity, and lysis buffer

added to equalize total volumes. This method can compensate for differences in labeling efficiency between different cell lines for comparison of relative rates of biosynthesis, but is poorly suited for normalizing total radioactivity during an extended chase. This is because radiolabeled proteins will be degraded at very different rates during the chase, so a gradual loss of total radiolabeled proteins over time is expected.

9. When handling multiple samples, it is simpler to dispense protein A/G sepharose and antibodies into tubes first, before adding the radiolabeled, precleared extract. Further variations of this step may be used. The antibody may be pre-incubated with the precleared extract for an hour and captured by subsequent incubation with protein A/G beads. Alternatively, if hybridoma culture supernatants are to be used as a source of mAbs for IP, the supernatants (0.5–1 ml per sample) may be pre-incubated with protein A or G-sepharose beads for 1 h in the cold, and washed once in PBS, before addition of radiolabeled cell extracts.
10. The recovered cell extracts may be disposed of as radioactive waste or recovered into fresh tubes, stored at –80°C, and used for sequential IPs of other molecules. For sequential IPs, thaw extracts at 4°C with thorough mixing, spin out any precipitated proteins at $\geq 10,000 \times g$ in a microfuge, and preclear at least once with PAS or PGS to minimize any carryover of antibody or immune complexes into subsequent IPs.
11. The initial washes contain most of the radiolabeled unbound proteins, and need to be disposed of and accounted for as radioactive waste. Later washes will contain far less radioactivity; as a result, drain disposal may be permissible in some jurisdictions. Processing of multiple samples is streamlined if the later washes are removed by vacuum aspiration, using an aspirator dedicated for radioactive work, fitted with filter traps that prevent escape of radioactivity into house vacuum systems or vacuum pumps.
12. Floppy dimers (apparent relative molecular mass (Mr) ~63–67 K) and compact dimers (Mr, ~56 K) were initially defined *in vitro* by studies of purified molecules (31). Size heterogeneity of SDS-stable class II molecules has also been observed in naturally occurring biosynthetic intermediates. Some MHC class II alleles form floppy-sized dimers when loaded with CLIP; others form SDS-unstable complexes. In contrast, DM-edited forms of MHC II loaded with stable peptides generally migrate as compact dimers (10, 24, 32), but the percentage of SDS-stable complexes varies between alleles and isotypes.
13. Molecular weight calibration will be most accurate if the markers are diluted in a buffer matched in composition to that of the

samples. For immunoprecipitates, a mixture of loading buffer and IP wash buffer, in similar ratios as in samples, works well.

14. Depending on the type and amount of detergent used in the IP wash buffer, the loading dye front may be broadened. Note that CLIP and other low-molecular weight peptides migrate ahead of the dye front. Their detection therefore requires electrophoresis to be stopped when the dye front is a few mm above the bottom of the gel. Note that such peptides can also be lost during standard gel fixation with methanol/acetic acid.

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References

1. Jamieson JD, Palade GE (1967) Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J Cell Biol* 34(2):577–596
2. Jamieson JD, Palade GE (1967) Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. *J Cell Biol* 34(2):597–615
3. De Riva A, Deery MJ, McDonald S, Lund T, Busch R (2010) Measurement of protein synthesis using heavy water labeling and peptide mass spectrometry: discrimination between major histocompatibility complex allotypes. *Anal Biochem* 403(1–2):1–12
4. Jones PP, Murphy DB, Hewgill D, McDevitt HO (1979) Detection of a common polypeptide chain in I-A and I-E sub-region immunoprecipitates. *Mol Immunol* 16(1):51–60
5. Machamer CE, Cresswell P (1982) Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J Immunol* 129(6):2564–2569
6. Busch R, Cloutier I, Sekaly RP, Hä默ling GJ (1996) Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J* 15(2):418–428
7. Bakke O, Dobberstein B (1990) MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell* 63(4):707–716
8. West MA, Lucocq JM, Watts C (1994) Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells. *Nature* 369(6476):147–151
9. Tulp A, Verwoerd D, Dobberstein B, Ploegh HL, Pieters J (1994) Isolation and characterization of the intracellular MHC class II compartment. *Nature* 369(6476):120–126
10. Qiu Y, Xu X, Wandinger-Ness A, Dalke DP, Pierce SK (1994) Separation of subcellular compartments containing distinct functional forms of MHC class II. *J Cell Biol* 125(3):595–605
11. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349(6311):669–676
12. Blum JS, Cresswell P (1988) Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc Natl Acad Sci USA* 85(11):3975–3979
13. Maric MA, Taylor MD, Blum JS (1994) Endosomal aspartic proteinases are required for invariant-chain processing. *Proc Natl Acad Sci USA* 91(6):2171–2175
14. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, Chapman HA (1996) Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* 4(4):357–366

15. Neefjes JJ, Ploegh HL (1992) Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS resistance alpha beta heterodimers in endosomes. *EMBO J* 11(2):411–416
16. Riberdy JM, Newcomb JR, Surman MJ, Barbosa JA, Cresswell P (1992) HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature* 360(6403):474–477
17. Sette A, Ceman S, Kubo RT, Sakaguchi K, Appella E, Hunt DF, Davis TA, Michel H, Shabanowitz J, Rudersdorf R et al (1992) Invariant chain peptides in most HLA-DR molecules of an antigen-processing mutant. *Science* 258(5089):1801–1804
18. Denzin LK, Cresswell P (1995) HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82(1):155–165
19. Sherman MA, Weber DA, Jensen PE (1995) DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* 3(2):197–205
20. Sloan VS, Cameron P, Porter G, Gammon M, Amaya M, Mellins E, Zaller DM (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375(6534):802–806
21. Lovitch SB, Pu Z, Unanue ER (2006) Amino-terminal flanking residues determine the conformation of a peptide-class II MHC complex. *J Immunol* 176(5):2958–2968
22. Germain RN, Rinker AG Jr (1993) Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules. *Nature* 363(6431):725–728
23. Bikoff EK, Huang LY, Episkopou V, van Meerwijk J, Germain RN, Robertson EJ (1993) Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. *J Exp Med* 177(6):1699–1712
24. Viville S, Neefjes J, Lotteau V, Dierich A, Lemeur M, Ploegh H, Benoist C, Mathis D (1993) Mice lacking the MHC class II-associated invariant chain. *Cell* 72(4):635–648
25. Busch R, Rinderknecht CH, Roh S, Lee AW, Harding JJ, Burster T, Hornell TM, Mellins ED (2005) Achieving stability through editing and chaperoning: regulation of MHC class II peptide binding and expression. *Immunol Rev* 207:242–260
26. Rinderknecht CH, Roh S, Pashine A, Belmares MP, Patil NS, Lu N, Truong P, Hou T, Macaubas C, Yoon T, Wang N, Busch R, Mellins ED (2010) DM influences the abundance of major histocompatibility complex class II alleles with low affinity for class II-associated invariant chain peptides via multiple mechanisms. *Immunology* 131(1):18–32
27. Busch R, Doebele RC, von Scheven E, Fahrni J, Mellins ED (1998) Aberrant intermolecular disulfide bonding in a mutant HLA-DM molecule: implications for assembly, maturation, and function. *J Immunol* 160(2):734–743
28. Pashine A, Busch R, Belmares MP, Munning JN, Doebele RC, Buckingham M, Nolan GP, Mellins ED (2003) Interaction of HLA-DR with an acidic face of HLA-DM disrupts sequence-dependent interactions with peptides. *Immunity* 19(2):183–192
29. Neefjes JJ, Hämmерling GJ, Momburg F (1993) Folding and assembly of major histocompatibility complex class I heterodimers in the endoplasmic reticulum of intact cells precedes the binding of peptide. *J Exp Med* 178(6):1971–1980
30. Nijenhuis M, Neefjes J (1994) Early events in the assembly of major histocompatibility complex class II heterotrimers from their free subunits. *Eur J Immunol* 24(1):247–256
31. Dornmair K, Rothenhäusler B, McConnell HM (1989) Structural intermediates in the reactions of antigenic peptides with MHC molecules. *Cold Spring Harb Symp Quant Biol* 54(Pt 1):409–416
32. Miyazaki T, Wolf P, Tourne S, Waltzinger C, Dierich A, Barois N, Ploegh H, Benoist C, Mathis D (1996) Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell* 84(4):531–541
33. Roche PA, Marks MS, Cresswell P (1991) Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* 354(6352):392–394
34. Roche PA, Teletski CL, Stang E, Bakke O, Long EO (1993) Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci USA* 90(18):8581–8585
35. Wraith CJ, van Endert P, Moller P, Lipp J, Ling NR, MacLennan IC, Koch N, Moldenhauer G (1990) Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem* 265(10):5787–5792
36. Avva RR, Cresswell P (1994) In vivo and in vitro formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. *Immunity* 1(9):763–774
37. Denzin LK, Robbins NF, Carboy-Newcomb C, Cresswell P (1994) Assembly and intracellular

- transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1(7):595–606
38. Doebele RC, Busch R, Scott HM, Pashine A, Mellins ED (2000) Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity* 13(4):517–527
39. Stang E, Guerra CB, Amaya M, Paterson Y, Bakke O, Mellins ED (1998) DR/CLIP (class II-associated invariant chain peptides) and DR/peptide complexes colocalize in prelysosomes in human B lymphoblastoid cells. *J Immunol* 160(10):4696–4707
40. Guy K, Van Heyningen V, Cohen BB, Deane DL, Steel CM (1982) Differential expression and serologically distinct subpopulations of human Ia antigens detected with monoclonal antibodies to Ia alpha and beta chains. *Eur J Immunol* 12(11):942–948
41. Denzin LK, Hammond C, Cresswell P (1996) HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J Exp Med* 184(6):2153–2165
42. Lee AW, Wang N, Hornell TM, Harding JJ, Deshpande C, Hertel L, Lacaille V, Pashine A, Macaubas C, Mocarski ES, Mellins ED (2011) Human cytomegalovirus decreases constitutive transcription of MHC class II genes in mature Langerhans cells by reducing CIITA transcript levels. *Mol Immunol* 48(9–10):1160–1167
43. Hitzel C, Gruneberg U, van Ham M, Trowsdale J, Koch N (1999) Sodium dodecyl sulfate-resistant HLA-DR “superdimer” bands are in some cases class II heterodimers bound to antibody. *J Immunol* 162(8):4671–4676
44. Lampson LA, Levy R (1980) Two populations of Ia-like molecules on a human B cell line. *J Immunol* 125(1):293–299
45. Guerra CB, Busch R, Doebele RC, Liu W, Sawada T, Kwok WW, Chang MD, Mellins ED (1998) Novel glycosylation of HLA-DRalpha disrupts antigen presentation without altering endosomal localization. *J Immunol* 160(9):4289–4297
46. Watanabe M, Suzuki T, Taniguchi M, Shinohara N (1983) Monoclonal anti-Ia murine alloantibodies crossreactive with the Ia-homologues of other mammalian species including humans. *Transplantation* 36(6):712–718
47. Carven GJ, Chitta S, Hilgert I, Rushe MM, Baggio RF, Palmer M, Arenas JE, Strominger JL, Horejsi V, Santambrogio L, Stern LJ (2004) Monoclonal antibodies specific for the empty conformation of HLA-DRI reveal aspects of the conformational change associated with peptide binding. *J Biol Chem* 279(16):16561–16570
48. Potolicchio I, Chitta S, Xu X, Fonseca D, Crisi G, Horejsi V, Strominger JL, Stern LJ, Raposo G, Santambrogio L (2005) Conformational variation of surface class II MHC proteins during myeloid dendritic cell differentiation accompanies structural changes in lysosomal MIIC. *J Immunol* 175(8):4935–4947
49. Johnson JP, Meo T, Riethmuller G, Schendel DJ, Wank R (1982) Direct demonstration of an HLA-DR allotypic determinant on the low molecular weight (beta) subunit using a mouse monoclonal antibody specific for DR3. *J Exp Med* 156(1):104–111
50. Johnson JP, Wank R (1984) Identification of two cis-encoded HLA-DQ molecules that carry distinct alloantigenic specificities. *J Exp Med* 160(5):1350–1359
51. Mellins E, Kempin S, Smith L, Monji T, Pious D (1991) A gene required for class II-restricted antigen presentation maps to the major histocompatibility complex. *J Exp Med* 174(6):1607–1615
52. Sanderson F, Thomas C, Neefjes J, Trowsdale J (1996) Association between HLA-DM and HLA-DR in vivo. *Immunity* 4(1):87–96
53. Verreck FA, Fargeas CA, Hämerling GJ (2001) Conformational alterations during biosynthesis of HLA-DR3 molecules controlled by invariant chain and HLA-DM. *Eur J Immunol* 31(4):1029–1036
54. Patil NS, Pashine A, Belmares MP, Liu W, Kaneshiro B, Rabinowitz J, McConnell H, Mellins ED (2001) Rheumatoid arthritis (RA)-associated HLA-DR alleles form less stable complexes with class II-associated invariant chain peptide than non-RA-associated HLA-DR alleles. *J Immunol* 167(12):7157–7168
55. Hou T, Macmillan H, Chen Z, Keech CL, Jin X, Sidney J, Strohman M, Yoon T, Mellins ED (2011) An insertion mutant in DQA1*0501 restores susceptibility to HLA-DM: implications for disease associations. *J Immunol* 187(5):2442–2452
56. Spits H, Borst J, Giphart M, Coligan J, Terhorst C, De Vries JE (1984) HLA-DC antigens can serve as recognition elements for human cytotoxic T lymphocytes. *Eur J Immunol* 14(4):299–304
57. Fallang LE, Roh S, Holm A, Bergseng E, Yoon T, Fleckenstein B, Bandyopadhyay A, Mellins ED, Sollid LM (2008) Complexes of two cohorts of CLIP peptides and HLA-DQ2 of the autoimmune DR3-DQ2 haplotype are poor substrates for HLA-DM. *J Immunol* 181(8):5451–5461
58. Huan J, Meza-Romero R, Mooney JL, Vandebark AA, Offner H, Burrows GG (2011) Single-chain recombinant HLA-DQ2.5/

- peptide molecules block alpha2-gliadin-specific pathogenic CD4+ T-cell proliferation and attenuate production of inflammatory cytokines: a potential therapy for celiac disease. *Mucosal Immunol* 4(1):112–120
59. Viken HD, Paulsen G, Sollid LM, Lundin KE, Tjonnfjord GE, Thorsby E, Gaudernack G (1995) Characterization of an HLA-DQ2-specific monoclonal antibody. Influence of amino acid substitutions in DQ beta 1*0202. *Hum Immunol* 42(4):319–327
 60. Shookster L, Matsuyama T, Burmester G, Winchester R (1987) Monoclonal antibody 1a3 recognizes a monomorphic epitope unique to DQ molecules. *Hum Immunol* 20(1): 59–70
 61. Robbins PA, Evans EL, Ding AH, Warner NL, Brodsky FM (1987) Monoclonal antibodies that distinguish between class II antigens (HLA-DP, DQ, and DR) in 14 haplotypes. *Hum Immunol* 18(4):301–313
 62. Radka SF, Machamer CE, Cresswell P (1984) Analysis of monoclonal antibodies reactive with human class II beta chains by two-dimensional electrophoresis and Western blotting. *Hum Immunol* 10(3):177–186
 63. Koch N, Koch S, Hä默ling GJ (1982) Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. *Nature* 299(5884):644–645
 64. Anderson MS, Miller J (1992) Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci USA* 89(6):2282–2286
 65. Germain RN, Hendrix LR (1991) MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* 353(6340):134–139. doi:[10.1038/353134a0](https://doi.org/10.1038/353134a0)
 66. Mehringer JH, Harris MR, Kindle CS, McCourt DW, Cullen SE (1991) Characterization of fragments of the murine Ia-associated invariant chain. *J Immunol* 146(3):920–927
 67. Bhattacharya A, Dorf ME, Springer TA (1981) A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J Immunol* 127(6):2488–2495
 68. Janeway CA Jr, Conrad PJ, Lerner EA, Babich J, Wettstein P, Murphy DB (1984) Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cellbound Ia antigens as targets of immunoregulatory T cells. *J Immunol* 132(2): 662–667
 69. Bikoff EK, Germain RN, Robertson EJ (1995) Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Immunity* 2(3):301–310
 70. Oi VT, Jones PP, Goding JW, Herzenberg LA (1978) Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr Top Microbiol Immunol* 81:115–120
 71. Koch N, Hä默ling GJ, Tada N, Kimura S, Hä默ling U (1982) Cross-blocking studies with monoclonal antibodies against I-A molecules of haplotypes b, d and k. *Eur J Immunol* 12(11):909–914
 72. Dang LH, Lien LL, Benacerraf B, Rock KL (1993) A mutant antigen-presenting cell defective in antigen presentation expresses class II MHC molecules with an altered conformation. *J Immunol* 150(10):4206–4217
 73. Kappler JW, Skidmore B, White J, Marrack P (1981) Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med* 153(5):1198–1214
 74. Steinman RM, Nogueira N, Witmer MD, Tydings JD, Mellman IS (1980) Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J Exp Med* 152(5):1248–1261
 75. Ozato K, Mayer NM, Sachs DH (1982) Monoclonal antibodies to mouse major histocompatibility complex antigens. *Transplantation* 34(3):113–120
 76. Rinderknecht CH, Belmares MP, Catanzarite TL, Bankovich AJ, Holmes TH, Garcia KC, Nanda NK, Busch R, Kovats S, Mellins ED (2007) Posttranslational regulation of I-E^d by affinity for CLIP. *J Immunol* 179(9):5907–5915

Chapter 32

Assembly of Matched Alpha/Beta Subunits to HLA Class II Peptide Receptors

Sebastian Temme, Nadine Kämper, and Norbert Koch

Abstract

Human antigen presenting cells express three human leukocyte antigen (HLA) class II isotypes (DR, DP, and DQ), which are composed of polymorphic α and β subunits. The combination of polymorphic α - and β -chains results in *cis* (encoded on the same chromosome) or *trans* (encoded on different chromosomes) combinations. Since some of the $\alpha-\beta$ combinations may yield mismatched non-functional $\alpha-\beta$ heterodimers, it is not entirely clear which type of HLA class II peptide receptors are found on the cell surface of antigen presenting cells. We have developed a combination of biochemical techniques for inspection of the assembly and intracellular transport of isotype matched and mismatched class II heterodimers.

Key words: MHC class II molecules, Invariant chain, Intracellular transport, Endoglycosidase H, SDS-stable MHCII dimers, Flow cytometry

1. Introduction

The HLA gene complex contains genes encoding class I and class II peptide receptors. Class II molecules are of critical importance for the induction and maintenance of immune responses against pathogens or tumors. The class II peptide receptors also play an important role in autoimmune diseases, such as type 1 diabetes or rheumatoid arthritis, and in rejection of transplants. Class II α - and β -chain encoding genes exhibit an unusually high polymorphism with more than 1,500 alleles (IMGT HLA database 2011). The genes encoding class II α and β subunits, which are located on one DNA strand, are evolutionarily selected to form matched class II heterodimers. Trans combination of α - and β -chains encoded on allelic chromosomes results in novel heterodimers. Some of these heterodimers are mismatched and are not expressed on the cell

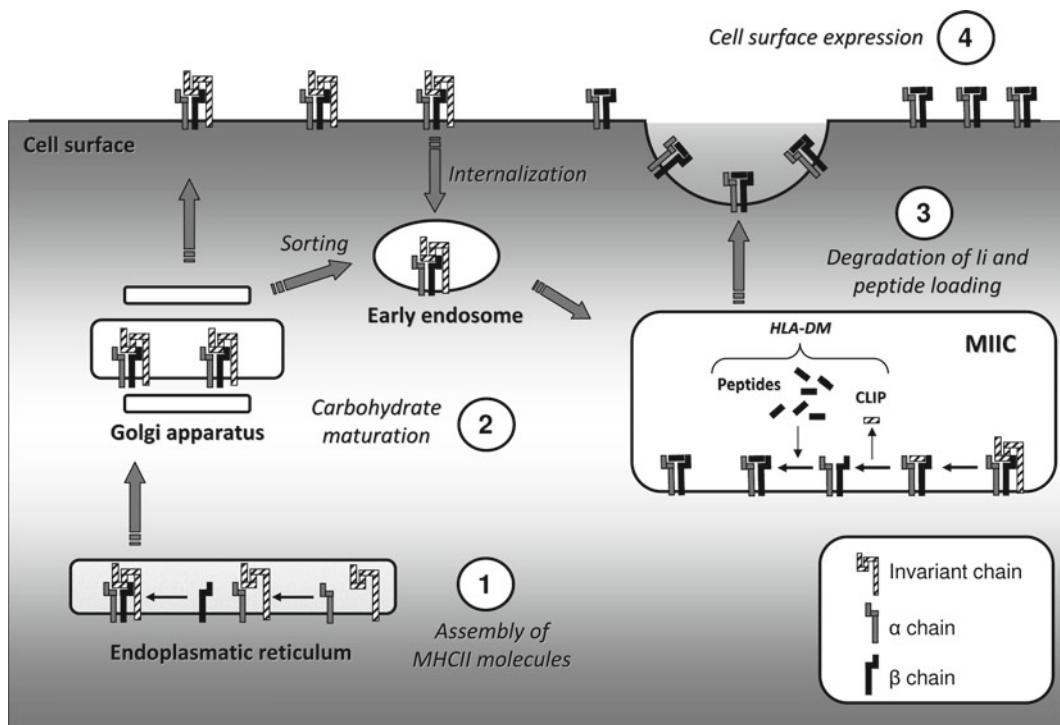


Fig. 1. Formation and intracellular transport of MHC class II molecules. Assembly of class II molecules in the endoplasmatic reticulum (ER) is chaperoned by invariant chain (1). Properly folded class II-Ii complexes exit the ER to the Golgi apparatus. In the *cis*-Golgi, high-mannose N-linked carbohydrates are processed to complex type glycans (2). Class II molecules are transported to class II loading compartments (MIIC) by vesicular sorting from the trans-Golgi to endosomes or by internalization of class II from the cell surface. Ii is sequentially degraded in endocytic compartments and MHCII molecules are loaded in MIICs with high-affinity peptides, which is mediated by HLA-DM (3). For activation of CD4+ T-cells, peptide-loaded class II molecules are displayed on the cell surface (4). *Ii* Invariant chain, *MIIC* MHCII loading compartment, *CLIP* Class II-associated Ii peptides.

surface of APC. At present, no systematic survey for matched or mismatched class II heterodimers is available. Information, which class II subunit combination yields functional peptide receptors, could be of critical importance for studies on class II antigen presentation. Recent research has revealed a modified model for class II assembly with invariant chain (1) (Fig. 1—1). Shortly after biosynthesis in the endoplasmatic reticulum (ER) class II α -chain binds to a trimer of invariant chain (Ii) and forms an α -Ii matrix. Subsequently, one of the various β -chains binds to the α -Ii matrix to form an α - β -Ii complex (1, 2). Ii promotes assembly of matched class II subunits by controlling formation of functional class II combinations (1). In addition, Ii abolishes loading of the class II peptide binding groove with unfolded polypeptides (3). Upon ER export, the class II-Ii complexes enter the Golgi apparatus (Fig. 1—2). From the *trans*-Golgi stack, class II-Ii complexes are sorted to endosomal class II loading compartments (MIICs) (4) (Fig. 1—3).

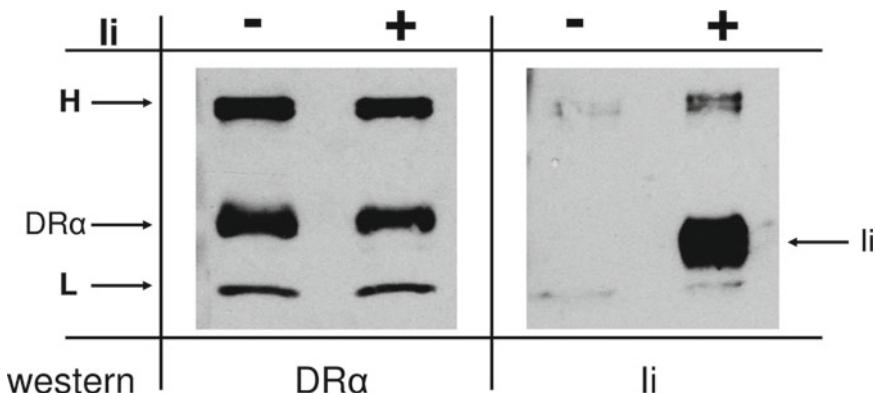


Fig. 2. Co-immunoprecipitation of DR and Ii. Lysates of cells transfected with DR α and DR β (–) or with DR α , DR β , and Ii (+) were subjected to immunoprecipitation using the DR-specific mAb ISCR3. Precipitates were split, separated by 12% SDS-PAGE, probed with mAbs TAL-1B5 (DR α) or Bu43 (Ii), and subsequently incubated with appropriate horseradish peroxidase conjugated secondary antibodies. The immunoglobulin heavy and light chains (H, L), DR α , and Ii are marked by arrows.

In an alternate pathway, class II–Ii complexes are internalized from the cell surface to endosomes (5, 6). In endocytic MIIC vesicles, Ii is sequentially degraded by proteases. The Ii-derived CLIP fragment remains bound to the peptide binding groove (7, 8). Subsequently, CLIP is removed through intervention by HLA-DM (9). In addition, HLA-DM functions as a peptide editor, facilitating loading of MHC class II heterodimers with high-affinity peptides (10, 11). These peptide-loaded class II molecules are then exposed at the cell surface, where they display antigenic peptides to T-cells (12).

Within the ER, class II molecules consist of non-covalently associated alpha and beta subunits attached to a trimer of Ii which facilitates proper folding and is important for intracellular transport. The association of class II subunits with Ii can be assessed by immunoprecipitation (IP; Fig. 2). During biosynthesis, the class II α - and β -chains are modified by appending oligosaccharides to asparagine (Asn) residues at the consensus sequence Asn-X-Ser/Thr (X can be any amino acid except proline) (Fig. 1—2; Fig. 3 *upper panel*). The α -chain binds two N-glycans, whereas the β -chain is modified by one N-linked carbohydrate (see Note 1). After arrival of class II glycoproteins to the Golgi stack, high-mannose carbohydrates are processed to complex type glycans (13). Hence, acquisition of complex type N-glycans indicates egress of class II glycoproteins from the ER to the Golgi compartment. To determine the nature of carbohydrate maturation, class II glycoproteins are digested with endoglycosidase H (EndoH). EndoH cleaves N-glycosides from the mannose-rich type, but does not cleave complex type glycans. In contrast to EndoH, N-glycosidase F (PNGaseF) cleaves mannose-rich as well

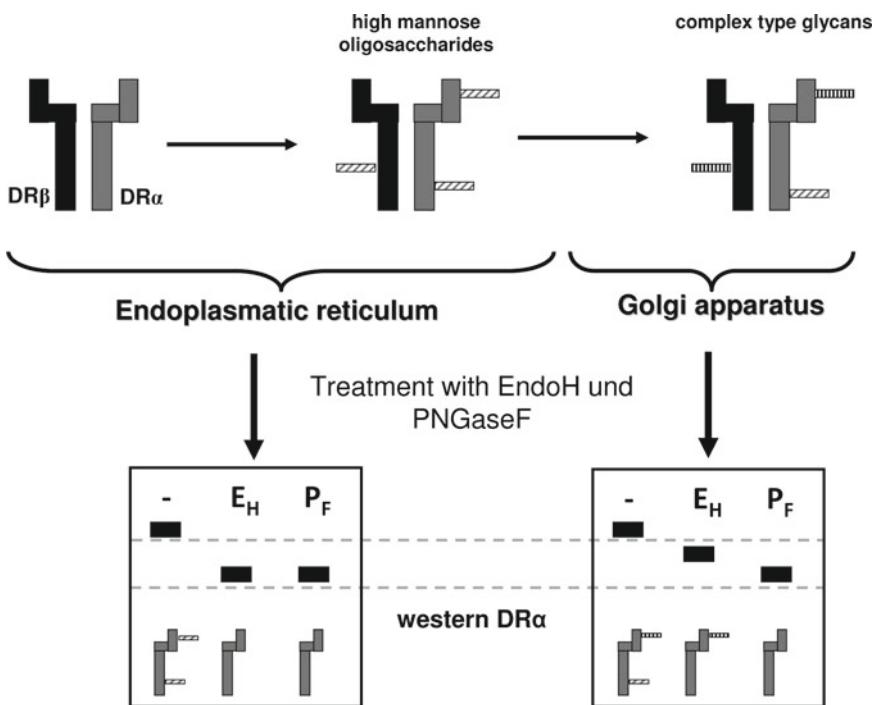


Fig. 3. Glycosidase digestion of class II subunits. In the ER, class II α - and β -chains are modified with high-mannose glycans at asparagine (Asn) containing motifs. After export of the glycoproteins from the ER to the *cis*-Golgi the mannose-rich N-glycan of class II the β -chain and one of the two N-glycans of the α -chain are processed to complex type carbohydrates. Endoglycosidase H (EndoH, E_H) treatment cleaves high-mannose oligosaccharides only, whereas *N*-glycosidase F (PNGaseF, P_F) removes mannose-rich and complex type N-linked carbohydrates. SDS-PAGE separated and EndoH digested DR α and DR β chains with complex glycans display a higher molecular weight compared to EndoH digested chains with high-mannose oligosaccharides (*lower panel*).

as complex type oligosaccharides. To identify properly folded MHCII molecules that left the ER to the *cis*-Golgi, class II subunits are treated with EndoH and analyzed by SDS-PAGE and western blotting (Fig. 3). Loading of MHC class II molecules with high-affinity peptides is catalyzed by the non-classical class II molecule HLA-DM (Fig. 1–3). The MHC class II heterodimer changes its conformation after peptide binding to a compact form. This class II peptide complex is resistant to treatment with 1% SDS for 30 min at 37°C (14, 15). SDS-stable α - β -peptide complexes can be visualized by western blotting and appear as a 55 kDa band (Fig. 4). To demonstrate the formation of SDS-stable class II dimers we used the class II and HLA-DM expressing MelJuSo cell line (see Note 2). Finally, cell surface expression is assessed by flow cytometry using MHC class II mAbs. Since class II antibodies are important tools for the study of class II molecules, we provide information of class II antibodies useful for western blotting, immunoprecipitation, or flow cytometry (Table 1).

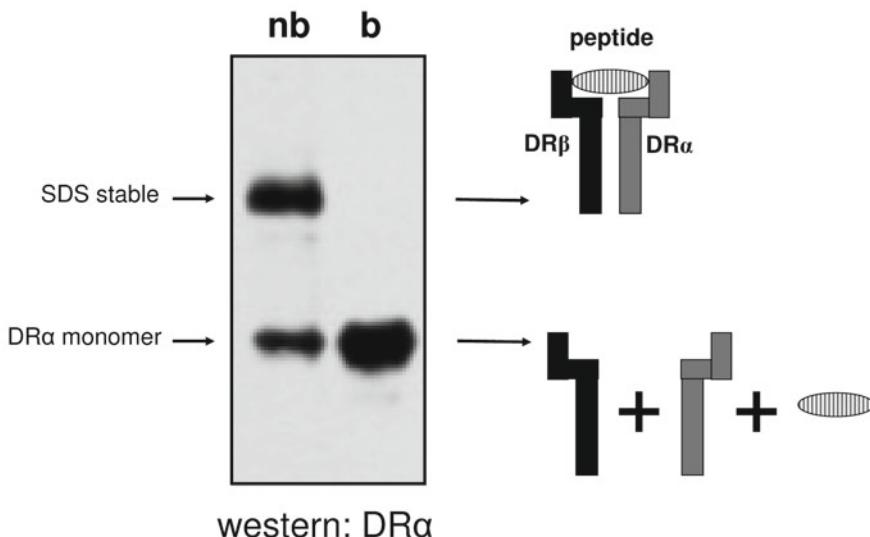


Fig. 4. Western blotting of SDS resistant class II peptide complexes. MelJuSo cell lysate was mixed with reducing SDS containing sample buffer and incubated for 30 min at room temperature. The sample was split and one-half was incubated at 95°C to dissociate class II subunits. After separation by SDS-PAGE, the membrane was probed with mAb TAL-1B5 for detection of DR α . The SDS-stable class II dimers appear as a 55 kDa band in the non-boiled sample and the DR α monomers at about 34 kDa. nb non-boiled, b boiled.

2. Materials

2.1. Transfection Reagents

- jetPEI transfection reagent (Peqlab) and sterile 150 mM NaCl solution (pH 7.5) (see Note 3).
- Plasmids: Class II cDNAs were TA-cloned into the eukaryotic expression vector pcDNA3.1/V5-HisTOPO (Invitrogen). This vector contains a CMV promoter 5' upstream and a V5-His sequence 3' of the multiple cloning site. The class II cDNAs were expressed as V5-His tagged fusion proteins or without protein tag.

2.2. Cell Culture

- COS-7, IMR90S, and MelJuSo cell line.
- DMEM (4.5 g/L) glucose containing 10% FCS, 1% antibiotics, sodium pyruvate, and HEPES (All PAA).
- 10 cm Culture dishes.
- PBS/EDTA: Prepare a 0.5 M EDTA solution and sterilize by autoclaving. Dilute 2 ml of 0.5 M EDTA in 500 ml sterile PBS to obtain a 2.5 mM EDTA/PBS solution.
- Trypsin/EDTA (PAA): Trypsin 5 mg/ml, EDTA 2.2 mg/ml.
- Lysis buffer (Saline-Tris (ST) buffer): 140 mM NaCl, 10 mM Tris-Base, pH 7.5, with 1× Complete protease inhibitors Roche Diagnostics and a final concentration of 0.5% Nonidet P-40 (NP40) (see Note 4).

Table 1
Properties of antibodies for the study of MHC class II molecules

Clone	Species	Isotype	Specificity	Epitope	FACS	Western	IP	Reference	Purchased by
<i>Class II mAbs</i>									
ISCR3	Mouse	IgG2b	DR	Conf.	Yes	No	Yes	(16)	Cosmo Bio Co.
I251SB	Mouse	IgG	DR, DP	Conf.	Yes	No	Yes	(17)	–
LGII-612.14	Mouse	IgG1	DR, DP, DQ	Lin., beta chains MHCI/pept.	Yes	Yes	Yes	(18)	–
L243	Mouse	IgG2a	DR	MHCI/pept.	Yes	Yes ^a	Yes	(19)	AbD Serotec
D1-12	Mouse	IgG2a	DR	MHCI/pept.	Yes	–	Yes	(20, 21)	–
2.06	Mouse	IgG1	DR, DP, DQ	Conf.	Yes	–	Yes	(22)	Santa Cruz Biotechnology
TAL-1B5	Mouse	IgG1	DR	Lin., DR α , C terminus	Yes ^b	Yes	Yes	(23)	Thermo Scientific
IC4.6	Mouse	IgG1	DR	Lin., DR α , N terminus	No	Yes	No	(24)	–
1a3	Mouse	IgG2a	DQ	Conf.	Yes	No	Yes	(25)	MyBiosource
SPV-L3	Mouse	IgG2a	DQ	–	Yes	–	Yes	(26)	AbD Serotec
<i>Li mAbs</i>									
Bu43	Mouse	IgM	Li	C terminus	–	Yes	Yes ^c	(27)	Santa Cruz Biotechnology
Bu45	Mouse	IgG1	Li	Trimer-domain	Yes	No	Yes	(27)	Novus Biologicals
CerCLIP	Mouse	IgG1	Li	CLIP	Yes	Yes	Yes	(28)	BD Pharmingen
<i>Tag mAbs</i>									
V5	Mouse	IgG2a	P/V protein	Lin. 14aa	Yes	Yes	Yes	(29)	Invitrogen, AbD Serotec
6D4	Mouse	IgG1	RT1-DM β	Lin. 10aa	Yes	Yes	Yes	(30, 31)	–
12B8	Mouse	IgG1	RT1-DM α	Lin 12aa	Yes	Yes	Yes	(31)	–

Conf. = conformational epitope, Lin. = linear epitope, pept. = peptide, aa = amino acid, – = unknown

^aMAb L243 can be used for detection of SDS class II dimers under non-boiled conditions (24)

^bIntracellular staining is required for flow cytometry with mAb TAL-1B5

^cIP with Bu43 requires secondary Ab for binding to Protein-A or -G Sepharose

2.3. Immuno-precipitation

1. CL4B Sepharose: 50 µl of CL4B Sepharose to 200 µl ST-buffer and spin for 30 s at 5,000×*g*. Remove supernatant carefully using a syringe (see Note 5).
2. Protein-A or -G Sepharose: add 10 µl of Protein-A or -G Sepharose suspension to 200 µl ST-buffer and spin for 30 s at 5,000×*g*. Remove supernatant carefully using a syringe (see Note 5).
3. Antibodies for immunoprecipitation: Purified antibodies or concentrated hybridoma supernatants can be employed. Approximately 0.5–1 µg of antibody are used for IP. For low affinity antibodies larger amounts of the precipitating antibody are required. Information about antibodies for IP is listed in Table 1.
4. IP washing buffer: ST-buffer with 0.25% NP40 and complete protease inhibitors (Roche Diagnostics).
5. Material for SDS-PAGE and immunoblotting.

2.4. Treatment with N-Glycosidases

1. Endoglycosidase H (EndoH): concentration of 500,000 U/ml (New England Biolabs).
2. N-Glycosidase F (PNGaseF): concentration of 500,000 U/ml (New England Biolabs).
3. Buffer G5 (50 mM sodium citrate, pH 5.5) and G7 (50 mM sodium phosphate, pH 7.5).
4. 10% NP40.
5. Reducing sample buffer: 4× concentrated reducing sample buffer (4× RSB, RotiLoad). To generate 1× RSB, the 4× RSB stock solution was diluted with ST-buffer (see Subheading 2.2).
6. Material for SDS-PAGE and immunoblotting.

2.5. Flow Cytometry

1. FACS buffer: PBS with 2% FCS and 0.01% sodium azide (see Note 6).
2. Antibody concentrations for flow cytometry were in the range of 0.5–10 µg/ml. The primary mAbs were diluted in FACS buffer.
3. Secondary antibodies are used at the same concentration as the primary mAbs. To stain dead cells, Propidium iodide (1 µg/ml final concentration) is added to the secondary mAb solution (see Note 7).
4. Flow cytometer.

3. Methods

3.1. Expression of Class II Molecules in the Absence or Presence of li

1. Detach cells with trypsin/EDTA and count cell number. Resuspend 3×10^6 cells in 500 μl of culture medium and transfer cell suspension into a round-bottom polystyrene tube. Deposit tubes at 37°C in a CO₂ incubator.
2. Dilute 1.5–3 μg of plasmid DNA (for example, DR α and DR β) in 250 μl of 150 mM NaCl solution (see Note 8). Mix 15 μl of jetPEI transfection reagent with 250 μl of 150 mM NaCl solution in a second tube. Transfer the jetPEI/NaCl solution to the DNA solution. Vortex briefly and incubate for 15 min at room temperature (RT).
3. Add 500 μl of the transfection mix to cells and incubate for 15 min at 37°C and mix with 300 rpm. Subsequently, resuspend the cells in 5 ml of fresh culture medium and transfer to a 10 cm culture dish. Incubate cells for 48 h at 37°C under CO₂ atmosphere.

3.2. Harvesting and Processing of Cells After Transfection

1. Discard cell culture medium from adherent cells and add 5 ml of PBS/EDTA solution per 10 cm plate (see Note 9). Incubate for 15 min at 37°C. Flick plate to detach cells.
2. Resuspend cells and transfer to a 15 ml tube. Add 10 ml of ice-cold FACS buffer and centrifuge at $1,000 \times g$ for 5 min. Remove supernatant and resuspend cells in 1.5 ml FACS buffer.
3. Take 250 μl of the cell suspension into a new tube for flow cytometry (see Subheading 3.6).
4. Centrifuge remaining cell suspension (1,250 μl ; s.o) and remove supernatant.
5. Resuspend cell pellet in 200 μl of lysis buffer and incubate for 30 min on ice. Remove cell debris and unsoluble components by centrifugation at $18,000 \times g$ and 4°C.
6. Transfer 150 μl of the lysate to a new tube, place on ice or store at -20°C.
7. 50 μl of each lysate is used for immunoprecipitation (see Subheading 3.3), for EndoH digestion (see Subheading 3.4), or for detection of SDS-stable class II dimers (see Subheading 3.5).

3.3. Detection of Class II-li Interaction by Immunoprecipitation

1. Pre-immunoprecipitation is performed to remove proteins with nonspecific binding to CL4B Sepharose:
 - (a) Transfer 50 μl of lysate to a 1.5 ml tube.
 - (b) Add 200 μl of lysis buffer and 50 μl of CL4B Sepharose beads and incubate for 30 min at 4°C under constant motion.
 - (c) Pellet the CL4B Sepharose beads by centrifugation and transfer the supernatant carefully to a new tube.

2. Immunoprecipitation:

- (a) Add 10 µl of protein-A or -G Sepharose suspension and 1 µg of the ISCR3 antibody (see Note 10) to the cell lysate and incubate for 1 h (or overnight) at 4°C under constant motion.
- (b) Spin down the Sepharose beads and discard supernatant.
- (c) Wash the protein-A Sepharose pellet three times with 500 µl of washing buffer and discard supernatant.
3. Resuspend the protein-A Sepharose pellet in 20–30 µl 1× reducing sample buffer and heat for 5 min at 95°C to elute the immunocomplexes from the protein-A Sepharose.
4. Separate proteins by 12% SDS-PAGE and detect class II/Ii subunits by western blotting using the mAbs 1B5 (specific for DR α) or Bu43 (binds to Ii) (Fig. 2; see Note 11).

3.4. Carbohydrate Maturation as a Marker for ER-Egress

For inspection of carbohydrate maturation, three experiments are performed:

- A control, which demonstrates the untreated N-linked glycosylation pattern of class II subunits.
- EndoH-treated lysates, where high-mannose N-linked carbohydrates are removed from the polypeptides.
- PNGaseF-treated samples, devoid of all N-linked carbohydrates.
 1. Prepare sample by mixing 45 µl of cell lysate with 15 µl of 4× reducing sample buffer and heat at 95°C for 5 min (see Note 12).
 2. Prepare enzyme digest in PCR tubes and incubate overnight at 37°C. Use a PCR thermocycler for incubation.
Control: 20 µl sample + 30 µl ST-buffer.
EndoH: 20 µl sample + 0.5 µl EndoH + 5 µl buffer G5 + 25 µl ST-buffer.
PNGaseF: 20 µl sample + 0.5 µl PNGaseF + 5 µl buffer G7 + 5 µl NP40 + 20 µl ST-buffer.
 3. After incubation, add 5 µl of 4× RSB to the digests and separate the samples on 12% SDS-PAGE. Use mAbs TAL-1B5 or anti-V5 for western blot detection of DR α or of V5-tagged class II chains (Fig. 3; see Note 13).

3.5. Peptide Loading Is Assessed by SDS-Resistance of Class II/Peptide Complexes

Formation of SDS-stable class II dimers requires the presence of HLA-DM. We show here the formation of SDS-stable class II dimers in MelJuSo cells containing endogenous HLA-DM (see Note 14).

1. Lyse 10 6 MelJuSo cells in 100 µl lysis buffer according to Subheading 3.2, step 5.

2. Prepare two samples by mixing 14 µl of cell lysate with 2 µl of 4× RSB (final concentration of SDS is 1%) and incubate for 15 min at room temperature.
3. Boil one of the samples for 5 min at 95°C to dissociate the class II subunits and the peptide. Keep the other sample on ice.
4. Separate both samples by 12% SDS-PAGE and detect DR α or DR β chains by western blotting (Fig. 4).

3.6. Cell Surface Expression of Class II Molecules is Detected by Flow Cytometry

1. Transfer 50 µl of cell suspension to a FACS tube, add 500 µl of FACS buffer, and spin cells down. Remove supernatant and resuspend cell pellet in 50–100 µl of primary mAb. Incubate for 20–30 min at 4°C.
2. Wash cells two times with 500 µl of FACS buffer.
3. Discard supernatant and resuspend cells in 50–100 µl of secondary mAb solution.
4. Incubate for 20–30 min at 4°C, wash cells twice with 500 µl FACS buffer, and resuspend cells in 200 µl of FACS buffer.
5. Analyze a minimum of 10,000 viable cells per experiment.

4. Notes

1. Ii is modified by two N-linked oligosaccharides. However, the Ii isoform Ii41 contains two additional N-glycans in the thyroglobulin domain (32).
2. We use COS-7, IMR90S cells, or the MelJuSo cell line to analyze assembly and transport of class II heterodimers. After transfection, COS-7 cells (*cercopithecus aethiops*) show high transgenic protein expression levels. COS-7 cells express the SV40 large T antigen, which facilitates the replication of plasmid vectors that contain an SV40 origin (for example, the pcDNA3.1-vectors (Invitrogen, Karlsruhe, Germany)). IMR90S is a human lung fibroblast cell line that also expresses the SV40 large T antigen. This cell line was derived from the SV40 large T antigen negative cell line IMR90 (ATCC: CCL-186). IMR90S cells responds to IFN gamma treatment with expression of components of the class II pathway (33). The class II expressing human melanoma MelJuSo cell line (34) is a commonly used model for studying the class II pathway of antigen presentation (35). The intracellular transport and peptide loading of transgenic class II chains was analyzed in MelJuSo cells (35, 36). We used the MelJuSo cell line to demonstrate formation of SDS-stable dimers (see Subheading 3.5).

3. The 150 mM NaCl solution is sterilized by autoclaving. Preparation of larger volumes of 150 mM NaCl solution is recommended to yield standardized transfection mixes.
4. The detergent NP40 is highly viscous. Prepare a 10% NP40 solution by weighting the detergent and diluting in ST-buffer.
5. Protein-A or -G Sepharose has a high antibody binding capacity. Only small amounts of protein-A or -G Sepharose suspension are required for immunoprecipitation. One critical step during IP is a loss of Sepharose beads by removing supernatant. To avoid loss of Sepharose beads, leave a small volume of fluid covering the Sepharose pellet. Use a syringe instead of pipette for removal of supernatant.
6. FACS buffer is stable at 4°C. A final concentration of 0.01% sodium azide in FACS buffer prevents bacterial growth. In addition, sodium azide inhibits internalization of cell surface molecules, which promotes detection of class II cell surface expression.
7. Propidium iodide (PI), 7-aminoactinomycin D (7-AAD), or DAPI (4,6-diamidino-2-phenylindole) can be used to label dead cells. 7-AAD and PI need longer incubation times (30 min) than DAPI (few seconds). DAPI requires a UV-laser for excitation, whereas PI and 7-AAD can be excited by laser light emitted by an argon laser.
8. We recommend to adjust plasmid DNA concentrations for transfection to 0.5–1 µg/µl. This dilution yields standardized transfection mixes and optimal transfection rates.
9. To avoid protein degradation by trypsin digestion, we use a solution of 2.5 mM EDTA in PBS for detachment of cells.
10. The choice of the antibody is important for successful IPs. MAb ISCR3 recognizes peptide-loaded or Ii-associated DR molecules. In contrast to ISCR3, the mAb L243 is specific for peptide-loaded class II molecules and does not co-isolate class II–Ii complexes. Information concerning class II mAbs is listed in Table 1.
11. The mAb Bu43 is of the IgM isoform. Most of the secondary HRP-coupled polyclonal antibodies recognize IgG but not IgM. For western blotting with Bu43, we employ a secondary Ab which recognizes IgM and IgG or IgM only.
12. We use reducing sample buffer (RotiLoad, Carl Roth) for protein denaturation prior to enzymatic cleavage of carbohydrates instead of denaturation buffer supplied by the manufacturer of the glycosidases.
13. Removal of one N-linked glycan yields a 5 kDa lower apparent molecular weight than undigested glycoproteins as determined by SDS-PAGE. Optimal separation of EndoH-treated samples is achieved by a long run on SDS gel.

