14

Localization and MHC Class II Presentation of Antigens Targeted for Macroautophagy

Dorothee Schmid and Christian Münz

Summary

Intracellular antigens can be presented on major histocompatibility complex (MHC) class II molecules after degradation via macroautophagy. To enhance MHC class II presentation of potential vaccine antigens, we have developed a method to target antigens for autophagic degradation via fusion to the Atg8/LC3 protein: Atg8/LC3 is specifically incorporated into autophagosomes via coupling to phosphatidylethanolamine, and subsequently degraded in MHC class II loading compartments (MIICs). Antigens fused to the N-terminus of Atg8/LC3 follow the same pathway and get preferentially presented on MHC class II molecules. The localization of Atg8/LC3 fusion antigens in MIICs can be visualized by confocal microscopy, and MHC class II presentation can be quantified in a presentation assay with antigen-specific $CD4^+$ T-cell clones. These assays are good measures of autophagosome formation and lysosomal degradation of macroautophagy cargo and therefore are useful for studying regulation of the autophagic pathway under various experimental conditions and physiological perturbations.

Key Words: Atg8/LC3; influenza matrix protein 1; MHC class II; MHC class II loading compartment (MIIC); confocal microscopy; $CD4^+$ T-cell clones; IFN-γ ELISA.

1. Introduction

Major histocompatibility complex (MHC) class II molecules present products of lysosomal proteolysis to $CD4^+$ T cells, which orchestrate the adaptive immune response and therefore are key to successful immune surveillance ***(1–3)***.

From: *Methods in Molecular Biology, vol. 445: Autophagosome and Phagosome*
Edited by: V. Deretic © Humana Press, Totowa, NJ

One pathway by which intracellular antigens gain access to lysosomal degradation and subsequent MHC class II presentation is macroautophagy (reviewed in **refs. *4*** and ***5***). Macroautophagy has been suggested to deliver viral, model, and tumor antigens for MHC class II presentation to $CD4^+$ T cells ***(6–9)***. More recently, we have shown that autophagosomes frequently fuse with MHC class II loading compartments (MIICs) and that this pathway can be targeted to improve MHC class II presentation of potential vaccine antigens ***(10)***.

Our targeting method takes advantage of the properties of the Atg8/LC3 protein: During autophagosome formation, Atg8/LC3 is covalently coupled to a lipid in the nascent autophagosome membrane via residue Gly_{120} and thus is incorporated into autophagosomes ***(11,12)***. After fusion of autophagosomes with lysosomes, Atg8/LC3 is degraded by lysosomal proteases ***(10)*** and Atg8/LC3-derived peptides can be found to be presented on MHC class II molecules ***(13)***. To target antigens for autophagic degradation via the same mechanism, we fused the Atg8/LC3 sequence to the 3´ end of the influenza A virus matrix proteins 1 (MP1) sequence. We could demonstrate that this fusion antigen was indeed targeted to MIICs and was presented on MHC class II molecules up to 20-fold more efficiently than MP1 itself ***(10)*** (**Figs. 1 and 2**). Furthermore, we could show that the targeting strategy was dependent on covalent coupling to the autophagosomal membrane, since mutation of Gly_{120} to Ala_{120} completely abrogated autophagosome targeting and eliminated enhanced MHC class II presentation of the fusion antigen ***(10)*** **(Fig. 2)**. In addition, we could show that MHC class I presentation was not affected, since the fusion protein was equally well recognized by MP1-specific $CD8^+$ T-cell clones ***(10)*** **(Fig. 2)**.

We propose that Atg8/LC3 fusion antigens are useful tools for studying the autophagy pathway under various experimental conditions and perturbations, since their MIIC localization and MHC class II presentation require an intact autophagic pathway, including autophagosome formation, fusion with late endosomes and lysosomal degradation of autophagic cargo. Any treatment that affects macroautophagy should also affect MIIC localization and MHC class II presentation of Atg8/LC3 fusion antigens and therefore these readouts can be used to measure autophagosome formation and lysosomal degradation.