14. To examine formation of SDS-stable class II dimers in COS-7 or IMR90S cells it is necessary to co-transfect cells with HLA class II α - and β -chains, with Ii and with HLA-DM encoding cDNAs.

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References

1. Koch N, McLellan AD, Neumann J (2007) A revised model for invariant chain-mediated assembly of MHC class II peptide receptors. *Trends Biochem Sci* 32(12):532–537
2. Neumann J, Koch N (2005) Assembly of major histocompatibility complex class II subunits with invariant chain. *FEBS Lett* 579(27):6055–6059
3. Busch R, Cloutier I, Sekaly RP, Hämmерling GJ (1996) Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J* 15(2):418–428
4. Davidson HW (1999) Direct transport of newly synthesized HLA-DR from the trans-Golgi network to major histocompatibility complex class II containing compartments (MIICS) demonstrated using a novel tyrosine-sulfated chimera. *J Biol Chem* 274(38):27315–27322
5. Koch N, Moldenhauer G, Hofmann WJ, Möller P (1991) Rapid intracellular pathway gives rise to cell surface expression of the MHC class II-associated invariant chain (CD74). *J Immunol* 147(8):2643–2651
6. McCormick PJ, Martina JA, Bonifacino JS (2005) Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc Natl Acad Sci U S A* 102(22):7910–7915
7. Avva RR, Cresswell P (1994) In vivo and in vitro formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. *Immunity* 1(9):763–774
8. Bryant PW, Lennon-Dumenil AM, Fiebiger E, Lagaudriere-Gesbert C, Ploegh HL (2002) Proteolysis and antigen presentation by MHC class II molecules. *Adv Immunol* 80:71–114
9. Sloan VS, Cameron P, Porter G, Gammon M, Amaya M, Mellins E, Zaller DM (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375(6534):802–806
10. Sadegh-Nasseri S, Chen M, Narayan K, Bouvier M (2008) The convergent roles of tapasin and HLA-DM in antigen presentation. *Trends Immunol* 29(3):141–147
11. Wubbolds R, Neefjes J (1999) Intracellular transport and peptide loading of MHC class II molecules: regulation by chaperones and motors. *Immunol Rev* 172:189–208
12. Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 317(6035):359–361
13. Shackelford DA, Strominger JL (1983) Analysis of the oligosaccharides on the HLA-DR and DC1 B cell antigens. *J Immunol* 130(1):274–282
14. Germain RN, Hendrix LR (1991) MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* 353(6340):134–139
15. Mellins E, Smith L, Arp B, Cotner T, Celis E, Pious D (1990) Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* 343(6253):71–74
16. Watanabe M, Suzuki T, Taniguchi M, Shinozaki N (1983) Monoclonal anti-Ia murine alloantibodies crossreactive with the Ia-homologues of other mammalian species including humans. *Transplantation* 36(6):712–718
17. Pesando JM, Graf L (1986) Differential expression of HLA-DR, -DQ, and -DP antigens on malignant B cells. *J Immunol* 136(11):4311–4318
18. Temponi M, Kekish U, Hamby CV, Nielsen H, Marboe CC, Ferrone S (1993) Characterization of anti-HLA class II monoclonal antibody LGII-612.14 reacting with formalin fixed tissues. *J Immunol Methods* 161(2):239–256

19. Lampson LA, Levy R (1980) Two populations of Ia-like molecules on a human B cell line. *J Immunol* 125(1):293–299
20. Accolla RS, Gross N, Carrel S, Corte G (1981) Distinct forms of both alpha and beta subunits are present in the human Ia molecular pool. *Proc Natl Acad Sci U S A* 78(7):4549–4551
21. Hitzel C, Grüneberg U, van Ham M, Trowsdale J, Koch N (1999) Sodium dodecyl sulfate-resistant HLA-DR “superdimer” bands are in some cases class II heterodimers bound to antibody. *J Immunol* 162(8):4671–4676
22. Charron DJ, McDevitt HO (1980) Characterization of HLA-D-region antigens by two-dimensional gel electrophoresis. Molecular genotyping. *J Exp Med* 152(2 Pt 2):18s–36s
23. Adams TE, Bodmer JG, Bodmer WF (1983) Production and characterization of monoclonal antibodies recognizing the alpha-chain subunits of human Ia alloantigens. *Immunology* 50(4):613–624
24. Ting YT, Temme S, Koch N, McLellan AD (2009) A new monoclonal antibody recognizing a linear determinant on the HLA-DR α chain N-terminus. *Hybridoma (Larchmt)* 28(6):423–429
25. Spits H, Borst J, Giphart M, Coligan J, Terhorst C, De Vries JE (1984) HLA-DC antigens can serve as recognition elements for human cytotoxic T lymphocytes. *Eur J Immunol* 14(4):299–304
26. Shookster L, Matsuyama T, Burmester G, Winchester R (1987) Monoclonal antibody 1a3 recognizes a monomorphic epitope unique to DQ molecules. *Hum Immunol* 20(1):59–70
27. Wright CJ, van Endert P, Moller P, Lipp J, Ling NR, MacLennan IC, Koch N, Moldenhauer G (1990) Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J Biol Chem* 265(10):5787–5792
28. Denzin LK, Robbins NF, Carboy-Newcomb C, Cresswell P (1994) Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1(7):595–606
29. Southern JA, Young DF, Heaney F, Baumgartner WK, Randall RE (1991) Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *J Gen Virol* 72(Pt 7):1551–1557
30. Kurth D, Neumann J, Demleitner K, Hildmann J, Mehlig M, Scheicher C, Wendling U, Sun D, Reske K (1997) Full length cDNA of rat RT1.DM α and RT1.DM β and expression of RT1.DM genes in dendritic and Langerhans cells. *Biol Chem* 378(9):1005–1012
31. Neumann J (2005) Novel antibody tags from the rat lysosomal protein RT1.DM for immunodetection of recombinant proteins. *J Immunol Methods* 301(1–2):66–76
32. Koch N (1988) Posttranslational modifications of the Ia-associated invariant protein p41 after gene transfer. *Biochemistry* 27(11):4097–4102
33. Kämper N, Franken S, Temme S, Koch S, Bieber T, Koch N (2012) {gamma}-Interferon-regulated chaperone governs human lymphocyte antigen class II expression. *FASEB J* 26(1):104–116
34. Johnson JP, Demmer-Dieckmann M, Meo T, Hadam MR, Riethmüller G (1981) Surface antigens of human melanoma cells defined by monoclonal antibodies. I. Biochemical characterization of two antigens found on cell lines and fresh tumors of diverse tissue origin. *Eur J Immunol* 11(10):825–831
35. Wubbolts R, Fernandez-Borja M, Oomen L, Verwoerd D, Janssen H, Calafat J, Tulp A, Dusseljee S, Neefjes J (1996) Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J Cell Biol* 135(3):611–622
36. Koch N, Zacharias M, König A, Temme S, Neumann J, Springer S (2011) Stoichiometry of HLA class II-invariant chain oligomers. *PLoS One* 6(2):e17257

Chapter 33

Studying MHC Class II Peptide Loading and Editing In Vitro

AeRyon Kim, Isabel Ishizuka, Isamu Hartman, Yuri Poluektov,
Kedar Narayan, and Scheherazade Sadegh-Nasseri

Abstract

HLA-DM is now known to have a major contribution to the selection of immunodominant epitopes. A better understanding of the mechanisms controlling epitope selection can be achieved by examination of the biophysical behavior of major histocompatibility complex (MHC) class II molecules upon binding of antigenic peptides and the effect of DM on the interactions. Using purified soluble molecules, in this chapter, we describe several in vitro methods for measuring peptide binding to HLA-DR molecules and the effects of HLA-DM on the interactions. A simple qualitative method, *Gentle SDS-PAGE Assay*, would assess the ability of peptides to form tight complexes with MHC class II molecules. Measuring binding kinetics is among the most informative approaches to understanding molecular mechanisms, and here we describe two different methods for measuring binding kinetics of peptide–MHC complexes. In one method, rates of association and dissociation of fluorescently labeled peptides to soluble MHC class II molecules can be determined using G50 spin columns to separate unbound peptides from those in complex with MHC molecules. In another method, association and dissociation of unlabeled peptides and MHC class II molecules can be determined in *real time* using BIAcore *surface plasmon resonance* (*SPR*). We also have described an Intrinsic Tryptophan Fluorescence Assay for studying transient interactions of DM and MHC class II molecules.

Key words: Kinetic studies, Peptide binding to HLA-DR, HLA-DM editing, BIAcore *surface plasmon resonance*, Tryptophan fluorescence assay, Gentle SDS-PAGE assay, Fluorescent peptide

1. Introduction

T-cell-mediated responses to protein antigens involve recognition by T-cell receptors (TCRs) of antigenic peptides bound to major histocompatibility complex (MHC) molecules. Proteolytic digestion of protein antigens during antigen processing generates many peptide fragments that can potentially bind to MHC molecules. However, only a small subset of these antigen fragments induce T-cell responses, and these are defined as being the *immunodominant*

epitopes of a protein antigen (1). Generation and selection of ligands for CD4 T helper cells recognition takes place in professional antigen presenting cells. Many elements control the selection of antigenic epitopes: One, HLA-DM (DM) (H2-M in mice), wields a particularly strong influence on the selection of peptides that bind to MHC class II molecules. DM assists in the maturation of the nascent MHC class II molecule by accelerating dissociation of the class II-associated invariant peptide (CLIP) from its peptide binding groove (2). DM influences the binding kinetics between MHC II and many other peptides as well, modulating both association and dissociation rates (3–5). DM accelerates peptide/MHC II binding by inducing MHC II to adopt a peptide-receptive conformation; it also accelerates the dissociation of some peptides from MHC II molecules, accomplished by exerting conformational changes in MHC class II/peptide complexes preventing the formation of a critical H-bond between the β His81 of the MHC II molecule and the peptide main chain (4, 6). A peptide-receptive MHC II molecule can quickly sample a large pool of peptides derived from exogenously acquired proteins, and DM acts as a peptide editor that helps in shaping epitope selection (1).

Over the years, there have been many studies to elucidate the mechanisms of epitope selection. At the level of antigen processing and presentation, epitope affinity for MHC class II molecules has been suggested to play an important role in determining immunodominance (7, 8). To arrive at a better understanding of mechanisms that govern epitope selection, it is useful to assess the biophysical parameters of binding between peptide and MHC class II and to characterize the effect of DM on this interaction. There are many *in vitro* methods available for studying the peptide–MHC class II interaction using purified proteins. To qualitatively assess the ability of a peptide to form tight complexes with MHC class II molecules, a gentle SDS-PAGE assay in which samples are not boiled prior to gel loading can be used. Structurally stable MHC class II/peptide complexes that resist SDS-induced dissociation into α - and β -chains allow for a simple way to assess peptide binding to some alleles of MHC II (9, 10). To quantitatively investigate the kinetics of peptide–MHC binding, we primarily rely on two methods: one is to measure the association and dissociation rates of fluorescently labeled peptides with soluble MHC class II molecules. The other is real-time detection of binding between peptide and MHC class II using a BIACore surface plasmon resonance (SPR) instrument, which allows for measuring very rapid interactions that are too fast to measure by other common methods (4). Similarly, studying the transient and low-affinity interactions between soluble DM and MHC class II molecules requires a sensitive assay; we use the burial of solvent-exposed Trp residues, as measured by a change in intrinsic Tryptophan fluorescence, as a readout for productive interactions between these molecules (4, 6).

The methods described in this chapter utilize purified recombinant forms of HLA-DRI (DRB1*0101) and HLA-DM, both of which have been rendered soluble by genetic removal of transmembrane domains. These methods have long been used in our laboratory for reliably and accurately measuring binding and dissociation of peptides to soluble HLA-DRI (sDRI) and have been applied to study peptide editing in the presence of DM in vitro.

2. Materials

2.1. Consumables and Lab Equipment

1. Purified MHC II:
 - (a) Soluble recombinant DRI protein in PBS, 0.05% NaN_3 , pH 7.4 (see Note 1).
 - (b) Soluble recombinant DM protein in Citrate Phosphate buffer pH 6.0 (see Notes 2 and 3).
2. Fluorescent Peptide Labeling with Fluorescein: dissolve 1–2 mg peptide modified with terminal cysteine residue (cysteine is normally added to the N-terminal end of the peptide (>90% pure) in 1 ml chilled PBS (see Note 4) and incubate for 1–3 h at 25°C with 10–20 μl of 75 mM fluorescein-5-maleimide (final concentration at 0.75 mM or 1.5 mM) (Pierce) in *N,N*-dimethylformamide. Concentrate samples to ~0.1–0.2 ml in a SpeedVac (Savant Instruments) and remove unbound fluorescein from the sample by passing through a Sephadex G-10 column (Sigma). Determine concentration by spectrophotometry according to extinction coefficient of fluorescein-5-maleimide ($83 \text{ mM}^{-1} \text{ cm}^{-1}$).
3. Citrate phosphate buffer at pH 5.0: 24.3 mM citric acid and 51.4 mM dibasic sodium phosphate (Na_2HPO_4) with 0.05% NaN_3 in nanopure water.
4. Citrate phosphate buffer at pH 5.4: 22.2 mM citric acid and 55.6 mM dibasic sodium phosphate (Na_2HPO_4) with 0.05% NaN_3 in nanopure water.
5. Citrate phosphate buffer at pH 6.0: 17.9 mM citric acid and 64.2 mM dibasic sodium phosphate (Na_2HPO_4) with 0.05% NaN_3 in nanopure water.
6. PBS/Z: PBS containing 0.05% NaN_3 .
7. Sephadex G50 Column.
8. Fluoromax3 spectrofluorometer (Horiba Jobin-Yvon).
9. BIACore systems.

2.2. SDS-PAGE Assay

1. 10 or 12% SDS-PAGE gel (Bio-Rad and Invitrogen).
2. 2× Sample buffer: 2.5 ml of 0.5 M Tris–HCl, pH 6.8, 2 ml of Glycerol, 0.2–2 ml of 10% SDS, 0.5 ml of 0.1% Bromophenol Blue. Adjust volume to 10 ml with nanopure water.
3. 10× SDS Running buffer: 30 g Tris Base, 144 g Glycine, 100 ml 10% SDS, or 10 g SDS powder. Adjust volume to 1 L with nanopure water.

2.3. Preparation of Sephadex G50 Column

1. Mix 0.9 g Sephadex G-50 (Sigma) in 10 ml buffer of choice (Let sephadex G-50 hydrate for at least 30 min before using).
2. Load 1 ml of gel suspension into Bio-Spin disposable chromatography columns (Bio-Rad) pre-rinsed with nanopure water.
3. Centrifuge columns at 3,000 rpm ($1,300 \times g$) at 25°C for 2 min 45 s (set at low brake or no brake, depending on your centrifuge) to remove excess buffer from the resin (see Note 5).
4. Transfer the column into a fresh reservoir tube (5 ml polypropylene round-bottom tube, Becton Dickinson).
5. Load samples directly and carefully onto the top of the gel bed. Centrifuge at $1,300 \times g$ at 25°C for 4 min (set at high brake) to pass samples through the gel bed.
6. Collect filtrate from bottom of the reservoir tube.

2.4. BIACore Experiments

Sensor chip CM5 (GE Healthcare).

1. N-Hydroxy-succinimide (NHS) (Thermo Scientific Pierce): dissolve 0.14 g in 10 ml nanopure water to make 122 mM stock solution. Aliquot in 1.5 ml centrifuge tubes and immediately store them at -80°C. When removing an aliquot from -80°C to use for activation of the CM5 chip surface, place tube in dry ice and thaw right before injection.
2. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Thermo Scientific Pierce): dissolve 0.8 g in 10 ml nanopure water to make 417 mM stock solution. Aliquot in 1.5 ml centrifuge tubes and immediately store at -80°C. When removing an aliquot from -80°C to use for activation of the CM5 chip surface, place tube in dry ice and thaw immediately before injection.
3. 1 M Ethylenediamine (EDA): It is advisable to prepare it fresh before each use, as the EDA solution is sensitive to air, when it starts to degrade the solution turns yellow. Weigh EDA and dissolve in nanopure water. Adjust pH to 6.0 by slowly adding 12 M HCl. Alternatively you can use the EDA-2HCl (Thermo Scientific Pierce) to dissolve in water and adjust the pH with a dilute solution of NaOH. Store the stocks at -20°C.
4. 75 mM Sulfo-succinimidyl 4-(*p*-maleimidophenyl)-butyrate (SMPB) (Thermo Scientific Pierce): Dissolve 50 mg SMPB in

- 1.8 ml DMF (Dimethylformamide) to make 75 mM stock solution. Store at -20°C for long-term storage.
5. Running Buffer: PBS or citrate phosphate pH 6.0 with 0.005–0.01% Tween 20. Filter and degas the running buffer before using. Note: degassing is very important.

3. Methods

3.1. Gentle SDS-PAGE

Formation of stable complexes between peptide and purified MHC class II molecules in the absence or presence of purified DM.

1. Incubate various combinations of purified MHC class II molecules (1 μM), DM (0.2–1 μM), and peptide (50–150 μM) in citrate phosphate buffer pH 5.0–6.0 with 0.05% NaN₃ for various time points (see Note 6) at 37°C.
2. Following incubations, readjust pH to 7.5 with 1 M Tris-HCl pH 8.0.
3. Add sample buffer with 0.1–1% SDS final concentration and no reducing agents. Do not boil. Let the samples sit for 10 min at room temperature and run them on a 10 or 12% polyacrylamide gel. Note that for native MHC II molecules isolated from cells one can use up to 1% SDS (11), but for our soluble forms of class II alleles we use 0.1% final concentration of SDS.
4. Silver-stain the gel (Fig. 1).

3.2. Peptide-Class II Association Kinetics

1. On ice, mix purified MHC II molecules (final concentration 1–2 μM) with fluorescently labeled peptide (final concentration at 50 μM) in a volume of 50–60 μl.
2. Use citrate phosphate buffer at pH 5.0–5.5 with 0.05% NaN₃ as the buffer when studying effect of DM (0.2–1 μM) on peptide binding, since DM is active at acidic pH.
3. Keep reactions on ice. At each indicated time point, transfer a tube to a 37°C water bath.
4. At the end of the incubation, take all the samples reaction tubes out from the 37°C water bath and place them on ice to stop further peptide binding to MHC class II molecules. The sample tube for the 0 time point is left on ice throughout and is never transferred to the 37°C water bath.
5. Spin sample reactions down on mini bench-top centrifuge.
6. For removal of excess unbound fluorescent peptides and buffer exchange from acidic pH to PBS, pH 7.4, load samples on a Sephadex G-50 spin column equilibrated in PBS/Z. Centrifuge at 1,300×g at 25°C for 4 min (set at high brake) to pass samples through the gel bed.

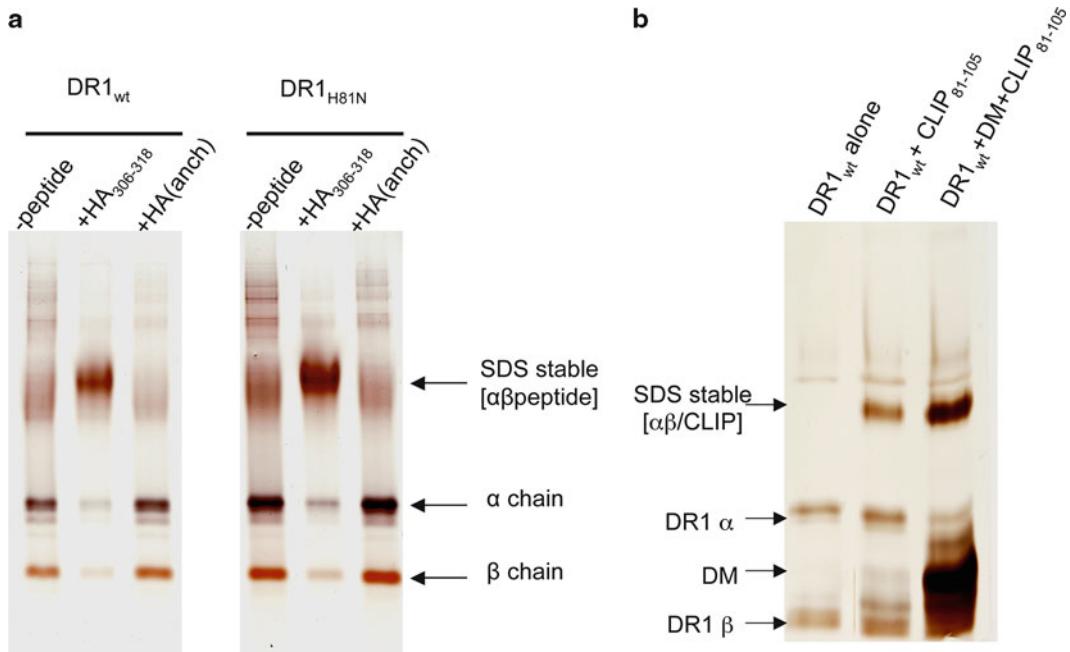


Fig. 1. SDS stability of DR1–peptide complex. (a) Wild-type DR1 and mutant DR1 (β H81N) (1 μ M) incubated for 24 h at 37°C with or without peptides (100 nM) in PBS, pH 7.4. (b) Wild-type DR1 was incubated for overnight at 37°C with long CLIP_{81–105} in the presence or absence of DM. The peptide/MHCII complexes were incubated in citrate phosphate buffer, pH 5.5. Reaction samples were neutralized first before being mixed with SDS-PAGE sample buffer containing 0.1% SDS (final concentration) and were incubated for 10 min at room temperature prior to loading. Samples were then run on 12% (a) or 10% (b) SDS-PAGE and gels were silver-stained.

7. Collect filtrate from bottom of the reservoir tube. Add 70–80 μ l PBS, pH 7.4 to each sample.
8. Measure the fluorescence emission of fluorescein-5-maleimide–peptide–MHC II complexes at 25°C and measure optimal emission wavelengths for fluorescein-5-maleimide at 514–516 nm with excitation at 492 nm on a Fluoromax3 spectrofluorometer with a slit width of 2 nm (Fig. 2).

3.3. Kinetic Analysis of Peptide Dissociation

1. Incubate soluble MHC class II (10 μ M) with fluorescence-labeled peptides (50 μ M) in PBS, pH 7.4 or in citrate phosphate buffer, pH 5.5 for 3 days at 37°C to form MHC II–peptide complexes.
2. After 3-day incubation, remove excess unbound fluorescence-labeled peptide by filtering through a Sephadex G-50 spin column equilibrated in citrate phosphate buffer pH 5 with 0.05% NaN₃.
3. Aliquot DR1–fluorescent peptide complexes into a number of tubes (one tube per time point).
4. Use citrate phosphate buffer, pH 5.5, as the sample buffer with the total reaction volume at 50–60 μ l. Keep all sample reaction

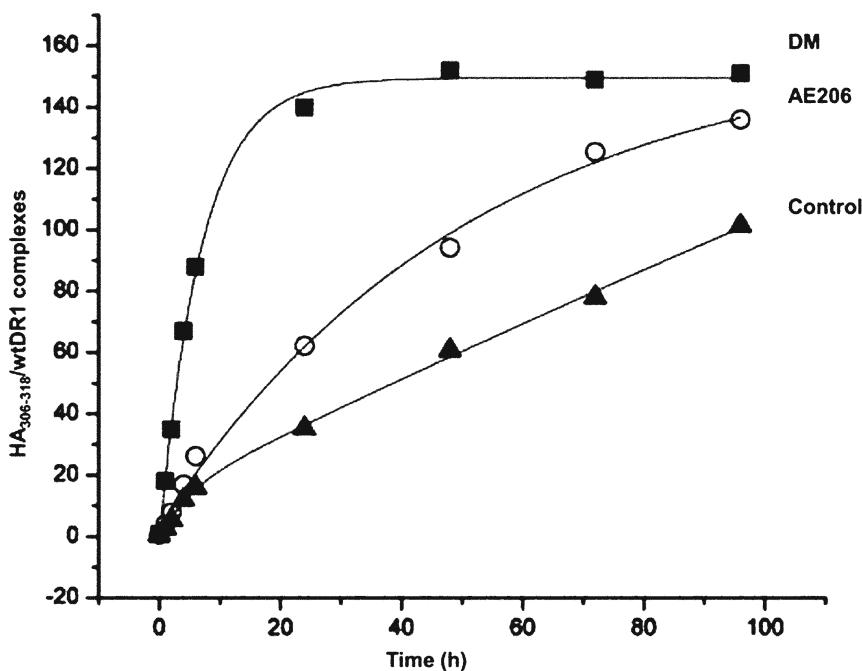


Fig. 2. Effects of DM, or peptide helper, AE206, on peptide association with DR1. Soluble DR1 (2.4 μ M) was incubated in the presence or absence of DM (0.5 μ M), AE206 (150 μ M) with fluorescently labeled HA₃₀₆₋₃₁₈ for various times in citrate phosphate, pH 5.5, at 37°C. Association of fluorescent peptide with DR1 is shown as a function of time in the absence (filled triangle), presence of AE206 (open circle), or DM (filled square) (12).

tubes on ice. At each incubation time, remove tubes from ice and transfer to 37°C water bath.

5. Incubate DR1–fluorescent peptide complexes (1 μ M) for the required length of time at 37°C with excess unlabeled competitor peptide (usually 50–100 μ M, to prevent rebinding of dissociated fluorescent peptide) in the presence or absence of DM.
6. After incubation, remove all reaction samples from the water bath and place on ice.
7. After removal of dissociated fluorescent peptide by passage through a Sephadex G-50 spin column equilibrated with PBS, pH 7.4, collect filtrate from bottom of the reservoir tube.
8. Add 70–80 μ l PBS, pH 7.4 to each sample.
9. Measure fluorescence emission of the fluorescein-5-maleimide labeled peptide/DR complexes at 25°C and 514–516 nm with excitation at 492 nm on a Fluoromax3 spectrofluorometer with a slit width of 2 nm (Fig. 3).

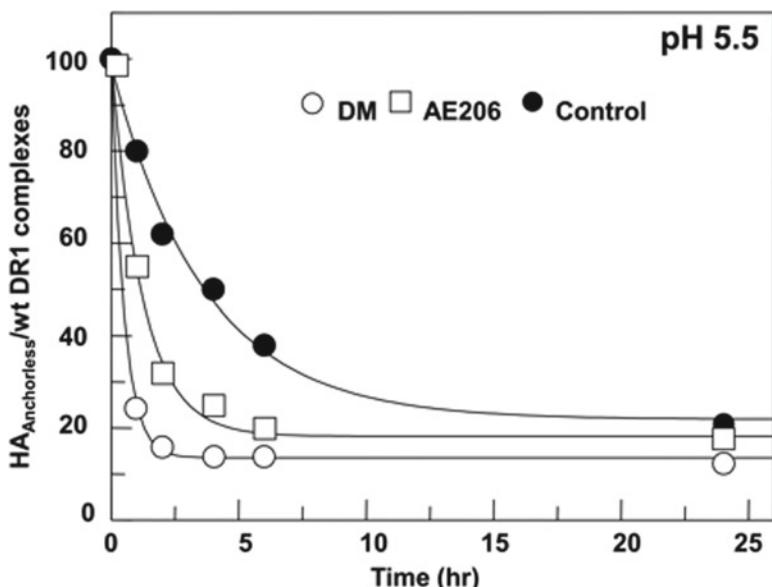


Fig. 3. Effects of DM or helper peptide, AE206, on peptide dissociation from peptide/DR1 complexes. Fluorescently labeled peptide/DR1 complexes were dissociated in the presence of 100 times molar excess of unlabeled HA_{306–318} at 37°C for the indicated times in the absence (filled circle), presence of AE206 (open square), or DM (open circle). The y-axis represents arbitrary fluorescence units (12).

3.4. Real-Time Kinetics via SPR

3.4.1. Peptide Immobilization to CM5 Chip

Perform immobilization of Cys-peptides on CM5 chip at room temperature:

1. Equilibrate Biosensor CM5 chip to room temperature before docking it into a BIACore 1000/2000/3000 instrument.
2. Before peptide immobilization, use PBS, pH 7.4 as running buffer at 10 µl/min flow speed (see Notes 7–9).
3. For peptide immobilization to the biosensor CM5 chip, activate the CM5 surface by injecting a mixture containing equal amounts of 0.06 M NHS and 0.2 M EDC at a low flow rate of 10 µl/min for 7 min (see Note 7).
4. Prepare extra volume of NHS and EDC mixture (If injecting 70 µl, prepare 100 µl of solutions). Mix freshly thawed NHS and EDC and use them immediately.
5. Generate Amino groups by injecting 80 µl of ethylenediamine hydrochloride (EDA) (1 M, pH 6.0) for 8 min at a flow rate of 10 µl/min. Use freshly prepared or thawed frozen stock of EDA.
6. To introduce maleimido groups, inject a solution of the heterobifunctional reagent, SMPB, over the surface at a flow rate of 5 µl/min for 10 min.
7. Dissolved the peptide in pre-chilled PBS immediately before the injection to prevent the formation of disulfide bonds and inject immediately!

8. Inject Cysteine peptides (100–200 μM) over the CM5 surface and observe the binding of peptide to the surface at a rate of 5 $\mu\text{l}/\text{min}$ for 20 min. After the injection, wash the flow cell for 15–20 min with running buffer.
9. After the peptide injection, stop the sensogram and change the buffer to citrate phosphate, pH 6.0 with 0.005% Tween 20. Wash out unbound Cys-peptides at a flow rate of 10 $\mu\text{l}/\text{min}$ overnight.

3.4.2. For DR1 Binding Experiments

1. Perform experiments either at room temperature or 37°C.
2. Perform all binding experiments in citrate phosphate buffer at pH 6.0 with 0.005% Tween 20. Degas running buffer to minimize air bubble formation during experiments (see Note 9).
3. Prepare peptide-receptive DR1. Pre-incubate soluble DR1 with 200 μM HA_{Y308A} peptide at 37°C for 36 h (see Notes 8, 10–12).
4. Isolate the sDR1/HA_{Y308A} complexes by size-exclusion HPLC (Superdex 200 gel filtration column; Amersham Pharmacia Biotech).
5. Collect the complex peak fractions and concentrate by centrifugal filter devices (Millipore).
6. Determine the concentration of the complex by measuring absorbance at 280 nm with the extinction coefficient of 77,000 $\text{M}^{-1} \text{cm}^{-1}$.
7. Alternatively, although not as good as fractionation by Superdex 200 gel filtration column, run sDR1/HA_{Y308A} complexes on a Sephadex G-50 spin column equilibrated in citrate phosphate buffer pH 6.0.
8. Incubate complexes at 37°C for 20 min with or without 1 μM DM.
9. Inject over the peptide-immobilized surfaces and collect association sensograms on a BIACore 2000 (Fig. 4a) (see Notes 13 and 14).

3.4.3. For Dissociation Experiment

1. Inject 9 μM DM in citrate buffer, pH 6.0, over the DR1/peptide complex surface.
2. Collect dissociation sensograms (Fig. 4b).

3.5. Detecting Interactions of DM and DR Using Intrinsic Tryptophan Fluorescence Assay

1. Generate MHC complex II/peptide complexes by incubating MHC class II molecules with an excess of specific peptides, followed by spin column separation, as above.
2. Incubate various MHC class II/peptide complexes (2 μM) at 37°C in citrate phosphate, pH 5.5, and measure the intrinsic tryptophan fluorescence of peptide/DR1 complexes alone

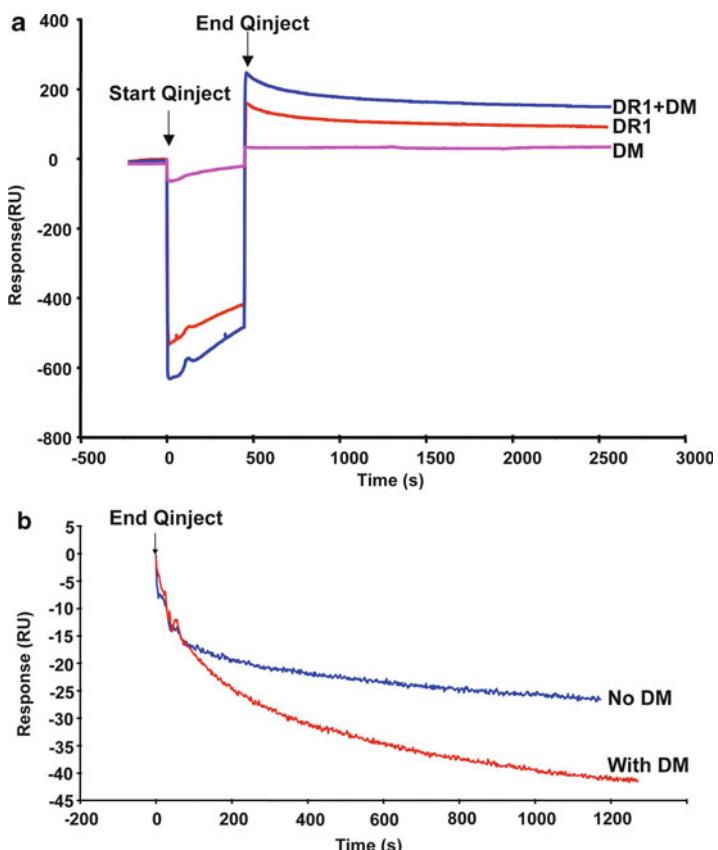


Fig. 4. Real-time binding and dissociation assay and measuring SDS stability of peptide/MHC II complexes using Biacore. (a) Detecting DR1 binding to peptides using Biacore. Cys-HA(anch) peptide was first immobilized on the surface of CM5 chip and sensograms were obtained by injection of soluble DM, DR1, and DR1 + DM (in citrate phosphate, pH 6). The binding curve is shown as RU (response unit) as a function of time. (b) Detecting dissociation of DR1 from peptide using Biacore. Cys-HA(anch) peptide was immobilized on SMPB-activated CM5 chip in a Biacore 2000. Preformed HA(Y308A) (PKAVKQNTLKLAT)/DR1 complexes were first allowed to bind on the HA(anch) peptide-immobilized surface. DM was then injected over the surface and sensograms of HA(anch)/DR1 complex dissociation were obtained in real time. The dissociation curve is shown as RU (response unit) as a function of time.

or of complexes immediately after the addition of DM (0.1–1 μ M).

3. There will be an increase in intrinsic tryptophan fluorescence upon addition of DM due to the greater probability of random interactions simply from the increase in protein concentration. Complexes refractory to DM, such as DR1/HA_{306–318}, will show this “baseline” readout. A further increase in the

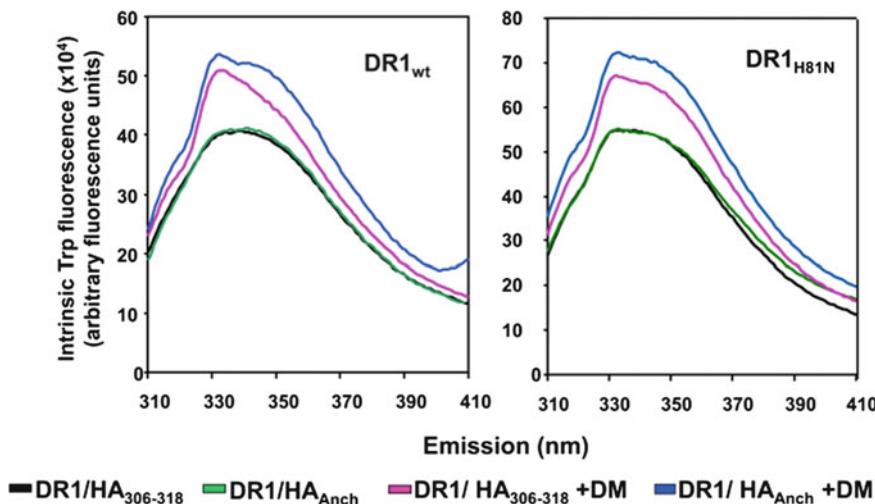


Fig. 5. Detecting the interaction of DR with DM. Wild-type DR1 and mutant DR1 (β H81N) (0.2 μ M) in complex with HA (anch) or $\text{HA}_{306-318}$ were pre-incubated in citrate phosphate, pH 5.5. Before and immediately after addition of DM, the samples were excited at 295 nm and tryptophan fluorescence of emission spectrum was monitored from 310 to 410 nm (6).

fluorescence readout, and a slight red shift in the emission peak, is indicative of specific interactions between DM and MHC class II/peptide complexes, e.g., DR1/HA(anch).

- Excite samples at 295 nm (to minimize interference from tyrosine residues) and monitor emission in a range of 310–410 nm (peak \sim 340 nm) on a temperature-controlled Fluoromax-3 fluorometer with a slit width of 5 nm (Fig. 5).

4. Notes

Protein expression and purification

- Express soluble recombinant DR1 and purify them as originally described (10). Cotransfect Baculovirus DNA (BaculoGold, BD Biosciences) and transfer vectors carrying the extracellular domain of DRB1*0101 genes into Sf9 insect cells (13) to produce recombinant viruses and infect Hi5 cells for protein production.
- Express soluble HLA-DM (DM), modified with a C-terminal FLAG epitope tag, in the same way as DR1. Purify DR1 and DM proteins from the Hy5 culture supernatant using an anti-DR1 mAb (L243) or M2 (α FLAG) mAb sepharose resin (Sigma), respectively. Elute DR1 at high pH and DM with 0.1 mg/ml FLAG peptide from the M2 (α FLAG) affinity column.

3. Upon elution concentrate via a 10 kDa cutoff centrifugal filter unit (Millipore Amicon Ultra REF#UFC901024) in PBS + 0.05% NaN₃ (pH 7.4) for DRI or Citrate Phosphate buffer + 0.05% NaN₃ (pH 6.0) for DM and store at -80°C in small aliquots.

Gentle SDS-Gel Assay and peptide binding kinetic measurements

4. In order to prevent or minimize disulfide bond formation of Cys-peptide, add fluorescein immediately after addition of chilled PBS to the peptides. Protect from light to prevent bleaching of fluorescein. Use amber-colored 0.6 ml tubes or protect samples from light.

The gel in the spin column should look uniformly moist after the first spin. At very high speed, the gel bed might dry too much and look cracked, which is what you do not want to happen.

5. Time points for the formation of SDS-Stable peptide/MHC II complexes may span from 30 min through 72 h. Generally when DM or helper peptides (12) are included the time points more than 10 h is not necessary.

BIACore experiments

6. For immobilization use Running Buffer without *Tween-20*).
7. Make sure that anything injected onto BIACore flow cell is free of particles or large aggregates. It is recommended by the company to filter everything before injection. We have used Sephadex G50 spin columns for our protein samples with success.
8. Degassing of all the buffers used is absolutely critical to avoid formation of air bubbles. We flow Argon gas to replace air in the degassed running buffer bottle.
9. Be sure that all samples to be diluted in the running buffer, otherwise, strange looking sensograms will occur.
10. Use of Tween 20 in running buffer is not mandatory, although is recommended by BIACore application scientists.
11. For binding of DR to immobilized peptide surface, special care must be taken to generate peptide-receptive conformers otherwise binding would not occur.
12. For binding to peptide surface it is recommended to have the system equilibrated to 33–37°C. Remember to warm up all your buffers to the same temperature.
13. A good control for peptide-receptive DR binding to peptide surface is to flow through a preformed DRI/H_A_{306–318} or any other peptides that form highly stable complexes with DRI, before injecting experimental samples. It is advisable to prepare parallel flow cells with similarly immobilized peptide surface to use as experimental versus control surfaces.

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References

1. Hartman IZ, Kim A, Cotter RJ, Walter K, Dalai SK et al (2010) A reductionist cell-free major histocompatibility complex class II antigen processing system identifies immunodominant epitopes. *Nat Med* 16: 1333–1340
2. Denzin LK, Cresswell P (1995) HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82:155–165
3. Sloan VS, Cameron P, Porter G, Gammon M, Amaya M et al (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375:802–806
4. Chou CL, Sadegh-Nasseri S (2000) HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J Exp Med* 192:1697–1706
5. Zarutskie JA, Busch R, Zavala-Ruiz Z, Rushe M, Mellins ED et al (2001) The kinetic basis of peptide exchange catalysis by HLA-DM. *Proc Natl Acad Sci USA* 98: 12450–12455
6. Narayan K, Chou CL, Kim A, Hartman IZ, Dalai S et al (2007) HLA-DM targets the hydrogen bond between the histidine at position beta81 and peptide to dissociate HLA-DR-peptide complexes. *Nat Immunol* 8:92–100
7. Lazarski CA, Chaves FA, Jenks SA, Wu S, Richards KA et al (2005) The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance. *Immunity* 23:29–40
8. Lazarski CA, Chaves FA, Sant AJ (2006) The impact of DM on MHC class II-restricted antigen presentation can be altered by manipulation of MHC-peptide kinetic stability. *J Exp Med* 203:1319–1328
9. Sadegh-Nasseri S, Germain RN (1991) A role for peptide in determining MHC class II structure. *Nature* 353:167–170
10. Natarajan SK, Stern LJ, Sadegh-Nasseri S (1999) Sodium dodecyl sulfate stability of HLA-DRI complexes correlates with burial of hydrophobic residues in pocket 1. *J Immunol* 162:3463–3470
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
12. Chou CL, Mirshahidi S, Su KW, Kim A, Narayan K et al (2008) Short peptide sequences mimic HLA-DM functions. *Mol Immunol* 45:1935–1943
13. Stern LJ, Wiley DC (1992) The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell* 68:465–477

Chapter 34

Study of the Allogeneic Response Induced by Endothelial Cells Expressing HLA Class II After Lentiviral Transduction

**Cécile Taflin*, Benoit Favier*, Dominique Charron,
Denis Glotz, and Nuala Mooney**

Abstract

Rejection is still the major cause of allograft loss following organ transplantation and a more complete comprehension of the alloimmune response is required in order to develop new therapeutic approaches. Allogenicity is primarily generated by the expression of major histocompatibility complex (MHC) molecules in the donor organ. Graft microvascular endothelial cells express both HLA class I and class II molecules. They are the first target of the allogeneic response because of their vascular localization and are also able to present antigen to recipient T cells. The endothelium can therefore be considered as both a stimulator of and a target for alloimmune responses and both aspects require further study. We have established a model of constitutive expression of HLA-DR by human microvascular endothelial cells following transduction with a lentiviral vector. This model was employed in a study demonstrating that endothelial cells can induce allogeneic expansion of regulatory and pro-inflammatory CD4⁺ T lymphocyte subsets. Because microvascular endothelial cells rapidly lose their expression of HLA-DR ex vivo, this experimental system of lentiviral-mediated expression of HLA-DR allows the study of alloantigen presentation without requiring addition of inflammatory cytokines and thus provides a model for the study of the intra-graft allogeneic CD4⁺ T cell response at the single-cell level.

Key words: Allogeneic response, CD4⁺ T cell subsets, Human endothelial cell, HLA class II

1. Introduction

While antigen presentation by hematopoietic cells has been extensively studied, less is known about the antigen presenting ability of non-hematopoietic cells which only express MHC class II proteins under certain circumstances such as inflammation. The heterogeneity of endothelial cells has resulted in some confusion regarding their expression of MHC class II antigens while histopathological

*Cécile Taflin and Benoit Favier made an equal contribution to this manuscript.

studies of human tissues have shown that microvascular endothelial cells constitutively express HLA-DR (1). This expression is of particular interest in the context of allograft transplantation wherein microvascular endothelial cells of the graft are the primary site of tissue damage (2–5). Indeed, endothelial cells are the first target of donor-specific antibodies leading to humoral rejection (6–9) and are also able to activate an allogeneic CD4⁺ T cell response leading to acute cellular rejection of the graft (10). In this context, we have recently studied the consequences of alloantigen presentation by HLA-DR expressing human microvascular endothelial cells under inflammatory conditions and observed proliferation of CD4⁺ T lymphocytes preceding expansion of both pro-inflammatory and regulatory memory CD4⁺ T subsets (11). The expansion of the pro-inflammatory Th17 subset was dependent on a pathway implicating IL-6 secretion by the endothelial cell and phosphorylation of STAT-3 while expansion of regulatory CD4⁺FOXP3^{bright} T cells was contact-dependent and required endothelial cell expression of CD54.

One problem relevant to microvascular endothelial cell expression of HLA-DR is that expression is downregulated ex vivo and recuperated only in the presence of inflammatory cytokines. In order to more closely reproduce physiological conditions, we established a model of microvascular endothelial cells constitutively expressing HLA-DR following lentiviral vector transduction. This allows the study of alloantigen presentation in the absence of addition of inflammatory cytokines. This model provides information about the nature of the intra-graft allogeneic CD4⁺ T cell response at the single-cell level generated by either resting microvascular endothelial cells constitutively expressing HLA-DR molecules as opposed to microvascular endothelial cells expressing HLA-DR as part of their response to inflammatory stimuli.

2. Materials

2.1. Endothelial Cells

1. HMEC-1 cells (Human dermal Microvascular Endothelial Cells transfected with a plasmid containing the coding region for the Simian Virus SV40) were used to stimulate an allogeneic CD4⁺ T cell response.
2. HMEC medium: MCDB-131 (Gibco/Invitrogen) supplemented with 15% fetal calf serum (FCS), 10 µg/ml of hydrocortisone (Sigma-Aldrich), 10 ng/ml of epidermal growth factor (Collaborative Biochemical Products Bedford), 4% of Glutamine, and 1% of Penicillin.
3. 75 and 25 cm² cell culture flasks (Falcon brand) or 96-well flat-bottom plates, without addition of extracellular matrix.

4. Solution of cell detachment solution Versene (Gibco/Invitrogen).
5. Phosphate-buffered saline (PBS), pH 7.4.
6. Recombinant IFN- γ : was reconstituted in PBS-0.1% BSA at 2 \times 10⁵ UI/ml and aliquoted at -20°C (R&D).