Here we describe in detail how LC3 fusion antigens can be expressed by transient transfection in suitable target cell lines. Furthermore, we describe a method to visualize MIIC localization of LC3 fusion antigens by confocal immunofluorescence microscopy. Finally, we describe how to set up MHC class II presentation assays with antigen specific $CD4^+$ T-cell clones and how their recognition of MHC class II presented epitopes, derived from autophagic cargo, can be measured by IFN-γ ELISA.

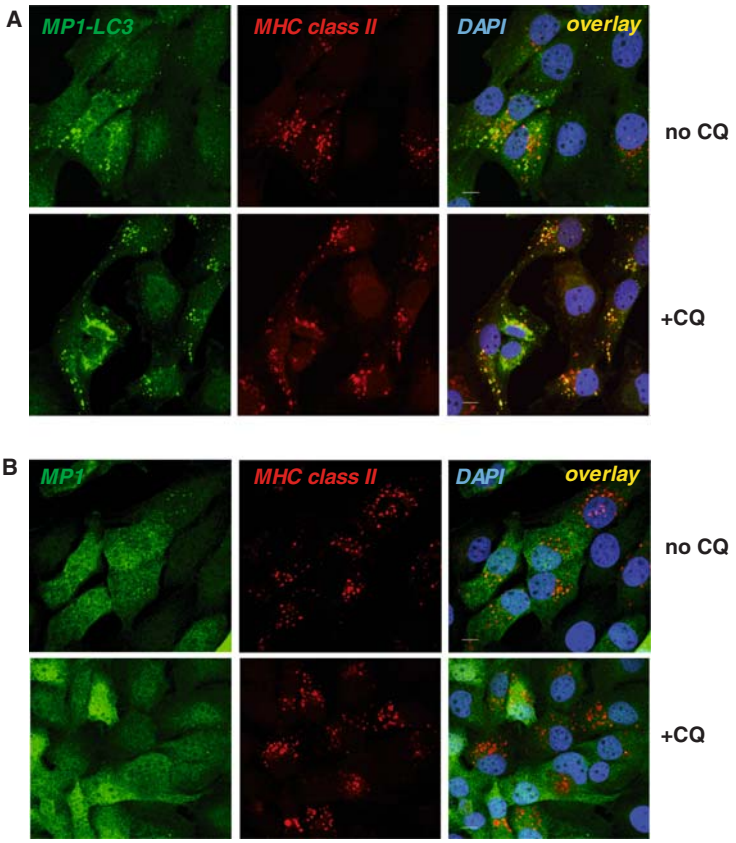

Fig. 1. Localization of MP1-LC3 in MHC class II loading compartments. **(A)** MDAMC cells transfected with the MP1-LC3 construct were treated with 200 U/mL IFN-γ for 36 h to upregulate MHC class II expression. To prevent degradation of MP1-LC3 by lysosomal proteases, cells were treated with 50 μ*M* chloroquine (CQ) during the last 6 h of the culture, where indicated (+CQ). Cells were fixed, stained with MP1- and MHC class II–specific antibodies and DAPI and analyzed by confocal microscopy. MP1-LC3 staining partially overlaps with the MHC class II staining, especially in CQ-treated cells, indicating that MP1-LC3 is degraded in MIICs. (Scale bar: 10 μm.) Representative fields from one experiment out of two are shown. (From **ref. *10*** with permission from Elsevier.) **(B)** As in **(A)**, except that cells were transfected with MP1 construct. In contrast to MP1-LC3, MP1 is homogeneously distributed in the cytoplasm and nucleus and even after CQ-treatment does not accumulate in MHC class II loading compartments. (Adapted from **ref. *10*** with permission from Elsevier.)