2.2. Lentiviral Vector Production

1. Lentiviral vectors (Addgene):
 - (a) Envelope plasmid pMD2.G.
 - (b) Packaging plasmid psPAX2.
 - (c) Expression plasmid pWPXL (see Note 1).
2. HEK293T cells were cultivated in DMEM containing 10% FCS and 1% Penicillin/Streptomycin.
3. 2.5 M CaCl₂: Dissolve 7.35 g of CaCl₂, 2H₂O in 20 ml of distilled water. Sterilize through a 0.2 μ m microfilter. Prepare aliquots and store at -20°C.
4. 2 \times HBS: 280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM Hepes, pH 7.05 precisely. Dissolve in almost 90 ml of distilled water, adjust pH to exactly 7.05 using 0.5 M NaOH and add water to a final volume of 100 ml. Sterilize by filtration through a 0.2 μ m filter. The solution is stable for several months at room temperature.
5. Amicon Ultra Millipore.

2.3. Human Allogeneic Peripheral Blood Mononuclear Cells and CD4⁺ T Cells

Blood samples from healthy volunteers should be obtained with their informed consent and in accordance with local and national guidelines and regulations. Healthy volunteers should be selected after HLA typing, with two mismatches on the HLA-DR loci compared with the endothelial cells.

1. Vacutainer blood collection tubes (sodium heparin).
 2. 50 and 15 ml Polypropylene tubes.
 3. Lymphocyte separation medium.
 4. RPMI medium.
 5. CD4⁺ T cell isolation kit (Miltenyi Biotec), columns (MS or LS), Vario MACS magnet, pre-separation filters, PBS with 0.5% bovine serum albumin (BSA), and 2 mM ethylenediaminetetraacetic acid (EDTA).
 6. CD4⁺ T medium: RPMI-1640 with 10% human AB serum (Biowest), 1% Glutamine, 1% Penicillin, and 1% Sodium Pyruvate.
-
1. Polystyrene tubes (5 ml) (Falcon brand).
 2. Fluorescence-activated cell sorting (FACS) buffer: PBS with 0.05% BSA, 10 mM Carboxyfluorescein Diacetate.

3. Succinimidyl ester (CFSE), reconstitute in DMSO, aliquots, and store at -20°C.
HMEC-1 staining: to confirm HLA-DR expression anti-HLA-DR-PhycoErythrin (PE) (Becton Dickinson).
1. Peripheral blood mononuclear cell (PBMC) and T cell staining:
 - (a) Anti-IFN- γ -FITC.
 - (b) Anti-CD45RA-PC-7.
 - (c) Anti-CD3-PerCP.
 - (d) Anti-CD4-PB and anti-CD4-PE.
 - (e) Anti-CD8-PB.
 - (f) Anti-CD3-Amcyan, anti-IL-2 or anti-IL-4-APC, anti-P-STAT-3-APC antibody (pS727).
 - (g) Anti-P-Akt-APC (pS473).
 - (h) Anti-P-ERK1/2-APC (pT202/pY204) (BD Biosciences).
 - (i) Anti-FoxP3-APC (clone PCH101; e-Bioscience).
 - (j) Anti-IL-17-APC (clone eBio64DEC17, e-Bioscience).
2. FACS Canto Cytometer (BD Biosciences) with computer interface (Macintosh or PC).
3. Data retrieval software: DIVA software (BD Biosciences) and FlowJo (TreeStar) or WinMDI version 2.8 software (The Scripps Research Institute).
4. Intracellular staining reagents:
 - (a) BD Cytofix/Cytoperm Kit for cytokine detection.
 - (b) BD Cytofix/Perm Buffer III for phospho-protein detection.
 - (c) e-Bioscience Fixation/Permeabilization kit for FoxP3 detection.
5. Polyclonal stimulation for cytokine detection:
 - (a) Phorbol 12-Myristate 13-Acetate (PMA) was dissolved in DMSO at 1 mg/ml.
 - (b) 1 mM Ionomycin was dissolved in Ethanol (Sigma-Aldrich).
 - (c) Golgi stop (BD Biosciences).

3. Methods

3.1. Endothelial Cell Culture

1. HMEC-1 cells were added to flasks at a density of 5,000 cells/cm² with 1 ml of HMEC medium per 5 cm² of flask area. Renew medium every 3 days.

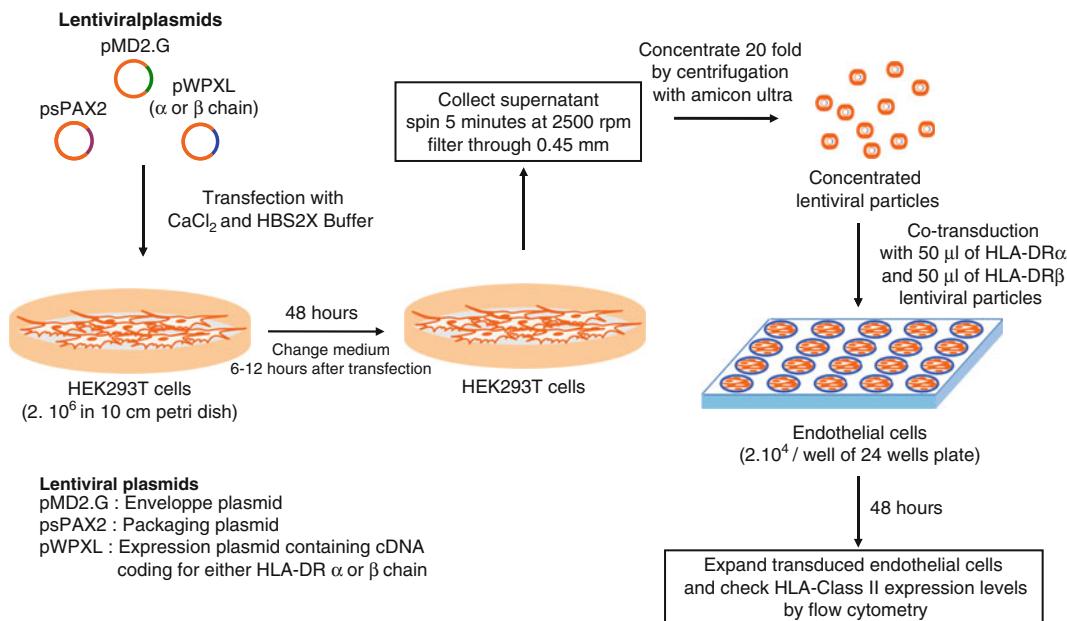


Fig. 1. Production of HLA-DR α and β lentiviral particles for transduction of microvascular endothelial cells. HEK293T cells were co-transfected with PMD2.G, psPAX2, and pWPXL (containing either HLA-DR α or β chain cDNA) lentiviral plasmids. Medium is changed after 6–12 h and the lentiviral particle-containing supernatant is collected after 48 h, centrifuged, and filtered before concentrating. Concentrated lentiviral particles containing HLA-DR α and β cDNA are simultaneously added to microvascular endothelial cells in 24-well plates (see right). After 48 h of incubation, transduced microvascular endothelial cells are expanded and surface HLA-DR expression molecules is measured by flow cytometry.

2. Subculture when cells reach 80% of confluence: thoroughly wash with PBS and incubate cells with 1 ml of Versen per 15 cm² of flask area at 37°C for 7 min (see Note 2). Collect the cells in a 15 ml polypropylene tube and wash twice with HMEC medium at 300× μ g for 5 min. Determine cell number and replate for cell culture (see step 1).

3. HMECs can be used until the 20th subculture.

3.2. Lentivirus Production and Endothelial Cell Transduction (Fig. 1)

3.2.1. Production of Lentiviral Vectors in HEK293T Cells

- On Day 1, add 2× 10⁶ HEK293T cells per 10 cm² plate with 6 ml of HEK medium and incubate overnight at 37°C, 5% CO₂.
- On Day 2: transfection of plasmid DNA:
 - Prepare a mix of DNA containing per plate: 3.8 µg psPAX2, 1.3 µg pMD2.G, and 5 µg pWPXL.
 - Add 50 µl of 2.5 M CaCl₂ and complete with H₂O to a final volume of 500 µl.
 - Add this solution to 500 µl 2× HBS drop-by-drop and simultaneously pass bubbles through the solution using a Pasteur pipette.

- (d) Incubate for 20 min at room temperature.
 - (e) Gently add this solution to HEK293T cells and gently shake the petri dish from right to left and back and forward before incubating overnight at 37°C, 5% CO₂.
 - (f) Change medium 6 to 12 h after transfection.
3. On Day 4: Collect and concentrate lentiviral supernatant:
- (a) Collect and spin the supernatant at 1,250 × g for 5 min at room temperature in order to remove cell debris.
 - (b) Filter through a 0.45 µm microfilter.
 - (c) Concentrate the lentiviral supernatant (20-fold) using an Amicon ultra Millipore.
4. Aliquot concentrated lentiviral supernatants and store at -80°C (do not repeatedly thaw and freeze aliquots).

3.2.2. Transduction of Endothelial Cells with Lentiviral Particles

1. The day before transduction, HMEC cells were added to a 24-well plate at a concentration of 20,000 cells/well in HMEC medium (1 ml final/well).
2. On the day of transduction remove 500 µl of medium and add 50 µl of concentrated lentiviral particles to endothelial cells (see Note 3).
3. Put the plate in the incubator at 37°C with 5% CO₂.
4. After 48 h change the medium.
5. Check the efficiency of transduction of endothelial cells by flow cytometry with an anti-HLA-DR antibody (e.g., clone L243) and expand HLA Class II⁺ endothelial cells.

3.3. Isolation of PBMCs and CD4⁺ T Cells

1. Collect blood samples in heparinized tubes and dilute 1:1 with RPMI.
Isolate PBMCs according to a standard protocol using lymphocyte separation medium density-gradient centrifugation. (Either freshly isolated or thawed, cryopreserved PBMCs may be used.)
2. Wash twice with RPMI medium and determine cell number.
3. Isolate CD4⁺ T cells by negative selection using the CD4⁺ T cell isolation kit for magnetic separation following the manufacturer's instructions.
4. Collect the effluent fraction which contains the enriched CD4⁺ T cells, wash twice with RPMI medium, and count cells.
5. After washing the PBMCs twice with buffer, add 5 µl of CD4⁺ T cell biotin-antibody cocktail with 20 µl of buffer per 10⁷ cells and incubate at 4°C for 10 min.
6. Add 10 µl of anti-biotin microbeads and 15 µl of buffer per 10⁷ cells for an additional incubation at 4°C for 15 min.

7. Wash cells with buffer and pass through a MACS magnet using LS or MS column (depending on cell number).

3.4. In Vitro Alloactivation of PBMCs and CD4⁺ T Cells by Endothelial Cells

1. Plate HMECs expressing HLA-DR (transduced HMECsαβ or HMECs activated by 72 h of IFN-γ treatment (100 UI/ml)) in 96-flat-bottom plates at a density of 5×10^4 /well and culture until confluent.
2. Irradiate (20 Gy) HMEC cells and wash the plate twice with PBS before co-culture (see Note 4).
3. Count allogeneic PBMC or purified CD4⁺ T cells.
4. Label half of PBMCs or purified CD4⁺ T cells with 1 μM CFSE: wash the cells twice with RPMI and incubate them for 15 min at 37°C in pre-warmed RPMI containing 1 μM of CFSE (see Note 5). Wash cells twice with RPMI and finally resuspend in RPMI-10% human AB serum.
5. Count final cell number, dilute cells to a concentration of 0.25×10^6 /ml in RPMI-10% human AB serum, and add 200 μl of the cell suspension per well of the 96-well plates containing irradiated endothelial cells (ratio of 1:1, see Note 6).
6. Co-culture for 7 days without adding exogenous cytokines.

3.5. Analysis of CD4⁺ T Cell Alloresponse

3.5.1. Alloproliferation of Memory CD4⁺ T Cells and Regulatory T cells

After 7 days of co-culture, collect PBMCs or CD4⁺ T cells in 5 ml polystyrene tubes for analysis by flow cytometry.

1. Use CFSE-labeled cells prepared as described in Subheading 3.4, step 4.
2. Wash cells twice with FACS buffer.
3. Stain cell pellet with the following mixture of antibodies at 4°C for 20 min: anti-CD3-Amcyan (3 μl), anti-CD4-PB (3 μl), anti-CD45RA-PC-7 (3 μl).
4. Wash cells and fix by addition of 500 μl of e-Bioscience fixation buffer for 30 min at 4°C.
5. Wash cells once with FACS buffer and once with 1 ml of e-Bio-science permeabilization buffer.
6. Stain cells by adding 3 μl of the anti-FoxP3-APC antibody to the pellet for 30 min at 4°C.
7. Wash and resuspend cells in 200 μl of FACS buffer before FACS analysis.
8. Before recording data for a sample, optimize the PMT voltages, compensations, and threshold settings for the cell type and fluorochrome:
 - (a) Create compensation controls with an unstained control sample and with samples for each fluorochrome used: CFSE (see Note 7), PC-7, APC, PB, and Amcyan.

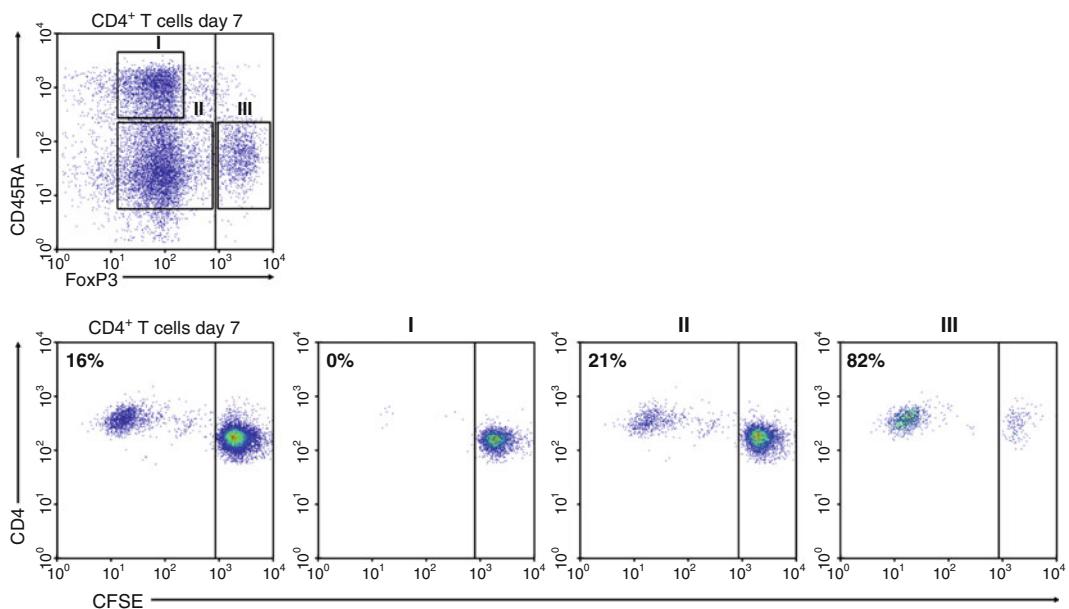


Fig. 2. Alloproliferation of CD4⁺ T cell populations induced by endothelial cells. The *top density plot* illustrates the proportion of naive (I, CD45RA⁺FoxP3⁻), memory (II, CD45RA⁻FoxP3⁻ or low), and Treg (III, CD45RA⁻FoxP3^{bright}) cells within the total CD4⁺ T cell population from an allogeneic donor, after 7 days of culture with endothelial cells. The *bottom density plots* show the proliferation (percentage of CFSE⁻ cells in total CD4⁺ T cells) of naive, memory, and regulatory CD4⁺ T cell subsets in one allogeneic donor after 7 days of co-culture with endothelial cells.

(b) Use the unstained cell control sample to adjust the FSC, SSC, voltages, and optimize each fluorescent voltage so that the negative population is situated within the first log decade.

(c) Record data for each single-stained control after adjusting the gate around the positive population and automatically calculate the compensation.

9. For each sample: gate on CD4⁺ T cells (CD3⁺CD4⁺).
10. Determine the proportion of memory CD4⁺ T cells (CD45RA⁻FoxP3⁻ or low), naive CD4⁺ T cells (CD45RA⁺FoxP3⁻), Treg cells (CD45RA⁻FoxP3^{bright}), and their respective proliferation (CFSE⁻) (Fig. 2).

3.5.2. Cytokine Secretion

1. Use the non-CFSE-labeled cells prepared as described in Subheading 3.4, step 4.
2. Stimulate cells with 20 ng/ml of PMA, 1 µM of Ionomycin in the presence of 1/1,500 Golgi stop in a total volume of 200 µl of RPMI-10% AB serum per tube for 4 h at 37°C.
3. Wash the cells twice with FACS buffer and add the following mixture of antibodies to the pellet at 4°C for 20 min for cell

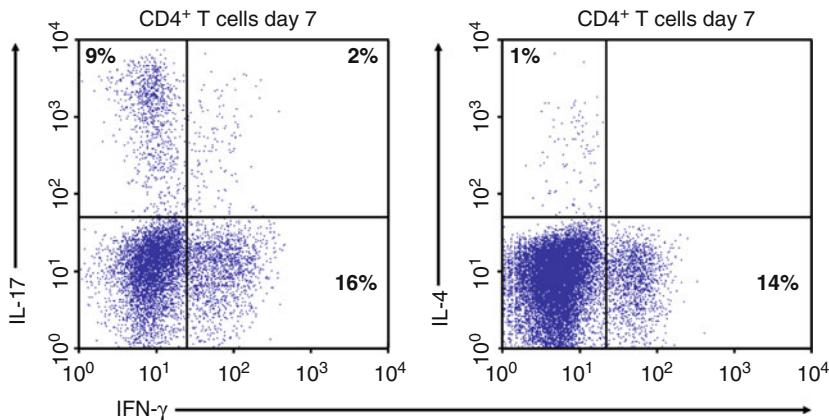


Fig. 3. Cytokine secretion by CD4⁺ T cells alloactivated by endothelial cells. Allogeneic CD4⁺ T cells were harvested after 7 days of culture with endothelial cells and stimulated for 4 h with PMA and ionomycin in the presence of Golgi stop before intracellular cytokine staining. The density plots are gated on CD4⁺ T cells and illustrate the proportion of Th1 cells (IFN- γ ⁺IL-17⁻), Th17 cells (IL-17⁺, IFN- γ or ⁺) (left plots), and Th2 cells (IL-4⁺IFN- γ) (right plots).

surface staining: anti-CD3-PerCP (3 μ l), anti-CD8-PB (3 μ l), anti-CD4-PE (3 μ l) (see Note 8) corrected.

4. After washing cells, fix with 100 μ l of BD Cytofix for 10 min at 37°C.
5. Wash cells once with FACS buffer and once with 1 ml of BD Cytoperm.
6. Stain cells with 3 μ l of the following antibodies: anti-IFN- γ -FITC and anti-IL-17-APC or anti-IFN- γ -FITC and anti-IL-4-APC or anti-IFN- γ -FITC and anti-IL-2-APC.
7. Wash cells and resuspend in 200 μ l of FACS buffer before FACS analysis.
8. Create compensation controls with an unstained control sample and with samples for each fluorochrome used: FITC, PE, PerCP, APC, and PB.
9. For each sample: gate on CD4⁺ T cells (CD3⁺CD4⁺ or CD3⁺CD8⁻).
10. Determine the proportion of Th1 cells (IFN- γ ⁺IL-17⁻), Th2 cells (IL-4⁺IFN- γ), and Th17 cells (IL-17⁺, IFN- γ or ⁺) (Fig. 3).

3.5.3. CD4⁺ T Cell Signaling Pathways

1. Collect CFSE-labeled cells.
2. Fix cells immediately by adding an equal volume of pre-warmed (37°C) BD Cytofix buffer to the cell suspension: approximately 200 μ l for 200 μ l of harvested cells (see Note 9). Incubate cells for 10 min at 37°C.

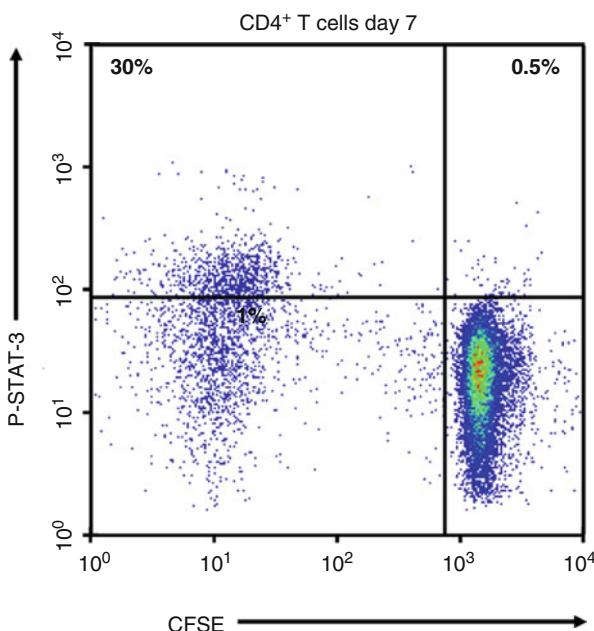


Fig. 4. Phosphorylation of STAT-3 within alloproliferating CD4⁺ T cells. Intracellular detection of P-STAT-3 was determined on fixed and permeabilized CFSE-labeled CD4⁺ T cells harvested immediately after 7 days of activation with endothelial cells. The density plot illustrates the phosphorylation of STAT-3 within proliferating (CFSE⁻) or nonproliferating (CFSE⁺) CD4⁺ T cells.

3. Wash the cells with FACS buffer.
4. Permeabilize cells by adding 500 µl of BD Phosflow Perm Buffer III and incubating for 30 min at 4°C.
5. Wash cells twice with FACS buffer and stain by adding 3 µl of the following antibodies: anti-CD45RA-PC-7, anti-CD4-PB, anti-CD3-Amcyan, anti-P-STAT-3-APC, or anti-P-ERK1/2-APC or anti-P-Akt-APC.
6. Incubate cells at room temperature for 30 min in the dark.
7. Wash cells and resuspend in 200 µl of FACS buffer before FACS analysis.
8. Create compensation controls with an unstained control sample and with samples for each fluorochrome used: CFSE, PC-7, APC, and PB.
9. For each sample: gate on CD4⁺ T cells (CD3⁺CD4⁺).
10. Determine the proportion of phospho-protein (P-STAT-3, P-ERK1/2, P-Akt) in proliferating CD4⁺ T cells (CFSE⁻) and in nonproliferating CD4⁺ T cells (CFSE⁺) (Fig. 4).

4. Notes

1. The cDNA coding for either HLA-DR α or HLA-DR β were cloned into pWPXL lentiviral vector (Addgene, Cambridge, USA) by replacing GFP cDNA using BamHI and NdeI cloning sites.
2. During cell subculture, if the endothelial cells do not readily detach from the plate, rinse again with PBS in order to completely remove growth factors and serum and incubate with Versen for up to 15 min at 37°C. Scraping is not recommended because it will damage the cells. Versen is preferred to Trypsin for this type of endothelial cell as it is less toxic and allows subculturing to a later passage.
3. Since HLA class II molecules are composed of two chains (alpha and beta), endothelial cells are transduced simultaneously with 50 μ l of each lentiviral construct.
4. The endothelial cell monolayer should be washed with PBS just before adding CD4⁺ T cells, otherwise the cell monolayer will detach.
5. The CFSE concentration used in proliferation assays should not exceed 1 μ M otherwise the DMSO diluent will be toxic and inhibit CD4⁺ T cell proliferation.
6. The optimal ratio for endothelial cell-induced CD4⁺ T cell alloproliferation is 1 stimulator to 1 responder. The minimum number of responder CD4⁺ T cells should be 5×10^5 /well in order to ensure cell contact in flat-bottom wells and to provide a critical number of responder T cell which is required for optimal activation.
7. The control CFSE sample used for compensation setting is control CFSE-labeled CD4⁺ T cells or PBMCs cultured during the same 7-day period as the responder cells.
8. Polyclonal stimulation with PMA downregulates CD4 expression. Therefore CD4⁺ T cell cytokine secretion is determined within the CD3⁺CD8⁻ population, using CD3 and CD8 as markers.
9. The cells should be immediately fixed by directly adding an equal volume of pre-warmed BD cytofix to the cell suspension in order to maintain phosphorylation.

Acknowledgements

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References

1. Muczynski KA, Ekle DM, Coder DM, Anderson SK (2003) Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression. *J Am Soc Nephrol* 14:1336–1348
2. Trpkov K, Campbell P, Pazderka F, Cockfield S, Solez K, Halloran PF (1996) Pathologic features of acute renal allograft rejection associated with donor-specific antibody, analysis using the Banff grading schema. *Transplantation* 61:1586–1592
3. Halloran PF, Wadgymar A, Ritchie S, Falk J, Solez K, Srinivasa NS (1990) The significance of the anti-class I antibody response. I. Clinical and pathologic features of anti-class I-mediated rejection. *Transplantation* 49:85–91
4. Halloran PF, Schlaut J, Solez K, Srinivasa NS (1992) The significance of the anti-class I response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. *Transplantation* 53:550–555
5. Racusen LC, Colvin RB, Solez K, Mihatsch MJ, Halloran PF, Campbell PM, Cecka MJ, Cosyns JP, Demetris AJ, Fishbein MC, Foggo A, Furness P, Gibson IW, Glotz D, Hayry P, Hunsicker L, Kashgarian M, Kerman R, Magil AJ, Montgomery R, Morozumi K, Nickeleit V, Randhawa P, Regele H, Seron D, Seshan S, Sund S, Trpkov K (2003) Antibody-mediated rejection criteria - an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant* 3:708–714
6. Susal C, Opelz G (2002) Kidney graft failure and presensitization against HLA class I and class II antigens. *Transplantation* 73:1269–1273
7. Gloor J, Cosio F, Lager DJ, Stegall MD (2008) The spectrum of antibody-mediated renal allograft injury: implications for treatment. *Am J Transplant* 8:1367–1373
8. Campos EF, Tedesco-Silva H, Machado PG, Franco M, Medina-Pestana JO, Gerbase-DeLima M (2006) Post-transplant anti-HLA class II antibodies as risk factor for late kidney allograft failure. *Am J Transplant* 6:2316–2320
9. Langan LL, Park LP, Hughes TL, Irish A, Luxton G, Witt CS, Christiansen FT (2007) Post-transplant HLA class II antibodies and high soluble CD30 levels are independently associated with poor kidney graft survival. *Am J Transplant* 7:847–856
10. Shiao SL, Kirkiles-Smith NC, Shepherd BR, McNiff JM, Carr EJ, Pober JS (2007) Human effector memory CD4+ T cells directly recognize allogeneic endothelial cells in vitro and in vivo. *J Immunol* 179:4397–4404
11. Taflin C, Favier B, Baudhuin J, Savenay A, Hemon P, Bensussan A, Charron D, Glotz D, Mooney N (2011) Human endothelial cells generate Th17 and regulatory T cells under inflammatory conditions. *Proc Natl Acad Sci U S A* 108(7):2891–2896

Chapter 35

Antigen Processing for MHC Presentation via Macroautophagy

Monique Gannage, Rosa Barreira da Silva, and Christian Münz

Abstract

Macroautophagy has recently emerged as an important catabolic process involved not only in innate immunity but also in adaptive immunity. Initially described to deliver intracellular antigens to MHC class II loading compartments, its molecular machinery has now also been described to enhance the delivery of extracellular antigens to MHC class II loading compartments by accelerating phagosome maturation. Therefore in pathological situations (viral or bacterial infections, tumorigenesis) the pathway might be involved in shaping CD4⁺ T cell responses.

In this chapter we describe three basic experiments for the monitoring and manipulation of macroautophagic antigen processing towards MHC class II presentation. Firstly, we will discuss how to monitor autophagic flux and autophagosome fusion with MHC class II loading compartments. Secondly, we will show how to target proteins to autophagosomes in order to monitor macroautophagy-dependent antigen processing via their enhanced presentation on MHC class II molecules to CD4⁺ T cells. And finally, we will describe how macroautophagy can be silenced in antigen presenting cells, like human monocyte-derived dendritic cells (DCs).

Key words: Macroautophagy, CD4⁺ T cells, MHC class II loading compartment

1. Introduction

Since antigen processing for MHC presentation to T cells relies on cellular proteolytic systems, like lysosomes for MHC class II and proteasomes for MHC class I presentation (1, 2), cellular transport pathways that deliver proteins to these catabolic machineries can contribute to immune surveillance by T cells. Autophagy is no exception. It describes at least three pathways, by which cytoplasmic constituents gain access to lysosomal degradation. These are chaperone-mediated, micro- and macroautophagy (3). Both chaperone-mediated and microautophagy rely on substrate recognition

by HSC70 chaperones, and proteins selected for it carry a pentameric recognition sequence (4, 5). For chaperone-mediated autophagy HSC70 docks to LAMP2a in the lysosomal membrane and the substrates are then translocated across this membrane with the help of luminal HSP70 chaperones. For microautophagy, substrate carrying HSC70 molecules attach to late endosomal membranes, which then bud into the endosomal lumen, and endosome–lysosome fusion then delivers this cargo for degradation. While chaperone-mediated and microautophagy degrade only soluble proteins, macroautophagy is able to deliver larger protein aggregates and whole cell organelles for lysosomal degradation (6). For this purpose a cup-shaped isolation membrane forms around the autophagic cargo to finally engulf it completely in a double membrane surrounded autophagosome. This autophagosome then fuses with lysosomes for degradation of its content and the inner autophagosomal membrane. This process requires more than 30 so-called autophagy related genes (atgs), which were originally described in yeast, but are now more and more also identified in higher eukaryotes (7). Of these, we will only discuss two complexes in this chapter, which are relevant for the described experiments, while a more complete description has been given in a recent review (8). The two Atg complexes that are primarily targeted for macroautophagy monitoring and manipulation, center around the two ubiquitin-like molecules Atg8 with its main mammalian homologue LC3 and Atg12. Atg12 gets conjugated to Atg5 by the two E1- and E2-like enzymes Atg7 and 10. The resulting conjugate then associates with Atg16L1 at the outer autophagosomal membrane. This complex catalyzes ligation of Atg8 to phosphatidylethanolamine (PE), a phospholipid in the autophagome membrane. Prior to this coupling, Atg8 gets cleaved by Atg4 to liberate a C-terminal glycine residue, which conjugates to PE, and is activated by the E1- and E2-like enzymes Atg7 and Atg3. Atg8 is thought to facilitate lipid fusion during isolation membrane generation (9) and substrate recruitment (10). While the Atg5/12/16 L1 complex and Atg8 are recycled from the outer autophagosome membrane upon vesicle completion, Atg8 stays attached to the inner autophagosomal membrane and gets degraded with it after fusion with lysosomes. This can be monitored for autophagosome visualization and turn-over. Furthermore, primarily Atg5 and 7 are targeted to inhibit macroautophagy.

Immunologists are mainly interested in macroautophagy for three reasons. It can degrade intracellular pathogens (11), modulates pathogen detection (12) and it assists in antigen processing for MHC presentation (13). Macroautophagy facilitates MHC presentation to T cells mainly via three pathways. It directly imports antigens into MHC class II loading compartments (14), it enhances exogenous antigen delivery for MHC class II presentation (15) and augments antigen packaging in antigen donor cells for efficient

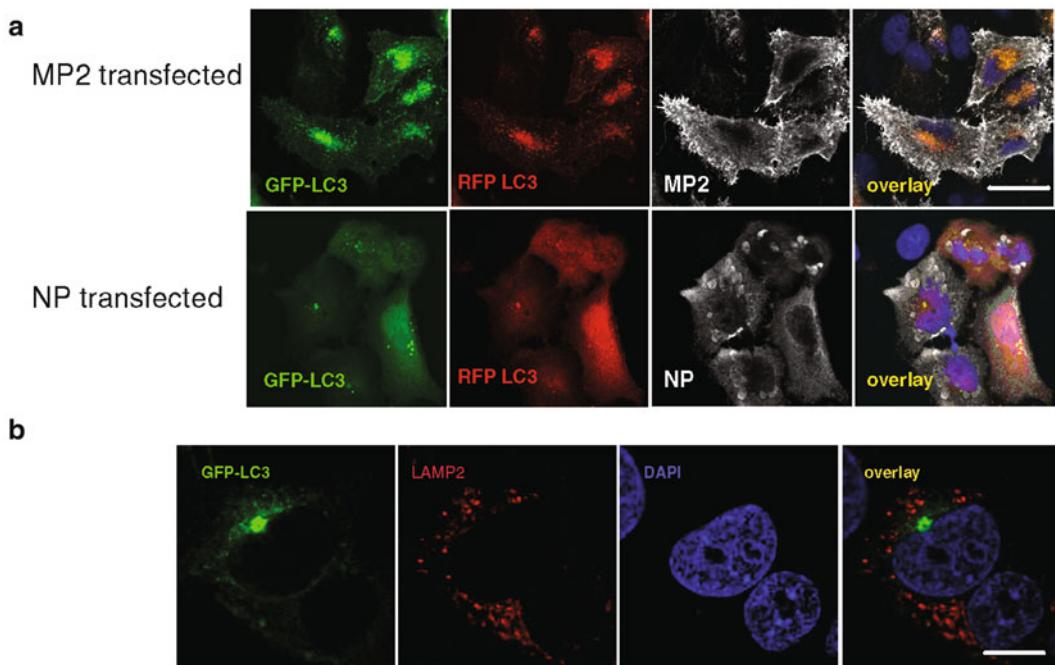


Fig. 1. The mRFP–GFP–LC3 reporter construct was used to monitor autophagosomes turnover. Lysosomal turnover of autophagosomes was impaired in influenza virus MP2-transfected cells, and autophagosomes did not colocalize with MHC class II loading compartment after influenza A virus infection. **(a)** The tandem reporter construct mRFP–GFP–Atg8/LC3 was transiently co-transfected with influenza A virus matrix protein 2 (MP2) or nucleoprotein (NP) into A549 human lung epithelial cells. GFP (sensitive to acidification and lysosomal degradation) and RFP fluorescence (insensitive to acidification and lysosomal degradation) of the reporter construct were analyzed by fluorescence microscopy. DAPI was used to stain nuclear DNA. Scale bar: 60 μ m. One of three experiments is shown. The GFP moiety of the tandem construct is sensitive to lysosomal proteolysis and quenching in acidic pH, while the mRFP is not. Therefore, the green fluorescent component of the composite yellow fluorescence for the mRFP–GFP–LC3 reporter was lost upon autophagosome fusion with lysosomes. This fluorescence change from yellow to red can be used to visualize lysosomal proteolysis and localization in acidified compartments of macroautophagy-targeted GFP. *In NP transfected lung epithelial cells* few yellow autophagosomes, but a high number of mRFP positive autolysosomes could be detected after transient transfection of mRFP–GFP–Atg8/LC3. *In MP2 transfected cells* in contrast, there is an accumulation of mRFP and GFP double positive vesicles, especially in the perinuclear region, suggesting impaired autophagosome fusion with lysosomes. **(b)** HLA-DR4⁺ HEK 293 cells were infected with influenza A virus for 24 h, cells were then fixed and stained with antibodies specific for MHC class II loading compartments (*in the upper panel* LAMP2). Autophagosomes were excluded from LAMP2 positive compartments.

cross-presentation (16, 17). Intracellular antigen processing via macroautophagy might account for 20–30% of natural MHC class II ligands that are derived from cytosolic or nuclear proteins (18), including self-protein-derived peptides that are involved in positive and negative selection of CD4⁺ T cells in the thymus (19). In addition to self-proteins, few viral and bacterial antigens have been reported to be processed via macroautophagy for MHC class II presentation (20–23). For the monitoring of this pathway we will describe three experiments. *Firstly*, we will discuss how the tandem GFP-RFP-Atg8/LC3 construct can be used to monitor autophagic flux and autophagosome fusion with MHC class II loading compartments (Fig. 1). *Secondly*, we will show how Atg8/LC3 fusion

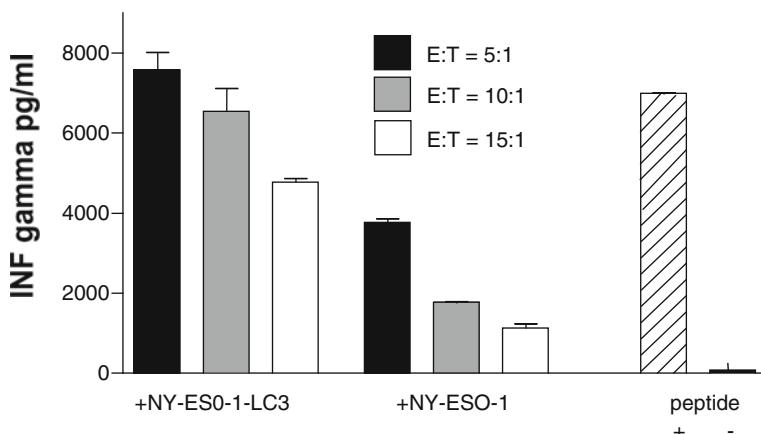


Fig. 2. MHC class II presentation assay using LC3-targeted antigen: example of NY-ESO-LC3 (unpublished). (a) NY-ESO-1-specific clonal CD4⁺ T cells were cocultured at various effector to target cell (E:T = 5:1, black bars; E:T = 10:1, gray bars; E:T = 15:1, white bars) ratios with the M199 melanoma cell line transfected with either NY-ESO-1 or NY-ESO-1-LC3 constructs. The next day, IFN- γ was measured in culture supernatants by ELISA to assess antigen presentation of NY-ESO-1 on MHC class II molecules. MHC class II presentation of the cognate NY-ESO-1 epitope was significantly enhanced by the Atg8/LC3 fusion. *Error bars* indicate standard deviations. One of three experiments is shown. Peptide pulsed targets (striped bars) and non-pulsed targets (far right) were used as positive and negative controls.

proteins can be used to monitor macroautophagy-dependent antigen processing via their enhanced presentation on MHC class II molecules to CD4⁺ T cells (Fig. 2). And finally, we will describe how macroautophagy can be silenced in antigen presenting cells, like human monocyte-derived dendritic cells (DCs), via Atg-specific siRNA (Fig. 3). These basic experiments allow for the monitoring and manipulation of macroautophagic antigen processing towards MHC class II presentation. They characterize this specific antigen processing pathway and the level of macroautophagy in antigen presenting cells in general.

2. Materials

2.1. Cell Culture

2.1.1. Cell Lines

(See Note 1)

1. Human embryonic kidney epithelial cell line, HLA-DR4⁺ HEK 293 (obtained from Dr. Rong-Fu Wang, Houston, Texas).
2. Human lung epithelium cell line A549 (obtained from Dr. Thomas Moran, New York, NY).
3. Human melanoma cell line M199 (obtained from Dr. Jean-Francois Fonteneau, Nantes, France).
4. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM, Gibco,) with 10% fetal bovine serum (FBS), 2 mM glutamine, 110 μ g/ml sodium pyruvate, and 2 μ g/ml gentamicin (Gibco).

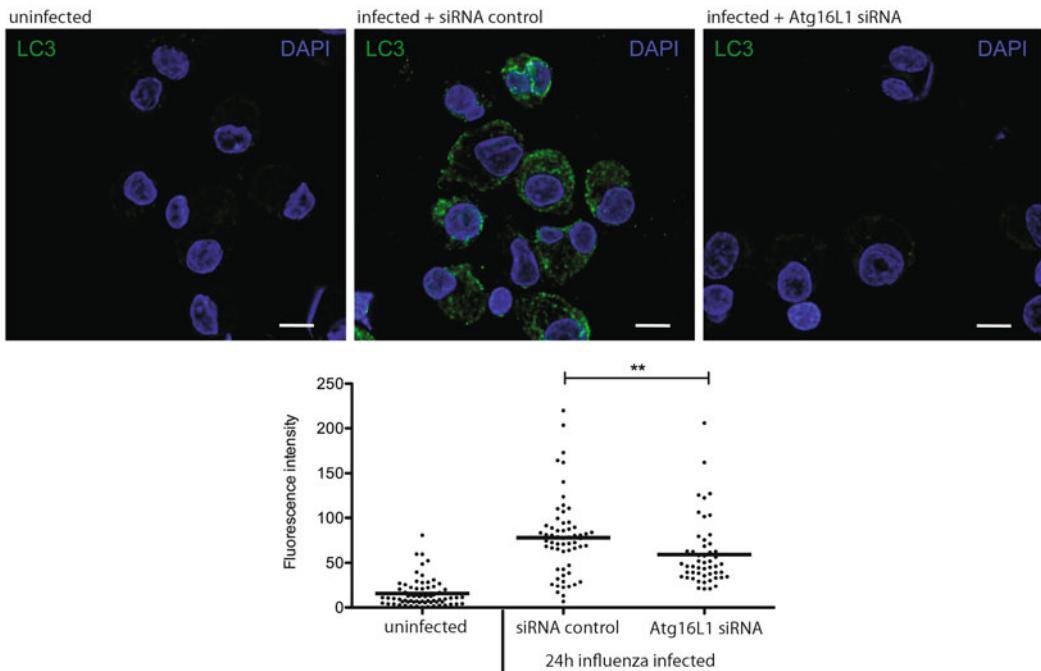


Fig. 3. Silencing macroautophagy in monocyte-derived dendritic cells. Immunofluorescence analysis of autophagosome accumulation (LC3) in immature DCs and influenza virus-infected DCs pretreated with Atg16L1 siRNA or control scrambled siRNA: Immature DCs were pretreated at day 4 with siRNA against Atg16L1 or with a control siRNA. At day 5, cells were infected with 0.1 HA unit of influenza A virus (PR8). At day 6, cells were harvested and fixed for immunostaining with a LC3-specific antibody. Upon treatment with Atg16L1 siRNA the accumulation of autophagosomes after influenza A virus infection was down-regulated. One experiment out of two is shown. Scale bars: 10 μ m.

2.1.2. Human Dendritic Cells

- PBMCs are isolated from leukocyte concentrates (Zurich Blood Center) by density-gradient centrifugation on Ficoll-Hypaque. CD14⁺ monocytes/macrophages are isolated by positive magnetic cell separation (Miltenyi Biotec) and differentiated to monocyte-derived DCs with IL-4 and GM-CSF and matured with appropriate stimuli as described in Subheading 3.1.2.
- Dendritic cell culture medium: RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen) and 2 μ g/ml gentamicin (Gibco).

2.1.3. Cytokines

- Recombinant human interferon-gamma (IFN- γ) (Peprotech) is reconstituted in sterile H₂O + 0.1% human serum albumin (HSA, Sigma) to a concentration of 10 ng/ μ l and frozen aliquots are kept at -20°C.
- Recombinant human interleukin-4 (IL-4) (Peprotech) is reconstituted to a concentration of 20 ng/ μ l and frozen aliquots are kept at -20°C.
- Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Invitrogen) is reconstituted to a concentration of 20 ng/ μ l and frozen aliquots are kept at -20°C.

2.2. Antibodies

2.2.1. Primary Antibodies

1. Maturation of monocyte-derived DCs is monitored by flow cytometry using anti-CD11c, -CD83, -CD86, -HLA-DR, and HLA-ABC antibodies (BD Biosciences).
2. Expression of MHC class II molecules:
 - (a) The mouse monoclonal HLA-DR/DP/DQ-specific hybridoma IVA12 (ATCC). The hybridoma is grown in RPMI-1640 with 10% FBS, 2 mM glutamine, 2 µg/ml gentamicin, and supernatant is harvested by spinning cells down at $300 \times g$ for 10 min. Supernatant is filtered through a 0.2-µm filter and stored at 4°C. Use at a 1:10 dilution.
 - (b) A mouse monoclonal antibody against human LAMP2 (lysosomal-associated membrane protein 2) (clone H4B4) antibody (eBioscience) is used at 0.5 µg/ml.
 - (c) A mouse monoclonal antibody against HLA-DR molecules (clone L243) (eBioscience) is used at 1 µg/ml.
3. Expression of LC3 is monitored with:
 - (a) Rabbit polyclonal anti-LC3 antibody (MBL) use at 1:1'000.
 - (b) Mouse monoclonal anti-LC3 antibody (Clone 5 F10) (Nanotools) use at 1:500.

2.2.2. Secondary Antibodies

1. Rhodamine-Red-X (RRX)-conjugated or Cy-3-conjugated donkey anti-mouse (Jackson ImmunoResearch).
2. Alexa fluor-555 conjugated goat anti-mouse: to be used at 1:500 dilution.
3. Alexa fluor-488 conjugated goat anti-rabbit: to be used at 1:500 dilution.
4. Alexa fluor-647 conjugated rat anti-mouse: to be used at 1:500 dilution.
5. Alexa fluor-647 conjugated goat anti-mouse: to be used at 1:500 dilution.

2.3. Expression of NY-ESO-LC3 Fusion Construct and the RFP-GFP-LC3 Tandem Construct by Transient Transfection

1. Plasmids DNA.

- NY-ESO/LC3 fusion construct is obtained by inserting the human Atg8/LC3 cDNA sequence (genebank entry NM_022818) and the human NY-ESO-1 cDNA sequence NM_001327.1 into the mammalian expression vector pEGFP-C2 (BD, Biosciences).
- NY-ESO-1 (pCMV6-XL4) (Origene) expression vector encoding for the human NY-ESO-1 is used in parallel as a control.
- The fusion mRFP–GFP tandem fluorescent LC3 (tfLC3) construct is obtained from Dr. Tamotsu Yoshimori (Osaka, Japan).

- For better transfection results, maxipreps of the different DNA plasmids should be prepared and DNA should be eluted in sterile DPBS. DNA concentration and purity is determined by OD260/OD280 absorption spectroscopy in a spectrophotometer. OD260/OD280 ratio should be greater than 1.8. DNA and is stored in aliquots at -20°C.

2. Transient transfection medium: cell culture medium without gentamicin (see Note 2).
3. Transfecting reagent: Lipofectamine 2000 (Invitrogen).
4. Transfecting medium: OptiMEM-I (Gibco) without Phenol Red.

2.4. Immunocyto-chemistry and Confocal Microscopy to Analyze MHC Class II Compartments

1. Circular 1.5-mm cover slips (Fisher) (see Note 3).
2. 70% (v/v) Ethanol.
3. Sterile DPBS.
4. Chloroquine (CQ) (Sigma) the stock is prepared in water at a concentration of 20 mM, and frozen aliquots are stored at -20°C, the working concentration is 50 µM (i.e., dilute the stock to 1:400).
5. Fixative solution 4% paraformaldehyde.
6. Permeabilization solution: 0.1% (v/v) Triton-X 100 in PBS.
7. Blocking buffer: PBS, 10% normal goat or donkey serum (NDS or NGS from Sigma), 0.1% saponin (Calbiochem) (see Note 4).
8. Staining buffer: PBS, 5% normal goat or donkey serum (NGS or NDS from Sigma), 0.1% saponin (Calbiochem).
9. Washing buffer: 1× PBS.
10. DAPI nucleic acid staining solution: Prepare a 5 mg/ml stock of 4,6-diamidino-2-phenylindole (DAPI, Invitrogen-Molecular Probes) in ddH₂O and store in aliquots at -20°C. Dilute 1:10'000 in PBS to freshly prepare a working solution.
11. Mounting medium: Prolong Gold Antifade Reagent (Invitrogen-Molecular Probes).
12. Confocal laser scanning microscope, using a high N.A. oil immersion lens (e.g., 63×/1.4 N.A.).
13. ImageJ software.