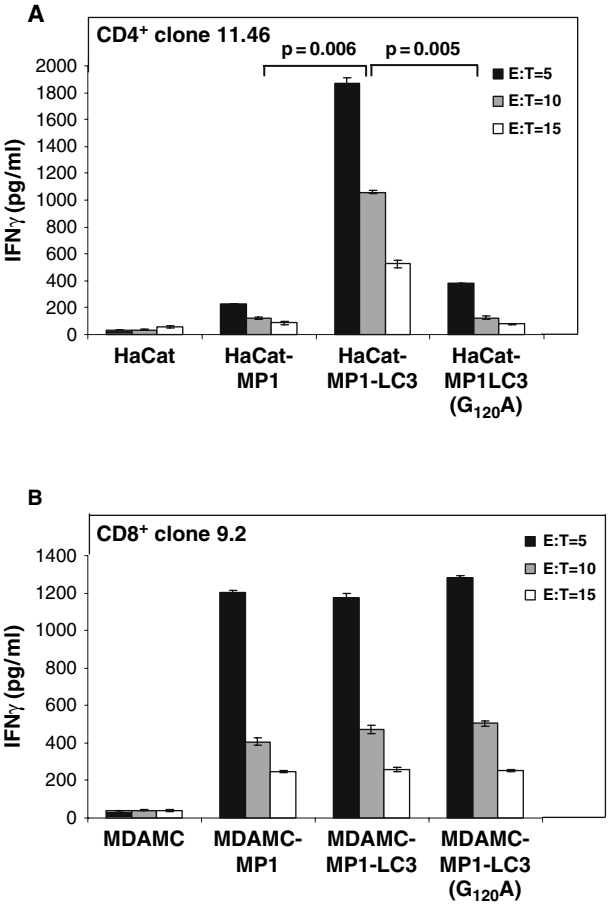

Fig. 2. MHC class II presentation assay with MP1-specific $CD4^+$ T-cell clones. **(A)** MP1-specific $CD4^+$ T-cell clone 11.46 was cocultured at various effector to target cell (ET) ratios with HaCat target cells transfected with either MP1, MP1-LC3, or MP1-LC3($G_{120}A$). The next day, IFN-γ in culture supernatants was measured by ELISA to assess presentation of MP1 on MHC class II. MHC class II presentation is strongly enhanced by the Atg8/LC3 fusion, and this is dependent on covalent coupling to the autophagosome membrane via Gly_{120}. Error bars indicate standard deviations and *p*-values for paired, one-tailed Student's *t*-test statistics across all E:T ratios are shown. One of two experiments is shown. **(B)** MHC class I presentation assay with MP1-specific $CD8^+$ T-cell clone 9.2. The setup was the same as in **(A)**, except that MDAMC cells transfected with MP1, MP1-LC3 or MP1-LC3($G_{120}A$) were used to assess presentation on MHC class I. This control experiment shows that MHC class I presentation is not affected by the Atg/LC3 fusion. (From **ref. *10*** with permission from Elsevier.)

2. Materials

2.1. Cell Culture

1. Cell lines: HaCat keratinocyte cell line, a gift of Rajiv Khanna, Brisbane, Australia. MDAMC human breast carcinoma cell line, a gift of Irene Joab, Paris, France. (For choice of cell lines, *see* **Note 1**).
2. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS; Sigma), 2 m*M* glutamine, 110 µg/mL sodium pyruvate, and 2 µg/mL gentamicin (Gibco).
3. Sterile Dulbecco's phosphate-buffered saline (DPBS; Gibco) with 0.5 m*M* ethylenediamine tetraacetic acid (EDTA; Sigma, St. Louis, MO). To prepare, make 0.5 *M* EDTA stock, pH 8.0 in H_2O, filter through a 0.2-µm filter to sterilize, and dilute 1:1000 in sterile DPBS.
4. Solution of 0.05% trypsin/0.53 m*M* EDTA (Gibco).
5. Recombinant human interferon-γ (IFN-γ; ProSpec-Tany TechnoGene LTD, Rehovot Israel). Reconstitute in sterile H_2O + 0.1% human serum albumin (HSA, Sigma) to prepare a 2×10^6 U/mL stock and freeze in aliquots at −20ºC.

2.2. Expression of MP1-LC3 Fusion Construct by Transient Transfection

1. Mammalian expression vector encoding for LC3 fusion protein of influenza A matrix protein 1 (MP1, genebank entry X08088), as described in **ref. *10***. The construct was designed by cloning the human Atg8/LC3 cDNA sequence (genebank entry NM_022818) into the mammalian expression vector pEGFP-C2 (BD Biosciences, San Jose, CA) and subsequently replacing the EGFP coding sequence with the MP1 coding sequence (without a Stop codon at the 3´ end). As controls, mammalian expression vectors encoding for MP1 or MP1-LC3($G_{120}A$) ***(10)*** should be used in parallel with MP1-LC3. These constructs were designed the same way, except that the MP1 construct contains a Stop codon at the 3´ end of the MP1 sequence and the MP1-LC3($G_{120}A$) construct contains a point mutation at nucleotide 358 of the Atg8/LC3 sequence (A instead of G). For best transfection results, maxipreps of the different DNA plasmids should be prepared and DNA should be eluted in sterile DPBS. Determine DNA concentration and purity by OD_{260}/OD_{280} reading in a spectrophotometer. OD_{260}/OD_{280} ratio should be greater than 1.8. Store DNA in aliquots at −20ºC.
2. Cell culture medium (*see* **Subheading 2.3.1.**) without gentamicin (*see* **Note 2**).
3. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA).
4. OptiMEM-I medium (Gibco).

2.3. Immunocytochemistry and Confocal Microscopy to Analyze Localization of MP1-LC3 in MHC Class II Compartments

1. Circular 1.5-mm cover slips (Fisher, Pittsburgh, PA).
2. 70% (v/v) ethanol and sterile DPBS.

3. Recombinant human IFN-γ (*see* **Subheading 2.1.5.**).
4. Chloroquine (CQ, Sigma). Prepare a 20 m*M* stock in ddH_2O and sterilize through a 0.2-µm filter. Freeze in aliquots at –20ºC. Use at a 1:400 dilution.
5. Phosphate-buffered saline (PBS): Prepare 10x stock (1.37 *M*NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4, adjust pH to 7.4 with HCl) and autoclave before storage at room temperature. Prepare 1x PBS by dilution of one part with nine parts ddH_2O.
6. 4% Paraformaldehyde (PFA): Prepare a 4% (w/v) solution in PBS by heating PFA (Sigma) in PBS in a covered jar until PFA dissolves (careful, since PFA is toxic and vapors should not be inhaled). Let cool to room temperature for immediate use or store in aliquots at –20ºC. Thaw a fresh aliquot for each experiment.
7. Permeabilization solution: 0.1% (v/v) Triton-X 100 in PBS.
8. Blocking buffer: PBS + 1% bovine serum albumin (BSA, Sigma) + 5% normal donkey serum (NDS) + 0.1% saponin (Calbiochem, San Diego, CA) (*see* **Note 3**).
9. Wash buffer: PBS + 0.1% saponin.
10. Primary antibodies: Rabbit polyclonal MP1-specific antiserum ***(14)*** (use at 1:2000) and mouse monoclonal HLA-DR/DP/DQ-specific hybridoma IVA12 (ATCC, Manassas, VA). Grow hybridoma in RPMI-1640 with 10% FBS, 2 m*M* glutamine, 2 µg/mL gentamicin and harvest supernatant by spinning cells down at 300*g* for 10 min. Filter supernatant through a 0.2-µm filter and store at 4ºC. Use at a 1:10 dilution.
11. Secondary antibodies: Donkey anti-rabbit IgG-Alexa 488 (Invitrogen-Molecular Probes, Carlsbad, CA, use at 1:500) and donkey anti-mouse IgG-Rhodamine-Red™-X (Jackson ImmunoResearch, West Grove, PA, use at 1:300) (*see* **Note 4**).
12. DAPI nucleic acid stain: Prepare a 5 mg/mL stock of 4,6-diamidino-2-phenylindole (DAPI, Invitrogen-Molecular Probes) in ddH_2O and store in aliquots at −20ºC. Dilute 1:10,000 in PBS to prepare working solution and store at 4ºC wrapped in aluminium foil.
13. Mounting medium: Prolong Gold Antifade Reagent (Invitrogen-Molecular Probes).