2.5. MHC Class II Presentation Assay Using an NY-ESO-1-Specific CD4⁺ T-Cell Clone

1. An NY-ESO-1-specific CD4⁺ T-cell clone for the 157–170 epitope of this tumor antigen (obtained from Dr. Jean-Francois Fonteneau, Nantes, France) is cultured in RPMI-1640 with 8% pooled human serum (PHS, Mediatech, Inc.), with 450 U/ml recombinant human IL-2 (Peprotech), 2 mM glutamine, 2 µg/ml gentamicin in round-bottom 96-well plates (see Note 5).

2. Recombinant human IFN- γ (Peprotech) as in Subheading 2.1.3.
3. Coculture medium: RPMI-1640 with 5% PHS, 2 mM glutamine, and 2 μ g/ml gentamicin.
4. As positive control the following stimuli is used: specific NY-ESO-1 peptide (1 mM stock in 10% DMSO, for T-cell stimulation dilute 1:1'000 in coculture medium) or phytohemagglutinin (PHA-L, Sigma, 1 mg/ml stock, for T-cell stimulation dilute 1:1'000 in coculture medium).

2.6. IFN- γ ELISA to Analyze Secretion of IFN- γ by NY-ESO-1-Specific Clonal CD4 $^{+}$ T Cells

1. High-protein-binding 96-well ELISA plates (e.g., Maxisorp, Nunc).
2. ELISA kit for human IFN- γ (Mabtech, Nacka Strand). A 10 μ g/ml stock of human recombinant IFN- γ provided with the kit is prepared and frozen in aliquots at -20°C. A freshly thawed aliquot should be used for each experiment.
3. coculture medium as in Subheading 2.5.
4. Blocking buffer: PBS, 1% BSA (Sigma).
5. Washing buffer: PBS, 0.05% Tween-20.
6. Incubation buffer: PBS, 0.1% BSA, 0.05% Tween-20.
7. TMB peroxidase substrate solution (Sigma).
8. Stop solution: 1 N sulfuric acid.

2.7. Silencing Macroautophagy in Dendritic Cells

1. Stealth siRNA: All Stealth™ siRNA duplexes (Invitrogen) are dissolved to a final concentration of 100 μ M according to the manufacturer's instructions. Scrambled siRNA are ordered for each target sequence. In our case, we used siRNA targeting the Atg16L1 sequence: Sense-5'GAG UUG UCU UCA GCC CUG AUG GCA G3' and Anti-Sense-5'CUG CCA UCA GGG CUG AAG ACAACUC3'.
2. Electroporation medium: Opti-MEM without phenol red (Invitrogen).
3. 4 mm Electroporation cuvette (Biorad).
4. Electroporator: ECM830 Square Porator TM.

3. Methods

3.1. Cell Culture

3.1.1. Cell Lines

1. A549, M199, and HLA-DR4 $^{+}$ HEK 293 cells are maintained in 100 mm plates until they approached confluence.
2. To split cells, monolayers are washed once with 5 ml of DPBS and incubated with 2 ml of 0.025% trypsin/EDTA solution at 37°C for 2–3 min to detach cells. To set up new maintenance

cultures, the cells are re-plated at 1:5 for M199 and A549, and at 1:10 for HLA-DR4⁺ HEK 293 and fresh culture medium is added. These cultures approach confluence after 2–3 days.

3. To induce expression of the MHC class II antigen processing machinery in the M199 cell line, cells are cultured with 200 U/ml of IFN- γ , for 48 h. At least 50% of cells express MHC class II on their cell surface after this treatment.

3.1.2. Monocyte-Derived Dendritic Cells

1. PBMCs are isolated from leukocyte concentrates by density-gradient centrifugation on Ficoll/Hyphaque.
2. CD14⁺ monocytes are isolated from PBMCs by positive magnetic cell separation (Miltenyi Biotec), then plated in 6-well plates at a density of $2\text{--}3 \times 10^6$ cells per well, in a final volume of 3 ml, and cultured for 5 days in dendritic cell medium (RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen)). GM-CSF and (IL)-4 are added to the culture on days 0, 2, and 4, each at a final concentration of 20 ng/ml.
3. On day 5 immature DCs are collected after pipetting up and down each well at least 20 times with a 1 ml pipette. Remaining adherent cells should not be collected.
4. Cells are then counted and plated in a 12-well plate at a concentration of 1 million cells/ml and matured with the appropriate stimuli (in our case influenza infection).
5. Matured dendritic cells are collected at day 6 and their maturation phenotype checked by FACS analysis.

3.2. Expression of NY-ESO-LC3 or the Tandem RFP-GFP-LC3 Fusion Construct by Transient Transfection

1. To set up cells for transfection, cells are detached as described in Subheading 3.1.1, step 2 and plated in 6-well tissue culture plates in transient transfection medium at a density of $2\text{--}3 \times 10^5$ cells/well (see Note 2).
2. The next day, cultures should be about 70–80% confluent.
 - (a) For each well to be transfected, 2.5 μg plasmid DNA are diluted in 250 μl OptiMEM-I medium and mixed by vortexing briefly.
 - (b) In a separate tube, 5 μl lipofectamine 2000 are diluted in 250 μl OptiMEM-I medium and vortexed briefly.
 - (c) Both tubes are incubated for 5 min at room temperature.
 - (d) Both solutions are combined and briefly vortexed, followed by incubation for 20 min at room temperature to allow formation of DNA–lipofectamine complexes.
3. Complexes are then added in a dropwise manner to the culture medium of cells in the 6-well plate and incubated at 37°C for 4 h.

**3.3. Immunocyto-
chemistry and
Confocal Microscopy
to Analyze
Autophagosome Flux
and MHC Class II
Compartment**

4. After 4 h, the complex-containing medium is replaced with fresh culture medium, and cells are cultured for 18–20 h at 37°C (see Note 6).
5. Twenty-four hours post-transfection, cells are ready to be split onto microscopy cover slips for localization analysis by confocal immunofluorescence microscopy (see Subheading 3.3) or for use in MHC presentation assays (see Subheading 3.4).
1. Round 1.5-mm microscopy cover slips are placed into 24-well tissue culture plate. Eight extra cover slips are used for control stainings (see Note 7).
2. Cover slips are then sterilized by washing once with 70% ethanol and twice with sterile DPBS. Any traces of ethanol are removed by completely aspirating ethanol and wash solutions with a vacuum suction flask.
3. Cells, transfected with the mRFP–GFP tandem fluorescent LC3 (tfLC3) construct (see Subheading 3.2), are trypsinized and plated onto sterilized cover slips in cell culture medium, at a density of 200,000 cells/well. Two wells of each sample should be plated, so that cells could be analyzed with and without chloroquine (CQ) treatment or Toll-like receptor stimulation.
4. During the last 24 h of the culture, one set of cells are treated with 50 µM CQ to prevent degradation of the tfLC3 construct by lysosomal proteases, or/and the cells are stimulated with the TLR agonist or infected with the pathogen of interest depending on the experimental set up (in our case influenza infection for 24 h). Leave at least two wells of cells untreated as a control (see Note 7).
5. Cells are then washed once in PBS (0.5 ml/well) and fixed in 4% paraformaldehyde (PFA, 200 µl/well) for 15 min at room temperature (see Note 8).
6. Then cells are washed once in PBS (0.5 ml/well) and permeabilized in 0.1% Triton X-100 (200 µl/well) for 5 min.
7. Next, cells are washed once in PBS (0.5 ml/well) and blocking buffer (200 µl/well) is added for 30 min.
8. The primary antibodies are diluted in blocking buffer and added to cells (200 µl/well) for 45 min at room temperature or for longer periods (up to overnight) at 4°C. *Co-staining of LC3 and MHC class II compartment is possible by using the anti-LC3 rabbit antibody and one of the mouse antibodies specific for MHC class II compartment.*
9. Then cells are washed three times in washing buffer (0.5 ml/well) and incubated for 5 min each time.
10. The secondary antibodies are diluted in blocking buffer and added to cells (200 µl/well) for 30 min.

11. Secondary antibody solutions are then aspirated and DAPI nucleic acid stain is added for 1 min (200 μ l/well). Afterwards, cells are immediately washed.
12. Cells are then washed once in PBS and cover slips are mounted on microscopy slides by inverting them onto a drop of mounting medium on a microscopy slide, up to three cover slips per slide. Next, cover slips are carefully press down, excess mounting medium is aspirated and slides dry at room temperature in the dark (see Note 9). Afterwards, slides could be stored in the dark at 4°C for several months.
13. Slides are then analyzed with a confocal laser scanning microscope, using a high N.A. oil immersion lens (e.g., 63 \times /1.4 N.A.). Excitation at 405 nm elicited DAPI fluorescence (blue emission), excitation at 488 nm Alexa 488 fluorescence, or GFP (green emission), and excitation at 543 nm Rhodamine-RedTM-X fluorescence or RFP or Alexa 555 fluorescence (red emission). Image J Software is used to overlay the different fluorescence channels and to quantify colocalization (see Note 10). Two experiments are shown as an example in Fig. 1: An experiment with influenza virus matrix protein 2 (MP2) transfection of A549 cells expressing the tandem RFP-GFP-LC3 construct is shown in Fig. 1a. In Fig. 1b an example of MHC class II staining of HEK 293 cells that were infected with influenza A virus is shown.

3.4. MHC Class II Presentation Assay Using an NY-ESO-1-Specific CD4 $^{+}$ T-Cell Clones

1. For MHC class II presentation assays, HLA-DR4 $^{+}$ HEK 293 or M199 cells transfected with the two different NY-ESO-1 constructs (see Subheading 2.3) in a 6-well format are used, as described in Subheading 3.2. M199 cells are treated with 200 U/ml IFN- γ for 48 h to initiate expression of the MHC class II antigen processing machinery.
2. Any traces of IFN- γ are removed from cells by washing cell monolayers three times in RPMI-1640 medium. Then cells are trypsinized to prepare a cell suspension, washed once in coculture medium, and counted with a hemacytometer. Cell suspensions are prepared in coculture medium at three different cell concentrations (2×10^5 , 10^5 , and 6.67×10^4 cells/ml) (see Note 11).
3. NY-ESO-1-specific clonal CD4 $^{+}$ T cells are collected from 96-well culture plates, washed once in coculture medium, and counted. The cell concentration is adjusted to 2×10^6 cells/ml.
4. Cocultures of T cells and target cells are set-up in duplicates (2 wells/condition) in a 96-well round-bottom plate. Per well, 50 μ l of T-cell suspension (10^5 cells/well) and 100 μ l of the different target cell suspensions (2×10^5 , 10^5 , and 6.67×10^4 cells/ml) are added. This results in effector to target (E:T) ratios of 5, 10, and 15. As a positive control, clonal T cells are

stimulated with the cognate NY-ESO-1 peptide (1 μ M) or PHA-L (1 μ g/ml). As a negative control, clonal T cells are exposed to coculture medium alone.

5. Cells are then cultured overnight (18–24 h) at 37°C.
1. One day prior to ELISA, high-protein-binding ELISA plates are coated with a primary anti-IFN- γ antibody (1-D1K, included in IFN- γ ELISA kit), diluted 1:500 in PBS, 100 μ l/well. Plates are then incubated overnight at 4°C.
2. The next day, the plates are washed two times with PBS (200 μ l/well) and blocked with blocking buffer (200 μ l/well) for 1 h at room temperature.
3. An aliquot of IFN- γ standard (10 μ g/ml) is thawed and serial dilutions are prepared in coculture medium (prepare 2'000, 1'000, 500, 250, 125, 62.5, 31.25 pg/ml standards, at least 300 μ l each).
4. To make sure that IFN- γ secreted by T cells is homogeneously distributed in culture supernatants, supernatants are mixed by pipetting up and down with a multichannel pipet and cells are then pelleted by centrifugation of plates at 300 $\times g$ for 5 min.
5. With a multichannel pipet, 120 μ l of supernatant are carefully removed from each well and transferred to a new 96-well plate.
6. ELISA plates are washed four times with washing buffer.
7. The coculture supernatants or IFN- γ standards (100 μ l/well) are then added and incubated for 2 h at room temperature. The remaining 20 μ l of supernatant are frozen at –20°C in case ELISA has to be repeated on diluted supernatants (see Note 13).
8. Plates are then washed as in step 6 and 100 μ l/well of secondary antibody (7-B6-1-biotin, provided in ELISA kit) are added, diluted 1:1'000 in incubation buffer. An incubation time of 1 h at room temperature follows.
9. Again, plates are washed as in step 6 and 100 μ l/well of streptavidin-HRP (provided in ELISA kit) are added, diluted 1:1'000 in incubation buffer. Another hour at room temperature is observed as incubation time.
10. As in step 6 plates are washed again and 100 μ l/well of TMB peroxidase substrate is added. The plates are incubated until blue reaction product has sufficiently developed, then the reaction is stopped by adding 100 μ l/well of Stop solution.
11. Optical density is measured at 450 nm (OD450) in an ELISA plate reader and OD450 values are converted into IFN- γ concentration in pg/ml, using the IFN- γ standards (see Note 12). An example of the produced results is shown in Fig. 2.

3.6. Silencing Macroautophagy in Monocytes-Derived Dendritic Cells

1. Electroporation of immature monocyte-derived DCs with siRNA targeting Atg16L1 or Atg5 or scrambled siRNA is performed *at day 4* (see Note 13). For this purpose, immature DCs are collected after pipetting up and down each well at least 20 times with a 1 ml pipette, and then washed once in RPMI without serum. Remaining adherent cells should not be collected.
2. Cells are adjusted to a final concentration of $3\text{--}4 \times 10^6$ immature DCs/in 200 μl Opti-MEM medium without phenol red, and transferred to an electroporation cuvette. The cells are kept at 4°C until the electroporation step.
3. One nmol of the siRNA of interest (Atg16L1 in our case or the scrambled control siRNA) is added to the cells directly in the 4 mm cuvette on ice just prior to electroporation.
4. The cells are then electroporated in an ECM830 Square Porator TM with a unique square wave pulse of 500 V 0.5 ms.
5. Cells are then plated at a density of 1 million cells/ml in a 12 well plate in R2 medium supplemented with GM-CSF and IL-4.
6. Maturation of these immature DCs is performed the next day, *at day 5*, using the maturation stimuli of interest (in our case influenza A infection).
7. At day 6 matured dendritic cells are collected for analysis. Maturation phenotype and viability of the cells are checked by FACS analysis (see Note 14).
8. Immunocytochemistry and microscopy are then performed as described in Subheading 3.3.

4. Notes

1. Cell lines are chosen based on their ability to upregulate MHC class II expression upon IFN- γ treatment and their MHC class II expression matching the restriction of the used T cell clone. The M199 cell line carries HLA-DP4 and the used CD4 $^{+}$ T cell clone is restricted by both DR4 and DP4 molecules. Therefore, HLA-DR4 $^{+}$ HEK293 cells are also used in some experiments.
2. For transfection with lipofectamine 2000, cell culture medium should not contain any antibiotics. Therefore, gentamicin is omitted for the transfection.
3. Non-adherent cells are cytospanned on 1.5 mm cover slips that are pre-coated with L-polylysine (Sigma).
4. Normal donkey or goat sera are used as blocking reagents because secondary antibodies are derived from donkey or goat.

For secondary antibodies from different species 5% normal serum from that species should be used as blocking reagent. Alternatively, 5% BSA could be employed as blocking solution.

5. T cell clones are expanded in RPMI-1640 + 8% PHS + 150 U/ml rhIL-2 (Chiron) + 1 µg/ml PHA-L (Sigma), in 96-well U bottom plates. Up to 1×10^4 clonal T cells / well, 10^5 irradiated allogeneic irradiated PBMCs/well, and 10^4 irradiated LCLs/well are added as feeder cells. T cell clones should not be used prior to 2 weeks after re-expansion to allow the T cells to rest after proliferation and avoid high background reactivity.
6. The transfection medium should be changed after 4 h not only to improve the viability of the transfected cells but also to prevent autophagosome accumulation due to an effect of the lipofectamine 2000 transfection reagent.
7. For correct interpretation of results, the following control stainings should be included:
 - (a) Primary and secondary antibodies should be replaced with blocking buffer. These stainings should be completely negative.
 - (b) Primary antibody should be replaced with blocking buffer, but secondary antibodies should be used. Background from secondary antibodies should be low. If the background turns out to be too high, the concentration of the secondary antibodies must be lowered.
 - (c) Untransfected cells and cells that are not treated with IFN- γ or with TLR/maturation stimuli are used as controls.
 - (d) Single-labeling of cells should be performed and signal bleed-through into nonspecific neighboring fluorescence channels should be checked (red channel for Alexa488 labeling and green channel for Rhodamine-Red X or Alexa555 labelings). There should be no signal in these channels.
8. From the fixation step onwards, cells could be handled outside sterile biosafety cabinets on a laboratory bench. Vacuum suction flasks can be used to change solutions, but the plastic tip of suction device should be exchanged between different solutions (e.g., antibodies). All incubation steps are done at room temperature, unless noted otherwise.
9. Prolong Gold antifade mounting medium (Invitrogen-Molecular Probes) should be allowed to dry at room temperature, in the dark, overnight. During this time, the mounting medium solidifies and its refractive index increases. Sealing of cover slips with nail polish is not necessary for Prolong Gold, but is recommended for other, water-based mounting media.

10. If a confocal microscope is not available, alternatively slides can be analyzed with a conventional wide-field fluorescence microscopes with a motorized z-stage. To remove out-of-focus light and accurately analyze colocalization of fluorochromes, z-stacks subsequently have to be deconvoluted using a deconvolution software.
11. The optimal number of target cells per clonal T cell may vary, depending on the T-cell clone and the type of target cell. Therefore it is recommended to try a range of different effector and target cell numbers, ranging from 10^4 to 2×10^5 T cells/well and 10^3 – 10^5 target cells/well.
12. In case IFN- γ levels in supernatants exceed the linear range of the ELISA (approximately 20–1'000 pg/ml), frozen supernatants are diluted 1:10 in coculture medium and the ELISA assays are repeated.
13. In case of ATG proteins, it is optimal to perform the knock down 48 h prior to the analysis, since the half life of these protein is usually >24 h.
14. Electroporation of dendritic cells can result in 30–50% of cell death. Therefore it is mandatory to control for cell viability and maturation phenotype of your DCs prior to any functional assay.

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References

1. van den Hoorn T, Paul P, Jongsma ML, Neefjes J (2011) Routes to manipulate MHC class II antigen presentation. *Curr Opin Immunol* 23:88–95
2. Sijts EJ, Kloetzel PM (2011) The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell Mol Life Sci* 68:1491–1502
3. Münz C (2009) Enhancing immunity through autophagy. *Annu Rev Immunol* 27: 423–429
4. Massey AC, Zhang C, Cuervo AM (2006) Chaperone-mediated autophagy in aging and disease. *Curr Top Dev Biol* 73:205–235
5. Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, Potolicchio I, Nieves E, Cuervo AM, Santambrogio L (2011) Microautophagy of cytosolic proteins by late endosomes. *Dev Cell* 20:131–139
6. Levine B, Mizushima N, Virgin HW (2011) Autophagy in immunity and inflammation. *Nature* 469:323–335

7. Yang Z, Klionsky DJ (2010) Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 22:124–131
8. Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 27:107–132
9. Nakatogawa H, Ichimura Y, Ohsumi Y (2007) Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* 130:165–178
10. Kirkin V, McEwan DG, Novak I, Dikic I (2009) A role for ubiquitin in selective autophagy. *Mol Cell* 34:259–269
11. Münz C (2011) Macroautophagy during innate immune activation. *Front Microbiol* 2:72
12. Romao S, Münz C (2011) Autophagy of pathogens alarms the immune system and participates in its effector functions. *Swiss Med Wkly* 141:w13198
13. Münz C (2011) Antigen processing by macroautophagy for MHC presentation. *Front Immunol* 2:1
14. Schmid D, Pypaert M, Münz C (2007) MHC class II antigen loading compartments continuously receive input from autophagosomes. *Immunity* 26:79–92
15. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, Mizushima N, Grinstein S, Iwasaki A (2010) In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* 32:227–239
16. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM (2008) Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res* 68:6889–6895
17. Uhl M, Kepp O, Jusforgues-Saklani H, Vicencio JM, Kroemer G, Albert ML (2009) Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. *Cell Death Differ* 16:991–1005
18. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Muller M, Kreymborg K, Altenberend F, Brandenburg J, Kalbacher H, Brock R, Driessens C, Rammensee HG, Stevanovic S (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc Natl Acad Sci U S A* 102:7922–7927
19. Nedjic J, Aichinger M, Emmerich J, Mizushima N, Klein L (2008) Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455:396–400
20. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, Münz C (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 307:593–596
21. Leung CS, Haigh TA, Mackay LK, Rickinson AB, Taylor GS (2010) Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. *Proc Natl Acad Sci U S A* 107:2165–2170
22. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL Jr, Eissa NT (2009) Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med* 15:267–276
23. Russmann H, Panthel K, Kohn B, Jellbauer S, Winter SE, Garbom S, Wolf-Watz H, Hoffmann S, Grauling-Halama S, Geginat G (2010) Alternative endogenous protein processing via an autophagy-dependent pathway compensates for *Yersinia*-mediated inhibition of endosomal major histocompatibility complex class II antigen presentation. *Infect Immun* 78:5138–5150

Chapter 36

Studying MHC Class II Transport in Dendritic Cells

Petra Paul and Jacques Neefjes

Abstract

Professional antigen presenting cells, such as dendritic cells, are effective in activating T lymphocytes due to their unique ability to present antigens in the context of both MHC class I and II molecules. After successful loading with antigenic peptides MHC class II molecules traffic from the late endosomal loading compartment to the plasma membrane to exert their function of presenting peptides to T helper lymphocytes. Various processes play a role in this event, which are only partly understood to date. The following protocols demonstrate a strategy of how to integrate high throughput datasets to select candidates possibly involved in MHC class II transport for in depth studies. A combination of proteomics, RNA interference and biochemical experimentation can uncover novel pathways regulating transport processes in primary dendritic cells.

Key words: MHC class II, Vesicle transport, Dendritic cells, RNA interference, High-throughput flow cytometry, Image analysis

1. Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) that regulate the adaptive immune response by stimulating naïve T cells (see ref. 1 for review). Expression of peptide-loaded MHC class II molecules at the cell surface is the result of tightly regulated transport processes from late endosomal compartments (called MIIC (2–4)), where antigen loading takes place. For a detailed description of the MHC class II pathway see ref. (5, 6). In DC MHC class II transport to the cell surface is enhanced when danger signals are encountered (7, 8). Various intracellular proteins have been associated with this process, such as iNOS and caspases (9). The ubiquitin ligase MARCH1 modifies MHC class II in human immature DC (imDC) (10, 11), clathrin and AP-2 orchestrate endocytosis (12), and cystatins regulate cathepsin

Table 1
Characteristics of flow cytometry versus microscopy

	Flow cytometry	Microscopy
<i>Readout</i>	Intracellular or PM	Mainly intracellular
<i>Analysis</i>	Simple	Complex, software programs needed
<i>Quantity</i>	Many data points (cells)	Few data points
<i>Detail</i>	Little insight	Precise localization, many features detected

activities (13). Despite these findings, many open questions remain when it comes to the control of MHC class II transport in DC. For example, which motors are involved in MIIC transport? Little is known about this. Myosin II has been reported to interact with invariant chain (Ii) to control MHC class II transport in B cells (14). Another actin-based motor, myosin 1E, has been recently implicated to be responsible for the positioning of MHC class II-positive vesicles in DC (15). The dynein motor is recruited to MIIC via the small GTPase Rab7 (16).

One way of identifying new players in the MHC class II transport route is to select candidates following an RNAinterference (RNAi) screen and other high-throughput cell biological assays. Gene knockdown is achieved by introducing small-interfering RNA molecules (siRNA) or short-hairpin RNA (shRNA) which are provided as either genome-wide or specialized libraries (kinases, phosphatases, G-protein-coupled receptors, etc.). The resulting candidates can then be tested in smaller scale in primary DC. RNAi screens can be performed by microscopy as well as flow cytometry when appropriate antibodies are available. For a comparison of the two methods see Table 1. For the RNAi screen described here, changes in cell surface expression of MHC class II detected by flow cytometry served as a primary read-out. In general, a robust read-out is essential. Negative and positive controls are required. See ref. 17 for a review on tips and tricks of RNAi screening. Secondary screens may subsequently be used to cluster candidates into phenotypically similar pathways, which is essential for creating new biology.

Primary immune cells are often unsuitable for screening. They are limited in number, difficult to manipulate and prone to differentiate. Cell lines can serve as substitutes. For a list of MHC class II-positive cell lines see Table 2. For any of these cell lines, functionality and transfection efficiency need to be determined and optimized before large-scale screening activities. In some cases activation with cytokines is required for efficient antigen processing and presentation.

Table 2
Possible cell lines for an MHC class II-related RNAi screen

Endogenous	Type	ATCC	References
<i>MUTZ-3</i>	Human myelomonocytic cell line with DC properties after cytokine stimulation		(25)
<i>KG-1</i>	Human myelomonocytic cell line with DC properties after cytokine stimulation	CCL-246	(26)
<i>THP-1</i>	Adherent, macrophage-like after stimulation with phorbol ester	TIB-202	(27)
<i>LCL</i>	Human Epstein Barr virus-transformed lymphoblastoid cell line		Transformation protocol (28)
<i>MelJuSo</i>	Adherent human melanoma cell line		(29)
Ectopic	Manipulation	ATCC	References
<i>RAW264.7</i>	Adherent murine monocyte/macrophage cell line transfected with MHC class II	TIB-71	(30)
<i>Fibroblasts</i>	Transfected with MHC class II and Ii		(31)
<i>HeLa</i>	Transfected with CIITA	CCL-2	(32)

Following the primary screen, microarray or deep sequencing studies on APCs provide information whether genes that influence MHC class II surface levels in cell lines are expressed in primary cells. The model cell line may be used for further high throughput (HT) experimentation: (a) Quantitative RT-PCR (qRT-PCR) following silencing of genes identified in the primary screen reveals whether the transcription rate of the MHC locus is altered. (b) Immunofluorescence and confocal microscopy provide vital information on alterations of the intracellular MHC class II distribution. The large amount of images generated can be analyzed and clustered based on similarity using open software programs such as Cell Profiler and CP Analyst (18, 19).

To select candidates for in-depth follow-up studies, stringent criteria must be set. When addressing the question of what regulates MHC class II transport in DC, the following key points need to be considered. Silencing a gene that is a positive regulator of transport might result in reduced MHC class II levels at the plasma membrane. Knocking down a negative regulator, on the other hand, will result in higher cell surface expression levels. If this regulator is involved in the maturation-induced outward transport of MHC class II, its expression level might be lower in mature (m)DC compared to imDC, information that can be deduced from the gene expression data set. Finally, the intracellular distribution of

MHC class II might be altered after silencing. For an example of HT data set integration and candidate selection strategy see ref. 15.

The protocols described below are dedicated at how candidates selected from HT data sets can be validated and investigated in DC. First, the effects of silencing the candidate genes observed in the screen need to be confirmed in the primary immune cell. Lentiviral transduction is an efficient tool for the introduction of shRNA into human monocytes which can then be differentiated into imDC. In principle, such viral particles can also be used to introduce overexpression constructs. Once candidates that alter the transport of MHC class II are confirmed and changes of MHC class II gene transcription are excluded, the search for interaction partners/effectors can begin. Different methods can be applied for this purpose depending on which type of protein is selected. Yeast Two Hybrid (Y2H) technologies have been proven to be useful when looking for effectors of small GTPases. In our experience, for effector molecules of other protein classes (e.g., scaffold proteins) GST pulldown might be a more suitable method (20). Kinases in general exceed very short lived interactions, which are difficult to detect in either assay. Any interaction identified using these methods needs to be confirmed by alternative methods, e.g., coimmunoprecipitation. All these techniques and considerations are required to build new pathways from high content screening data.

An RNAi screen only helps identifying potential candidate genes controlling a biological phenomenon. To understand the cell biology at a high content level, secondary HT screens can be applied. Subsequently, these are integrated with other datasets such as those from microarray, yeast two-hybrid and proteomics. When properly done, new pathways can be generated allowing definition of the control of MHC class II transport in primary DC.

2. Materials

2.1. Lentiviral Transduction of DC (See Also Chapter 30)

1. Human monocytes isolated from peripheral blood (see Chapter 30 in this book).
2. Cell line and appropriate medium for lentivirus production (e.g., human 293T cells) and all necessary materials for cell culture (flasks, tissue culture dishes, trypsin-EDTA, DMEM, fetal calf serum (FCS)).
3. Cellgro medium (Cellgenix) or other medium for serum-free cultivation of dendritic cells.
4. Cellgro supplemented: Cellgro medium supplemented with 800 U/ml IL-4, 1,000 U/ml GM-CSF, and 4 µg/ml Polybrene.

5. Phosphate-buffered saline (PBS): dissolve one tablet in 500 ml of distilled water (GIBCO) pH 7.4.
6. Maturation trigger:
 - (a) Lipopolysaccharide LPS (Invitrogen).
 - (b) IFN- γ (Immukine, Boehringer Ingelheim).
7. Transfection reagent: Fugene 6 (Roche) or alike.
8. Plasmids:
 - (a) Packaging constructs (e.g., pMDLg/pRRE, pRSV-Rev, and pCMV-VSV-G (21)).
 - (b) Control vectors (e.g., pLKO.1empty, pLKO.1shEGFP, pLKO.1shLuciferase, pLKO.1shSCRAMBLE).
 - (c) Lentiviral plasmid containing shRNA of choice (pLKO.1 vectors from Open Biosystems, Thermo Scientific, usually 4–5 different sequences per gene).
9. 0.45 μ m Filter units (Millipore).
10. Polybrene (Millipore) dissolved in PBS.
11. Ultracentrifugation tubes (Beckman Coulter).
12. Ultracentrifuge (Beckman Coulter Rotor SW28).

2.2. Flow Cytometry

1. Wash Buffer: PBS containing 2% FCS.
2. Antibodies diluted to 1–10 μ g/ml in Wash Buffer:
 - (a) PE anti-human HLA-DR (L243).
 - (b) FITC anti-human CD14.
 - (c) FITC anti-human CD83.
 - (d) PE anti-human CD80.
 - (e) APC anti-human CD86.
 - (f) APC anti-human CD40.
 - (g) APC anti-human DC-SIGN.
3. FACS Calibur flow cytometer (BD Biosciences).

2.3. Immuno-fluorescence

1. Cover glasses or μ -Slide 18-well plates (IBIDI).
2. Coating: Fibronectin (Invitrogen) diluted to 20 μ g/ml in PBS.
3. Fixative solution: PBS with 3.75% formaldehyde (free from acid, Merck).
4. Permeabilization: PBS with 0.1% Triton X-100 (Sigma).
5. Blocking solution: 0.5% bovine serum albumin (BSA) in PBS.
6. Primary Antibody solution: mouse anti-human CD63 (or any other late endosomal marker) and rabbit anti-HLA-DR (2) diluted together in blocking buffer.

7. Secondary Antibody solution: Fluorophore-conjugated Secondary Antibody diluted in blocking buffer (e.g., goat anti-mouse IgG Alexa 488 and goat anti-rabbit IgG Alexa 647), 2 µg/ml HOECHST 33342 nuclear dye, 0.4 U/ml Phalloidin-Alexa568.
8. Mounting medium: Vectashield (Vector Laboratories) or 80% glycerol in PBS.
9. AOBS confocal microscope (Leica).

2.4. qRT-PCR

1. Roche mRNA Capture Kit (Roche).
2. Transcriptor High Fidelity cDNA Synthesis Sample Kit (Roche).
3. Lightcycler 480 SYBR Green 1 Master (Roche).
4. PCR-grade, RNase-free water: DEPC-treated water.
5. Plates (Lightcycler 480 Multiwell 96, Roche).
6. 10× primer solution at 3.3 µM of both forward and reverse primer in PCR-grade water:
 - (a) 18S rRNA reference primers: Forward 5'-CGGCTACCA CATCCAAGGAA-3', Reverse 5'-GCTGGAATTACCGC GGCT-3'.
 - (b) HLA-DR α-chain primers: Forward 5'-CATGGGCTAT CAAAGAAGAAC-3', Reverse 5'-CTTGAGCCTCAAAG CTGGC-3'.
7. PCR machine Thermocycler (Peltier Thermal Cycler, MJ Research).
8. Light Cycler 480 Detection System (Roche).

2.5. Y2H and Glutathione-S-Transferase Pulldown

1. cDNA clone of gene of interest (e.g., IMAGE clone).
2. Y2H suitable vector: e.g., pGBT9 (<http://www.dkfz.de/gpcf/y2h.html>).
3. Cells of choice for glutathione-S-transferase (GST) pulldown, e.g., monocyte-derived imDC or mDC, peripheral blood mononuclear cells (PBMC).
4. Lysis buffer: 50 mM NaCl (can be up to 500 mM to reduce unspecific binding), 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 0.8% NP-40 and protease inhibitors (EDTA-free, Roche Diagnostics) in water.
5. Wash buffer: same as lysis buffer, but only 0.08% NP-40 and without protease inhibitors.
6. Recombinant GST-tagged protein of choice plus free GST for control purposes.
7. Glutathione Sepharose beads 4 G (GE Healthcare).

2.6. Coimmunoprecipitation

1. Cell line to be transfected with proteins identified to interact (e.g., MelJuSo or 293T) or primary immune cell type to study interaction of endogenous proteins.
2. Expression vectors encoding tagged proteins of interest (possible tags: GFP, HA, myc, etc.).
3. Lysis and wash buffers: see Subheading 2.5, steps 4–5.
4. Antibodies: anti-GFP, anti-HA, anti-myc, or antibodies raised against the proteins of interest.
5. Protein G Sepharose beads 4 Fast Flow (GE Healthcare).

2.7. SDS-PAGE and Western Blot

1. 2× Sample Buffer: 4 ml distilled water (dH_2O), 10 ml 0.5 M Tris–HCl pH 6.8, 8 ml glycerol, 16 ml 10% SDS, few flakes of Bromphenol blue, store aliquots at -20°C , add 400 μl of β -mercaptoethanol per 3.8 ml of sample buffer before use.
2. Resolving gel buffer: 1.5 M Tris–HCl pH 8.8, store at 4°C .
3. Stacking gel buffer: 0.5 M Tris–HCl pH 6.8, store at 4°C .
4. 10% Sodium dodecylsulfate (SDS) in dH_2O .
5. 40% Acrylamide/Bis-acrylamide: ratio 37.5:1 (Biorad).
6. TEMED (*N,N,N,N*-Tetramethylethylenediamine, Sigma).
7. Initiator: 10% ammonium persulfate solution (APS) in dH_2O , prepare just prior to use.
8. *n*-Butanol, water saturated: add dH_2O , shake, let phases separate, use upper layer.
9. Electrophoresis buffer: dilute 10× TGS (Biorad) with dH_2O to obtain 1× TGS final (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3).
10. Molecular weight standard: Page Ruler prestained protein ladder (Thermo Scientific).
11. Gel fixative: 40% methanol and 10% acetic acid in dH_2O .
12. Silver stain kit: SilverQuest (Invitrogen).
13. Transfer buffer: dilute 10× TG (Biorad) with dH_2O to obtain 1× TG final (25 mM Tris, 192 mM glycine, pH 8.3) and add 20% methanol.
14. PVDF membrane (Immobilon-P, Millipore).
15. Blotting paper: Whatman (GE Healthcare).
16. PBS/T: 0.1% Tween-20 (Sigma) in PBS.
17. Blocking buffer: 5% skim milk powder in PBS/T.
18. Primary antibody: diluted in 1% blocking buffer.
19. Secondary antibody: e.g., swine anti-rabbit IgG horse radish peroxidase-conjugated (Dako), diluted 1:5,000 in 1% blocking buffer.

20. Detection: Amersham ECL Detection Reagents (GE Healthcare).
21. Chemiluminescence film and cassette.
22. Biorad Mini Protean and Transfer System.

3. Methods

3.1. Lentivirus Production

1. 293 T cells are grown in 150 cm² flasks in DMEM supplemented with 10% FCS.
2. Cells are washed with PBS, trypsinized, and seeded at 3.5×10^6 per 10 cm tissue culture dish. Per lentiviral shRNA construct four dishes are required.
3. After 24 h cells are transfected. The three packaging constructs are mixed 1:1:1. The ratio of this packaging trio to shRNA construct (pLKO.1) is 1:1.
 - (a) To 1.4 ml of serum-free DMEM (room temperature) add 56 µl of Fugene 6.
 - (b) In a separate tube (see Note 1) mix 7 µg (see Note 2) of packaging vectors with 7 µg of pLKO.1shRNA.
 - (c) Combine Fugene/DMEM and DNA, incubate for 30 min at room temperature.
 - (d) Replace the medium in the 10 cm dishes with 12.6 ml of DMEM—10% FCS.
 - (e) Add DNA/Fugene dropwise.
4. After 24 h change medium to 8 ml Cellgro medium. The cells should be 50% confluent. Handle them carefully as they detach very easily.
5. After another 24 h harvest the supernatant.
 - (a) Filter through a 0.45 µm filter.
 - (b) Concentrate the viral supernatant 100× by ultracentrifugation at $20,000 \times g$ at room temperature for 2 h.
 - (c) Resuspend the viral pellet in 360 µl Cellgro medium (see Note 3) and snap freeze in liquid nitrogen. Store at -80°C (see Note 4). The viral suspension obtained is enough to perform transduction of at least three different monocyte donors.
6. Add another 7 ml of Cellgro medium to the 293T cells. A second harvest can be performed the day after like in step 5.

3.2. Silencing Candidate Genes in DC

Human monocytes are transduced with lentivirus carrying shRNA constructs targeting the selected candidate genes followed by differentiation into imDC. Several control vectors should be used.

An average of their phenotype serves as a reference point. The experiment should be performed in several donors as a big difference between individuals is usually observed when working with primary cells. General effects can be easily separated from MHC class II-specific ones by including additional cell surface markers such as MHC class I (for effects on the entire MHC locus), CD63 (for effects on recycling between PM and late endosomes), and transferrin receptor (for recycling in early endosomes).

1. Thaw frozen monocytes. Wash two times with Cellgro medium. Plate cells at 1×10^6 in 12-well plate in 900 μl Cellgro supplemented. Add 100 μl of 100 \times viral supernatant.
2. After 24 h remove 400 μl of medium and refresh with 1.4 ml of Cellgro supplemented.
3. On day 5 mature a well of untreated imDC with LPS (2.5 $\mu\text{g}/\text{ml}$) and IFN- γ (1,000 U/ml) to use as an mDC control.
4. After 24 h determine the cell surface levels of MHC class II and other maturation markers by flow cytometry (see Subheading 3.3) and the intracellular MHC class II distribution by immunofluorescence (see Subheading 3.4). To ensure that imDC were successfully generated monitor the reduction of CD14 and the increase of DC-SIGN cell surface levels compared to monocytes by flow cytometry. Furthermore, the cells are subjected to qRT-PCR to establish the level of knockdown and to rule out any effects on MHC class II gene transcription (see Subheading 3.5).

3.3. Phenotyping DC by Flow Cytometry (See Also Chapter 26)

1. DC are semi-adherent and can be detached by repetitive pipetting and flushing the well.
2. Approx. 50% of cells remain after differentiation from monocytes to imDC. Transfer approx. 100,000 DC per staining to a round-bottom 96-well plate. Spin cells down and discard supernatant. Wash cells one time with wash buffer and spin down.
3. Add 20 μl of antibody cocktail (e.g., FITC-CD83, PE-MHCII, APC-CD40) and incubate for 30 min on ice in the dark. Wash cells with buffer followed by centrifugation.
4. Resuspend pellet in 100 μl of wash buffer. Measure the cell surface expression of the respective markers using a plate reader attached to the flow cytometer.

3.4. Intracellular MHC Class II Distribution

In imDC MHC class II mainly resides in late endosomes where it colocalizes with CD63. When exposed to danger signals DC undergo striking morphological changes one of which is a redistribution of MHC class II to the plasma membrane. To determine whether any of the silenced candidate genes is responsible for maintaining MHC class II's endosomal location the colocalization between CD63 and MHC class II is being determined (Fig. 1).

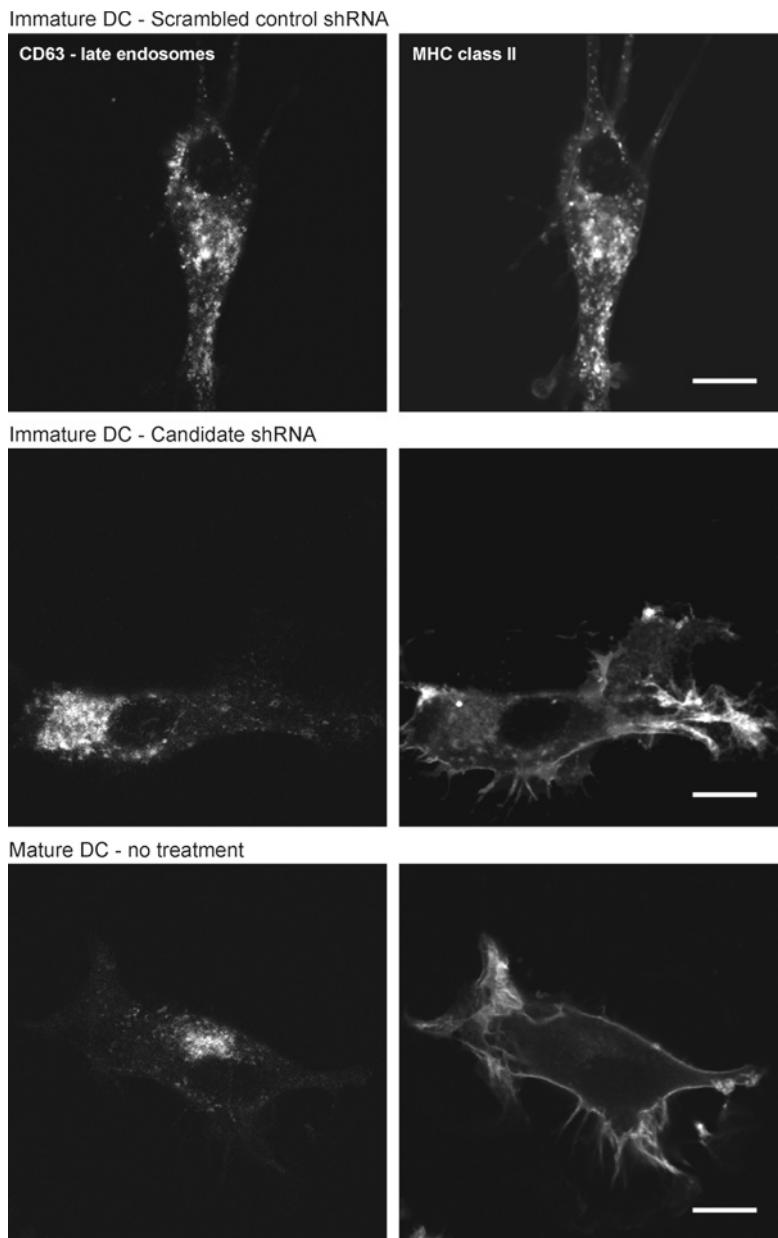


Fig. 1. Redistribution of MHC class II after candidate gene silencing. While MHC class II colocalizes with the late endosomal marker CD63 on intracellular vesicles in immature DC (imDC, *upper panel*), most of MHC class II resides at the plasma membrane (PM) in mature DC (mDC, *lower panel*). When factors negatively influencing this transport process are silenced MHC class II molecules are redistributed to the PM, which resembles the phenotype of an mDC, while other maturation markers remain negative as is typical for an imDC (determined by flow cytometry). DC are fixed and stained with antibodies against CD63 and MHC class II. Fluorophore-conjugated secondary antibodies are used to visualize the localization of the two molecules by confocal microscopy. Bar = 10 μ m.

1. Coat μ -slides for 30 min at 37°C with fibronectin and rinse with PBS.
2. Add 30 μ l DC suspension and incubate in a wet chamber at 37°C for 6–7 h.

All the following steps are carried out at room temperature.
3. Fix cells with fixative solution for 15 min and wash by immersing the slide in a chamber filled with PBS. Slides may be stored for several days in PBS at 4°C.
4. Permeabilize the cells with Triton-X for 10 min and wash with PBS.
5. Unspecific binding is prevented by blocking with blocking solution for 45 min.
6. Add the primary antibody solution and incubate for 60 min. 15 μ l of solution are enough to cover the entire surface area of one well.
7. Wash the slides three times for 5 min by placing the PBS-filled chamber on a shaking platform.
8. Add the secondary antibody solution containing HOECHST as a nuclear dye and phalloidin to stain the actin cytoskeleton and incubate for 30 min in the dark.
9. Wash the slides again three times for 5 min and fill each well with 80% glycerol. The slides can be stored at 4°C for a few days until analysis with a confocal microscope. When using individual cover glasses perform all washing steps in multi-well plates and mount the glasses in the end using a drop of mounting medium on a glass slide. Remove excess mounting medium by pressing the glass slide on tissue.
10. Analyze the MHC class II distribution in DC by confocal microscopy.

3.5. Determination of mRNA Levels in DC by qRT-PCR

This method is used to determine the knockdown efficiency on one hand and to rule out any effects on transcriptional regulation of MHC class II on the other hand (see Note 5).

1. Wash 50,000 DC two times with cold PBS (see Note 6) and lyse in 50 μ l lysis buffer (see Note 7).
2. Add 4 μ l of biotinylated oligo dT primer and anneal for 5 min at 37°C. Transfer lysate to a streptavidin-coated tube and incubate for another 5 min at 37°C (see Note 8).
3. Add 200 μ l of wash buffer and discard. Repeat washing step three times. Leave the liquid of the last wash in the tube while preparing the cDNA synthesis master mix following manufacturer's instructions.
4. Discard wash buffer completely. When pipetting away the liquid avoid touching the walls of the tube as this might destroy

the layer of streptavidin. Add 30 µl of RT mix following manufacturer's instructions.

5. Reverse transcribe the captured mRNA into cDNA with a PCR program of 30 min 50°C and 5 min 85°C. Stop at 99°C. At such high temperature the bond between biotin and streptavidin is broken and the cDNA can be removed.
6. Quickly remove the 30 µl of liquid and replace them with 50 µl of PCR-grade water. Incubate for 3 min at 99°C. Remove the liquid and combine it with the previous 30 µl. The cDNA can be stored at -20°C.
7. For the qRT-PCR reaction 96-well plates are used. Per well add 5 µl of Lightcycler 480 SYBR Green 1 master, 1 µl of 10× primer solution (3.3 µM each) and 1.5 µl of water. Add 2.5 µl of cDNA. Seal the plate and spin it to collect all fluid at the bottom of each well.
8. Insert the plate in the LightCycler and start the run, which will take about 1 h 20 min.
9. Analyze using the comparative CT method ($\Delta\Delta CT$). Relate the results to the 18S rRNA values and normalize it to the average of control shRNA-treated cells.