2.4. MHC Class II Presentation Assay Using MP1-Specific $CD4^+$ T-Cell Clones

1. MP1-specific $CD4^+$ T-cell clones, generated as described in **ref. *15*** and cultured in RPMI-1640 with 8% pooled human serum (PHS, Mediatech Inc.), 450 U/mL recombinant human IL-2 (Chiron, Vacaville, CA), 2 m*M* glutamine, 2 µg/mL gentamicin in round-bottom 96-well plates (*see* **Note 5**).
2. Recombinant human IFN-γ (s*ee* **Subheading 2.1., step 5.**).
3. RPMI-1640.
4. Coculture medium: RPMI-1640 with 5% PHS, 2 m*M* glutamine, 2 µg/mL gentamicin.

5. Positive control stimulus: Specific MP1 peptide (1 m*M* stock in 10% DMSO, for T-cell stimulation dilute 1:1000 in coculture medium) or phytohemagglutinin (PHA-L, Sigma, 1 mg/mL stock, for T-cell stimulation dilute 1:1000 in coculture medium).

2.5. IFN-γ ELISA to Analyze Secretion of IFN-γ by MP1-Specific $CD4^+$ T-Cell Clones

1. High-protein-binding 96-well ELISA plate (e.g., Maxisorp from Nunc, Rochester, NY).
2. ELISA kit for human IFN-γ (Mabtech, Nacka Strand, Sweden). Prepare a 10 μg/mL stock of human recombinant IFN-γ provided with kit and freeze in aliquots at -20ºC. Use a freshly thawed aliquot for each experiment.
3. PBS (*see* **Subheading 2.3.**).
4. Coculture medium (*see* **Subheading 2.4.**).
5. Blocking buffer: PBS + 1% BSA (Sigma).
6. Wash buffer: PBS + 0.05% Tween-20.
7. Incubation buffer: PBS + 0.1% BSA + 0.05% Tween-20.
8. TMB peroxidase substrate solution (Sigma) and Stop solution (1 *N* sulfuric acid).

3. Methods

3.1. Cell Culture

1. HaCat and MDAMC cell lines are maintained in DMEM + 10% FBS medium in 75-cm^2 tissue culture flasks or 100-mm plates until they approach confluence.
2. To split cells, wash monolayer once with 5 mL of DPBS/0.5 m*M* EDTA and incubate with 2 mL of trypin/EDTA solution at 37ºC for 2–3 min (MDAMC) or 10–15 min (HaCat) to detach cells. To set up new maintenance cultures, replate 1/20 of cells and add fresh culture medium. These cultures will approach confluence after 2–3 d.
3. To induce expression of MHC class II machinery, cells have to be cultured with 200 U/mL IFN-γ for 24 h (for HaCat) or 36 h (for MDAMC). At least 50% of cells then will express MHC class II on their cell surface.

3.2. Expression of MP1-LC3 Fusion Construct by Transient Transfection

1. To set cells up for transfection, detach cells as described in **Subheading 3.1.2.** and plate onto a 6-well tissue culture plate in antibiotic-free culture medium at a density of 2×10^5 cells/well (*see* **Note 2**).
2. The next day, cultures should be about 70–80% confluent. For each well to be transfected, dilute 2.5 μg plasmid DNA in 250 μL OptiMEM-I medium and mix by vortexing briefly. In a separate tube, dilute 7.5 μL lipofectamine 2000 in

250 µL OptiMEM-I medium and vortex briefly. Incubate both tubes for 5 min at room temperature.
3. Combine both solutions, vortex briefly and incubate 20 min at room temperature to allow formation of DNA–lipofectamine complexes.
4. Add complexes in a dropwise manner to the culture medium of cells in the 6-well plate and incubate at 37ºC for 4–6 h.
5. After 4–6 h, replace the complex-containing medium with fresh culture medium and culture cells for 18–20 h at 37ºC (*see* **Note 6**).
6. Twenty-four hours posttransfection, 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.**).

3.3. Immunocytochemistry and Confocal Microscopy to Analyze Localization of MP1-LC3 in MHC Class II Compartments

1. Place round 1.5-mm microscopy cover slips into 24-well tissue culture plate. Use eight extra cover slips for control stainings (s*ee* **Note 7**).
2. Sterilize cover slips by washing once with 70% ethanol and twice with sterile DPBS. Remove any traces of ethanol by completely aspirating off ethanol and wash solutions with vacuum suction flask.
3. Trypsinize cells transfected with different MP1 constructs (*see* **Subheading 3.2.**) and plate onto sterilized cover slips in cell culture medium, at a density of 5×10^4 cells/well. Plate two wells of each sample, so that cells can be analyzed with and without chloroquine treatment (*see* **Note 8**).
4. Treat cells with 200 U/mL IFN-γ for 24 h (HaCat cells) or 36 h (MDAMC cells) to induce expression of MHC class II molecules.
5. During the last 6 h of the culture, treat one set of cells with 50 µ*M* chloroquine (CQ), to prevent degradation of MP1-LC3 by lysosomal proteases. Leave the other set of cells untreated (*see* **Note 8**).
6. Wash cells once in PBS (0.5 mL/well) and fix in 4% paraformaldehyde (PFA, 200 µL/well) for 15 min at room temperature (*see* **Note 9**).
7. Wash cells once in PBS (0.5 mL/well) and permeabilize in 0.1% Triton X-100 (200 µL/well) for 5 min.
8. Wash cells once in PBS (0.5 mL/well) and add blocking buffer (200 µL/well) for 30 min.
9. Dilute primary antibodies in blocking buffer and add to cells (200 µL/well) for 30 min at room temperature or for longer periods (up to overnight) at 4ºC.
10. Wash cells three times in wash buffer (0.5 mL/well), incubate for 5 min each time.
11. Dilute secondary antibodies in blocking buffer and add to cells (200 µL/well) for 30 min.
12. Aspirate secondary antibody solutions and add DAPI nucleic acid stain for 20–30 sec (200 µL/well). Afterwards, immediately wash cells as in **step 9**.

13. Wash cells once in PBS and mount cover slips by inverting them onto a drop of mounting medium on a microscope slide, up to four cover slips per slide. Carefully press down on cover slip, aspirate excess mounting medium and let dry at room temperature (*see* **Note 10**). Afterwards, slides can be stored in the dark at 4ºC for several months.
14. Analyze slides with a confocal laser scanning microscope, using a high N.A. oil immersion lens (e.g., 63x/1.4 N.A.). Excitation at 405 nm induces DAPI fluorescence (blue emission), excitation at 488 nm induces Alexa 488 fluorescence (green emission), and excitation at 543 nm induces Rhodamine-Red^{TM}-X fluorescence (red emission). Software can be used to overlay the different fluorescence channels and to quantify colocalization (*see* **Note 11**). An experiment with MP1-LC3 and MP1-expressing MDAMC cells is shown as an example in **Fig. 1**.

3.4. MHC Class II Presentation Assay Using MP1-Specific CD4$^+$ T-Cell Clones

1. For MHC class II presentation assay, use HaCat cells transfected with different MP1 constructs in a 6-well format, as described in **Subheading 3.2**. Treat cells with 200 U/mL IFN-γ for 24 h to initiate expression of the MHC class II machinery.
2. Remove any traces of IFN-γ from cells by washing cell monolayers three times in RPMI-1640 medium. Trypsinize cells to prepare a cell suspension, wash once in coculture medium and count with hemacytometer. Prepare cell suspensions in coculture medium at three different cell concentrations (2×10^5, 10^5, and 6.67×10^4 cells/mL) (*see* **Note 12**).
3. Collect MP1-specific CD4$^+$ T-cell clones from 96-well culture plates, wash once in coculture medium and count. Adjust cell concentration to 2×10^6 cells/mL.
4. Set up cocultures of T cells and target cells in doublets (2 wells/condition) in a 96-well round-bottom plate. Per well, plate 50 µL of T-cell suspension (10^5 cells/well) and 100 µL of the different target cell suspensions (either 2×10^4, 10^4, or 6.67×10^3 cells/well). This will result in effector to target (E:T) ratios of 5, 10 and 15. As a positive control, stimulate T-cell clones with specific MP1 peptide (1 µ*M*) or PHA-L (1 µg/mL). As negative control, stimulate T-cell clones with coculture medium only.
5. Culture cells overnight (18–24 h) at 37ºC.