3.6. Identification of Interaction Partners/Effectors by Y2H

1. The Y2H analysis can be outsourced (e.g., <http://www.dkfz.de/gpcf/y2h.html>). Alternatively, a protocol on how to perform Y2H can be found here (22).
2. To prepare the sample for Y2H clone the cDNA of your gene of interest into a Y2H-combatible vector.
3. Choose an appropriate cDNA library to screen (e.g., lymph node). Choose a cell type/tissue where your gene of interest is expressed under normal conditions. Information about tissue distribution can be found at <http://biogps.org> (23).

3.7. Identification of Interaction Partners/Effectors by GST Pulldown

1. To prepare a protein for GST pulldown clone the cDNA of your gene of interest into a GST expression vector. Produce the recombinant GST-tagged protein as well as free GST in, e.g., *Escherichia coli* and purify them.
2. Wash 120 µl of glutathione beads three times in lysis buffer. Spin at $500 \times g$ for 3 min. Remove liquid carefully (see Note 9).
3. Couple 50 µg of recombinant protein dissolved in lysis buffer to the beads for 1 h. All subsequent incubation steps are performed at 4°C on a spinning wheel. Wash beads three times in lysis buffer (see Note 10).
4. Lyse 200×10^6 PBMC (see Note 11) in 750 µl lysis buffer for 30 min. Centrifuge for 10 min at maximum speed.
5. Incubate beads with the supernatant of lysed cells for 1 h or overnight. Wash one time in lysis buffer and three times in wash buffer (see Note 10).

6. Remove all liquid (see Note 9). Add 25 or 50 μ l of 1 \times sample buffer (prepare by mixing equal volumes of water and 2 \times sample buffer), boil for 5 min at 100°C, centrifuge at max. speed and load supernatant on one or two gels, respectively.
1. Wash glass plates, clean with 70% ethanol and dry.
2. Prepare two 1 mm 12% resolving gels by mixing 4.35 ml water with 2.5 ml resolving gel buffer, 0.1 ml 10% SDS solution, and 3 ml acrylamide/bis-acrylamide solution. Start the polymerization by adding 50 μ l of 10% APS solution and 5 μ l of TEMED. Pour the gel leaving space for the stacking gel. Overlay with *n*-butanol.
3. After polymerization is complete pour away the butanol and rinse the gel with water. Absorb residual water using blotting paper.
4. Prepare 4% stacking gel mixing 3.22 ml water with 1.25 ml stacking gel buffer and 50 μ l 10% SDS solution and 0.5 ml acrylamide/bis-acrylamide solution. Start the polymerization by adding 25 μ l of 10% APS solution and 5 μ l of TEMED. Pour the liquid on top of the resolving gel and insert the comb.
5. When polymerization is complete, assemble the gel unit, remove the combs, fill the unit with 1 \times electrophoresis buffer and flush each sample well using a long slender tip or syringe with needle.
6. Load samples. Fill empty wells with 1 \times sample buffer. Include a weight marker in one lane.
7. Close the unit and connect it to a power supply. Set the power supply to constant voltage and run at 90 V. When the sample front has passed the stacking gel the voltage may be increased to 110 V.
8. When the SDS-PAGE run is finished, remove the gel, and fix it for at least 20 min in gel fixative before proceeding to the silver staining.
9. Perform mass spectrometric analysis to determine the proteins that have interacted with your protein of choice but not with GST alone (see Note 12).

3.9. Confirmation of Interaction by Coimmunoprecipitation

Interactions identified by Y2H or GST pulldown need to be confirmed. If antibodies are available it is better to perform the coimmunoprecipitation (CoIP) with endogenous proteins in, e.g., imDC or mDC. If such antibodies are not available ectopic expression of the proteins of interest in a cell line may be applied. By tagging the proteins with two different tags interactions can be studied.

1. For CoIP either isolate the cell type of choice to confirm the interaction or transfet a cell line with plasmids encoding tagged versions of the two putative interaction partners. Use,

e.g., Fugene 6 for the transfection following Subheading 3.1, steps 1–3.

2. Wash 50 µl of protein G beads three times in lysis buffer. Spin at $500 \times g$ for 3 min. Remove liquid carefully (see Note 9).
3. Couple 5 µl (the amount might be reduced to as little as 1 µl depending on the strength of the antibody) of antibody (against interaction partner one or against the tag of interaction partner one) diluted in lysis buffer to the beads for 1 h. All subsequent incubation steps are performed at 4°C on a spinning wheel. Wash beads three times (see Note 10).
4. Lyse 5×10^6 transiently transfected cells (more primary cells are necessary when detecting endogenous proteins) in 750 µl lysis buffer for 30 min. Centrifuge for 10 min at maximum speed.
5. Mix 50 µl of supernatant with an equal volume of 2× sample buffer, boil for 5 min at 100°C, centrifuge, and store at -20°C. Load on a gel as a total lysate control.
6. Incubate beads with the remaining supernatant of lysed cells for 1 h or overnight. Wash one time in lysis buffer and three times in wash buffer (see Note 10).
7. Remove all liquid (see Note 9). Add 25 µl of 1× sample buffer (prepare by mixing equal volumes of water and 2× sample buffer), boil for 5 min at 100°C, centrifuge, and load supernatant on a gel.
8. Perform SDS-PAGE as described in Subheading 3.8.
9. Prepare pieces of blotting paper and PVDF membrane slightly larger than the gel and soak the membrane in 100% methanol for 2–3 min.
10. Fill a tray with 1× transfer buffer.
 - (a) Submerge the transfer cassette. Stack a foam pad and three pieces of blotting paper on the dark plastic side of the cassette.
 - (b) Remove the gel from the electrophoresis chamber, rinse with water, and place onto the filter paper.
 - (c) Place the membrane on the gel (avoid the introduction of bubbles). Add three pieces of blotting paper prewet in transfer buffer. Remove any bubbles in between the different layers by rolling a serological pipette over the stack.
 - (d) Place wet foam pad on top and close the cassette.
11. Insert the cassette into the transfer tank. Black side of the cassette facing the black side of the tank. Double check that the membrane is placed between the gel and the anode (+). Proteins will migrate towards the anode. If inserted the opposite way proteins will be lost in the buffer.

12. Place an ice block in the transfer tank and fill the tank with 1× transfer buffer. Add a magnetic stirrer.
13. Place tank on a stirrer platform, close lid and perform transfer at constant current of 150 mA for 1.5 h. Alternatively, the transfer can be performed at constant 20 mA in the cold room overnight.
14. Remove the PVDF membrane from the transfer unit (see Note 13). The prestained molecular weight marker should be clearly visible.
15. Block the membrane in 10 ml of blocking buffer for 1 h at room temperature rocking on a shaking platform. Wash the membrane three times with PBS/T for 5 min.
16. Add the primary antibody (against interaction partner two or tag of interaction partner two) and incubate for at least 1 h at room temperature or overnight at 4°C. Wash the membrane three times with PBS/T for 5 min.
17. Add the secondary antibody and incubate for 1 h at room temperature. Wash the membrane three times with PBS/T for 10 min.
18. Prepare the ECL reagents by mixing equal volumes of the two components. Remove the membrane from the washing solution. Remove excess buffer by blotting one corner of the membrane on tissue. Place membrane on clear plastic foil, cover with ECL mix, and wrap in foil. Ensure complete coverage of the membrane by striking the liquid from one side to the other repeatedly.
19. After 1 min remove excess liquid and wrap membrane in clean foil. Place membrane with chemiluminescence film in a film cassette.
20. In a dark room expose X-ray film to the chemiluminescence signals and develop afterwards. The exposure time needs to be adjusted to the signal strength.

3.10. Colocalization Studies on Putative Interaction Partners Using Immunofluorescence Microscopy

Colocalization observed by immunofluorescence (IF) represents an alternative method to support protein interaction data.

1. Prepare primary cells as described under Subheading 3.4 or transiently transfected cells as described under Subheading 3.6.
2. Determine the localization of the two putatively interacting proteins by staining them with antibodies directly or indirectly (anti-tag). See Subheading 3.4 for procedure.
3. To place these interactions in context with MHC class II transport stain also for MHC class II or for the compartments involved in MHC class II antigen presentation (e.g., CD63 for

late endosomes). Phalloidin is a stain for the actin cytoskeleton and will help to determine whether transport occurs along actin filaments. Furthermore, the microtubule network may be visualized using anti-tubulin antibodies.

3.11. Conclusion

An RNAi screen often results in a list of genes that are somehow affecting a biological process. To classify these “hits” HT follow-up screens are needed. When all these individual datasets are integrated and stringent criteria are set a list of candidates emerges that might play a role in, e.g., the control of MHC class II transport. To study and confirm these candidates experiments in primary immune cells (DC) need to be performed. Silencing can be achieved by introduction of shRNA constructs using lentivirus transduction. Changes in cell surface levels of MHC class II and its intracellular distribution are measured. To extend the findings of individual new players to whole pathways interaction partners and effectors can be identified using Y2H and proteomic approaches. All new findings require further confirmation applying alternative methods. Including appropriate controls ensures that the new findings are MHC class II-specific and do not affect any general transport or protein secretion pathway.

4. Notes

1. All tubes should be made from polyethylene to limit the rate of liposomes sticking to the tube’s wall.
2. In order to monitor the transfection efficiency 0.5 µg of pEG-FPC1 (Clontech) may be added to the packaging vector mixture. The amount of pLKO.1 shRNA construct should be reduced to 6.5 µg, respectively. More than 90% of 293 T cells should be GFP-positive after 48 h.
3. Add Cellgro to pellet and leave it standing for a few minutes before pipetting up and down gently (try not to introduce bubbles).
4. To be able to set a certain mode of infection (MOI) the viral titer of your lentivirus preparation needs to be determined as transducing units per ml (TU/ml). Produce GFP-expressing lentiviral particles by introducing the pLKO.1TURBOGFP construct. Infect monocytes with decreasing volume of virus supernatant and determine the amount of GFP-positive colonies by microscopy after 48 h. Multiply this number with the dilution factor.
5. A useful program to design primers against your gene of interest for qRT-PCR is Perlprimer ([24](#)). Adjust the settings according to what is being recommended with the SYBR Green you are

using. BLAST the primer sequences to ensure they only recognize one gene.

6. To ensure RNase-free working conditions wipe surfaces and tools with 70% ethanol. Use filter tips and RNase-free tubes and reagents. Avoid working in a laminar flow hood as the flow of air will favor contamination with RNases.
7. The solution is very viscous. One freeze-thaw cycle at -80°C helps to complete the lysis.
8. When working with the SA-coated tubes avoid the introduction of bubbles and scratching the wall of the tubes while pipetting!
9. Use a needle of diameter 0.4 mm. Liquid can be entirely removed without loss of beads.
10. Transfer beads to a fresh tube during the last washing step. This will help eliminate proteins that unspecifically stick to the tube's wall.
11. The cell number may vary depending on (a) the expression level of the protein of interest and (b) the availability of the cell type you want to use.
12. Contamination of gels with keratine can cause problems during mass spectrometry. To ensure keratine-free conditions pre-cast gels can be used.
13. Use forceps to handle the membrane. The transfer of the prestained marker to the membrane gives an indication of the quality of protein transfer. To get an overview of protein transfer across the entire membrane stain with Ponceau S for a few minutes. Destain by rinsing with excess of water.

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References

1. Lipscomb MF, Masten BJ (2002) Dendritic cells: immune regulators in health and disease. *Physiol Rev* 82:97–130
2. Neefjes JJ, Stollorz V, Peters PJ, Geuze HJ, Ploegh HL (1990) The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* 61:171–183
3. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349:669–676
4. Roche PA, Cresswell P (1990) Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* 345:615–618
5. van den Hoorn T, Paul P, Jongsma ML, Neefjes J (2011) Routes to manipulate MHC class II antigen presentation. *Curr Opin Immunol* 23:88–95

6. Neefjes J, Jongsma ML, Paul P, Bakke O (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 11:11(12): 823–836
7. Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388:782–787
8. Pierre P, Turley SJ, Gatti E, Hull M, Meltzer J, Mirza A, Inaba K, Steinman RM, Mellman I (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 388:787–792
9. Wong SH, Santambrogio L, Strominger JL (2004) Caspases and nitric oxide broadly regulate dendritic cell maturation and surface expression of class II MHC proteins. *Proc Natl Acad Sci U S A* 101:17783–17788
10. de Gassart A, Camossetto V, Thibodeau J, Ceppi M, Catalan N, Pierre P, Gatti E (2008) MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH 1 down-regulation. *Proc Natl Acad Sci U S A* 105:3491–3496
11. van Niel G, Wubbolds R, ten Broeke T, Buschow SI, Ossendorp FA, Melief CJ, Raposo G, van Balkom BW, Stoorvogel W (2006) Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity* 25:885–894
12. McCormick PJ, Martina JA, Bonifacino JS (2005) Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to the antigen-processing compartments. *Proc Natl Acad Sci U S A* 102:7910–7915
13. Pierre P, Mellman I (1998) Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* 93:1135–1145
14. Vascotto F, Lankar D, Faure-Andre G, Vargas P, Diaz J, Le Roux D, Yuseff MI, Sibarita JB, Boes M, Raposo G, Mougneau E, Glaichenhaus N, Bonnerot C, Manoury B, Lennon-Dumenil AM (2007) The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *J Cell Biol* 176:1007–1019
15. Paul P, van den Hoorn T, Jongsma ML, Bakker MJ, Hengeveld R, Janssen L, Cresswell P, Egan DA, van Ham M, Ten Brinke A, Ovaa H, Beijersbergen RL, Kuijl C, Neefjes J (2011) A Genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell* 145:268–283
16. Rocha N, Kuijl C, van der Kant R, Janssen L, Houben D, Janssen H, Zwart W, Neefjes J (2009) Cholesterol sensor ORP1L contacts the ER protein VAP to control Rab7-RILP-p150 Glued and late endosome positioning. *J Cell Biol* 185:1209–1225
17. Sharma S, Rao A (2009) RNAi screening: tips and techniques. *Nat Immunol* 10:799–804
18. Carpenter A, Jones T, Lamprecht M, Clarke C, Kang I, Friman O, Guertin D, Chang J, Lindquist R, Moffat J, Golland P, Sabatini D (2006) Cell Profiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* 7:R100
19. Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, Castoreno AB, Eggert US, Root DE, Golland P, Sabatini DM (2009) Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A* 106:1826–1831
20. Abu-Farha M, Elisma F, Figge D (2008) Identification of protein-protein interactions by mass spectrometry coupled techniques. *Adv Biochem Eng Biotechnol* 110:67–80
21. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72: 8463–8471
22. Kail M, Barnekow A (2008) Identification and characterization of interacting partners of Rab GTPases by yeast two-hybrid analyses. *Methods Mol Biol* 440:111–125
23. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW III, Su AI (2009) BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 10:R130
24. Marshall OJ (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* 20:2471–2472
25. Masterson AJ, Sombroek CC, De Gruyl TD, Graus YM, van der Vliet HJ, Lougheed SM, van den Eertwegh AJ, Pinedo HM, Schepers RJ (2002) MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. *Blood* 100:701–703
26. Berges C, Naujokat C, Tinapp S, Wieczorek H, Hoh A, Sadeghi M, Opelz G, Daniel V (2005) A cell line model for the differentiation of human dendritic cells. *Biochem Biophys Res Commun* 333:896–907
27. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, Tada K (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol ester. *Cancer Res* 42:1530–1536

28. Harding CV, Canaday D, Ramachandra L (2010) Choosing and preparing antigen-presenting cells. *Curr Protoc Immunol* Chapter 16, Unit
29. Johnson JP, Demmer-Dieckmann M, Meo T, Hadam MR, Riethmuller G (1981) Surface antigens of human melanoma cells defined by monoclonal antibodies. I. Biochemical characterization of two antigens found on cell lines and fresh tumors of diverse tissue origin. *Eur J Immunol* 11:825–831
30. Hockett RD, Cook JR, Findlay K, Harding CV (1996) Interferon-gamma differentially regulates antigen-processing functions in distinct endocytic compartments of macrophages with constitutive expression of class II major histocompatibility complex molecules. *Immunology* 88:68–75
31. Stockinger B, Pessara U, Lin RH, Habicht J, Grez M, Koch N (1989) A role of Ia-associated invariant chains in antigen processing and presentation. *Cell* 56:683–689
32. Poloso NJ, Denzin LK, Roche PA (2006) CDw78 defines MHC class II-peptide complexes that require Ii chain-dependent lysosomal trafficking, not localization to a specific tetraspanin membrane microdomain. *J Immunol* 177:5451–5458

Chapter 37

In Vitro Digestion with Proteases Producing MHC Class II Ligands

Mira Tohmé, Sophia Maschalidi, and Bénédicte Manoury

Abstract

Proteases generate peptides that bind to MHC class II molecules to interact with a wide diversity of CD4⁺ T cells. They are expressed in dedicated organelles: endosomes and lysosomes of professional antigen presenting cells (pAPCs) such as B cells, macrophages, and dendritic cells. The identification of endosomal proteases which produce antigenic peptides is important, for example, for better vaccination and to prevent autoimmune diseases. Here, we describe a panel of technics (in vitro digestion assays of protein with recombinant proteases or purified endosomes/lysosomes, T cell stimulation) to monitor the production of MHC class II ligands.

Key words: MHC-II, Proteases, Endosomes/lysosomes, Antigen processing, pAPCs

1. Introduction

MHC class II molecules associate in the endoplasmic reticulum with a chaperone protein: the invariant chain ([1](#)). It has many functions; one of them is to deliver MHC class II complexes to endosomal/lysosomal compartments ([2](#)). In these compartments, which also receive exogenous antigens, proteases must perform two tasks: the generation of peptides that will be loaded on MHC class II molecules and the proteolysis of Ii. Proteolytic enzymes release amino acids and peptides from proteins and can be grouped in different families based on their catalytic activities. They belong to three major classes: aspartic, serine, and cysteine families and are usually dependent on acidic pH for their activities ([3](#)). The main enzymes present in the endosomal/lysosomal pathway are the cathepsins (cathepsins B, C, D, E, F, H, K, L, S) and AEP for asparagine endopeptidase. Processing of Ii is mainly performed by cathepsin S in professional antigen presenting cells (pAPCs) ([4, 5](#)) and cathepsin L in cortical thymic epithelial cells ([6](#)). For antigen

processing, it appears that there is no clear dependency on a single protease to generate a dedicate peptide but it remains to be fully elucidated (7). Nonetheless, few exceptions have shown that, for example, toxin tetanus C-fragment (TTCF) processing requires AEP (8). Proteases instead of generating peptides can also have the opposite effect which is to destroy MHC class II ligands. Indeed, the immunodominant peptide of a self-antigen, myelin basic protein, is destroyed by AEP and a myoglobin epitope is cleaved by cathepsins D (9, 10). Here, in this chapter, we demonstrate that the production of MHC class II ligands generated by in vitro digestion with proteases either purified from lysosomes or bought as recombinant proteins can be tested by incubating fixed APCs together with recombinant or radiolabeled antigens predigested with proteases and T cells.

2. Materials

We will take the example of the antigen TTCF (for Tetanus Toxin C-fragment) and use the CD4⁺ T cell hybridomas as a readout but in principle any antigen with the specific T cell hybridoma can be used with the same method.

2.1. Common Consumables and Lab Equipment

1. Recombinant proteins such as TTCF or ovalbumin are either bought or purified from *Escherichia coli* using standard procedures (11, 12).
2. T cell hybridoma: CD4⁺ T cells hybridoma are generated from standard procedures (13). For example, TTCF-specific T cell hybridomas were obtained by fusion between spleens from TTCF-injected mice and a thymoma lacking the expression of the T cell receptor (14).

2.2. Purification of Endosomes and Lysosomes

1. Dentritic cells were generated from bone marrow by culturing precursors for 7–10 days in GM-CSF-supplemented medium as previously described (15).
2. Complete medium: IMDM (Gibco) supplemented with 10% FCS, 1% PS, 1% GLN, and 10 ng/ml of rGM-CSF (Preprotech).
3. Magnetic nanoparticles (Turbobeads).
4. Homogenization buffer (HB): 3 mM imidazole, 8% sucrose, 2 mM DTT, and 5 µg/ml of DNase.
5. Wash buffer: PBS, 0.1% BSA.
6. Lysis buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP40, 2 mM MgCl₂, and a tablet of cocktail inhibitors (Roche).
7. Magnetic stand for Eppendorf tubes (Eppendorf).

2.3. In Vitro Digestion Assays

1. TNT T7 or SP6 quick Coupled Transcription/Translation system (Promega).
2. ^{35}S methionine (Perkin Elmer).
3. 50 mM citrate buffer: mix 23.25 mL of 0.05 M citric acid pH 5.5 with 76.75 mL of 0.05 M trisodium citrate, pH 5.5.
4. Digestion buffer: 50 mM citrate buffer, pH 5.5, 0.1% CHAPS, 1 mM DTT.
5. Lysis buffer: 4 \times Laemli buffer supplemented with 8% β -mercaptoethanol.
6. Specific inhibitors for:
 - (a) AEP: 20 μM MV026630.
 - (b) Cathepsins B, L, S: 10 nM LHVS.
 - (c) Cathepsin K: 10 μM Boc-Phe-Leu-NHNH-CO-NHNH-Leu-Z.
 - (d) Cathepsin G: 10 μM inhibitor I (Calbiochem, cat: 219372).
 - (e) Cathepsins D, E: 10 μM pepstatin A.

2.4. Fluorometric Assays

1. Recombinant proteases are supplied (Calbiochem).
2. Citrate buffer: 50 mM citrate buffer, pH 5.5, 1 mM DTT.
3. Mithras LB940 (Berthold technologies). Excitation/Emission wavelength: 360/460 nm.
4. Specific fluorometric substrates for:
 - (a) AEP: Z-Ala-Ala-Asn-NHMec.
 - (b) CatB: Z-Arg-Arg-NHMec.
 - (c) CatB/L: Z-Phe-Arg-NHMec.
 - (d) CatG: Suc-Ala-Ala-Ala-Pro-Phe-NHMec.
 - (e) CatH: Z-Arg-NHMec.
 - (f) CatS: Z-Val-Val-Arg-NHMec.
 - (g) CatK: Z-Gly-Pro-Arg-NHMec.
 - (h) Standard curve: NHMec from 0.2 to 1 μM , 50 mM stock in DMSO.

2.5. 10% Acrylamide Gels for SDS-PAGE

1. Stock Acrylamide/Bis 30%/0.8%.
2. 10% Ammonium persulfate solution (APS) in dH₂O.
3. TEMED (*N,N,N,N*-Tetramethylethylenediamine).
4. 4 \times Lower Buffer: 1.5 M Tris, 0.4% SDS, pH 8.8.
5. 4 \times Upper Buffer: 0.5 M Tris, 0.4% SDS, pH 6.8.
6. Running buffer: Tris 10 g, Glycine 144 g, SDS 10 g, in 1 L of water.

7. Staining buffer: dissolve 0.6 g of Blue Coomassie (Sigma) in 195 ml of water and add 75 ml of propanol-2 together with 30 ml acetic acid.
8. Fixation solution: in 195 ml of water, add 75 ml of propanol-2 with 30 ml acetic acid.
9. Enhancer solution (Perkin Elmer).
10. Destained buffer: 10% acetic acid, 20% propanol-2, and 70% water.
11. Autoradiographic film and cassette.

2.6. T Cell Activation

1. 0.1% Glutaraldehyde-PBS.
2. 0.2 M Glycine, pH 7.4.
3. RPMI 1640 supplemented with 1% FCS.
4. 96 Flat-bottom well plates.
5. IL-2 ELISA kit (e-biosciences).
6. Plate reader.

3. Methods

3.1. Endosome and Lysosome Purification

Endosomes and lysosomes are purified using magnetic nanoparticles of 20 nm of diameter.

1. 10^7 Dendritic cells are incubated with 60 μ l of magnetic nanoparticles (10 mg/ml) for 30 min at 4°C.
2. Pulsed for 20 min at 37°C in 1 ml of IMDM medium alone.
3. Chased for 100 min in 3 ml of complete medium.
4. Cells are then washed three times with cold wash buffer and resuspend in 1 ml of HB.
5. Cells are mechanically disrupted by passing them through a 25 G needle for about 30 times in order to have 80% mortality.
6. Endosomes and lysosomes are purified by magnetic separation at 4°C on a magnetic Eppendorf stand.
 - (a) They are washed ten times with cold PBS supplemented with a tablet of cocktail inhibitors (Roche).
 - (b) Lyse in 50 μ l of lysis buffer for 20 min on ice.
7. The endosomes/lysosomes are then centrifuged at $13,000 \times g$ at 4°C for 10 min to get rid of the magnetic particles and can be either frozen or used immediately.

3.2. Digestion Assay

1. cDNAs (0.5 µg) encoding the protein of interest are in vitro translated with the kit following manufacturer's instructions and 10 µCi of methionine labeled with ^{35}S . Alternatively recombinant purified proteins can be used as substrates.
2. Radiolabeled proteins (5 µl of the reaction) or recombinant purified proteins (10 µg) are incubated with proteases (1–15 U) or with purified endosomes/lysosomes (1 µg) from wild-type cells or cells lacking different cathepsins (B or D or G or K or L or S or AEP, etc.) for 2–4 h in a final volume of 30 µl in digestion buffer.
3. The reaction is either stopped by addition of 10 µl of lysis buffer and SDS-PAGE gel is then performed (see Subheading 3.3) to monitor degradation of the antigen or by addition of specific proteases inhibitors and production of complexes MHC-peptides is detected using a T cell activation assay (see Subheading 3.4 and Note 1).
4. Activity of the proteases is assessed using specific fluorometric substrates (see Subheading 2.4, step 4) by measuring the release of fluorescent *N*-Acetyl-Methyl-Coumarin (NHmec) in citrate buffer (pH 5.5) supplemented with 1 mM of DTT at 37°C. 10 ng of cathepsins B, D, G, K, L, S, or AEP are incubated with their specific substrates, respectively (20 µM), for different times (from 10 to 240 min) in 200 µl. To calculate the specific activity of each protease (µmole of substrate release per minute), a standard curve is obtained using different concentrations of NHmec (from 0.2 to 2 µM) in 200 µl in citrate buffer. The same specific activity of each proteases (e.g.: 1 U) is used to digest in vitro the antigen.

3.3. SDS-PAGE

1. Prepare 10% poor resolving gel by mixing 6.7 ml Stock Acrylamide/Bis with 5 ml 4× Lower buffer, 8.3 ml water, 70 µl 10% APS, and 10 µl TEMED. Add EtOH on top of the gel. It should take 30 min to polymerize at room temperature.
2. Once resolving gel is set, pour off EtOH. Rinse with dH₂O.
3. Prepare 4% poor stacking gel: mix 1.7 ml Stock Acrylamide/Bis with 1.25 ml 4× Upper buffer, 7 ml water, 60 µl 10% APS, and 20 µl TEMED. Load stacking solution onto the top of the resolving gel. Insert gel comb carefully. It should take about 10–15 min to polymerize at room temperature.
4. When polymerization is complete, assemble the gel unit, remove the combs, and fill the unit with running buffer.
5. Heat aliquots at 95°C for 10 min. Load samples (30 µl).
6. Following electrophoresis (1 h, 180 V, 400 mA), open the gel plates with a spatula.

7. For radiolabeled samples,
 - (a) Rinse the gel with water and incubate in a fixation solution for 30 min to 1 h.
 - (b) Rinse the gel again with dH₂O and add the enhancer solution for 30 min.
 - (c) Rinse the gel with dH₂O again and dry it for 1 h at 80°C.
 - (d) Develop the autoradiographic film after few hours or an overnight exposure at -80°C.
8. For recombinant proteins digested in vitro, the gels are incubated in the staining buffer for 2 h and wash extensively with destained buffer.

3.4. T Cell Activation

1. In vitro digestion assays (see Subheading 3.2) are stopped by adding the respective protease inhibitors (see Subheading 2.3, step 6 and see Note 2).
2. Cell preparation:
 - (a) 2×10^6 of pAPCs (dendritic cells, for example) are fixed with 200 µl of 0.1% glutaraldehyde-PBS for 30 s at room temperature and the reaction is stopped by adding 200 µl of 0.2 M Glycine, pH 7.4.
 - (b) Cells are extensively washed with a solution of RPMI 1640 supplemented with 1% FCS.
 - (c) They are then distributed in 96 flat-bottom well plates at 5×10^4 cells in 50 µl per well.
3. The in vitro digestion assays (50 µl) are added to the antigen presenting cells (50 µl) together with 100 µl of 10^5 T cells (ratio 2:1) for 24 h.
4. Stimulation of T cells is measured by release of interleukine 2 using an ELISA kit.

4. Notes

1. The in vitro digestion assays are most of the times performed in an acidic pH (citrate buffer, pH 5.5). When added to the pAPCs, it can kill them. To avoid this problem, you have to raise the pH of your buffer by adding few drops of NaCl.
2. To assess that the protease inhibitor you have used is indeed specific for the protease you want to block, you can use the Fluorometric assay to test it (see Subheading 3.2, step 3).

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References

1. Kvist S, Wiman K, Claesson L, Peterson PA, Dobberstein B (1982) Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell* 29:61–69
2. Lotteau V, Teyton L, Peleraux A, Nilsson T, Karlsson L, Schmid SL, Quaranta V, Peterson PA (1990) Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* 348:600–605
3. Rawlings ND, Barret AJ (1993) Evolutionary families of peptidases. *Biochem J* 290: 205–208
4. Nakagawa T, Brissette WH, Lira PD, Griffiths RJ, Petrushova N, Stock J, McNeish JD, Eastman SE, Howard ED, Clarke SRM et al (1999) Impaired invariant chain degradation and antigen presentation and diminished collagen induced arthritis in cathepsin S null mice. *Immunity* 10:207–217
5. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, Chapman HA (1996) Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* 4:357–366
6. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY (1998) Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* 280:450–453
7. Watts C (2011) The endosome-lysosomes pathway and information generation in the immune system. *Biochim Biophys Acta* 8:4–6
8. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C (1998) An asparagine endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* 396:695–699
9. Manoury B, Mazzeo D, Fugger L, Viner N, Ponsford M, Streeter H, Mazza G, Wraith DC, Watts C (2002) Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat Immunol* 3:169–174
10. Moss CX, Villadangos JA, Watts C (2005) Destructive potential of the aspartyl protease cathepsin D in MHC class-restricted antigen processing. *Eur J Immunol* 35:3442–3451
11. Schein CH (1989) Production of soluble recombinant proteins in bacteria. *Nat Biotechnol* 7:1141–1149
12. Mitraki A, King J (1989) Protein folding intermediates and inclusion bodies formation. *Nat Biotechnol* 7:690–697
13. Lai MZ, Ross DT, Guillet J-G, Briner TJ, Gedter ML, Smith JA (1987) T lymphocyte response to bacteriophage 1 repressor CI protein: recognition of the same peptide presented by Ia molecules of different haplotypes. *J Immunol* 139:3973–3977
14. Matthews SP, Werber I, Deussing C, Peters C, Reinheckel T, Watts C (2010) Distinct proteases requirement for antigen presentation in vitro and in vivo. *J Immunol* 184:2423–2431
15. Savina A, Jancic C, Hugues S, Guermonprez P, Vargas P, Moura IC, Lennon-Duménil AM, Seabra MC, Raposo G, Amigorena S (2006) NOX2 controls phagosome pH to regulate antigen processing during cross presentation by dendritic cells. *Cell* 126:205–218

Chapter 38

MHC-II Ubiquitination

Aude De Gassart, Francesca De Angelis Rigotti, and Evelina Gatti

Abstract

Ubiquitinated protein detection is often troublesome since in most cases this modification reduces the half-life of targeted proteins, inducing their degradation. Furthermore, ubiquitination is reversible thanks to the action of highly specific deubiquitinases present in all eukaryotic cells. MHC molecules ubiquitination has been demonstrated to be a key event in the regulation of the potent immunostimulatory properties of activated human dendritic cells.

Key words: Ubiquitination, MARCH1 ubiquitin ligase, Antigen presentation molecules, Dendritic cells, Immunoprecipitation

1. Introduction

MHC class II surface expression is tightly regulated in professional antigen presenting cells, mainly through the control of its intracellular trafficking. In the endoplasmic reticulum, neosynthesized MHC class II is associated to a small transmembrane molecule called invariant chain (Ii). Ii prevents premature binding of peptides to the MHC-II peptide-binding-groove and promotes exit from the ER and transport through the Golgi to the cell surface as well as towards the MHC-II containing compartments (MIIC). In lysosomes, invariant chain degradation leads to MHC class II loading with specific peptides (mature loaded MHC-II) (1). In 2007, we and others described that at the cell surface peptide-loaded MHC class II molecules can be ubiquitinated on lysine 225 of their cytoplasmic domain. Ubiquitination reduces MHC-II stability at the cell surface and provokes its internalization (2–4).

Ubiquitin (Ub) is a small, 76-residue, protein (8.5 kDa) found both as free monomer and covalently attached to itself and other

proteins in eukaryotic cells (5). Ubiquitination, the covalent attachment of ubiquitin to target proteins, is a posttranslational modification carried out by a set of three enzymes. They include ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3. Unlike to E1 and E2, E3 ubiquitin ligases display substrate specificity. On the other hand, numerous deubiquitylating enzymes have roles in processing polyubiquitinated proteins (5). Ubiquitination can result in change of protein stability, cellular localization, and biological activity. It represents a key molecular mechanism of regulation for many fundamental cellular processes and has been recently implicated in the control of the immune response (3, 6–10).

In response to Toll-like receptor (TLR) ligands, dendritic cells (DCs) enhance their antigen presentation capacity by stabilizing at the cell surface MHC molecules bearing antigenic peptides (1). In DCs, MHC class II molecules are sorted towards the endocytic pathway prior loading with antigenic peptides and cell surface appearance. In non-activated human DCs, a membrane-associated RING-CH ubiquitin E3 ligase called MARCH1 promotes the selective ubiquitination of peptide-loaded MHC class II molecules, inducing their internalization from the cell surface. This internalization leads to the lysosomal targeting of MHC-II molecules and impairs antigen presentation in the absence of an appropriate microbial stimulus. Upon TLR engagement, MARCH1 expression is lost, allowing MHC class II molecules stabilization at the plasma membrane and productive interaction of dendritic cells with T cells (2). MHC molecules ubiquitination appears to be a key event in the regulation of the potent immunostimulatory properties of activated human dendritic cells.

Ubiquitinated protein detection is often troublesome since in most cases this modification reduces the half-life of targeted proteins, inducing their degradation. Furthermore, ubiquitination is reversible thanks to the action of highly specific deubiquitinases present in all eukaryotic cells. It is therefore recommended to keep cell lysates on ice and to use lysis buffers complemented with cysteine protease and proteasome inhibitors (*N*-ethylmaleimide and MG132, respectively).

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C) and analytical grade reagents.

2.1. Cell Culture

- RPMI complete cell culture medium: RPMI 1640 medium supplemented with 10% FCS, non-essential amino acids, 100 ng/mL penicillin/streptomycin (>1,000 U/mL), 20 ng/mL recombinant human IL-4 (>100 U/mL), and 100 ng/mL recombinant human GM-CSF (Peprotech).
- Ficoll-PaqueTM PLUS (Amersham Biosciences).
- LPS: *Escherichia coli* type 026:B6.
- DMEM complete cell culture medium: DMEM, supplemented with 10% fetal calf serum (FCS) and 100 ng/mL penicillin/streptomycin (>1,000 U/mL) under hygromycin selection (11).
- Lipofectamine2000 Reagents (Invitrogen).
- 1× Ice-cold phosphate buffer saline (PBS): 200 mg/L KCl, 200 mg/L KH₂PO₄, 8,000 mg/L NaCl, 2,160 mg/L Na₂HPO₄·7H₂O.
- Freezing medium: 40% RPMI, 50% FCS, and 10% DMSO.
- MACS running buffer: 1× PBS, 2 mM EDTA, 0.5% FCS. Prepare 0.5 M EDTA solution, pH 8. Dissolve 93 g of EDTA in 500 mL of water and adjust pH with NaOH solution. Mix 500 mL PBS to 2 mL of 0.5 M EDTA solution and 2.5 mL of FCS (see Note 1).
- CD14 MicroBeads (Miltenyi Biotec).
- AutoMACS System (Miltenyi Biotec).

2.2. Cell Lysis

- PBS–5 mM EDTA.
- Lysis buffer: 10 mM Tris–HCl pH = 7.6, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100 (see Note 2).
- Complete lysis buffer: freshly supplement 10 mL lysis buffer with 20 mM *N*-ethylmaleimide (25 mg), one tablet of complete protease inhibitor cocktail (Roche Molecular Biochemicals), and 5 µM MG132 (Enzo Life Sciences) (see Note 3).
- BCA protein assay.

2.3. Immuno-precipitation

- Protein-A agarose (see Note 4).
- Anti-peptide-loaded HLA-DR antibody (L243 clone, BD PharMingen) (see Note 5) (12).
- Non-denaturing SDS sample buffer: 2.5% SDS, 25 mM Tris–HCl [pH 6.8], 3% Glycerol, 0.00625% bromophenol blue. Mix 2.5 mL of stacking gel buffer (see Subheading 2.3), 12.5 mL of 20% SDS solution, 3 mL of glycerol, 6.25 mg of bromophenol blue, and add water to 100 mL. Prepare aliquots of 1 mL and store at –20°C.

2.4. SDS-PAGE and Immunoblotting

Solution of acrylamide 30%/bis-acrylamide 0.8%.

- Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Dissolve 181.7 g of Tris (UltraPure Tris, Invitrogen) in 0.9 L of water. Mix, adjust pH with HCl, and add water to 1 L. Store at 4°C.
- Stacking gel buffer: 1 M Tris–HCl, pH 6.8. Dissolve 121.1 g of Tris in 0.9 L of water, adjust pH, and add water to 1 L. Store at RT.
- 20% SDS solution in water. Dissolve 10 g of SDS (UltraPure Sodium Dodecyl Sulfate, Invitrogen) in 50 mL of water. Store at RT.
- *N,N,N,N*-Tetramethyl-ethylenediamine (TEMED).
- Ammonium persulfate: 10% solution in water. Dissolve 10 g of APS in 100 mL of water. Freeze aliquots of 1 mL at -20°C and once thawed, keep it for maximum 1 week at 4°C.
- SDS-PAGE running buffer: 25 mM Tris–HCl, 192 mM glycine, 0.1% SDS (see Note 6).
- Mini-Protean Tetra Cell (Bio-Rad system).
- Nitrocellulose membranes, Immobilon-P transfer membrane, PVDF (Millipore).
- Western blot transfer buffer: 50 mM Tris–HCl, 384 mM glycine, 0.1% SDS, and 20% ethanol. Weight 11.8 g of Tris, 58 g of glycine (UltraPure Glycine, Invitrogen), and 2 g of SDS. Add 0.2 L ethanol and make up to 2 L with water. Store at 4°C.
- Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad system).
- Tris buffered saline (TBS): 150 mM NaCl, 20 mM Tris–HCl, pH 7.5 (see Note 7).
- TBS-T: TBS 1× containing 0.1% Tween-20.
- Blocking solution: 5% milk in TBS-T.
- Immunoblotting solution: 1% BSA in TBS-T.
- Anti-HLA-DR β chain specific (clone XD5; home made from a hybridoma ([13](#))).
- Anti-ubiquitin (clone P4D1, Covance).
- Goat anti-mouse IgG HRP-conjugated Ab (Jackson ImmunoResearch Europe).
- Super Signal West Pico Luminol/Enhancer Solution (Thermo Scientific).
- Stripping Buffer: 60 μ M Tris, pH 6.8, 2% SDS and freshly add 0.1% of 2-mercaptoethanol. Weight 20 g of SDS, add 60 mL of 1 M Tris–HCl pH 6.8 (stacking buffer), and add water to 1 L. The solution can be stored at RT. Add 2-mercaptoethanol (dilution 1:1,000) immediately before use.

3. Methods

3.1. Cells

We studied MHC-II ubiquitination during maturation of monocyte-derived dendritic cells (MoDC) (see Subheading 3.1.1). As alternative cellular model, we used HeLa-CIITA (see Subheading 3.1.2), a HeLa cell line stably expressing the MHC class II transactivator CIITA, which drives the coordinated expression of MHC class II molecules and Ii chain in APCs (1).

3.1.1. Monocyte-Derived DCs

We prepared monocyte-derived DCs as previously described (4) from fresh human leukapheresis products by Ficoll and CD14⁺ cells were immunomagnetically purified with AutoMACS system following the protocol of the manufacturer. In detail:

1. Prepare 50 mL Falcon tubes containing 15 mL of Ficoll-Paque PLUS.
2. Carefully layer 35 mL of human leukapheresis product on the Ficoll (see Note 8). The two solutions do not have to be mixed.
3. Centrifuge at $800 \times g$ for 25 min at room temperature. Do not use the brake during centrifugation (see Note 9).
4. Collect the white ring of mononuclear cells. Pour two rings in a new 50 mL Falcon tube and fill it up with RPMI. Centrifuge at $200 \times g$ for 10 min at room temperature.
5. Wash cells with RPMI, at least five times. Each time poor two pellets for tube, until only one tube is left. After the last washing, resuspend cells by pipetting up and down or vortex gently, if necessary.
6. Count cells.

From now on, work on ice, with pre-chilled solutions.

7. Centrifuge 2×10^9 PBMC at $300 \times g$ for 10 min at 4°C. Resuspend pellet in 8 mL of MACS running buffer, add 2 mL of CD14 MicroBeads (2 mL are sufficient for 2×10^9 of total PBMC). Incubate on a rotating wheel for 15 min in the cold room.
8. Full fill the Falcon tubes with MACS running buffer, centrifuge at $300 \times g$ for 10 min at 4°C and resuspend pellet in 15–20 mL of MACS running buffer.
9. Perform magnetic separation with autoMACS™ Separator. For separation choose the positive selection “possel.” Collect positive fraction (outlet port “pos1”). This is the purified CD14⁺ fraction (typically CD14⁺ cells are around 30% of PBMC, therefore you should obtain around 0.5×10^9 cells).
10. To promote differentiation into immature dendritic cells, the purified CD14⁺ cells (0.5×10^6 cells/mL) are plated in 6-well

plates (2×10^6 cells/well) and cultured in RPMI complete cell culture medium (see Note 10).

11. At days 2 and 4, half of the volume of the medium is replaced by fresh RPMI complete cell culture medium. For DC maturation, 5 µg/mL LPS is added to the cells.

3.1.2. HeLa-CIITA

HeLa-CIITA cells were maintained in DMEM complete cell culture medium. Cells were transfected with a plasmid expressing MARCH1 E3-ubiquitin ligase fused to -eGFP, using Lipofectamine2000 Reagents and following the manufacturer's instructions. Cells were harvested 24 h after transfection to analyze MHC-II ubiquitination (see Note 11).

3.2. Cell lysate and IP

The whole cell lysis and IP procedures were done at 4°C.

1. Harvest cells using PBS–5 mM EDTA (around 3×10^6 immature MoDCs or MARCH1 transfected HeLa-CIITA cells).
2. Wash two times with ice-cold PBS.
3. Saturate 1.5 mL Eppendorf tubes with PBS–1% FCS or complete medium.
4. Pellet cells in saturated 1.5 mL Eppendorf tubes.
5. Resuspend cell pellets in 100 µL lysis buffer and incubate at 4°C for 20 min (see Note 12).
6. Remove nuclei by centrifugation at $16,000 \times g$ for 10 min at 4°C.
7. Harvest the supernatant.
8. Resuspend the pellet in 50 µL complete lysis buffer.
9. Centrifuge at $16,000 \times g$ for 10 min at 4°C.
10. Pool this supernatant with previous one to obtain about 150 µL total cell lysate (post-nuclear supernatant, PNS).
11. Perform BCA protein assay following manufacturer's instructions to measure the total protein amount of each sample (for 3×10^6 cells, 250–300 µg of total protein is expected).
12. Adjust samples to the same protein concentration.
13. Add 20 µL pre-equilibrated Protein A agarose beads to PNS, to diminish unspecific binding to beads (pre-clearing step).
14. Rock 1 h at 4°C.
15. Centrifuge at $500 \times g$ for 2 min, to pool-down beads and harvest supernatant. Be careful to keep sample free of beads.
16. Add 1 µg of anti-HLA-DR (L243 clone) to pre-cleared cell lysate.
17. Incubate overnight at 4°C on a stirring wheel.
18. Add 20 µL of pre-equilibrated beads to each sample.

19. Incubate 2 h at 4°C.
20. Wash beads four times with lysis buffer.
21. Aspirate completely lysis buffer.
22. Add 30 µL non-denaturating SDS sample buffer. Boil for 5 min, centrifuge to pool-down beads, and separate samples on SDS-PAGE (see Note 13).

3.3. 8% SDS Polyacrylamide Gel

Carry out all procedures at room temperature unless otherwise specified.

1. For one mini gel (Biorad MINIPROTEAN III system) 8% (see Note 14): mix 2.5 mL of resolving buffer, 2.7 mL of acrylamide mixture, 4.6 mL water, 100 µL of 10% SDS solution, 100 µL of ammonium persulfate, and 10 µL of TEMED. Cast 7.5 mL of gel mixture within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Gently overlay with water and wait that the gel mixture is polymerized.
2. Remove the water from the gel cassette and cast 3 mL of stacking gel. To prepare the stacking gel mix 0.5 mL of stacking gel buffer, 0.67 mL of acrylamide mixture, 2.7 mL of water, 100 µL of 10% SDS, 100 µL of ammonium persulfate, and 5 µL of TEMED.
3. Insert a 10-well gel comb immediately without introducing air bubbles.
4. Electrophoresis is performed at constant amperage with SDS-PAGE running buffer. Start with 15 mA until the sample has entered the gel and then continue at 25 mA till the dye front has reached the bottom of the gel.
5. Cut a nitrocellulose membrane to the size of the gel and immerse it in ethanol. Rinse once with transfer buffer.
6. Immediately after electrophoresis, separate the gel plates with the help of a spatula or similar tool.
7. Rinse the gel carefully with transfer buffer.
8. Perform wet transfer with Mini Trans-Blot Electrophoretic Transfer Cell, using standard procedure with transfer buffer at 400 mA for 2 h at 4°C.

3.4. Immunoblot

3.4.1. Blot for Ubiquitin

1. Block the membrane with blocking solution overnight at 4°C under agitation.
2. Wash three times with TBS-T, 5 min each time.
3. Incubate the membrane with P4D1 (mouse anti-ubiquitin mAb) in TBS-T-1% BSA (dilution 1/2,000) for 4 h at room temperature.
4. Wash the membrane with TBS-T for 15 min.