3.5. IFN-γ ELISA to Analyze Secretion of IFN-γ by MP1-Specific CD4$^+$ T-Cell Clones

1. One day prior to ELISA, coat high-protein-binding ELISA plate with primary anti-IFN-γ antibody (1-D1K, included in IFN-γ ELISA kit), diluted 1:500 in PBS, 100 µL/well. Incubate plate overnight at 4ºC.
2. The next day, wash plate two times with PBS (200 µL/well) and block with blocking buffer (200 µL/well) for 1 h at room temperature.

3. Thaw an aliquot of IFN-γ standard (10 μg/mL) and prepare serial dilutions in coculture medium (prepare 2000, 1000, 500, 250, 125, 62.5, 31.25, 0 pg/mL standards, at least 300 μL each).
4. To make sure that IFN-γ secreted by T cells is homogeneously distributed in culture supernatants, mix supernatants by pipetting up and down with a multichannel pipet and pellet cells by centrifugation of plates at 300*g* for 5 min.
5. With a multichannel pipet, carefully remove 120 μL of supernatant from each well and transfer to a new 96-well plate.
6. Wash ELISA plate four times with wash buffer.
7. Add supernatants or IFN-γ standards (100 μL/well) and incubate for 2 h at room temperature. Freeze remaining 20 μL of supernatant at –20ºC in case ELISA has to be repeated on diluted supernatants (*see* **Note 13**).
8. Wash as in **step 6** and add 100 μL/well of secondary antibody (7-B6-1-biotin, provided in ELISA kit), diluted 1:1000 in incubation buffer. Incubate one hour at room temperature.
9. Wash as in **step 6** and add 100 μL/well of streptavidin-HRP (provided in ELISA kit), diluted 1:1000 in incubation buffer. Incubate one hour at room temperature.
10. Wash as in **step 6** and add 100 μL/well of TMB peroxidase substrate. Incubate until blue reaction product has sufficiently developed, then stop reaction by adding 100 μL/well of Stop solution.
11. Measure optical density at 450 nm (OD_{450}) in an ELISA plate reader and convert OD_{450} values into IFN-γconcentration in pg/mL (*see* **Note 13**). An example of the results produced is shown in **Fig. 2**.

4. Notes

1. Cell lines were chosen based on their ability to upregulate MHC class II expression upon IFN-γ treatment ***(10)*** and their HLA haplotype. HaCat is HLA-DRB1*15, -DRB1*04, -DQBI*03 and -DQB1*03 positive, and therefore could be used in MHC class II presentation assays with MP1-specific $CD4^+$ T-cell clones from a healthy lab donor with the following MHC haplotype: HLA-A*0201, -A*6801, -B*4402, -B*0702, -C*0501, -C*0702, -DRB1*1501, -DRB1*0401, -DRB5*01, -DRB4*01, -DQBI*0602 and -DQB1*0301. MDAMC is HLA-A*02-positive and therefore could be used in MHC class I presentation assays with HLA-A2-restricted MP1-specific $CD8^+$ T-cell clones derived from the same donor. If other cell lines have to be used, determine MHC class II expression/upregulation upon IFN-γ treatment and compare their HLA haplotype with that of T-cell donors.
2. For transfection with lipofectamine 2000, cell culture medium should not contain any antibiotics. Therefore, omit gentamicin.
3. Normal donkey serum is used as blocking reagent because secondary antibodies are derived from donkey. If secondary antibodies come from a different species, use 5% normal serum from that species as blocking reagent.