5. Wash with TBS-T for 5 min. Repeat this step three times.
6. Add anti-mouse IgG HRP-conjugate (dilution 1:20,000 in TBS-T-1% BSA) and incubate for 1 h at room temperature.
7. Wash with TBS-T: once for 15 min and four times for 5 min.
8. Reveal using the enhanced chemiluminescent kit (ECL) following the manufacturer's instructions.

3.4.2. Western Blot Stripping

1. Heat stripping buffer to 50°C in water bath.
2. Incubate membrane with pre-warmed stripping buffer for 20 min with shaking.
3. Rinse membrane four times with TBS-T (10 min each time).

3.4.3. Blot for MHC Class II

1. Block membranes with blocking solution overnight at 4°C under agitation.
2. Incubate membranes with 1.5 µg/mL of XD5 (mouse anti-HLA-DR β chain mAb) in TBS-T-1% BSA, for 2 h at room temperature.
3. Wash with TBS-T: once for 15 min and four times for 5 min.
4. Add anti-mouse IgG HRP-conjugate (dilution 1:20,000 in TBS-T-1% BSA) and incubate for 1 h at room temperature.
5. Wash with TBS-T: once for 15 min and four times for 5 min.
6. Reveal using the enhanced chemiluminescent kit (ECL) following the manufacturer's instructions (Fig. 1).

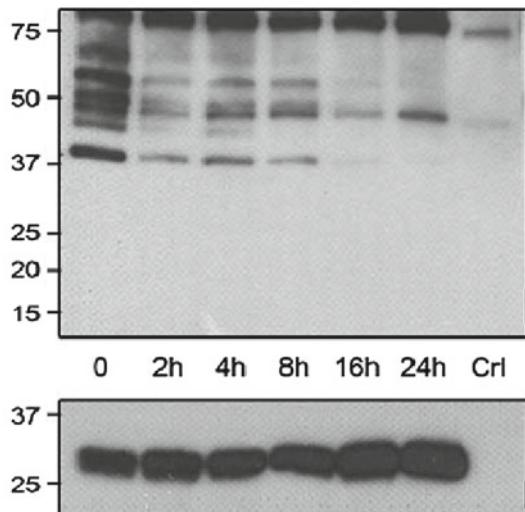


Fig. 1. HLA-DR ubiquitination is downregulated during MoDC maturation. Cells were stimulated with LPS, and HLA-DR molecules were immunoprecipitated with the L243 antibody, then immunoblotted either with anti-ubiquitin (*top panel*) or with anti-MHC-II (XD5) antibodies (*lower panel*).

4. Notes

1. Prepare each time fresh MACS running buffer and filter it to avoid contamination.
2. Prepare a solution 1 M Tris, pH=7.6 (dissolve 60.6 g of Tris in 500 mL of water and adjust the pH), a solution 5 M NaCl (dissolve 146 g NaCl in 500 mL of water), and a solution 1 M MgCl₂ (dissolve 47.6 g of MgCl₂ in 500 mL of water). These solutions can be kept at RT. To prepare the lysis buffer, mix 5 mL of the 1 M Tris pH=7.6 solution, 10 mL of 5 M NaCL solution, 2.5 mL of 1 M MgCl₂ solution, add 5 mL of Triton X-100, and make up to 500 mL with water. Use pre-chilled Lysis buffer.
3. To detect protein ubiquitination, the use of complete lysis buffer is crucial. Indeed, ubiquitination is a reversible modification that is controlled by ubiquitin ligases on one side and deubiquitinases on the other one. To inhibit deubiquitinases, which are cysteine proteases, we use *N*-ethylmaleimide. Moreover, ubiquitination may induce lysosomal or proteosomal degradation of the protein, respectively, inhibited by the complete protease inhibitor cocktail and MG132.

To prepare MG132, resuspend it at a concentration of 10 µM and store small aliquots (20 µL) at -20°C. Dilute it 1:2,000 (5 µL in 10 mL).

4. Before use, equilibrate beads with lysis buffer. Transfer 1 mL of beads (50% slurry, 500 µL final volume of beads) in a 15 mL Falcon and add 10 mL lysis buffer; mix by inverting the tube 4–5 times and pool-down beads by centrifugation (500 ×*g* for 2 min). Repeat washes 2–3 times and adjust the final volume to 1 mL with lysis buffer (50% slurry). Pre-equilibrated beads can be stored at 4°C for 1–2 weeks.
5. In humans, only mature peptide-loaded MHC-II complexes are ubiquitinated (2). Therefore, if you change antibody for the immunoprecipitation, use one specific for the mature form of MHC-II, like L243.
6. Prepare 10× SDS-PAGE running buffer. Weight 30.3 g of Tris, 144 g of glycine (UltraPure Glycine, Invitrogen, 15527-013), and 10 g of SDS and add water to 1 L. Store at RT. Working solution is 1×.
7. Prepare 10× TBS: weight 48.4 g of Tris, 175.3 g NaCl, and add water to 2 L. Adjust pH to 7.5 and store solution at RT. Working solution is 1×.
8. Dilute the leukapheresis product with RPMI medium in order to obtain even number of Falcon tubes and correct balance of the centrifuge.

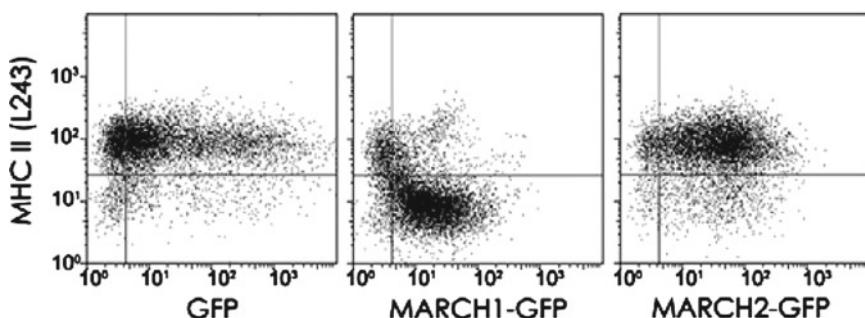


Fig. 2. MARCH1 overexpression downregulates surface MHC class II through ubiquitination.

9. After centrifugation, Ficoll-Paque PLUS and human leuka-pheresis product will form four distinct phases (plasma phase; white mononuclear cell layer containing lymphocytes, monocytes, and platelets; Ficoll; granulocytes and erythrocytes phase). Care should be taken during manipulation of Falcon tubes in order not to disturb the mononuclear cell layer.
10. CD14⁺ cells can also be frozen: 15×10^6 cells for vial in freezing medium. Keep at -80°C for short periods of storage and for longer one transfer them in liquid nitrogen. Each vial can be used for one 6-well plate.
11. Transfection efficiency was evaluated by FACS measuring eGFP fluorescence and MHC class II surface expression levels, as showed in Fig. 2.
12. If you wish to stop the protocol and continue later, freeze the cell pellets at -20°C . Do not keep it for more than 1 month. On the contrary, once you have performed the lysis proceed immediately with immunoprecipitation, do not freeze lysates.
13. Denaturing sample buffers reduce the antibody of the immunoprecipitation in heavy and light chain, respectively, of 50 and 25 kDa. These unspecific bands may mask the one of interest.
14. The use of 8% SDS polyacrylamide gel is not mandatory. However, for efficient separation of bands, use a gel which allows a good discrimination between 25 and 80 kDa.

References

1. Pierre P et al (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 388(6644):787–792
2. de Gassart A et al (2008) MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH1 down-regulation. *Proc Natl Acad Sci* 105(9):3491–3496
3. Shin J-S et al (2006) Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature* 444(7115):115–118
4. van Niel G et al (2006) Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity* 25(6):885–894

5. Komander D (2009) The emerging complexity of protein ubiquitination. *Biochem Soc Trans* 37(Pt 5):937–953
6. Baravalle G et al (2011) Ubiquitination of CD86 is a key mechanism in regulating antigen presentation by dendritic cells. *J Immunol* 187(6):2966–2973
7. Bartee E et al (2004) Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J Virol* 78(3):1109–1120
8. Goto E et al (2003) c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. *J Biol Chem* 278(17):14657–14668
9. Ishido S et al (2009) E3 ubiquitin ligases for MHC molecules. *Curr Opin Immunol* 21(1):78–83
10. Toyomoto M et al (2011) Anti-arthritic effect of E3 ubiquitin ligase, c-MIR, expression in the joints. *Int Immunopharmacol* 23(3):177–183
11. Bania J et al (2003) Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. *Proc Natl Acad Sci U S A* 100(11):6664–6669
12. Roche PA et al (1990) Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* 345(6276):615–618
13. Radka SF et al (1984) Analysis of monoclonal antibodies reactive with human class II beta chains by two-dimensional electrophoresis and Western blotting. *Hum Immunol* 10(3):177–186

Chapter 39

Studying MHC class II Presentation of Immobilized Antigen by B Lymphocytes

M.I. Yuseff and A.M. Lennon-Dumenil

Abstract

The ability of B lymphocytes to capture external antigens (Ag) and present them as peptide fragments, loaded on Major Histocompatibility complex (MHC) class II molecules, to CD4⁺ T cells is a crucial part of the adaptive immune response. This allows T-B cooperation, a cellular communication that is required for B cells to develop into germinal centers (GC) and form mature high-affinity antibody producing cells and to further develop B cell memory. MHC class II antigen presentation by B lymphocytes is a multistep process involving (1) Recognition and capture of external Ag by B lymphocytes through their B cell receptor (BCR); (2) Ag processing, which comprises the degradation of Ag in internal compartments within the B cell and loading of the corresponding peptide fragments on MHC class II molecules and (3) Presentation of MHCII-peptide complexes to CD4⁺ T cells. Here, we describe how to study MHC class II antigen presentation by B lymphocytes at these three major levels.

Key words: B lymphocytes, Immune synapse, Antigen extraction, Processing and presentation, MTOC polarization, Lysosome secretion

Abbreviations

Ag	Antigen
BCR	B cell receptor
BSA	Bovine serum albumin
FBS	Fetal bovine serum
GC	Germinal center
Ig	Immunoglobulin
IS	Immune synapse
MHC	Major histocompatibility complex
MTOC	Microtubule organizing center
OVA	Ovalbumin

1. Introduction

The onset of an adaptive immune response requires the activation of B lymphocytes. This is initiated upon the engagement of Ag to the cell surface B cell receptor (BCR) which is composed of a transmembrane Immunoglobulin (Ig) coupled to a signaling module formed by the Ig α /Ig β dimer (1, 2). BCR engagement induces a complex cascade of signaling events that ultimately leads to cell proliferation and initiation of GC development. To complete GC formation, activated B lymphocytes must present BCR-uptaken Ag as peptide fragments, loaded on MHC class II molecules to primed CD4 T cells (3). Productive Ag presentation relies on the convergence of BCR-Ag complexes with lysosomal-like compartments where processing of the Ag and peptide loading on MHC II molecules occur (4).

In vivo, B cell activation mainly depends on their recognition of Ag tethered to the surface of neighboring cells within the lymph nodes, which leads to the formation of an immune synapse (IS) between the B cell and the Ag-bearing cell (macrophages or dendritic cells) (5–7). Synapse formation allows sustained BCR signaling (8, 9), which controls the reorganization of the B cell actin cytoskeleton to promote efficient extraction of Ag at the IS through a two-phase membrane spreading and cell contraction response (10). Additionally, polarization of the microtubule network, highlighted by the repositioning of the microtubule-organizing center (MTOC) (11) at the site of Ag encounter is necessary to direct the transport of MHC II containing lysosomal vesicles towards the IS. There vesicles undergo exocytosis and release extracellular hydrolases, generating the acidification of the synaptic interface where the extraction of the immobilized Ag occurs. The morphological changes that characterize these initial stages of B lymphocyte activation and Ag extraction can be visualized by microscopy upon immunofluorescent labeling of these intracellular compartments. For this purpose, B cells can be stimulated with soluble multivalent BCR ligands (12, 13), where polarization of the actin cortex and the convergence of the uptaken Ag with Lamp-1 $^{+}$ /MHC II vesicles is observed. However, by using Ag-coated latex beads one can mimic the encounter of B cells with immobilized Ag, where a synapse is formed. Under these conditions, polarization of the MTOC, actin cytoskeleton and recruitment of Lamp-1 $^{+}$ /MHC II vesicles towards the Ag-coated bead (synaptic interface) can easily be studied. Additionally, this *in vitro* model is useful to quantify the amount of Ag extracted and to study the acidification of the synaptic interface by using latex beads coated with Ag coupled to a pH-sensitive dye.

Upon extraction, internalized BCR-Ag complexes are efficiently transported to Lamp-1 $^{+}$ late endo-lysosomal compartments, where

they converge with the Ag processing machinery (14) including, proteases (15), MHC class II, and H2-DM molecules (4). MHC class II products consist of two chains that dimerize shortly after synthesis in the endoplasmic reticulum (ER) where they associate with the molecular chaperone, invariant chain (Ii) to promote their transport to the endocytic pathway (16). There, Ii is removed by proteolysis (17, 18) and antigenic peptides are loaded, through a reaction catalyzed by the chaperone H2-DM (19). The loading of antigenic peptides loaded on MHC II molecules can be studied by using conformational antibodies capable of detecting such complexes. We use a mAb 2C44 that specifically recognizes the complexes formed between MHC II ($I-A^d$) molecules and the 156–173 peptide from *Leishmania major* Ag (LACK) but does not bind to any of the free components (12, 13, 20). By coupling BCR ligands and Lack protein on the same latex beads, this Ag can be targeted for BCR-mediated uptake. The appearance of $I-A^d$ -LACK156–173 complexes in intracellular compartments of B cells can be detected by immunofluorescent labeling and visualized by microscopy. Once Ag has been processed and loaded on MHC class II molecules these complexes are transported to the cell surface where they are presented to the corresponding T cell ($CD4^+$), which will stimulate the B cell by secreting cytokines, such as IL-2. Consequently, this last step can be quantified by measuring the activation of a Lack responding T cell hybridoma (11–13) by B cells that have previously uptaken Lack protein. Therefore, the use of these specific tools provides a direct and precise approach to study the efficiency of Ag processing and presentation by B lymphocytes.

Overall, Ag extraction, processing, and presentation are all essential steps of MHC class II antigen presentation by B lymphocytes. We here describe methods to study each of these components and use an *in vitro* model, which consists of Ag-coated latex beads that mimic how B cells encounter Ag *in vivo*.

2. Materials

2.1. Cells and Culture

1. The mouse B lymphoma II A1.6 cell line is an Fc γ R-defective variant of A20 cells and has the phenotype of quiescent mature B-cells expressing surface IgG2a as a BCR (21).
2. Primary B cells:
 - (a) Resting mature B cells containing cell surface IgM $^+$ /IgD $^+$ (BCR) are obtained from spleens of 8–12-week-old mice. These are purified by negative selection from a single-cell suspension generated by the mechanical disruption of the spleen (Miltenyi Biotec, see manufacturer's instructions).

- (b) Primary B-cells can also be purified from the spleen of I-A β -green fluorescent protein (GFP) knockin (referred to as MHC II-GFP) mice (22). By using these cells, endogenous MHC II molecules can be visualized directly by fluorescence microscopy.
- 3. The LMR7.5 Lack T cell hybridoma, which recognizes I-A d /LACK₁₅₆₋₁₇₃ complexes, is used for the antigen presentation assay (12, 13).
- 4. Culture medium: RPMI 1640 supplemented with 10 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-mercaptoethanol (2-ME), 5 mM sodium pyruvate, and 10% fetal bovine serum (FBS) (BioWest).
- 5. 1× PBS sterile.
- 6. FBS.

2.2. Antibodies

2.2.1. Primary Antibodies

- 1. Rabbit anti-mouse H2-DM (obtained by immunizing rabbits with a synthetic peptide corresponding to the cytoplasmic tail of the β chain (4)).
- 2. Biotinylated 2C44 mAb restricted to I-Ad/LACK156-173 complexes (20)
- 3. Rat anti-mouse CD107a (LAMP-1) (BD Biosciences).
- 4. Rabbit anti- γ Tubulin (provided by Michel Bornens, Institut Curie, Paris, France).
- 5. Rabbit anti-OVA (Sigma).
- 6. Phalloidin conjugated to FluoroProbes 547 (Molecular Probes) is used to stain actin.

2.2.2. Secondary Antibodies

- 1. F(ab')2 Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch).
- 2. F(ab')2 Cy5-conjugated donkey anti-rat (Jackson ImmunoResearch).
- 3. F(ab')2 Alexa488-conjugated donkey anti-rat (Molecular Probes).
- 4. F(ab')2 Alexa488-conjugated anti-rabbit (Molecular Probes).
- 5. F(ab')2 Alexa647-conjugated anti-rabbit (Molecular Probes).
- 6. Tyramide amplification kit containing streptavidin conjugated to HRP and Alexa546-tyramide (TSA Kit, Molecular Probes).

2.3. B Cell Activation (See Note 1)

- 1. F(ab')₂ Donkey anti-goat IgG (Jackson ImmunoResearch Laboratories).
- 2. F(ab')₂ goat anti-mouse-IgG (MP Biomedicals).
- 3. F(ab')₂ goat anti-mouse-IgM (MP Biomedicals).
- 4. 10× solution of the immune complex:

- (a) For IIA1.6 mouse lymphoma B cells: Mix 200 µg/ml of F(ab')₂ Donkey anti-goat IgG and 100 µg/ml F(ab')2 goat anti-mouse-IgG in RPMI. Incubate at 37°C for 30 min.
- (b) For primary spleen B cells: Mix 200 µg/ml of F(ab')₂ Donkey anti-goat IgG and 100 µg/ml F(ab')2 goat anti-mouse-IgM in RPMI. Incubate at 37°C for 30 min.
5. 3 µm Latex NH2-beads (Polyscience), for covalent linking of Ag.
6. 3 µm Latex polystyrene-beads (Polyscience), for passive adsorption of Ag.
7. 8% Glutaraldehyde in PBS.
8. Ovalbumin (Sigma).
9. Leishmania major recombinant protein (LACK) ([20](#)).
10. Fibronectin (Sigma).

2.4. Immuno-fluorescence

1. 3% Paraformaldehyde(PFA).
2. 10 mM Glycine.
3. Permeabilizing solution: 1× PBS, 0.2% BSA, 0.05% saponin.
4. Poly-L Lysine-coated cover slips.
5. Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL.).
6. Confocal Microscope (LSM Axiovert 720; Carl Zeiss MicroImaging, Inc.).
7. Metamorph software.

2.5. Time-Lapse Using Fluorescence Microscopy

1. Cells expressing the appropriate fluorescent markers:
 - (a) Spleen-derived primary B cells from MHC II-GFP mice to observe lysosomal compartments.
 - (b) The IIA1.6 cell line transfected with plasmids encoding centrin-GFP to label the MTOC and/or Cathepsin D-mRFP, which can be used as a live cell marker of MHCII⁺/LAMP-1⁺ vesicles ([11](#)). Transfection can be performed by using the Nucleofactor kit R (Lonza), according to manufacturer's instructions.
2. 35 mm Iwaki type chamber (Fluorodish).
3. Spinning disk microscope (Nikon Eclipse TE2000-U microscope).

2.6. Ag Extraction

1. Buffer for flow cytometry: PBS, 3% FBS.
2. Lysis buffer: 50 mM Tris, pH 7.0.5% NP-40, 150 mM NaCl, cocktail of protease inhibitors (Roche), DNase I (10-100U), 1 µM DTT.

3. PBS-2% BSA.
4. Rabbit anti-OVA antibody (Sigma).
5. Fluorophore-conjugated donkey anti-rabbit (see Subheading 2.1).
6. 96-well v-bottom plates.
7. FACS tubes.
8. Flow cytometer.

2.7. Measurement of Acidification at the Immune Synapse by Fluorescence Microscopy

1. 3 μ m Polystyrene beads (Polyscience) coated with F(ab')₂ goat anti-mouse-IgG conjugated to the dye Cypher5E Mono NHS ester (Amersham).
2. 35 mm Iwaki type chamber (Fluorodish).
3. Spinning disk microscope (Nikon Eclipse TE2000-U microscope).

2.8. Antigen Presentation Assay: ELISA

1. 24- and 96-well plates.
2. B lymphocytes: II A1.6 mouse B lymphoma or primary B cells obtained from I-A^d mice.
3. T lymphocytes: LMR7.5 Lack T cell hybridoma.
4. 3 μ m Latex NH2-beads covalently coated with the following Antigens:
 - (a) Lack protein/F(ab')₂ goat anti-mouse-IgG.
 - (b) Lack protein/F(ab')₂ goat anti-mouse-IgM.
 - (c) Lack protein (negative control).
5. Lack peptide 156–173 (20).
6. kit IL2 Elisa (BD-Bioscience).
7. ELISA plate reader.

If using fixation before adding T cells, the following reagents are also required:

8. Cold PBS.
9. Cold PBS/0.01%-glutaraldehyde.
10. Cold 100 μ M Glycine.

3. Methods

3.1. Preparation of Immobilized Ag: Coating Latex Amino Beads with Ag

1. Wash 3 μ m latex NH2-beads with PBS in an Eppendorf tube. 100 μ l (1.7×10^8 beads) can be used to prepare four different sets of Ag-coated beads. More beads can be activated if required.
2. Activate NH2 groups by incubating the beads in 500 μ l of 8% glutaraldehyde and spin on a wheel for 2 h at room temperature.

3. Centrifuge beads at $10,000 \times g$ for 5 min at 4°C and eliminate glutaraldehyde. Add 1 ml of PBS to wash beads and centrifuge again. Repeat two times and resuspend activated beads in 200 μ l of PBS.
4. In different Eppendorf tubes, dilute each Ag to 20 μ g/ml in 150 μ l of PBS:
 - (a) For the morphological analysis of activated B cells, two different sets of beads are coated with either F(ab')₂, goat anti-mouse-IgM or IgG.
 - (b) To study the extraction of Ag (model protein OVA), two different sets of beads are coated with F(ab')₂, goat anti-mouse-IgM or IgG plus OVA protein.
 - (c) To analyze the processing of Lack protein and its presentation to T cells, two sets of beads are coated with F(ab')₂, goat anti-mouse-IgM or IgG plus Lack protein.
 - (d) As a negative control one set of beads coated with Fibronectin (FN) to 2 μ g/ml, is used.
5. Add 50 μ l of activated beads (from Subheading 3.1, step 3) in each Eppendorf tube and incubate in a wheel overnight at 4°C.
6. Add 800 μ l of cold PBS to each tube and centrifuge beads at $10,000 \times g$ for 5 min at 4°C to eliminate free Ag. Repeat washes two times with PBS.
7. Leave Ag-coated beads in 40 μ l of PBS to obtain a suspension of beads at $1 \times 10^6/\mu$ l.

3.2. Activation of B Lymphocytes

3.2.1. Activation with Soluble Multivalent BCR Ligands

3.2.2. Activation with Ag-coated NH2 Beads

3.3. Morphological Analysis of Activated B Lymphocytes

3.3.1. Immunofluorescence

1. Dilute cells to the following concentrations: 2×10^6 cells/ml of IIA1.6 cells or 10^7 cells/ml of primary B cells in RPMI-5% FBS. This can be done in 1.5 ml Eppendorf or 15 ml Falcon tubes (see Note 2).
2. Add the immune complex final to 1x: To a solution of 900 μ l of cells add 100 μ l of the 10x immune complex.
1. Dilute cells to the following concentrations: 2×10^6 cells/ml of IIA1.6 cells or 10^7 cells/ml of primary B cells in RPMI-5% FBS. This can be done in Eppendorf (1.5 ml) or Falcon tubes (15 ml) (see Note 2).
2. Add Ag-coated NH2-beads at a 1:1 ratio with cells (see Note 3). Use the Ag-coated beads according to each type of experiment (see Subheading 3.1, step 4).
1. Plate 100 μ l of activated cells, obtained as described in Subheading 3.2, on poly-L-lysine-coated glass coverslips and incubate for different time points in an cell culture incubator (37°C/5% CO₂). The typical time points used in this assay are 0, 1 and 2 h. Cover the slides, so that they do not dry out (see Note 4).

The following steps can be carried out at RT.

2. Stop activation and fix the cells by gently removing supernatant from slides (cells should be adhered on slide) and replacing it with 100 µl of 3% PFA. Incubate for 10 min. This should be done under a hood.
3. Carefully remove the PFA and rinse three times with PBS. This is done with a plastic dropping pipette and then aspirated with a vacuum system on the bench (see Note 5).
4. Add 1 mM glycine to each slide with the plastic dropping pipette and incubate for 10 min. Rinse two times with PBS, as described in step 3.
5. In order to perform intracellular staining of Ag processing compartment and the microtubule cytoskeleton, permeabilize fixed cells by adding permeabilizing solution to each slide and incubate for 20 min.
6. Stain fixed cells with antibodies diluted in permeabilizing solution. Add 100 µl/slide and leave at RT for 1 h. Label the following intracellular structures/compartments:
 - (a) Microtubule Organizing Center (MTOC): Use Anti- γ Tubulin at a dilution of 1/100 (see Note 6).
 - (b) The lysosomal compartment where Ag is processed: This is positive for MHC II, H2-DM, and Lamp-1. If primary B cells from MHC II-GFP mice are used in this study MHCII molecules can be directly visualized by fluorescence microscopy. Use Rat anti-Lamp-1 at a dilution of 1/250 and/or H2-DM at a dilution of 1/250.
 - (c) Actin cytoskeleton: polymerized actin can be visualized by staining with phalloidin conjugated to FluoroProbes 547.
7. Wash three times with permeabilizing solution with the plastic dropping pipette and incubate for 45 min with 100 µl of secondary antibodies at a dilution of 1/200 in permeabilizing solution or according to manufacturer's instructions. Use the appropriate secondary antibodies according to the primary antibodies used (see Notes 7 and 8).
8. Mount coverslips onto a slide using Fluoromount-G and dry for 30 min at 37°C.
9. Acquire immunofluorescence images on a confocal microscope with a 63 \times 1.4 NA oil immersion objective. Acquire cells with transmission and different channels to detect fluorescent staining.
10. Image analysis.
 - (a) Calculation of the MTOC Polarity Index. MTOC polarization is quantified by calculating a "polarity index" corresponding to the distance between the

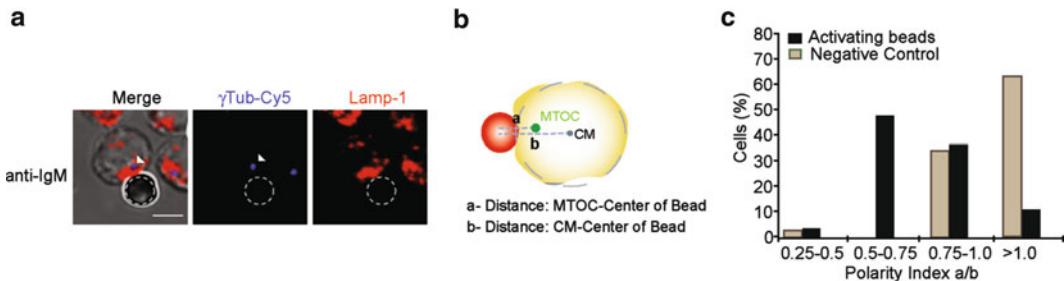


Fig. 1. (a) Spleen-derived primary B cell activated with an Ag-coated bead (anti-IgM) for 1 h. Staining for γ -tubulin (MTOC) in blue and Lamp-1 (lysosomes) in red is shown, scale bar = 3 μ m. Arrow head indicates the MTOC. (b) Scheme depicting how to evaluate MTOC polarization. The distances between (a) the MTOC or (b) cell center of mass (CM) and the Ag-coated bead CM are shown. The polarity index = the ratio of a/b. (c) MTOC polarization can be represented in a graph showing the distribution of polarity indexes.

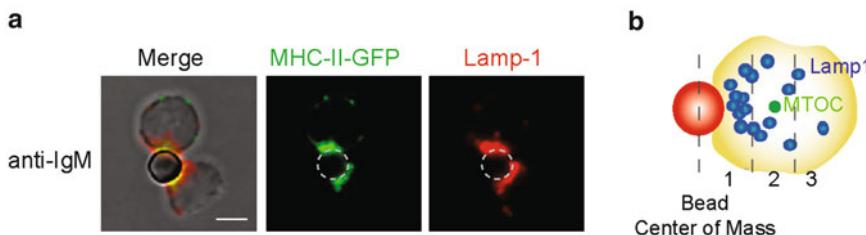


Fig. 2. (a) Spleen-derived primary B cell isolated from an MHCII-GFP mouse activated with an Ag-coated bead (anti-IgM) for 1 h. Lamp-1 (lysosomes) staining in red colocalizes with MHC II GFP, scale bar = 3 μ m. (b) Scheme illustrating how to quantify the redistribution of Lamp-1⁺ vesicles towards the site of Ag interaction (IS).

MTOC (γ -Tubulin staining) and the bead center of mass divided by the distance between both the cell and the bead centers of mass (Fig. 1). The distance between the MTOC or cell center of mass (CM) and the bead center of mass can be measured by metamorph software. The ratio between these two values gives the polarity index for each cell and represents the degree of polarization of the cell. Cell incubated with nonactivating beads should display a polarity index similar or higher than 1, indicating that their MTOC is located near the cell center. In contrast, B cells treated with activating beads exhibit polarity indexes between 0.5 and 0.75, which means that their MTOC had been positioned toward the IS.

- (b) Quantification of LAMP-1 recruitment to the IS.
By using metamorph software, a line-scan from the bead CM to the end of the cell is performed in B cell-bead conjugates stained for LAMP-1. The percentage of LAMP-1 recruited to the Ag-coated bead is calculated from the amount of LAMP-1 staining detected within the first third of the cell with respect to the bead and relative to the total amount of LAMP-1 staining across the entire cell (Fig. 2).

3.3.2. Time-Lapse

The dynamic polarization of the MTOC and lysosomes at the IS can be studied by time-lapse fluorescence microscopy. With this objective, cells expressing the appropriate fluorescent markers should be used (see Subheading 2.5).

- Spleen-derived primary B cells from MHC II-GFP mice to observe lysosomal compartments.
- The IIA1.6 cell line transfected with plasmids encoding centrin-GFP to label the MTOC and/or Cathepsin D-mRFP, which can be used as a live cell marker of MHCII⁺/LAMP-1⁺ vesicles (11). Transfection can be performed by using the Nucleofactor kit R (Lonza), according to manufacturer's instructions.
 1. Prepare a suspension of cells expressing the appropriate fluorescent markers at 2×10^6 cells/ml for the IIA1.6 cell line transfected or 6×10^6 cells/ml for spleen-derived primary B cells in RPMI-5% FBS.
 2. Plate 500 μ l of cells on poly-L-lysine-coated 35 mm Fluorodish and incubate for 20 min at 37°C to attach cells.
 3. Rinse gently with RPMI-5% FBS to eliminate unbound cells and fill the Fluorodish with 1.5 ml of medium.
 4. Observe live cells in a confocal spinning disk at 37°C and 5% CO₂. Once cells are in focus, add 1 μ l of Ag-coated beads prepared as described in Subheading 3.1, step 4.
 5. Focus on a cell that expresses the fluorescent marker of choice and that is engaged to an Ag-coated bead. Acquire sequential images every 30 s–1 min in transmission and the appropriate channels (see Note 9).
 6. Process images to visualize the polarized recruitment of both the MTOC and lysosomes to the site of contact with the Ag-coated bead.

3.4. Ag Extraction**3.4.1. Quantifying Ag-Extraction by Immunofluorescence**

1. Incubate cells incubated in a 1:1 ratio with Ag-coated beads (OVA/F(ab')₂, anti-mouse-IgM or anti-mouse-IgG, in equal concentrations, see Subheading 3.2.2). This is done in Eppendorf tubes in RPMI-5% FBS.
2. Following steps are identical to Subheading 3.3.1, steps 2–5.
3. Stain fixed cells with anti-OVA at a dilution of 1/100 and anti-Lamp-1 at a dilution of 1/250 diluted in permeabilizing solution. Add 100 μ l/slides and leave at RT for 1 h.
4. Wash three times with permeabilizing solution with the plastic dropping pipette and incubate for 45 min with 100 μ l of secondary antibodies diluted at 1/200 in permeabilizing solution or according to manufacturer's instructions. Use a F(ab')₂ Cy3-conjugated donkey anti-rabbit and a F(ab')₂ Alexa488-

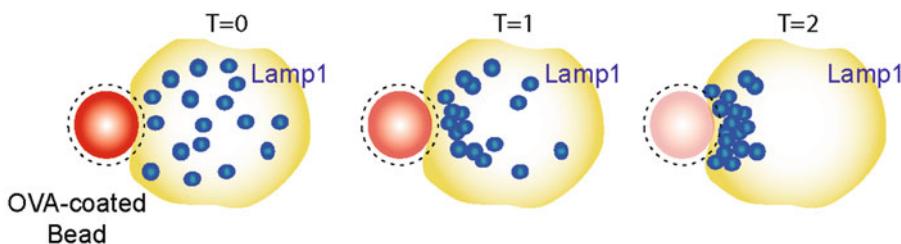


Fig. 3. Scheme showing how to quantify Ag extraction. The amount of Ag (OVA) remaining on the bead after incubation with B cells at different time points is represented. The disappearance of Ag from the bead correlates with the recruitment of lysosomal Lamp-1⁺ vesicles towards the synaptic interface.

conjugated donkey anti-rat for the OVA and Lamp1 staining, respectively (see Notes 7 and 8).

5. Following steps are identical to Subheading 3.3.1, steps 8–9.
6. Image analysis.

The amount of OVA present on the beads after each time point is calculated by using metamorph software, by establishing a fixed area around beads in contact with cells and the fluorescence intensity is measured on 3D projections obtained from the sum of each plane (Fig. 3).

3.4.2. Quantifying Ag-Extraction by Flow Cytometry

1. Prepare the mix of cell-bead conjugates (use beads described in Subheading 3.1, step 4) at a 2:1 ratio according to the number of points in the experiment. Use 2.0×10^6 cells with 1.0×10^6 antigen-coated beads per ml in RPMI/5% FBS. For five time points prepare 5 ml of bead-cell conjugates per condition:
 - (a) Condition 1: IIA1.6 cells incubated with F(ab')₂ goat anti-mouse-IgG/OVA.
 - (b) Condition 2: IIA1.6 cells incubated with F(ab')₂ goat anti-mouse-IgM/OVA.
2. Add 1 ml of cell-bead conjugates in 12-well plates and incubate for 0, 1, 2, 4, and 6 h at 37°C.
3. After each time point cells interacting with beads are recovered from each well and transferred to 1.5 ml Eppendorf tubes, placed on ice and washed two times with cold PBS + protease inhibitors, in order to stop protein degradation.
4. Cells are finally resuspended in 100 µl of PBS and put in v-bottom 96-well plates. Cells from different time points are progressively accumulated in the 96-well plates, which always remains on ice.
5. Next, cells are lysed in 100 µl of lysis buffer on ice for 30 min and centrifuged (in the plate), at $2,000 \times g$ for 5 min. After centrifugation, a cluster of cell debris can be observed at the

top of each well and can be carefully eliminated with a pipette tip. The beads should remain at the bottom of the plate.

6. Wash beads two times with 200 μ l of cold PBS-2% BSA and finally incubate the beads with 100 μ l of primary antibody rabbit polyclonal anti-OVA diluted at 1/500 in PBS-2% BSA for 1 h on ice.
7. Wash by adding 100 μ l of PBS-2% BSA and centrifuge at 2,000 $\times g$ for 5 min and then repeat another wash with 200 μ l of PBS-2% BSA.
8. Incubate beads with 100 μ l of the secondary antibody diluted at 1/250 in PBS-2% BSA. An anti-rabbit conjugated to Alexa 647 or Alexa488 can be used. Leave 30 min on ice in the dark. Wash, as previously described in step 5.
9. Analyze by FACS samples resuspended in 200 μ l of buffer for flow cytometry (see Note 10).
10. Read samples in by Flow Cytometry to obtain the Mean Fluorescence Intensity (MFI) of beads from each condition and calculate the percentage of Ag degradation relative to time 0.

*3.4.3. Acidification
at the IS: Analysis by
Fluorescence Microscopy*

1. Couple Cypher5E dye to F(ab')₂ goat anti-mouse-IgG, following manufacturer's instructions (Amersham).
2. Passively adsorb the Cypher5E-conjugated F(ab')₂ goat anti-mouse-IgG onto polystyrene 3.0 μ m beads by incubating 50 μ l of beads with 10 μ g/ml of F(ab')₂ fragments in 200 μ l of PBS at RT while spinning on a wheel. Wash two times to eliminate unbound ligand and resuspend in 100 μ l of PBS. Store at 4°C covered from light until use.
3. Prepare the mix of cell-bead conjugates at a 2:1 ratio in RPMI/5%-FBS. Use 2.0 $\times 10^6$ cells with 1.0 $\times 10^6$ antigen-coated beads containing F(ab')₂ goat anti-mouse-IgG coupled to Cypher5E per ml of RPMI-5% FBS.
4. Plate 500 μ l of activated cells on poly-L-lysine-coated 35 mm Fluorodish and incubate for 90 min at 37°C.
5. Remove the supernatant (cells should be adhered on the dish) and add 1.5 ml of warm RPMI-5%FBS.
6. Acquire images of live cells in a confocal spinning disk at 37°C.
7. Image analysis.

When the synaptic interface surrounding the Cypher5E/Ag-coated bead becomes acidic, the fluorescence intensity of the Cypher5E dye increases. This can be quantified by calculating the percentage of anti-IgG-Cypher beads associated to cells that are positive for Cypher fluorescence (MFI of the bead >10% above background levels).

3.5. Antigen Processing

3.5.1. Antigen Processing: Detecting processed Ag in Lysosomal Compartments by Immunofluorescence

1. Incubate cells at a 1:1 ratio with Ag-coated beads (Lack/ $F(ab')_2$ anti-mouse-IgM or Lack/ $F(ab')_2$ anti-mouse-IgG, in equal concentrations, see Subheading 3.2.2). This is done in Eppendorf tubes in RPMI 5% FBS.
2. Following steps are identical to Subheading 3.3.1, steps 2–5.
3. Stain fixed cells with Biotinylated 2C44 mAb restricted to I-A^d/LACK_{156–173} complexes at a dilution of 1/100 and anti-Lamp-1 at a dilution of 1/250 diluted in permeabilizing solution. Add 100 μ l/slide and leave at RT for 1 h.
4. Wash three times with permeabilizing solution with the plastic dropping pipette and incubate for 45 min with 100 μ l of secondary antibodies diluted in permeabilizing solution. Use $F(ab')_2$ Alexa488-conjugated donkey anti-rat diluted at 1/200 solution for Lamp1 staining and streptavidin-HRP for detection of I-A^d/LACK_{156–173} complexes, according to manufacturer's instructions (see Note 6).
5. Following steps are identical to Subheading 3.3.1, steps 8–9.
6. Image analysis.

Confocal images of B lymphocytes incubated with anti-IgG or IgM /Lack-beads for 0, 1, 2, and 4 h at 37°C. Lack complexes appear between 2–4 h.

3.6. Ag Presentation Assay

1. Prepare the mix of cells (spleen B cells obtained from Balbc WT mice (I-A^d haplotype) or IIA1.6 cells (I-A^d haplotype)). Prepare two sets of cells at 1.0×10^6 /ml in RPMI-5% FBS:
 - (a) Activate one set with Lack-coated beads (obtained as described in Subheading 3.1) at a 1:1 ratio.
 - (b) Activate the second set with $F(ab')_2$ anti-mouse-IgM and anti-mouse-IgG beads at a 1:1 ratio. These will be used for the peptide control.
2. Add 100 μ l of the cell-bead conjugate mix in 96-well plates and incubate for 4 h at 37°C to allow uptake and processing of Lack antigen. After 3 h add increasing concentrations of Lack peptides to cells preincubated only with $F(ab')_2$ anti-mouse-IgM or anti-mouse-IgG beads. Use 0, 2, 5, 10 and 20 μ M of the peptide.
3. Wash B cells two times by adding 200 μ l of cold PBS and centrifuging the plate at $300 \times g$ for 5 min. Next, proceed to fixation. This part is optional if you want to stop the processing of the Ag. Otherwise you can continue from step 7.
4. Eliminate the PBS and add 100 μ l of cold PBS-0.01% glutaraldehyde. Incubate 1 min on ice.
5. Add 100 μ l of cold PBS-100 μ M Glycine and centrifuge plate at $500 \times g$ at 4°C for 5 min.

6. Wash one time with PBS and one time with RPMI-5% FBS.
7. Add 150 μ l of Lack T hybridoma cells ($0.75 \times 10^6/\text{ml}$) per well and incubate at 37°C for another 4 h.
8. Recover supernatants from each condition and measure levels of IL-2 following manufacturer's instructions.

4. Notes

1. Given that the BCR is a cell surface immunoglobulin, a F(ab')₂ fragment raised against a mouse Ig can be used to engage the BCR from the mouse B cells and mimic the interaction of an Ag. This is also referred to as Ag.
2. Preparation of 1 ml suspensions of cells yields ten slides used for morphological studies.
3. As negative controls: use the IgM immune complex for IIA1.6 cells and the IgG immune complex for primary B cells.
4. For time 0, incubate cells on cover slips for 10 min at RT.
5. Fixed cells on slides can remain in PBS until 24 h. Samples performed at different time points can be accumulated at this step before continuing to the next one.
6. To optimize staining for γ Tubulin, preincubate fixed cells on slides with cold methanol for 30 s. Rinse with permeabilizing solution and then add the antibody.
7. Different options are available when using secondary antibodies. Use the ones that are more suited to the microscope available.
8. Incubate samples in the dark.
9. Upon engagement with immobilized Ag, polarization of lysosomes and MTOC at the IS occur within 1 h.
10. Control for staining: use beads labeled only with the secondary antibody.

References

1. Cambier JC, Pleiman CM, Clark MR (1994) Signal transduction by the B cell antigen receptor and its coreceptors. *Annu Rev Immunol* 12:457–486. doi:[10.1146/annurev.ij.12.040194.002325](https://doi.org/10.1146/annurev.ij.12.040194.002325)
2. Reth M, Wienands J (1997) Initiation and processing of signals from the B cell antigen receptor. *Annu Rev Immunol* 15:453–479. doi:[10.1146/annurev.immunol.15.1.453](https://doi.org/10.1146/annurev.immunol.15.1.453)
3. Mitchison NA (2004) T-cell-B-cell cooperation. *Nat Rev Immunol* 4(4):308–312. doi:[10.1038/nri1334](https://doi.org/10.1038/nri1334)
4. Lankar D, Vincent-Schneider H, Briken V, Yokozeki T, Raposo G, Bonnerot C (2002) Dynamics of major histocompatibility complex class II compartments during B cell receptor-mediated cell activation. *J Exp Med* 195(4):461–472

5. Carrasco YR, Batista FD (2007) B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* 27(1):160–171. doi:[S1074-7613\(07\)00334-2](#) (pii)
6. Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, Fink K, Henrickson SE, Shayakhmetov DM, Di Paolo NC, van Rooijen N, Mempel TR, Whelan SP, von Andrian UH (2007) Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 450(7166):110–114. doi:[nature06287](#) (pii)
7. Suzuki K, Grigorova I, Phan TG, Kelly LM, Cyster JG (2009) Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med* 206(7):1485–1493. doi:[jem.20090209](#) (pii)
8. Harwood NE, Batista FD (2008) New insights into the early molecular events underlying B cell activation. *Immunity* 28(5):609–619. doi:[S1074-7613\(08\)00197-0](#) (pii)
9. Tolar P, Sohn HW, Pierce SK (2008) Viewing the antigen-induced initiation of B-cell activation in living cells. *Immunol Rev* 221:64–76. doi:[IMR583](#) (pii)
10. Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD (2006) B cell ligand discrimination through a spreading and contraction response. *Science* 312(5774):738–741. doi:[312/5774/738](#) (pii)
11. Yuseff MI, Reversat A, Lankar D, Diaz J, Fanget I, Pierobon P, Randrian V, Larochette N, Vascotto F, Desdouets C, Jauffred B, Bellaiche Y, Gasman S, Darchen F, Desnos C, Lennon-Dumenil AM (2011) Polarized secretion of lysosomes at the B cell synapse couples antigen extraction to processing and presentation. *Immunity* 35(3):361–374. doi:[S1074-7613\(11\)00276-7](#) (pii)
12. Vascotto F, Lankar D, Faure-Andre G, Vargas P, Diaz J, Le Roux D, Yuseff MI, Sibarita JB, Boes M, Raposo G, Mougneau E, Glaichenhaus N, Bonnerot C, Manoury B, Lennon-Dumenil AM (2007) The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *J Cell Biol* 176(7):1007–1019. doi:[jcb.200611147](#) (pii)
13. Le Roux D, Lankar D, Yuseff MI, Vascotto F, Yokozeki T, Faure-Andre G, Mougneau E, Glaichenhaus N, Manoury B, Bonnerot C, Lennon-Dumenil AM (2007) Syk-dependent actin dynamics regulate endocytic trafficking and processing of antigens internalized through the B-cell receptor. *Mol Biol Cell* 18(9):3451–3462. doi:[E06-12-1114](#) (pii)
14. Aluvihare VR, Khamlich AA, Williams GT, Adorini L, Neuberger MS (1997) Acceleration of intracellular targeting of antigen by the B-cell antigen receptor: importance depends on the nature of the antigen-antibody interaction. *EMBO J* 16(12):3553–3562. doi:[10.1093/emboj/16.12.3553](#)
15. Lennon-Dumenil AM, Bakker AH, Wolf-Bryant P, Ploegh HL, Lagaudriere-Gesbert C (2002) A closer look at proteolysis and MHC-class-II-restricted antigen presentation. *Curr Opin Immunol* 14(1):15–21. doi:[S0952-7915\(01\)00293X](#) (pii)
16. Wolf PR, Ploegh HL (1995) How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu Rev Cell Dev Biol* 11:267–306. doi:[10.1146/annurev.cb.11.110195.001411](#)
17. Villadangos JA, Bryant RA, Deussing J, Driessens C, Lennon-Dumenil AM, Riese RJ, Roth W, Saftig P, Shi GP, Chapman HA, Peters C, Ploegh HL (1999) Proteases involved in MHC class II antigen presentation. *Immunol Rev* 172:109–120
18. Watts C (2001) Antigen processing in the endocytic compartment. *Curr Opin Immunol* 13(1):26–31. doi:[S0952-7915\(00\)00177-1](#) (pii)
19. Denzin LK, Cresswell P (1995) HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82(1):155–165. doi:[0092-8674\(95\)90061-6](#) (pii)
20. Muraille E, Gounon P, Cazareth J, Hoebke J, Lippuner C, Davalos-Misslitz A, Aebscher T, Muller S, Glaichenhaus N, Mougneau E (2010) Direct visualization of peptide/MHC complexes at the surface and in the intracellular compartments of cells infected in vivo by Leishmania major. *PLoS Pathog* 6(10):e1001154. doi:[10.1371/journal.ppat.1001154](#)
21. Lankar D, Briken V, Adler K, Weiser P, Cassard S, Blank U, Viguer M, Bonnerot C (1998) Syk tyrosine kinase and B cell antigen receptor (BCR) immunoglobulin-alpha subunit determine BCR-mediated major histocompatibility complex class II-restricted antigen presentation. *J Exp Med* 188(5):819–831
22. Boes M, Cerny J, Massol R, Op den Brouw M, Kirchhausen T, Chen J, Ploegh HL (2002) T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* 418(6901):983–988. doi:[10.1038/nature01004](#)

Chapter 40

Production of Primary Human CD4⁺ T Cell Lines and Clones

Jessica Matthis and Helena Reijonen

Abstract

Tetramer staining of CD4⁺ T cells is a valuable technique in immunology for detecting rare autoreactive T cells. Generating clones or cell lines from autoantigen tetramer-positive CD4⁺ T cells allows further characterization and phenotyping of autoreactive cells.

Key words: CD4⁺, Tetramer, Clones, Cell lines, Antigen specific, Cell culture, Autoreactive T cells

1. Introduction

MHC class II tetramers provide a powerful tool for cloning of antigen-specific CD4⁺ T cells (1–15). The use of tetramers in the search of disease-specific CD4⁺ T cells is based on the presumption that the peptide on the tetramer corresponds to an immunogenic epitope of interest that is prevalent in the disease process. Therefore, the prerequisite to use class II tetramers in the detection and cloning of autoimmune T cells is knowledge of the autoantigen, its immunodominant antigen-derived peptides, and the binding of these peptides to the class II molecule playing a role in the disease process. Bearing in mind that there are differences in the epitope processing between individuals and different antigen presenting cells, the selection of disease-relevant T cells could potentially limit the utility of the approach described here. Nevertheless, utilization of tetramer techniques in the detection of CD4⁺ T cells allows isolation of live cells with a single, defined, antigen specificity. T cell clones used in functional assays provide an excellent tool to gain insight into mechanisms and molecular basis of the disease, which otherwise would be possible only in experimental animal models.

Furthermore, this approach has many clinical applications ranging from vaccine design to antigen-specific therapies for prevention of, and intervention into, autoimmunity.

2. Materials

2.1. Reagents

2.1.1. Cell Culture Reagents

1. 1× PBS (Ca/Mg free).
2. Human PBMCS.
3. FICOLL.
4. Peptides.
5. Tritiated thymidine.
6. CD4 Isolation Kit II, Miltenyi Biotec.
7. PHA-P.
8. IL-2 (Aldesleukin Proleukin) for injection 22 million IU, 1.3 mg from local pharmacy (or other commercial preparation).
9. 15% Pooled Human Serum (PHS) T cell media: RPMI 1640 (with 25 mM HEPES), 15% b.v. Human AB Serum, Omega Scientific, 1% b.v. Penicillin-Streptomycin, 1% b.v. Sodium Pyruvate, and 1% b.v. L-Glutamine (see Note 1).

2.1.2. Flow Cytometry Reagents

1. Appropriate PE-labeled class II tetramer (see Note 2).
2. Anti-human CD3 antibodies in all fluors to be used.
3. Anti-human CD4-APC.
4. Mouse IgG₁ (Isotype match) in all fluors of to be used.
5. Running Buffer: 1× PBS, 2 mM EDTA, and 0.5% b.v. Neonatal Calf Serum (NCS) (see Note 1).
6. MACS Buffer: 1× PBS, 2 mM EDTA, and 0.5% Bovine Serum Albumin (BSA) (see Note 1).
7. FACS Buffer: 1× PBS, 1% b.v. NCS, and 0.1% b.v. sodium azide (see Note 1).

2.2. Equipment and Supplies

1. 48-Well plates, 96-well plates.
2. Transfer pipette.
3. AutoMACS, Miltenyi Biotec.
4. Irradiator.
5. FACS tubes and sterile capped FACS tubes.
6. Flow cytometer with cell sorting facility (FACS Vantage or Aria).
7. TomTec Harvester.
8. FACS Buffer: 1× PBS, 1% b.v. FBS, and 0.1% b.v. sodium azide (see Note 1).

3. Methods

3.1. Tetramer Assay

In Vitro

3.1.1. Primary Stimulation of the PBMC (See Note 3)

1. Warm MACS buffer in 37°C water bath for 2–3 h.
2. Isolate PBMC from each blood sample via Ficoll separation.
3. If the total cell count for a sample is less than 10×10^6 cells manually separate CD4⁺ T cells from PBMC using magnetic beads.
4. Labeling PBMC for AutoMACS purification.
 - (a) Count cells.
 - (b) If $<10 \times 10^6$ cells are present, proceed to step 6.e and aliquot PBMC rather than CD4⁺ cells or perform manual CD4⁺ separation.
 - (c) If $\geq 10 \times 10^6$ Isolate CD4⁺ cells using CD4 Isolation Kit II according to manufacturer's instructions (or perform manual CD4⁺ separation if AutoMACS not available).
5. AutoMACS purification of cells from PBMC.
 - (a) Label two 15 mL conical tubes for the collection of CD4⁺ and CD4⁻ cell fractions.
 - (b) Initialize AutoMACS by attaching buffers and running the *Clean Cycle* program. Place the CD4⁺ tube under the negative port and the CD4⁻ under the positive port.
 - (c) Run the *Deplete* program to separate the cells into CD4⁺ and CD4⁻ fractions.
 - (d) Just before the AutoMACS takes up the last of the cell sample, rinse sample tube with 1 mL of running buffer.
 - (e) Bring volume of each fraction up to 10 mL with T cell media and collect a 40 µL aliquot of each fraction for counting and count cells.
 - (f) The total number of CD4⁺ cells will determine the number of wells to plate. Each well of a 48-well plate will contain $1-2 \times 10^6$ cells.
6. Plate the cells and stimulate with the peptide(s) of interest (see Note 4).
 - (a) Centrifuge at 1,000 rpm ($250 \times g$) for 5 min (use low brake setting) at room temperature.
 - (b) Resuspend CD4⁻ cells at a concentration of 10×10^6 cells/mL and CD4⁺ cells at a concentration of 5×10^6 cells/mL.
 - (c) For each well add the appropriate amount of CD4⁺ cells ($3-4 \times 10^6$ cells per well for 48-well plates) and incubate for 1 h in a 37°C, 5% CO₂ incubator to allow cells to adhere.
 - (d) Following the 1 h incubation, fill each well with T cell media and use a transfer pipette to gently wash away all nonadherent cells. The remaining adherent cells are antigen

presenting cell population (APC). Optional: the APC can be irradiated (5,000 rad).

- (e) For each well add the appropriate amount of CD4⁺ cells ($1\text{--}2 \times 10^6$ cells for each well of a 48-well plate) and bring volume of each well up to 1 mL with T cell media.
- (f) Add 10 µg/mL of the appropriate peptide to each well. If there are only enough cells for a single well, pool the peptides together. Each peptide is used at final concentration of 10 µg/mL. Do not pool more than maximum of five peptides.
- (g) Incubate cells in a 37°C, 5% CO₂ incubator for 5–7 days.
- (h) Between days 5 and 7 of the culture, remove 400–500 µL of supernatant from each well. Add a corresponding amount of fresh T cell media and 10 IU of IL-2 to every well.
- (i) Monitor the expansion of the stimulated cells. If the cells in any well surpass 100% confluence, resuspend the cells and split into two wells. If the media yellows on a well that does not require splitting, remove 400–500 µL of supernatant from that well and add a corresponding amount of fresh T cell media with IL-2.
- (j) Incubate cells for a total of 13–15 days.

3.1.2. Tetramer and Antibody Staining of Stimulated Cells and Controls

1. Harvest cells for tetramer staining.

- (a) Remove supernatant from each well until the remaining volume is approximately 50 µL for each staining tube (tetramer) (plus some extra for single-color staining controls).
- (b) Resuspend the cells in the well using a transfer pipette.
- (c) If wells have been split, combine corresponding samples into one well and mix.
- (d) Transfer 50 µL aliquots of cell suspension from each well into labeled FACS tubes and designate for staining with the appropriate tetramers. This should include one or more relevant tetramers and an irrelevant tetramer for each sample ID. The relevant tetramers contain the peptides that were used to stimulate the cells in Subheading 3.1.1, step 6.f. If more than one class II allele is tested for a given sample ID, an irrelevant tetramer is needed for each allele (see Note 5).
- (e) Pool together all remaining cells and aliquot into labeled FACS tubes for the single-color staining controls.

2. Tetramer Staining.

- (a) Add 1 µL of the appropriate PE-labeled Class II tetramer (10 µg/mL final concentration) into each labeled FACS tube.
- (b) Incubate the cells with tetramer for 2 h in the dark at 37°C.

3. Antibody Staining of Single-Color Controls.

- (a) Take the staining control tubes and incubate on ice for 3 min (see Note 6).
- (b) Add 5 µL α-CD3 antibody for each fluor to be used to the appropriate single control tubes. Include one tube unstained with antibody.
- (c) Add 5 µL isotype control antibodies in each fluor to the isotype control tube.
- (d) Incubate in the dark at 4°C for 30 min.

4. Antibody Staining of PBMC Samples.

- (a) Place all sample tubes on ice for 3 min.
- (b) Add 5 µL of CD4 APC and 5 µL of CD3 FITC to each tube.
- (c) Incubate in the dark at 4°C for 30 min.
- (d) Wash samples and controls.
- (e) Add 2 mL of cold FACS buffer to each tube.
- (f) Spin down cells at 1,000 rpm ($250 \times g$) for 5 min at 4°C, low brake.
- (g) Remove most of the supernatant, leaving approximately 200 µL FACS buffer to resuspend the cells by gently tapping the tube.
- (h) Store all tubes in a covered ice container for subsequent FACS acquisition.

3.2. Generating CD4⁺ T Cell Clones**3.2.1. Single Cell Sorting of Tetramer-Positive Cells**

1. Perform tetramer and antibody staining of stimulated cells and controls according to Subheading 3.1.2, steps 1–4.
2. Single cell sort CD4⁺ tetramer-positive cells into a round bottom 96-well plate containing 100 µL media per well using a FACS Vantage or Aria (see Note 7 and Fig. 1).
3. After sorting you should have 1 tetramer-positive cell/well.
4. Add 100,000 irradiated (5,000 rad) HLA-mismatched fresh feeder cells/well in a volume of 100 µL 30% PHS T cell media (see Notes 8 and 9).
5. Incubate cells in a 37°C, 5% CO₂ incubator for 1 day.
6. Add IL-2 10 IU/mL and PHA 5 µg/mL the day after sorting in a volume of 25 µL T cell media.
7. Incubate cells for 9 more days.
8. Check plates every other day for possible contamination during the sorting process.

3.2.2. Expansion of Single Cell Sorted Cells

1. After 10 days of incubation stimulate the cells with HLA-matched fresh feeder cells and peptide (see Note 8).
2. Pulse the feeder PBMC with 10 µg/mL of the peptide of interest. Use a small volume when pulsing, example 10×10^6 /mL.

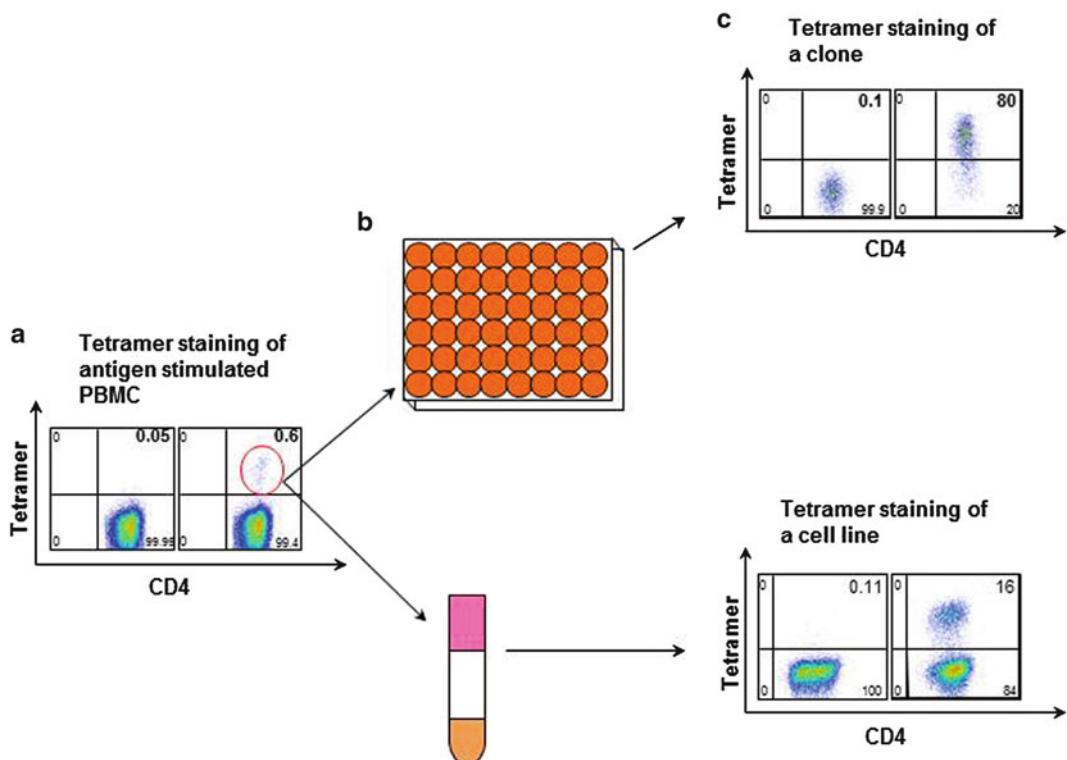


Fig 1. Summary of Subheadings 3.2 and 3.3, generating CD4⁺ T cell clones and cell lines. (a) After primary stimulation of PBMC stain cultures with tetramer and antibody. (b) Single cell sort CD4⁺ tetramer-positive cells into a 96-well plate to generate a clone or bulk the cells into a sterile capped FACS tube and plate to generate a cell line. (c) After stimulation and expansion test antigen specificity of cells with tetramer staining, as well as proliferation assays and cytokine staining (not shown).

3. Pulse for 2–3 h at 37°C, 5% CO₂.
4. Wash the peptide out with 1× PBS and resuspend in T cell media. Calculate the volume, so that you put 100,000 cells/100 µL/well.
5. Irradiate the feeder cells at 5,000 rad.
6. Take out about 120 µL supernatant from the wells and add 100 µL of the peptide pulsed 100,000 feeder cells/well.
7. Add 10 IU/mL of IL-2 to the wells next day in a volume of 25 µL of T cell media.
8. Incubate cells for 9 more days. Visually inspect: The cell pellets should grow larger.
9. After 10 days test the specificity of the growing clones in a proliferation assay and with tetramer staining FACS analysis if there are enough cells (you can take a small aliquot from some wells for a quick count). If there are not enough cells for a functional assay expand them further.
10. Once the cells are dense and look confluent under a microscope transfer them to flat bottom 96-well plate. Remove

100 µL supernatant and transfer 100 µL of cells. Add fresh T cell media and IL-2 (see Note 10).

11. Once the cells fill the well and are in contact with each other transfer them to a 48-well plate for a second unspecific stimulation.
12. Plate 250,000 clones/well with $0.75\text{--}1.0 \times 10^6$ irradiated HLA-mismatched feeder cells.
13. Incubate cells for 1 day.
14. Add IL-2 10 IU/mL and PHA 5 µg/mL to each well.
15. Check cells every 2–3 days. Once cells are confluent you may transfer them to a 24-well plate or split them 1:1 and add fresh T cell media and IL-2.
16. After 10–14 days in culture the cells should be tested again for specificity, restimulated with peptide, and/or frozen (see Note 11).

3.3. Generating CD4⁺ T Cell Lines

1. Perform tetramer and antibody staining of stimulated cells and controls according to Subheading 3.1.2, steps 1–4.
2. Sorting can be done using a FACS Vantage or Aria.
3. Sort the entire CD4⁺ tetramer positive into a 5 mL sterile capped FACS tube containing 500 µL of T cell media (see Fig. 1).
4. Take a count of the cells, or use the flow sort counts to plate $1\text{--}2 \times 10^6$ cells/well in a 48-well plate.
5. Add $3\text{--}4 \times 10^6$ irradiated PBMC/well and bring up volume of each well to 1 mL with T cell media.
6. Incubate cells for 1 day.
7. Add IL-2 10 IU/mL and PHA 5 µg/mL to each well.
8. Check cells every 2–3 days. Once cells are confluent you may transfer them to a 24-well plate or split them 1:1 and add fresh T cell media and IL-2.
9. After 10–14 days in culture the cells may be tested for specificity and restimulated according to the methods in Subheading 3.2.2.

3.4. Testing CD4⁺ T Cell Lines and Clones Specificity in a Proliferation Assay

1. Count the clones (see Subheading 3.2) or lines (see Subheading 3.3) to be tested.
2. Calculate the total volume of cells needed to get 25–50,000 cells/well × 7 wells (3 with peptide, 3 without peptide, and 1 with purified α-CD3 antibody) (see Note 12).
3. Plate cells (25–50,000/well) in a 96-well round bottom plate. Bring up the volume to 100 µL with T cell media in each well.
4. Pulse antigen presenting cells (APC=HLA-matched PBMC) cells with 10 µg/mL peptide for 2–3 h at 37°C, 5% CO₂.

As with specific stimulation, use a small volume when pulsing, such as $10 \times 10^6/\text{mL}$ (see Note 13).

5. Incubate APC to be added to the no antigen wells, but do not add peptide.
6. Irradiate the APC at 5,000 rad.
7. Wash the peptide out of the APC with 1× PBS and resuspend in T cell media at a concentration of 150,000 cells/100 μL .
8. Plate 150,000 APC/well. Use un-pulsed feeders in the α -CD3 well and add 10 $\mu\text{g}/\text{mL}$ purified α -CD3 antibody as a positive control.
9. Incubate for 48 h at 37°C, 5% CO₂.
10. Remove 50 μL of supernatant from the wells and store in clean 96-well plate at -80°C for cytokine assessment.
11. Add 25 $\mu\text{L}/\text{well}$ (1 $\mu\text{Ci}/25 \mu\text{L}/\text{well}$) of tritiated thymidine (H³).
12. Incubate further 8–18 h and harvest on TomTec Harvester according to manufacturer instructions.

3.5. Testing the Specificity of the Clones by Tetramer Staining

1. Count the clones and lines to be tested.
2. Take an aliquot of 50–100,000 cells and adjust the volume to 100 μL with T cell media.
3. Transfer 50 μL aliquots into two vials which will be stained with specific and irrelevant tetramers as in Subheading 3.1.2, step 2 at 37°C for 45 min–1 h (note: a shorter staining time) (see Note 13 and Fig. 1).

4. Notes

1. All buffers and media should be sterile filtered using a 0.2 μm bottle top filter and stored in the accompanying 500 mL bottle.
2. Information for class II tetramers can be obtained from Benaroya Research Institute's Tetramer Core Laboratory Web site <http://www.benaroyaresearch.org/our-research/core-resources/tetramer-core-laboratory>.
3. *Option 1:* Whole PBMC can be used instead of stimulation of the purified CD4⁺ T cells. If whole PBMC is used plate 3×10^6 PBMC on 48-well and proceed to Subheading 3.1.1, step 6.f.
Option 2: In order to distinguish memory and naïve T cell populations the cells can be further separated by CD45RO+/-.
4. The optimal number of PBMC for T cell cloning by MHC class II tetramers is variable. If the expected tetramer staining is strong (>2%) 2×10^6 CD4⁺ T cells should be sufficient. If the

staining is expected to weak (<2%), it is advisable to set up multiple culture wells especially if cloning of CD4⁺ T cells specific for several different peptides is attempted. T cell responses to self-antigens are generally weaker than responses to foreign antigens. Strong tetramer staining can be expected with flu or other vaccine-induced T cell responses and with responses to some immunodominant self-epitopes. The peptides of interest used to stimulate the cells should be selected by the HLA class II (DR, DQ, DP) genotype of the donor of the PBMC.

5. The irrelevant tetramers are used as negative controls in the staining of cells stimulated with the specific peptides. Empty (unloaded) tetramers or tetramers loaded with peptides that bind to the given MHC class II and were not used in the culture can be used as the negative staining controls. The cutoff value (% of CD4⁺/tetramer+ cells) for staining with a negative control tetramer is usually set at 0.1–0.5%.
6. In cell sorting protocol all cell- and reagent-handling steps should be performed in a laminar flow biosafety cabinet.
7. For flu stimulated CD4⁺ T cells, usually there are 4–15% tetramer-positive cells after 14 days of primary culture. For autoreactive CD4⁺ cells the frequency of tetramer binding cells is much lower, 0.5–4%.
8. Feeder cells are PBMC from HLA-matched (=APC, used in specific stimulation with a peptide) or HLA-mismatched (used in unspecific stimulations with PHA/IL-2) blood donors. PBMC isolated from fresh blood is preferred in T cell clone expansion but frozen PBMC can be used in T cell proliferation assays. In unspecific stimulation of the clones the MHC class II of the feeder PBMC should be mismatched for both HLA alleles. Feeder PBMC can be combined from different donors.
9. Use T cell media with a higher concentration of PHS when performing single-cell sorting. Thirty percent PHS is the standard. When expanding cells on subsequent days the cells can be weaned back to 10% PHS T cell media.
10. After single-cell sorting it is sometimes necessary to incubate the cells for longer than 10 days to generate enough clones to perform specificity testing. Look at the cells under the microscope: Activated and dividing T cells look elongated and form clusters.
11. It is important that the cells have time to rest (i.e. no IL-2 added) for a minimum 72 h before the next stimulation or any functional assay. The cells should be also washed well to remove any residual IL-2 from the culture media. Resting T cells look roundish rather than elongated. It is always important to freeze aliquots of the expanded clones early.

12. Primary specificity testing can be performed in single or duplicate wells if the cell number is limiting. Negative no antigen control should always be included in the assay. When sufficient number of cells is available proliferation assay should be performed in decreasing concentrations of peptide.
13. HLA-matched B-LCL can be used as APC if HLA-matched PBMC from suitable blood donors are not available. Lower number of B-LCL (50–100,000 cells/well) is sufficient. Optimal irradiation time should be tested for each B-LCL since usually higher irradiation dose (>5,000 rad) is needed for a complete blocking of proliferation.
14. Tetramer staining of T cell clone is dependent on the avidity of the T cell and can vary from 0–100%. Some low avidity T cell clones may not stain with a specific tetramer or stain very weakly but display proliferation and cytokine production in a dose-dependent manner when stimulated with a specific peptide. Avidity of tetramer-positive T cell clones can be assessed further by staining at decreasing concentrations of tetramer.

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References

1. Reichstetter S, Ettinger RA, Liu AW, Gebe JA, Nepom GT, Kwok WW (2000) Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response. *J Immunol* 165(12):6994–6998
2. Reijonen H, Novak EJ, Kochik S, Heninger AK, Liu AW, Kwok WW, Nepom GT (2002) Detection of GAD65 specific T-cells by MHC Class II tetramers in Type 1 diabetes patients and at-risk subjects. *Diabetes* 51:1375–1382
3. Nepom GT, Buckner JH, Novak EJ, Reichstetter S, Reijonen H, Gebe J, Wang R, Swanson E, Kwok WW (2002) HLA class II tetramers: tools for direct analysis of antigen-specific CD4+ T cells. *Arthritis Rheum* 46(1):5–12
4. Buckner JH, Holzer U, Novak EJ, Reijonen H, Kwok WW, Nepom GT (2002) Defining antigen-specific responses with human MHC class II tetramers. *J Allergy Clin Immunol* 110:199–208
5. Reijonen H, Kwok WW (2003) Use of HLA class II tetramers in tracking antigen-specific T-cells and mapping of T-cell epitopes. *Methods* 29(3):282–288
6. Reijonen H, Kwok WW, Nepom GT (2003) Detection of CD4+ autoreactive T-cells in T1D using HLA class II tetramers. *NYAS 6th IDS: immunology in diabetes II. Annu NY Acad Sci* 1005:82–87
7. Reijonen H, Mallone R, Heninger AK, Laughlin EM, Kochik SA, Falk BA, Kwok WW, Greenbaum C, Nepom GT (2004) GAD65 specific CD4+ T cells with high antigen avidity are prevalent in peripheral blood of type 1 diabetes patients. *Diabetes* 53:1987–1994
8. Mallone R, Kochik SA, Laughlin EM, Gersuk VH, Reijonen H, Kwok WW, Nepom GT (2004) Differential recognition and activation thresholds in human autoreactive GAD-specific T-cells. *Diabetes* 53(4):971–977

9. Mallone R, Kochik SA, Reijonen H, Carson B, Ziegler SF, Kwok W, Nepom GT (2005) Functional avidity directs T-cell fate in autoreactive CD4+ T-cells. *Blood* 106:2798–2805
10. Yang J, Danke NA, Berger D, Reichstetter S, Reijonen H, Greenbaum C, Pihoker C, James EA, Kwok WW (2006) Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD4+ T cells in human subjects. *J Immunol* 176(5):2781–2789
11. Laughlin E, Burke G, Pugliese A, Falk B, Nepom G (2008) Recurrence of autoreactive antigen-specific CD4+ T cells in autoimmune diabetes after pancreas transplantation. *Clin Immunol* 128:23–30
12. Vendrame F, Pileggi A, Laughlin E, Allende G, Martin-Pagola A, Molano RD, Diamantopoulos S, Standifer N, Geubtner K, Falk BA, Ichii H, Takahashi H, Snowwhite I, Chen Z, Mendez A, Chen L, Sageshima J, Ruiz P, Ciancio G, Ricordi C, Reijonen H, Nepom GT, Burke GW 3rd, Pugliese A (2010) Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, associated with autoantibodies and pathogenic autoreactive CD4+ T-cells. *Diabetes* 59(4): 947–957
13. Öling V, Geubtner K, Ilonen J, Reijonen H (2010) A low antigen dose selectively promotes expansion of high avidity autoreactive T cells with distinct phenotypic characteristics: A study of human autoreactive CD4+ T cells specific for GAD65. *Autoimmunity* 43(8): 573–582
14. Hänninen A, Soili-Hänninen M, Hampe CS, Deptula A, Geubtner K, Ilonen J, Knip M, Reijonen H (2010) Characterization of CD4+ T cells specific for glutamic acid decarboxylase (GAD65) and proinsulin in a patient with stiff-person syndrome but without type 1 diabetes. *Diabetes Metab Res Rev* 26(4):271–279
15. Robins H, Desmarais C, Matthijs J, Livingston R, Andriesen J, Reijonen H, Carlson C, Nepom G, Yee C, Cerosaletti K (2012) Ultra-sensitive detection of rare T cell clones. *J Immunol Methods* 375(1–2):14–19

Chapter 41

Analyzing Antigen Recognition by Natural Killer T Cells

**Sebastian Zeissig, Torsten Olszak, Espen Melum,
and Richard S. Blumberg**

Abstract

Natural Killer T (NKT) cells are a subset of T lymphocytes that recognize a wide variety of lipid antigens presented by CD1 molecules. NKT cells exhibit rapid activation after recognition of cognate antigens, secrete abundant amounts of T helper (Th) 1, Th2, and Th17 cytokines within hours of activation and shape the immune response through subsequent activation of dendritic, NK, T and B cells. NKT cells therefore play central roles in antimicrobial and anticancer immunity and in modulation of various autoimmune disorders. Consequently, recent research has focused on the discovery of microbial and self-antigens involved in NKT cell activation. In this chapter, we discuss different strategies for studying antigen recognition by NKT cells including CD1d tetramer-based approaches and in vitro assays characterizing NKT cell activation in response to lipid antigen presentation. While toll-like receptor (TLR) agonists and cytokines such as IL-12 are critical for NKT cell activation *in vivo*, particularly in the context of microbial infection, methods for detection of TLR- and cytokine-dependent NKT cell activation will not be discussed in this section.

Key words: CD1d, Natural Killer T cells, Lipid antigen presentation, Tetramer, α -Galactosylceramide

1. Introduction

The CD1 gene family consists of five members in humans and two highly related members in mouse (1, 2). In humans, these consist of type 1 (CD1a–c) and type 2 (CD1d) that are expressed on the cell surface and which differentially traffic through the endolysosomal system for sampling the lipid milieu within specific cellular locales (1, 2). The fifth human CD1 member, CD1e, is not expressed on the cell surface and plays a role in the regulation of the other CD1 family members (3). In mouse, only type 2 CD1 proteins are expressed and include two highly related CD1d molecules, CD1d1 and CD1d2 (for genomic organization please refer to Fig. 1) (4). CD1d1 and CD1d2 are highly homologous and in certain strains

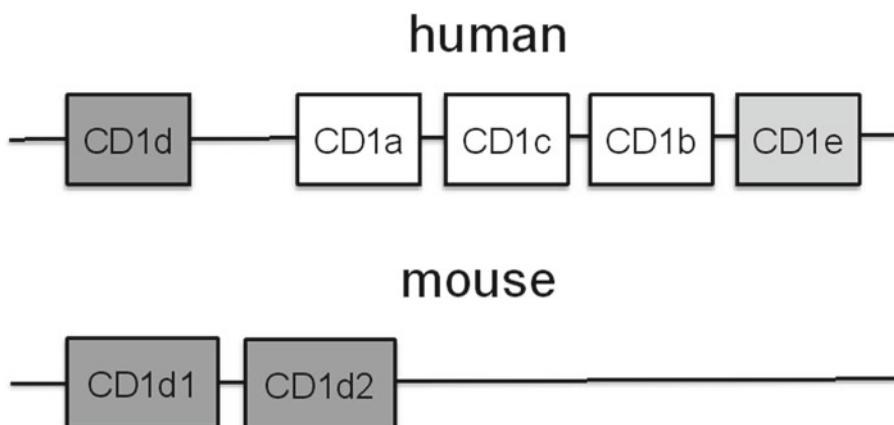


Fig. 1. Genomic organization of the human and murine CD1 locus.

Table 1
Subtypes of NKT cells

NKT cell	T cell receptor (TCR)	Detection/deletion
Invariant (Type I)	V α 14J α 18 in mice V α 24J α 18 in humans Biased V β repertoire	Detected by α GalCer/CD1d tetramers Deleted in J α 18 $^{-/-}$ mice (TCR J α 18 deletion) Deleted in CD1d $^{-/-}$ mice (impaired thymic selection)
Noninvariant (Type II)	Oligoclonal TCR repertoire	Subgroup detected by sulfatide/CD1d tetramers Not deleted in J α 18 $^{-/-}$ mice Deleted in CD1d $^{-/-}$ mice (impaired thymic selection)

of mice such as C57Bl/6 the CD1d2 gene product is nonfunctional due to a loss-of-function mutation in its open reading frame (4).

All of the CD1 proteins are characteristically involved in presenting endogenous (host) and exogenous (microbial) lipid antigens to distinct subsets of T cells (1, 2). There are two types of T cells which are involved in recognizing lipid antigens in the context of CD1 on an antigen presenting cell (APC). The T cells which recognize CD1d in mouse and human are considered as natural killer T cells (NKT cells) in view of their expression of both NK (NK1.1) and T cell (e.g., T cell receptor) markers (5, 6). Further, NKT cells include those that express an invariant T cell receptor (TCR) alpha chain in association with a variety of TCR- β chains (Table 1). These so-called invariant (i) NKT cells are the variety that are currently accessible to careful interrogation since they recognize an antigen, α -galactosylceramide (α -GalCer), in the

context of CD1d (18). A second class of NKT cells exist which express a variable, but restricted, array of TCR- α and TCR- β chains and are considered to be noninvariant (ni) NKT cells (Table 1) (8, 9). Although these niNKT cells are restricted by CD1d and certain antigens have been defined as determinant of recognition in the context of CD1d (e.g., sulfatides), these are less amenable to study as will be discussed further below given the relative range of antigens that have been identified to date which are responsible for their recognition and activation. We will not discuss cells that are restricted by human CD1a–c which also express a variety of TCR- α and TCR- β chains and which are responsible for recognizing a variety of poorly characterized endogenous antigens as well as a wide variety of exogenous microbial antigens from mycobacterial and non-mycobacterial sources (1). With the recent development of type 1 CD1 tetramers, the comments below will likely be extensible to these subsets of T cells as well (10).

CD1d is synthesized as a non-covalent heterodimer with β_2 -microglobulin (β_2 m) in the endoplasmic reticulum (ER), where it is loaded and presumably stabilized by endogenous lipid antigens (1, 11). CD1d then traffics to the cell surface and presents lipids acquired in the secretory pathway through the actions of microsomal triglyceride transfer protein (MTP), an ER resident lipid transfer protein (1, 12–15). Subsequently, CD1d undergoes endocytosis and reaches endosomes (murine CD1d (mCD1d) and human (hCD1d)) and lysosomes (mCD1d only) where lipid transfer and editing molecules assist in the exchange of bound antigens against other endogenous and exogenous lipids (1). CD1d thus surveys the cell for lipid antigens and presents a wide variety of self and foreign lipids acquired in diverse subcellular compartments (1).

Methods for characterization of antigen recognition by NKT cells are based on CD1d and rely either on direct detection of CD1d-lipid complexes bound to the NKT cell receptor or on measurement of NKT cell activation and cytokine secretion in response to CD1d-restricted lipid antigen presentation (16). Direct detection of antigen recognition by NKT cells is based on high-affinity multimers, usually tetramers, consisting of streptavidin-bound CD1d/ β_2 m complexes loaded with the lipid of interest. Conjugation of fluorophores to streptavidin allows detection of CD1d-lipid complex binding to the NKT cell receptor by flow cytometry. Using this approach, CD1d tetramers loaded with the marine sponge glycosphingolipid α -galactosylceramide (α GalCer) or its analogues (e.g., PBS57) allow sensitive and specific detection of murine and human iNKT cells, a subgroup of NKT cells expressing a semi-invariant TCR consisting of an invariant TCR alpha chain (V α 14J α 18 (17) in mice, V α 24J α 18 in humans) paired with a restricted set of TCR- β chains (Table 1 and Fig. 2) (18). In addition, sulfatide-loaded CD1d tetramers have been shown to allow

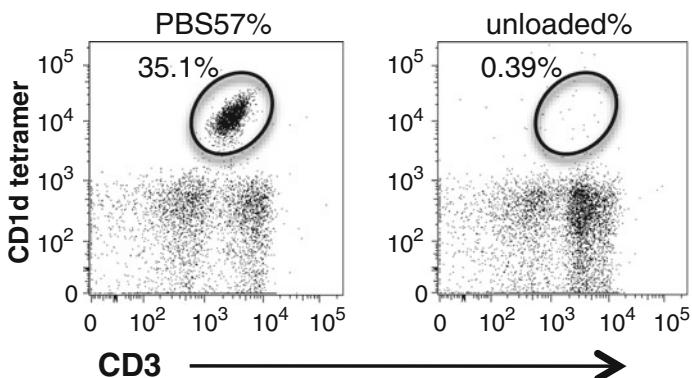


Fig. 2. Flow cytometry staining of iNKT cells. Invariant NKT cells are shown as PBS57/CD1d tetramer-positive, CD3^{intermediate} T cells (*left panel*). Unloaded CD1d tetramers served as control (*right panel*).

detection of a population of niNKT cells, a subgroup of NKT cells expressing a more diverse though still biased TCR repertoire as discussed above (19, 20). While CD1d tetramers allow for unambiguous direct detection of NKT cell antigen recognition, NKT cell-activating CD1d-restricted lipids can fail in tetramer-based approaches due to the inability to form stable complexes with CD1d (e.g., lysophospholipids) or low affinity to the T cell receptor (e.g., microbial-derived diacylglycerols).

In cases where potential antigens fail in generation of tetramers, antigen recognition by NKT cells can be demonstrated by upregulation of activation markers or secretion of cytokines by NKT cells in response to CD1d-restricted presentation of these lipids. To this end, CD1d-expressing antigen presenting cells (APC) are loaded with the respective lipids and cocultured with NKT cells *in vitro* or, alternatively, injected into mice for analysis of antigen presentation *in vivo*. Many lipids will in addition to functioning as antigens act as bioactive molecules that induce lipid signaling, metabolic alterations, and changes in lipid raft structure; effects that can be largely prevented in cell-free APC assays. In this approach, recombinant CD1d is coated onto plates, loaded with the lipids of interest and subsequently tested for presentation of these lipids to NKT cells.

In the following sections, we will discuss the generation of custom-made CD1d tetramers, isolation of NKT cells, and detection of NKT cells by tetramers. We will also outline strategies for characterization of antigen recognition by NKT cells based on their activation upon CD1d-restricted presentation of lipid antigens.

2. Materials

2.1. Consumables and Lab Equipment

1. Donor mice.
2. Borosilicate glass tubes (e.g., Pyrex®).
3. Syringes, 25 G needles.
4. 15 and 50 mL Conical tubes and Flow cytometry tubes.
5. 150 mm Non-plugged Pasteur pipettes.
6. Nylon mesh (e.g., BD Falcon cell strainers 40 and 70 µm).
7. 96-Well flat bottom plates.
8. Flow cytometer with possibility of cell sorting.

2.2. Generation of CD1d-Lipid Tetramers

1. PE- or APC-conjugated preloaded murine and human CD1d tetramers (NIH Tetramer Core Facility or different commercial vendors) or custom-made CD1d tetramers (see Subheading 3.1). For instructions on NIH tetramer orders please refer to <http://tetramer.yerkes.emory.edu>.
2. Biotinylated mouse or human CD1d monomer (NIH Tetramer Core Facility).
3. Streptavidin-PE or Streptavidin-APC.
4. Synthetic or (ideally HPLC-) purified lipids (various sources, e.g., Avanti Polar Lipids, Inc.).

2.3. Cell Lines

1. Primary APCs; CD1d-transfected and untransfected cell lines.
2. Primary NKT cells or NKT cell lines/clones/hybridomas.
3. Tissue culture medium: DMEM or RPMI-1640 containing 10% fetal bovine serum and 2 mM L-glutamine.
4. Percoll Plus.

2.4. Buffers

1. 1× PBS sterile.
2. ACK lysis buffer: 0.15 M ammonium chloride (8.29 g NH₄Cl), 10 mM potassium bicarbonate (1 g KHCO₃) and 0.1 mM Na₂EDTA (37.2 mg), add 800 mL H₂O and adjust pH to 7.2–7.4 with 1 N HCl. Finally, add H₂O to 1 L and filter through a 0.2-µm filter to sterilize and store at room temperature.
3. Staining buffer: 1× PBS, 1% BSA.

2.5. Antibodies and Reagents

1. 6B11 Anti-human iNKT TCR antibody (e.g., eBioscience).
2. Directly conjugated antibodies of choice for surface and intracellular staining of NKT cells.
3. Unlabeled streptavidin.
4. Cytofix/Cytoperm Plus kit (BD Biosciences).
5. Reagents for detection of NKT cell activation such as ELISA kits or flow cytometry antibodies.

3. Methods

3.1. Generation of CD1d–Lipid Tetramers

Tetramers of CD1d allow high-affinity single-cell detection of T cells that respond to CD1d-restricted lipid antigens. Strategies rely on loading of CD1d with the respective lipids, followed by streptavidin-mediated tetramerization of biotinylated CD1d/β₂m monomers and flow cytometry-based detection of CD1d/lipid complexes bound to the NKT cell receptor. Here, we describe the generation of lipid-loaded CD1d tetramers. For ready-to-use CD1d tetramers, refer to Note 1.

For each staining between 0.1 and 4 µg CD1d tetramer are required and are loaded with a 40-fold molar excess of the lipid of interest. The amount of lipid required can be calculated as follows:

$$\text{Amount of antigen} (\mu\text{g}) = \frac{\text{mCD1d} \times \text{ME} \times \text{MwAntigen}}{\text{MwCD1d}}$$

mCD1d = Microgram of biotinylated CD1d (0.1–4 µg/staining)

ME = Molar excess of lipid antigen (e.g., 40-fold)

MwAntigen = Molecular weight of antigen

MwCD1d = Molecular weight of biotinylated CD1d (48,579 Da)

For example, for the generation of 4 µg of CD1d loaded with α-galactosylceramide (α-GalCer), the following calculation would apply

$$\text{Amount of } \alpha\text{GC} = \frac{4\mu\text{g} \times 40 \times 858\text{d}}{48,579 \text{ d}} = 2.8\mu\text{g}$$

If lipids are resuspended in nonpolar solvents, transfer the required amount of lipid to a sonication-resistant borosilicate glass tube and dry the lipid under nitrogen stream. Add 1× PBS to obtain a 0.1–2 mg/mL solution of the lipid (see Note 2).

1. Mix 1–4 µg of biotinylated CD1d with the required amount of lipid and incubate at room temperature for 12–16 h (see Note 3).
2. For tetramerization, add aliquots of 1 µL (500 ng) of streptavidin–PE or streptavidin–APC every 10 min for a total of ten times. Mix well after each addition (see Note 4).
3. In case of stable complexes such as α-galactosylceramide/CD1d, tetramers can be stored at 4°C for weeks. However, in other cases, such as sulfatide/CD1d complexes, tetramers should be used immediately.

3.2. Extraction of Mononuclear Cells for NKT Cell Analysis

A general protocol for extraction of mononuclear cells from liver, spleen, and thymus is outlined. For an overview of expected cell numbers, see Note 5.

1. Immediately after euthanization of a mouse, open the peritoneal cavity and perfuse the liver with 10 mL of 1× PBS through the portal vein using a 25 G needle. After starting perfusion the inferior vena cava should be cut to facilitate flow. This step will remove blood cells but maintain liver mononuclear cells. Immediately upon perfusion, the liver should turn pale as a sign of successful perfusion (Fig. 3). Otherwise relocate the needle to make sure it is placed in the portal vein. The thymus and spleen are dissected out and all tissues are placed in a separate tube with cold PBS.
2. Harvest the liver and press gently through a nylon mesh (70 µm for liver, 40 µm for thymus and spleen) using the back of a 3 mL syringe plunger. Collect the filtered solution, transfer to a 50 mL conical tube, add 1× PBS for a total of 50 mL, and centrifuge at $400 \times g$ for 5 min at 4°C.
3. For the liver preparation only.
 - (a) Carefully aspirate the supernatant, fill the tube with 50 mL of 1× PBS, and centrifuge at $400 \times g$ for 5 min at 4°C.
 - (b) Aspirate supernatant and resuspend the pellet in 4 mL of 40% Percoll. Transfer to a conical 15 mL tube.
 - (c) Carefully underlay 2 mL of 60% Percoll ensuring that 40% and 60% Percoll are not mixed (see Note 6).
 - (d) Centrifuge tubes at $700 \times g$ for 20 min at 4°C. *Important:* Make sure the brake of the centrifuge is switched off since this will otherwise lead to a mixture of the two Percoll layers.
 - (e) After centrifugation, hepatocytes are on top and liver mononuclear cells are at the interphase of the Percoll gradient (at the 2 mL level).
 - (f) Remove hepatocytes from top by careful aspiration avoiding inadvertent aspiration of the interphase or a mixture of both layers.
 - (g) Using a 5 mL pipette, collect LMNCs by aspiration at the interphase. Transfer cells to a 50 mL tube, add 1× PBS for a total of 50 mL, and centrifuge at $400 \times g$ for 5 min at 4°C.
4. Aspirate the supernatant and resuspend pelleted cells in ACK lysis buffer for lysis of red blood cells (not required for thymus). Incubate cells for 5 min at room temperature, then add 45 mL of 1× PBS and centrifuge at $400 \times g$ for 5 min at 4°C. Repeat washing step with another 50 mL of 1× PBS.
5. Resuspend cells in staining buffer.

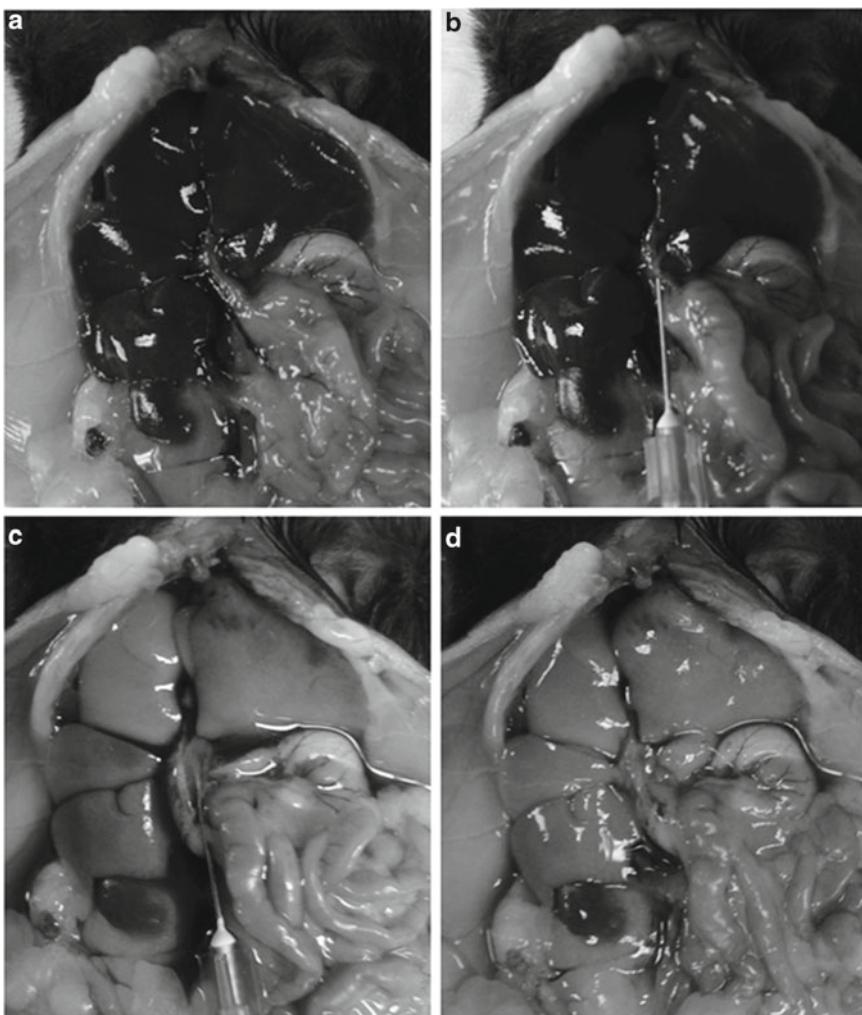


Fig. 3. Perfusion of a mouse liver. (a) Situation before perfusion with portal (*right*) and inferior cava (*left*) vein entering the liver. (b) Insertion of a needle into the portal vein. (c) Start of perfusion of the liver. (d) Situation after perfusion of the liver. Please note the change in liver color after perfusion as a sign of successful removal of blood cells.

3.3. Tetramer and Antibody Staining of iNKT Cells

A protocol for flow cytometry-based detection of iNKT cells is outlined. For an overview of methods for NKT detection, please see Note 7. For an high-throughput adaptation, please see Note 8).

1. Pellet mononuclear cells by centrifugation at $400 \times g$ for 5 min at 4°C.
2. Carefully aspirate supernatant and add CD1d-lipid tetramer in 50 µL of staining buffer. Incubate for 30 min at 4°C.
3. Without washing, add fluorochrome-conjugated antibodies (see Notes 9 and 10) for surface staining at pre-triturated

concentrations (usually 0.1–5 µg/mL) in 50 µL of staining buffer. Incubate for an additional 30 min at 4°C.

4. For washing, add 2 mL of staining buffer and centrifuge at 400 ×*g* for 5 min at 4°C. Carefully aspirate supernatant.
5. For direct flow cytometry analysis, resuspend cells in staining buffer and analyze.
6. For analysis of intracellular molecules such as transcriptional regulators or cytokines (see Note 11).
 - (a) Resuspend cells in 250 µL Cytofix/Cytoperm buffer for 20 min at 4°C for fixation and permeabilization.
 - (b) Wash cells by the addition of 2 mL of 1×Perm/Wash buffer and centrifugation at 600 ×*g* for 5 min at 4°C.
 - (c) Add pre-titrated directly conjugated antibodies (usually 0.1–5 µg/mL) for intracellular staining in 100 µL of 1×Perm/Wash buffer and incubate for 30 min at 4°C.
 - (d) Wash cells by addition of 2 mL of 1×Perm/Wash buffer and centrifugation at 600 ×*g* for 5 min at 4°C.
 - (e) Resuspend cells in staining buffer for flow cytometry analysis.

3.4. Characterization of Functional NKT Cell Responses to Lipid Antigens Using a Coculture Approach

1. Transfer APCs (for choice of APCs please see Note 12) in the appropriate cell culture medium to a suitable tube and add lipids of interest aiming for a series of tenfold dilutions with final lipid concentrations of 10 µg/mL to 1 ng/mL (see Note 13).
2. Incubate for 4–16 h at 37°C in a tissue culture incubator (see Note 14).
3. Count APCs and add 2×10^4 to 1×10^5 APCs per well in 100 µL of the appropriate cell culture medium to 96-well flat bottom plates (see Notes 15 and 16). Use triplicates for each condition.
4. Remove unbound lipid by 3–5 washing steps using the appropriate tissue culture medium. In case of non-adherent APCs, centrifugation at 400 ×*g* for 5 min is required for washing (see Note 17).
5. After the last washing step, aspirate supernatant and add 100 µL of the appropriate tissue culture medium.
6. Resuspend NKT cells in the same tissue culture medium used for the APCs and add 2×10^5 primary iNKT cells or 5×10^4 cells from an iNKT cell clone or hybridoma in 100 µL (see Note 18).
7. Incubate at 37°C in a tissue culture incubator (see Note 19).

8. Analyze NKT cell activation by ELISA- or flow cytometry-based detection of cytokine secretion or flow cytometry-based detection of upregulation of NKT activation markers (see Note 20).

3.5. Characterization of Functional NKT Cell Responses to Lipid Antigens Using an APC-Free Assay

APC-free assays for lipid antigen presentation are based on CD1d- β -monomers that are bound to tissue culture plates and allow for loading of lipids and presentation to NKT cells.

1. Load 0.25 μ g of CD1d in 100 μ L 1 \times PBS per well onto 96-well flat bottom tissue culture plates. Use triplicates for each experimental condition.
2. Incubate for 16 h at 37°C in a humidified incubator to allow for binding of CD1d to the plate (see Note 21).
3. Wash three times with 200 μ L of 1 \times PBS to remove unbound CD1d.
4. Add lipids in triplicates in a series of tenfold dilutions in 100 μ L of 1 \times PBS per well as described in Subheading 3.4, step 1.
5. Incubate for 16 h at 37°C in a humidified incubator to allow for spontaneous lipid loading onto CD1d (see Note 22).
6. Wash five times with 200 μ L of 1 \times PBS to remove unbound lipid.
7. Add 2×10^5 primary iNKT cells or 5×10^4 cells from an iNKT cell clone or hybridoma in 200 μ L of tissue culture medium.
8. Analyze NKT cell activation as described in Subheading 3.4, step 8.

4. Notes

1. Fluorescently labeled CD1d tetramers loaded with α -galactosylceramide (α -GalCer) analogues are available from the National Institutes of Health (NIH) Tetramer Core Facility (http://tetramer.yerkes.emory.edu/available/nonclassical_cd1d) and other commercial sources and allow for sensitive and specific detection of iNKT cells.
2. Depending on the solubility of the lipid of interest, detergents such as Tween or Triton-X might have to be added in low concentrations and the aqueous solution might have to be sonicated for 10 min in a water bath at room temperature or 37°C to ensure that lipids dissolve in PBS. However, addition of detergents might interfere with subsequent loading of lipids onto CD1d and prevent the formation of stable CD1d/lipid complexes.

3. It is noteworthy that recombinant CD1d, probably independent of the choice of expression system, is likely to contain endogenous lipids bound to the CD1d groove. Loading of CD1d with exogenous lipids will therefore rely on their ability to replace the endogenous lipid(s) bound to “unloaded” CD1d.
4. Phycoerythrin (PE) and Allophycocyanin (APC) are recommended as fluorophores since they exhibit strong fluorescence allowing detection of binding of low affinity antigen tetramers. Use of the respective fluorophores will also depend on the configuration of the flow cytometer.
5. Tissues vary significantly in the relative and absolute numbers of iNKT cells (18). However, little is known about the distribution of niNKT cells. The relative abundance of iNKT cells is highest in murine livers with up to 30–40% of T cells representing iNKT cells based upon analysis with α GalCer-loaded CD1d tetramers (18). In contrast, other human and murine lymphoid and non-lymphoid tissues contain low relative numbers of iNKT cells, usually less than two percent of T cells. Still, due to differences in the total T cell count, absolute numbers of iNKT cells are higher in spleen, thymus, and intestine compared to the liver. Thus, a murine liver yields about $1\text{--}2 \times 10^6$ mononuclear cells, with about $0.25\text{--}0.5 \times 10^6$ iNKT cells assuming 60% T cells and 40% of T cells expressing the iNKT TCR. From spleen, about 1×10^6 iNKT cells can be extracted assuming 1×10^8 total cells, 50% T cells, and 2% of T cells expressing the iNKT TCR.
6. The easiest way to achieve this is to put a Pasteur pipette at the bottom of the tube and to fill it with 2 mL of 60% Percoll. The Pasteur pipette will ensure slow release of Percoll and avoid a mixture of the two layers. In case the Pasteur pipette does not release the Percoll, carefully lift it so that it does not touch the bottom of the tube.
7. Invariant NKT cells can be detected in a sensitive and highly specific manner by CD1d tetramers loaded with the marine sponge glycosphingolipid α -GalCer (18) or its analogue PBS-57. Such tetramers are available in preloaded, fluorophore-conjugated formats from the NIH Tetramer Core Facility and different commercial vendors. Consistent with cross-species reactivity of CD1d, murine α -GalCer/CD1d tetramers can be used for staining of human iNKT cells (15). In addition to α -GalCer/CD1d tetramers, human but not mouse iNKT cells can be detected with a monoclonal antibody directed against a unique determinant in the complementarity determining region (CDR) 3 region of the human invariant (V α 24-J α 18) TCR chain (21) or by direct staining of the human iNKT TCR using antibodies against TCR V α 24 and TCR V β 11. Furthermore, CD1d can be loaded with lipids of interest for

tetramer-based characterization of antigen recognition by iNKT cells as described above.

8. We usually stain cells in 5 mL flow cytometry tubes. However, in case of multiple stainings, cells can also be stained in 96-well round or V-bottom plates. In this case, washing steps only allow lower volumes of washing buffer (200 µL). Accordingly, two instead of one washing cycles should be performed at each step.
9. Antibodies against T cell markers such as CD3ε or TCRβ need be included to detect invariant NKT cells as CD1d/lipid-tetramer-positive CD3ε^{int} or TCRβ^{int} (int=intermediate signal strength) cells. Inclusion of additional antibodies depends on the number and choice of flow cytometry detectors and the focus of interest. For basic characterization of lipid-reactive NKT cells, the following markers should be considered:
 - (a) Molecules such as CD4 (mouse and human) and CD8 (human) to distinguish CD4-CD8- double negative from CD4+ and CD8+ iNKT cells.
 - (b) NK cell markers typically expressed by NKT cells such as CD161 (human), NK1.1 (selected inbred mouse strains including C57BL/6, FVB/N, and NZB, but not AKR, BALB/c, CBA/J, C3H, DBA/1, DBA/2, NOD, SJL, and 129) and CD49b (most inbred mouse strains including BALB/c, C57BL/6, C3H, CBA, DBA, AKR, SJL, and 129).
 - (c) Differentiation (NK1.1, CD44, CD122), activation (CD69, CD38, CD25, CD95), effector memory markers (CD45RO^{hi}, CD62L^{lo}) expressed by NKT cells ([21, 22](#)). It is noteworthy that some of these markers, such as CD69, are acquired during thymic development of NKT cells and are largely independent of interaction with CD1d following thymic emigration thus serving as a marker for central NKT development and maturation ([23](#)). In contrast, other markers, such as NK1.1 are at least partially acquired in the periphery after thymic emigration, are dependent on CD1d-restricted antigen presentation in peripheral lymphoid and non-lymphoid organs and thus serve as markers for post-thymic NKT maturation and homeostasis ([23](#)). For additional information, please refer to excellent reviews on this topic ([22, 24, 25](#)).
 - (d) Dead cell markers such as 7-AAD, propidium iodide or 4-,6-Diamidin-2-phenylindol (DAPI).
10. In addition to αGC-loaded CD1d tetramers, human iNKT cells can be identified by the 6B11 antibody that recognizes a unique determinant in the complementarity determining

region (CDR) 3 region of the human invariant ($\text{V}\alpha 24\text{-J}\alpha 18$) TCR chain (21). Alternatively, iNKT cells can be detected by direct staining of the human iNKT TCR using antibodies against TCR $\text{V}\alpha 24$ and TCR $\text{V}\beta 11$ but this approach is less specific compared to $\alpha\text{GC/CD1d-tetramer}$ - and 6B11-staining (26). In contrast to human iNKT cells, detection of mouse iNKT cells is dependent on $\alpha\text{GC/CD1d-tetramers}$ since iNKT TCR antibodies are not commercially available.

11. For intracellular staining, transcriptional regulators such as the largely NKT cell-specific promyelocytic leukemia zinc finger (PLZF) (27, 28), the IL-17-lineage marker ROR γ t (7), the regulatory marker FoxP3 (29), and many other transcription factors expressed by NKT cells (24) should be considered. In addition, cytokines produced by NKT cells such as IFN- γ , IL-4, IL-10, IL-13, and IL-17 can be detected by intracellular cytokine staining, particularly after activation of NKT cells *in vitro* or *in vivo*.
12. For coculture systems, the choice of APC is most relevant. When synthetic or purified exogenous lipid antigens are used, the monomorphic nature of CD1d allows the use of many different types of APCs including primary CD1d-expressing APCs such as DCs, monocytes, and B cells; CD1d transfectants such as murine fibroblasts (L-CD1d), murine T cell lymphoma (RMAS-CD1d), murine B cell lymphoma (A20-CD1d), and human CD1d-transfected C1R B cells, 293 human embryonic kidney cells, and HeLa cervical cancer cells. While CD1d-transfected cell lines are readily available and easy to handle, lipids that require processing for loading onto CD1d might be presented more efficiently by primary APCs that are capable of extensive endolysosomal processing such as DCs.
13. In case hydrophobicity prevents direct addition of lipids to aqueous solutions, proceed as described in step 1 of Subheading 3.1, transfer lipids from nonpolar solvents to a sonication-resistant borosilicate glass tube, dry lipids under nitrogen stream, and dissolve in tissue culture medium at the final concentration by sonication in a water bath.
14. CD1d lipids that load at the cell surface require less time for efficient transfer onto CD1d than lipids dependent on endolysosomal loading (30). Similarly, processing-dependent antigens require longer incubation times than non-processing-dependent lipids.
15. It is critical to include a CD1d-negative control to demonstrate CD1d-restriction of NKT cell activation. In case of CD1d-transfected cell lines, mock-transfected cells serve as control. In case of primary APCs, antibody-mediated CD1d blocking is the method of choice. To this end, block CD1d by

the addition of monoclonal, unlabeled, azide-free antibodies between steps 5 and 6. An antibody concentration of 10 µg/mL is usually sufficient. Clones 19 G11 for murine CD1d and 51.1 for human CD1d block CD1d-restricted antigen presentation of a variety of different APCs (15, 31). It should be noted, however, that antibody blocking of CD1d is cell-type specific despite the monomorphic structure of CD1d (31). Notable exceptions of monoclonal antibodies with universal CD1d blocking across different cell types, such as 19 G11, exist (31).

16. The optimal ratio of APCs to primary iNKT cells and iNKT cell clones/hybridomas varies depending on the kind of APC and NKT cell used for coculture experiments. Pilot studies should be performed with different relative and absolute amounts of APCs and NKT cells.
17. Extensive washing is preferred in order to ensure that all unloaded lipid antigen is removed in order to avoid processing and presentation by CD1d bearing T cells when analyzing murine T cells. Human CD1d is not typically expressed on T cells so is usually not an issue when analyzing human systems (32).
18. Primary NKT cells, NKT cell clones and lines, and NKT cell hybridomas can be used. Primary NKT cells are difficult to extract in sufficient quantities but allow for analysis of polyclonal NKT cells producing of a wide variety of NKT cell cytokines. In contrast, NKT cell clones and particularly hybridomas can be easily expanded and maintained in culture but are restricted in the diversity of secreted cytokines (in hybridomas usually restricted to IL-2). In addition, it is important to note that iNKT cells express a semi-invariant TCR with various possible TCR-β chains that affect antigen recognition. Primary NKT cells thus offer a source of oligoclonal NKT cells with various TCR-β chains, while iNKT cell clones and hybridomas are monoclonal and limited to one particular TCR-β chain.
19. The required time for coculture is dependent on the type of assay used for the detection of NKT cell activation. Intracellular cytokines and upregulation of activation markers such as CD69 can often be detected after 12–24 h of stimulation by flow cytometry. In addition, secretion of IL-4, as detected by ELISA, reaches its peak at 24 h, while IFN-γ peaks at 72 h of coculture. For ELISpot assays, 16–24 h of coculture are sufficient.
20. Primary NKT cells usually secrete abundant amounts of IFN-γ and IL-4 that can be detected in ELISA- and flow cytometry-based approaches. NKT cell clones and particularly hybridomas often lose the ability to secrete IFN-γ and IL-4. In these cases, IL-2 is the preferred cytokine for detection of activation. As an activation marker, CD69 shows upregulation of NKT

cell surface expression within hours of activation and can be used for indirect detection of lipid antigen recognition by NKT cells.

21. Alternative approaches for enhanced CD1d binding such as streptavidin-coated plates or the use of CD1d-Fc fusion proteins on protein G-coated plates exist but are usually not necessary for activation of NKT cells by plate bound CD1d.
22. The addition of purified lipid transfer proteins such as microsomal triglyceride transfer protein (commercially available from different vendors) might facilitate the transfer of lipids onto CD1d (13).

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References

1. Cohen NR, Garg S, Brenner MB (2009) Antigen presentation by CD1 lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol* 102:1–94
2. Barral DC, Brenner MB (2007) CD1 antigen presentation: how it works. *Nat Rev Immunol* 7:929–941
3. de la Salle H et al (2005) Assistance of microbial glycolipid antigen processing by CD1e. *Science* 310:1321–1324
4. Park SH, Roark JH, Bendelac A (1998) Tissue-specific recognition of mouse CD1 molecules. *J Immunol* 160:3128–3134
5. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L (2004) NKT cells: what's in a name? *Nat Rev Immunol* 4: 231–237
6. Zeissig S, Kaser A, Dougan SK, Nieuwenhuis EE, Blumberg RS (2007) Role of NKT cells in the digestive system. III. Role of NKT cells in intestinal immunity. *Am J Physiol Gastrointest Liver Physiol* 293:G1101–G1105
7. Michel ML et al (2007) Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* 204:995–1001
8. Cardell S et al (1995) CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* 182:993–1004
9. Chiu YH et al (1999) Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. *J Exp Med* 189:103–110
10. Kasmar AG et al (2011) CD1b tetramers bind alpha₁beta₁ T cell receptors to identify a mycobacterial glycolipid-reactive T cell repertoire in humans. *J Exp Med* 208(9):1741–1747
11. Odyniec AN et al (2010) Regulation of CD1 antigen-presenting complex stability. *J Biol Chem* 285:11937–11947
12. Dougan SK, Rava P, Hussain MM, Blumberg RS (2007) MTP regulated by an alternate promoter is essential for NKT cell development. *J Exp Med* 204:533–545

13. Dougan SK et al (2005) Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells. *J Exp Med* 202:529–539
14. Kaser A et al (2008) Microsomal triglyceride transfer protein regulates endogenous and exogenous antigen presentation by group 1 CD1 molecules. *Eur J Immunol* 38:2351–2359
15. Zeissig S et al (2010) Primary deficiency of microsomal triglyceride transfer protein in human abetalipoproteinemia is associated with loss of CD1 function. *J Clin Invest* 120:2889–2899
16. Tupin E, Kronenberg M (2006) Activation of natural killer T cells by glycolipids. *Methods Enzymol* 417:185–201
17. Koseki H et al (1990) Homogenous junctional sequence of the V14+ T-cell antigen receptor alpha chain expanded in unprimed mice. *Proc Natl Acad Sci U S A* 87:5248–5252
18. Matsuda JL et al (2000) Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741–754
19. Halder RC, Aguilera C, Maricic I, Kumar V (2007) Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *J Clin Invest* 117:2302–2312
20. Jahng A et al (2004) Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* 199:947–957
21. Montoya CJ et al (2007) Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology* 122:1–14
22. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. *Annu Rev Immunol* 25:297–336
23. McNab FW et al (2005) The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol* 175:3762–3768
24. Das R, Sant'Angelo DB, Sant'Angelo DB, Nichols KE (2010) Transcriptional control of invariant NKT cell development. *Immunol Rev* 238:195–215
25. Godfrey DI, Berzins SP (2007) Control points in NKT-cell development. *Nat Rev Immunol* 7:505–518
26. Lee PT et al (2002) Testing the NKT cell hypothesis of human IDDM pathogenesis. *J Clin Invest* 110:793–800
27. Kovalovsky D et al (2008) The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* 9:1055–1064
28. Savage AK et al (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29:391–403
29. Monteiro M et al (2010) Identification of regulatory Foxp3+ invariant NKT cells induced by TGF-beta. *J Immunol* 185:2157–2163
30. Yu KO et al (2007) Production and characterization of monoclonal antibodies against complexes of the NKT cell ligand alpha-galactosylceramide bound to mouse CD1d. *J Immunol Methods* 323:11–23
31. Roark JH et al (1998) CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160:3121–3127
32. Dougan SK, Kaser A, Blumberg RS (2007) CD1 expression on antigen-presenting cells. *Curr Top Microbiol Immunol* 314:113–141

Chapter 42

Chromatofocusing Purification of CD1b–Antigen Complexes and Their Analysis by Isoelectric Focusing

Luis Fernando Garcia-Alles and Henri de la Salle

Abstract

The presentation of lipid antigens to T cells is mediated by the CD1 proteins. Purified functional CD1/lipid complexes are valuable tools to investigate such immune processes. Here, we describe how these complexes can be prepared in vitro, how they can be purified by chromatofocusing and how to control their antigen-loading status by isoelectric focusing.

Key words: Antigen presentation, CD1 proteins, Protein–lipid complex, Chromatofocusing, Isoelectric focusing

1. Introduction

Purified recombinant soluble MHC/peptide complexes are precious tools for immunologists. One of their major applications is tetramer staining, which is widely used to define the frequencies of antigen-specific T lymphocytes and to isolate them, thus permitting a more complete characterization of their immunological functions (1). Although the production of recombinant soluble CD1 molecules, as either natural heterodimers or engineered single chain constructs, in a variety of expression systems does not represent a major technical problem, analogous investigations using CD1/lipid complexes has proved difficult and until recently, has been mostly restricted to the CD1d isoform. Among the several technical bottlenecks which contribute to these difficulties, the preparation of CD1/lipid complexes is often hampered by the poor solubility of lipid antigens in aqueous media. This effect is especially pronounced in the case of mycobacterial lipid antigens having long and/or structurally complex hydrophobic chains.

In addition, the CD1 ligands must gain access to the intricate antigen-binding grooves, which are preoccupied by endogenous eukaryotic lipids (2). In spite of these obstacles, recent studies have demonstrated that CD1/microbial lipid complexes prepared in vitro can be successfully used to stimulate antigen-specific T cells (3, 4), to isolate them from peripheral blood (3) or, to perform biochemical studies (5, 6). This protocol describes a procedure enabling the preparation of functional CD1b/lipid complexes, their separation from unreacted CD1b molecules and the visualization of their loading yield by isoelectric focusing.

2. Materials

All solutions must be prepared with analytical grade reagents and using milli-Q water (deionized to attain an electrical resistivity of less than 18 MΩ cm at 25°C). Unless otherwise indicated, all solutions must be filtered through 0.22 µm membrane disk filters (Pall Life Sciences) and stored at 4°C. All established regulations must be respected when disposing of waste materials.

2.1. Common Consumables and Lab Equipment

1. Standard centrifugation equipment is required throughout this procedure. The centrifuge rotor may be either a swing-bucket or a fixed-angle type, but must accommodate conical bottomed concentrator tubes (17 mm).
2. A pH meter (Metrohm) and magnetic stirrer.
3. Glass insert vials (Atlanticlabo ICS).
4. Glass test tubes.
5. Thermomixer compact unit (Eppendorf).
6. Vivaspin 2 concentrator (10 kDa molecular weight cut-off, VivaScience, see Note 1).
7. Standard UV-Vis spectrophotometer (see Note 2).

2.2. Preparation of shCD1b-SGL12 Complexes

1. Solutions of 1 M Na acetate (pH 5.0), 1 M Bis-Tris (pH 6.5), 2 M NaCl, and 200 mM Na EDTA (pH 7.3): Respectively, add 41 g of sodium acetate anhydrous, 104.6 g of Bis-Tris, 58.4 g of sodium chloride, or 29.2 g of ethylenediaminetetraacetic acid (all from Sigma-Aldrich) to a 0.5 L graduated cylinder containing 450 mL of water. Stir magnetically. Adjust the pH of the Na acetate and Bis-Tris solutions to 5.0 and 6.5, using 37% HCl (see Note 3). The EDTA-containing cylinder should be refrigerated in an ice bath, while solid NaOH pellets are added (see Note 3) to drive the pH to 7.3. This solution will become transparent when pH > 6. Make all solutions up to 0.5 L.
2. Buffer A: 10 mM Na acetate, 50 mM NaCl, 1 mM Na EDTA, approximate solution pH 5.0. Pipette the following solutions

(volume) into a 0.1 L graduated cylinder containing about 80 mL of water: 1 M Na acetate, pH 5.0 (1 mL), 2 M NaCl (2.5 mL), and 200 mM EDTA, pH 7.3 (500 μ L). Make up to 100 mL.

3. Reaction buffer: 500 mM Na acetate, 300 mM NaCl, 20 mM EDTA, 10 mM DTT, approximate pH 5.0. Pipette the following solutions (volume) into a 1.5 mL Eppendorf tube: 1 M Na acetate, pH 5.0 (500 μ L), 2 M NaCl (150 μ L), 200 mM EDTA, pH 7.3 (100 μ L), 100 mM DTT (100 μ L), and water (150 μ L).
4. Buffer B: 10 mM Bis-Tris/HCl, 10 mM NaCl, pH 6.5. Pipette the following solutions (volume) into a 1.5 mL Eppendorf tube: 1 M Bis-Tris (10 μ L, pH 6.5 adjusted with HCl), 2 M NaCl (5 μ L). Add 985 μ L of water.
5. Soluble domain of human CD1b: shCD1b. The protein expressed in mouse cells (see Note 4) is purified by affinity chromatography using an anti-CD1b (BCD1b3.1) column, as described in ref. 2. After pooling and concentration, dilute tenfold by pipetting 200 μ L of the shCD1b sample ($OD_{280nm} = 3.0$) into a Vivaspin 2 concentrator containing 1.8 mL of buffer A. Mix thoroughly and spin at 5,000 $\times g$ and 4°C for 3–5 min until the volume is reduced to ca. 200 μ L. The tenfold dilution step with buffer A is repeated and the sample finally concentrated to 250 μ L (approximate measured $OD_{280nm} = 2.2$).
6. 500 μ M SGL12 antigen vesicles, prepared as described under Subheading 3.1, step 1.
7. 400 mM Taurocholate: weigh 107 mg of Na taurocholate (Sigma-Aldrich) in an Eppendorf tube and add 500 μ L of water. Vortex vigorously until the solution becomes transparent.

2.3. Chromatofocusing

1. FPLC purification system equipped with a 1 mL sample injection loop (see Note 5).
2. Plastic syringe (1 mL) with needle.
3. MonoP 5/200 GL column (GE Healthcare). The column should be stocked at 4°C, conditioned in 0.22 μ m-prefiltered 20% ethanol/water (v/v).
4. Buffer C: 25 mM Bis-Tris/HCl, pH 6.1. Weigh 2.6 g of Bis-Tris and add it to a 0.5 L graduated cylinder containing about 400 mL of water and a magnetic bar. Stir magnetically and adjust the pH to 6.1 with 37% HCl (see Note 3). Make up to 0.5 L.
5. Buffer D: Tenfold diluted PB74/HCl, pH 4.0. Place 25 mL of Polybuffer 74 (GE Healthcare) in a 250 mL graduated cylinder and add about 200 mL of water. Adjust the pH to 4.0 with 37% HCl (see Note 3) and make up to 250 mL.

6. PBS (10×): Add to a 1 L graduated cylinder 2 g of KCl, 2.4 g of anhydrous KH₂PO₄, 36.6 g of Na₂HPO₄·12H₂O and 80 g of NaCl. Add about 950 mL water, a magnetic bar and stir vigorously. If necessary, adjust to pH 7.3. Make up to 1 L, filter through 0.22 µm membranes and store at 4°C.
7. Buffer E: 10 mM Bis-Tris/HCl, 100 mM NaCl, 1 mM EDTA, approximate pH 6.5. Pipette the following solutions (volume) into a 0.1 L graduated cylinder containing about 80 mL of water: 1 M Bis-Tris/HCl, pH 6.5 (1 mL), 2 M NaCl (5 mL), and 200 mM EDTA, pH 7.3 (500 µL). Make up to 100 mL.

2.4. Isoelectric Focusing

1. PhastSystem separation and control unit (GE Healthcare).
2. PhastSystem development unit (GE Healthcare).
3. PhastGel isoelectric focusing (IEF) 4–6.5 media (GE Healthcare).
4. 20% Aqueous Trichloroacetic acid (TCA) (see Note 6): weigh 17 g of TCA (Sigma-Aldrich) in a 0.1 L glass bottle and add 85 mL of water (see Note 3). Dip the tubing connected to the IN-port 1 of the PhastSystem development unit into the bottle until it touches the bottom. Stop with parafilm and leave at room temperature.
5. Washing/destaining solution: add into a 1 L graduated cylinder 600 mL of water, 300 mL of methanol, and 100 mL of acetic acid. Mix well and transfer 0.5 L of the solution into a 0.5 L glass bottle. Immerse the tubing connected to the IN-port 2 of the PhastSystem development unit in the solution in the bottle, block with a stopper and keep at room temperature.
6. Staining stock solution: dissolve a PhastGel Blue R tablet (GE Healthcare) in 80 mL of water. Stir vigorously with a magnetic bar for 5–10 min, add 120 mL of methanol and continue to mix for 2 min. This solution should be stored at 4°C and used within 6 weeks.
7. CuSO₄ solution: add 500 mg of CuSO₄·5H₂O (Sigma-Aldrich) to a 0.5 L glass bottle containing 0.5 L of washing/destaining solution (see Note 7). Stir vigorously with a magnetic bar for 1 h until the blue solid dissolves. Block with a stopper and store at room temperature.
8. Final staining solution: dispense 72 mL of CuSO₄ solution into a 0.1 L glass bottle. Using a 10 mL plastic syringe (without needle), draw up about 8 mL of staining stock solution and filter through a 0.2 µm membrane (Sarstedt) into the 0.1 L bottle (see Note 8). Dip the tubing connected to the IN-port 3 of the PhastSystem development unit down to the bottom of the bottle. Prepare this solution the day of the experiment and do not reuse it.

3. Methods

3.1. Preparation of shCD1b–SGL12 Complexes and Purification by Chromatofocusing

1. Production of SGL12 antigen vesicles (500 μ M): with a glass syringe, transfer 200 μ g of synthetic sulfoglycolipid (SGL, 1,060 Da molecular weight, see Note 9) dissolved in chloroform/methanol into a glass test tube (see Note 10). Dry until total evaporation under a nitrogen stream (see Note 11). Cover the tube with aluminum foil and place it in a high-vacuum (lyophilization) system for 1 h. Add 375 μ L of buffer B to the tube, keep it at room temperature for 1 h and vortex gently regularly. Cover the mouth of the tube with a Teflon stopper (see Note 12). Dip the closed tube containing the solution in a Dewar filled with liquid nitrogen while gently shaking with the hand (see Note 13). Once the solution frozen, transfer the tube into a water bath kept at 40–50°C. Hand-shake until the solids melt and solution warms up. Repeat such freeze-thaw step for at least eight cycles (see Note 14).
2. Mix 70 μ L of 500 μ M SGL12 vesicles with 8 μ L of 400 mM taurocholate solution in a glass insert vial. Stop the vial with parafilm and shake at 400 rpm and room temperature for 1–2 days, in a Thermomixer compact unit (see Note 15).
3. Add the SGL12/taurocholate mixture to a glass test tube containing 250 μ L of shCD1b solution (approximate OD_{280nm} = 2.2, conditioned in buffer A) and 17 μ L of reaction buffer. Seal the tube with parafilm and shake for 3 h at 200 rpm and 37°C (see Note 16).
4. Connect the Mono P column to the FPLC system (see Note 17). To prepare the column for sample injection, run 15 mL of buffer C at a flow rate of 0.5 mL/min (see Note 18). Take a plastic syringe, wash the loop by passing through 3 \times 1 ml of 2 M NaCl, and finally inject 1 mL of 2 M NaCl to the column. Continue the equilibration with buffer C until the effluent pH is stable and approximately 6.1 as in the starting buffer.
5. After column equilibration, ensure that the injection loop is filled with buffer C: carry out 3–4 successive loadings of the loop using the same 1 mL plastic syringe, previously rinsed with water to remove traces of NaCl.
6. After the 3 h incubation, cool the shCD1b/SGL12 solution by placing the tube on ice for 15 min. Withdraw 5 μ L to be stored in a glass insert vial at 4°C for subsequent IEF analysis (sample BP1 in Fig. 1b). Transfer the rest to a Vivaspin 2 concentrator containing 1.5 mL of buffer C, mix thoroughly and concentrate the solution to ca 300 μ L by centrifuging at 4°C and 5,000 $\times g$ for 5 min. After homogenizing the concentrate, measure its OD_{280nm}, which should be around 1.4.

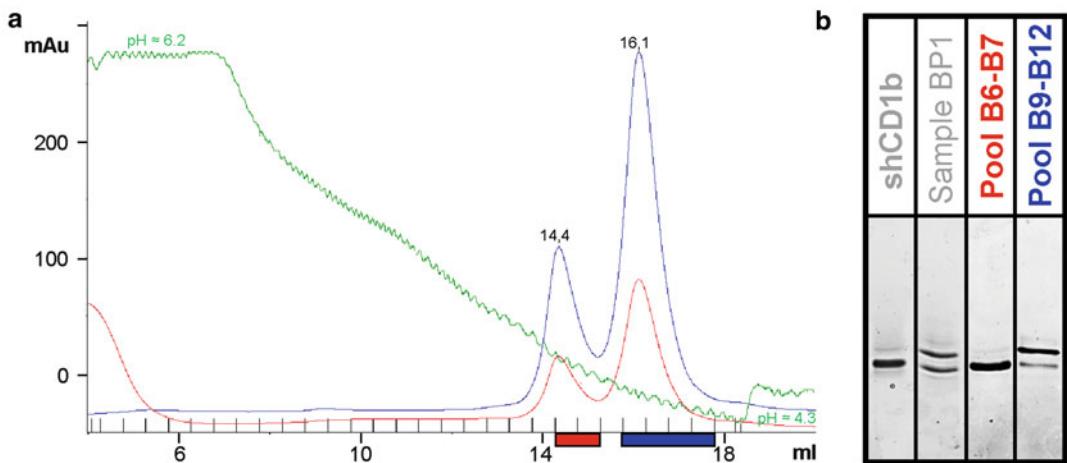


Fig. 1. Chromatofocusing and IEF characterization of shCD1b–SGL12 complexes. **(a)** Chromatogram showing eluate absorbance at 280 nm (blue line) and 254 nm (red). The injection step, i.e., the first 4 mL, is omitted. In green is shown the purification pH profile. Two protein samples were subsequently prepared after pooling fractions B6–B7 and B9–B12, highlighted by the red and blue boxes, respectively. **(b)** IEF profile of purified shCD1b–SGL12 complexes. Samples purified by chromatofocusing [shown in **(a)**] were applied after concentration and Polybuffer exchange in lanes 3 (3 µL) and 4 (2 µL) on an IEF 4–6.5 Phastgel. Lipid-untreated shCD1b and the BP1 sample withdrawn after incubation with SGL12 but before chromatofocusing were applied in lanes 1 (2 µL) and 2 (2 µL) for comparison.

7. Load the shCD1b/SGL12 solution into the FPLC injection loop using the 1 mL plastic syringe. Run directly with buffer D at a flow rate of 1 mL/min. First, inject the contents of the loop into the Mono P column. After a total injection volume of 6 mL, continue the elution for 18 mL while starting to collect 0.5 mL fractions in Eppendorf tubes (see Note 19). The monitored pH should steadily decrease from 6.1 to about 4.0, as shown in Fig. 1a (see Note 20).
8. Prerinse two Vivaspin 2 concentrators with buffer E. Pool fractions corresponding to each major chromatographic peak in each concentrator (see Note 21). If the total volume to be pooled exceeds the maximal 2 mL capacity of the concentrator, start with the less concentrated fractions (according to OD_{280nm} readings). Concentrate to 200–300 µL by centrifuging at 4°C and 5,000 × g for 6 min. Continue to pool fractions if necessary, but mixing thoroughly with a pipette before each new centrifugation. In the case of the purification described here, which furnished the elution profile shown in Fig. 1a, two pools were prepared corresponding to fractions B6–B7 and B9–B12.
9. Polybuffer removal: using the same concentrators, make up the solution to a total volume of approximately 2 mL with buffer E, homogenize and concentrate to 200 µL by centrifuging at 4°C and 5,000 × g for 6–8 min. Repeat this tenfold dilution/concentration step at least twice more (see Note 22).

10. Continue to carefully concentrate the samples until the absorbance at 280 nm lies between 1.3 and 2.5 (or about 0.6–1.1 mg/mL shCD1b). Transfer the solution with a pipette (measure the volume) to an Eppendorf vial. Maximal recovery is attained using thin gel loader pipette tips. Otherwise, reverse spin the Vivaspin concentrator to collect the concentrate into the recovery cap and transfer the volume to an Eppendorf vial.
11. Measure the final OD_{280nm} of each purified sample and store at 4°C. Two samples were obtained in the purification shown in Fig. 1a: pool B6–B7 (50 μ L, OD_{280nm} 1.3) and pool B9–B12 (100 μ L, OD_{280nm} 1.7). These samples were subsequently analyzed by IEF as described below (see also Fig. 1b).

3.2. Monitoring of the shCD1b–SGL12 Complex Purity by IEF

1. Switch the PhastSystem separation and control unit on and set the standby temperature to 15°C. The separation bed normally reaches this temperature after 2–5 min (see Note 23).
2. Raise to the vertical position the electrode assembly, including the PhastGel IEF gel cover on its underside (see Note 24).
3. Place 70 μ L of water in the middle of the area delimited by the red lines on the separation bed. Take one PhastGel IEF 4–6.5 stored at 4°C (see Note 25), open carefully the package and transfer the gel with a pair of forceps to the separation bed (see Notes 26–28). Position the gel so that the edges are well inside the box defined by the red lines. Lower the electrode assembly, run one thumb gently along the top of the electrodes to ensure good contact with the gel surface and close the separation compartment lid.
4. Start the programmed run for IEF (see Notes 29–30):
 - (a) Prefocusing step at 2,000 V, with 2.5 mA and 3.5 W for 75 volt hours (Vh).
 - (b) Sample application step (preceded by an alarm): programmed to last for 15 Vh, the voltage should fall to 200 V, while other parameters remain the same. When the alarm sounds, wait until the voltage drops to 200 V. Then open the lid (see Note 31) and use a pipette (preferably with a tip conditioned at 4°C, see Note 23) to apply a 1 μ L sample to the gel surface through the lowest slot of the PhastGel IEF cover (see Notes 32–33). Similarly, apply other samples at different horizontal emplacements of the gel through the same slot(s) (see Note 34). Close the lid and leave to run until 84 accumulated Vh is displayed. Open the lid again and repeat the process of sample application at the same emplacements as before (see Notes 33 and 35). Close the lid and leave to run.

- (c) Focusing step, under the same conditions as the prefocusing step, but running for at least 410 Vh (or 500 AVh), which takes about 20 additional minutes.
5. Stop the run, remove the gel with the forceps making use of the bent plastic tab (see Note 28) and proceed with development (see Note 36).
6. Slide the gel into the lower position of the gel holder with the gel surface facing up and close the development unit. Start the development (see Note 29):
 - (a) Fixing step: the gel is immersed in 20% TCA solution for 5 min at 20°C (see Note 6).
 - (b) Washing step with the washing/destaining solution, for 2 min at 20°C. After this step the solution is recycled back to the same bottle.
 - (c) Staining step: the gel is immersed in the final staining solution for 10 min at 50°C. The solution should be dispensed into a glass bottle for waste.
 - (d) Destaining step: final washing for 10 min at 50°C with the washing/destaining solution. The solution is collected in the same glass bottle for waste.
7. Turn the PhastSystem separation and control unit off. Recover the developed IEF gel with the forceps and transfer it into water. Scan the gel immediately after gently rinsing with ethanol the water drops from its surface (see Note 28). For the experiment described herein, the scan of the resulting gel is shown in Fig. 1b.
8. After immersion of the gel in water, it is left to dry at room temperature (over several days) inside a non-hermetically covered box.

4. Notes

1. Prerinse the concentrator device to remove trace amounts of glycerine and sodium azide, according to the manufacturer's instructions. Fill the device with 2 mL of buffer or water and centrifuge for 2 min at 1,000×g and 4°C.
2. In view of the small protein sample volumes handled in this procedure, a Nanodrop 1000 Spectrophotometer (Thermo Scientific) or similar system is recommended, since it permits straightforward measurements on 2 µL samples, which in addition can be recovered and pooled back into the original solution.
3. Wear glasses and protective gloves.

4. In our experience, shCD1b expressed in J558 plasmacytoma cells results in sharper IEF bands than shCD1b expressed in S2 Drosophila cells. Therefore, we selected the former for easier monitoring of lipid loading by IEF and to obtain well-resolved chromatofocusing profiles.
5. The FPLC system should be equipped with a UV detector permitting continuous monitoring of the absorbance at 280 nm. Although dispensable, a pH probe also proves useful. The system operates at 4°C with cold pre-filtered solutions.
6. This solution can be recycled at least six times.
7. CuSO₄ is added to decrease the background staining of gels.
8. The syringe can be recycled indefinitely, the filtering device 2–4 times.
9. This procedure can be used to purify CD1b–lipid complexes formed with other anionic glycolipid ligands of CD1b, e.g., phosphatidylinositol or GM1 gangliosides.
10. Select glass test tubes designed to withstand freeze-thaw cycles.
11. This should be done under a fume hood.
12. The Teflon stopper can be replaced by a double layered aluminum foil stopper covered with parafilm. The tube should not be sealed with parafilm alone, since it often implodes during the freeze-thaw cycles, thus contaminating the lipid solution.
13. Wear glasses and cryoprotective gloves.
14. Depending on the lipid structure, the solution might become clearer during the freeze-thaw cycles.
15. Premixing with detergent is to disrupt the lipid vesicles, thereby increasing the accessibility to lipid monomers of shCD1b.
16. At this stage, it is recommended to analyze the samples by IEF after incubation, because depending on the lipid the loading yield can be sufficiently good for the chromatofocusing purification to be deemed unnecessary.
17. The Mono P column should be stored clean, ready for use.
18. Set the pressure limit to 3 MPa to ensure that back pressure in the system will not damage the column. The flow rate can be increased progressively up to 1 mL/min as soon as the storage solution has been replaced by buffer C and the system pressure declines.
19. The solubility of the protein could be compromised at a pH similar to its isoelectric point (pI). Since proteins leave chromatofocusing columns at pH ≈ pI, 50 µL of PBS (10×, pH 7.3) is added per 0.5 mL of protein-containing fraction immediately after elution, to prevent precipitation and aggregation.

20. After elution, rapidly proceed with cleaning and regeneration of the Mono P column (see manufacturer's instructions). Start a reverse flow at 0.3 mL/min, running with buffer C. Inject 0.5 mL of 2 M NaCl and rinse with 20 mL of buffer C. Inject 0.5 mL of 2 M NaOH and rinse until pH < 8. Inject 0.5 mL of 75% acetic acid in water and rinse until pH > 6. Inject 0.5 mL of 2 M NaOH and rinse until pH < 8. Finally, inject 1 mL of 2 M NaCl and rinse until the pH reaches that of buffer C. The system pressure must be monitored throughout all these steps, especially during the first 4 mL after each injection. Flow rate can be increased to 0.7–1 mL/min once the pressure has dropped to values measured before each new injection.
21. Take into account the potential delay between OD_{280nm} readings and fraction attribution, which is often due to tubing volume between the UV detector and the collector outlet.
22. Additional dilution/concentration steps may be necessary to adequately remove Polybuffer components, depending on subsequent sample use. For instance, 6–8 such steps are necessary to prevent interference from Polybuffer components in the analysis of samples by electrospray ionization native mass spectrometry.
23. Before starting the run, place the required number of pipette tips for sample application in a tip box at 4°C. Applying the samples at as low temperature as possible permits one to limit drop spreading on the gel surface.
24. The red eccentric levers at either side of the electrode assembly should be in the “down” position. In this manner, the electrode assembly will be able to reach its lowest position, aligned evenly with the separation bed.
25. PhastGel media should be handled while wearing gloves. Pay attention not to exert pressure on the gel surface.
26. In order to facilitate gel handling, bend the plastic tab up using the forceps.
27. Note the presence of a thin plastic film protecting the gel. When the package is opened, the film often sticks to the package interior surface. The gel can be transferred to the separation bed with or without the film on it. However, the film must be removed to allow subsequent sample deposition.
28. Once the protective film has been removed, take great care to prevent the upper surface of the gel from touching anything, since the gel is extremely fragile. This precaution holds until the gel has been fully dried.
29. For further details on how to program a separation or development, please refer to the instructions provided with the PhastSystem instrumentation.

30. The temperature of the separation bed must remain at 15°C throughout the run.
31. A warning alarm will sound. Although electrodes are automatically turned off when the lid is lifted, avoid touching the electrodes, gel or system as much as possible.
32. To limit potential precipitation of the protein under study, samples should be applied at gel positions which differ maximally from the protein pI (see Note 19). The pI of the shCD1b construct studied here is between 5 and 5.5. Therefore, our shCD1b samples are best applied through the top and/or bottom slots of PhastGel IEF 4–6.5 media but not through the middle slots. Other constructs might differ depending on charged state of tags or glycans. Keep the samples refrigerated at 4°C throughout the time of sample application.
33. If the sample concentration is low (e.g., $OD_{280nm} < 1$), the volume applied can be doubled by loading an additional 1 μ L at the same horizontal emplacement through the uppermost slot (see Note 35). Once a sample applied, put the pipette tip back in the tip box, ready for next application.
34. Up to nine samples can be applied on the same gel.
35. This allows one to increase the total sample volume applied per lane to up to 4 μ L, thus improving the subsequent detection.
36. Wash the electrodes immediately after launching the development of the gel. Rinse the electrodes sequentially with water and ethanol, rubbing them dry with wiping paper.

References

1. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274(5284):94–96
2. Garcia-Alles LF, Versluis K, Maveyraud L, Vallina AT, Sansano S, Bello NF, Gober HJ, Guillet V, de la Salle H, Puzo G, Mori L, Heck AJ, De Libero G, Mourey L (2006) Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *EMBO J* 25(15):3684–3692
3. Kasmar AG, van Rhijn I, Cheng TY, Turner M, Seshadri C, Schieffner A, Kalathur RC, Annand JW, de Jong A, Shires J, Leon L, Brenner M, Wilson IA, Altman JD, Moody DB (2011) CD1b tetramers bind alpha}{beta T cell receptors to identify a mycobacterial glycolipid-reactive T cell repertoire in humans. *J Exp Med* 208(9):1741–1747
4. Garcia-Alles LF, Collmann A, Versluis C, Lindner B, Guiard J, Maveyraud L, Huc E, Im JS, Sansano S, Brando T, Julien S, Prandi J, Gilleron M, Porcelli SA, de la Salle H, Heck AJ, Mori L, Puzo G, Mourey L, De Libero G (2011) Structural reorganization of the antigen-binding groove of human CD1b for presentation of mycobacterial sulfoglycolipids. *Proc Natl Acad Sci USA* 108(43):17755–17760
5. Garcia-Alles LF, Giacometti G, Versluis C, Maveyraud L, de Paepe D, Guiard J, Tranier S, Gilleron M, Prandi J, Hanau D, Heck AJ, Mori L, De Libero G, Puzo G, Mourey L, de la Salle H (2011) Crystal structure of human CD1e reveals a groove suited for lipid-exchange processes. *Proc Natl Acad Sci U S A* 108(32):13230–13235
6. Facciotti F, Cavallari M, Angenieux C, Garcia-Alles LF, Signorino-Gelo F, Angman L, Gilleron M, Prandi J, Puzo G, Panza L, Xia C, Wang PG, Dellabona P, Casorati G, Porcelli SA, de la Salle H, Mori L, De Libero G (2011) Fine tuning by human CD1e of lipid-specific immune responses. *Proc Natl Acad Sci U S A* 108(34):14228–14233

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