4. For choice of fluorophores, consider which lasers and filter sets are available for your confocal microscope. For colocalization analysis it is important that emission spectra of the green and red fluorochromes do not overlap. Also consider brightness and photostability of fluorophores.
5. For generation of MP1-specific $CD4^+$ T-cell clones, $CD14^-$cells isolated from a healthy lab donor were stimulated with autologous mature DCs that were electroporated with 10 µg of in vitro transcribed MP1-RNA. On day 8, the stimulation was repeated and 10 U/mL IL-2 were added to enhance T-cell survival. On day 21, the surviving cells were cloned by limiting dilution at 10, 1, or 0.3 cells/well and expanded in RPMI-1640 + 8% PHS + 150 U/mL rhIL-2 (Chiron) + 1 µg/mL PHA-L (Sigma). 10^5 irradiated PBMCs/well and 10^4 irradiated LCLs/well were added as feeder cells. On day 40, expanded cells were tested in split-well IFN-γ ELISPOT assays for recognition of an MP1 peptide mix. T-cell clones can be frozen and stored for several years in liquid nitrogen (about 10^6 cells/aliquot). Frozen cultures can be reexpanded as described above.
6. Removal of complex-containing medium 4–6 h after transfection was found to improve viability of MDAMC and HaCat cells lines.
7. For correct interpretation of results, the following control stainings should be included:
    a) Replace primary and secondary antibodies with blocking buffer. These stainings should be completely negative.
    b) Replace primary antibody with blocking buffer, but use secondary antibodies. Background from secondary antibodies should be low. If background turns out to be too high, titer down concentration of secondary antibodies.
    c) Use untransfected cells and cells that were not treated with IFN-γ Stainings should be negative.
    d) Do single-labeling of cells and check signal in the "wrong channel" (red channel for Alexa488 labeling and green channel for Rhodamine-Red X-labeling). There should be no bleed-through into the wrong channel.
    e) Do single-labeling with "wrong" secondary antibodies. There should be no cross-reactivity with the wrong species.
8. Chloroquine inhibits lysosomal acidification and thus prevents degradation of lysosomal substrates, including LC3 fusion proteins (*see* **ref.** ***10***). Without inhibition of lysosomal proteases, MP1-LC3 is rapidly degraded and its detection in lysosomal compartments is more difficult. However, when cells are treated with chloroquine, the fusion protein accumulates in lysosomal compartments/MIICs and now can be visualized much more readily (*see* **Fig. 1** and **ref.** ***10***). Therefore, it is recommended that one set of cells be treated with chloroquine for 6 h prior to the staining.
9. From the fixation step onwards, cells can be handled outside sterile hood on a laboratory bench. Use a vacuum suction flask to change solutions, exchange plastic tip of suction device when handling different solutions (e.g., antibodies). All incubation steps are done at room temperature, unless noted otherwise.

10. Prolong Gold antifade mounting medium (Invitrogen-Molecular Probes) should be allowed to dry at room temperature overnight. During this time, the mounting medium will gel 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.
11. 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 deconvolved using deconvolution software.
12. The optimal number of target cells and T-cell clones 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.
13. In case IFN-γ levels in supernatants exceed the linear range of the ELISA (approximately 20–1000 pg/mL), dilute frozen supernatants 1:10 in coculture medium and repeat ELISA.

Acknowledgments

We thank Rajiv Khanna and Irene Joab for the gift of cell lines and Ari Helenius for the gift of the MP1-specific antiserum. This work was supported by the Arnold and Mabel Beckman Foundation, the Alexandrine and Alexander Sinsheimer Foundation, the Burroughs Wellcome Fund, the National Cancer Institute (R01CA108609 and R01CA101741) (to C.M.), and in part by a General Clinical Research Center grant (M01-RR00102) from the National Center for Research Resources at the National Institutes of Health (to the Rockefeller University Hospital). D.S. is a recipient of a predoctoral fellowship from the Schering Foundation.

References

1. Chapman, H. A. (2006) Endosomal proteases in antigen presentation. *Curr. Opin. Immunol.* **18,** 78–84.
2. Bryant, P., and Ploegh H. (2004) Class II MHC peptide loading by the professionals. *Curr. Opin. Immunol.* **16,** 96–102.
3. Bevan, M. J. (2004) Helping the $CD8^+$ T-cell response. *Nat. Rev. Immunol.* **4,** 595–602.
4. Schmid, D., Dengjel, J., Schoor, O., Stevanovic, S., and Münz. C. (2006) Autophagy in innate and adaptive immunity against intracellular pathogens. *J. Mol. Med.* **84,** 194–202.
5. Münz, C. (2006) Autophagy and antigen presentation. *Cell Microbiol.* **8,** 891–898.

6. Brazil, M. I., Weiss, S., and Stockinger, B. (1997) Excessive degradation of intracellular protein in macrophages prevents presentation in the context of major histocompatibility complex class II molecules. *Eur. J. Immunol.* **27,** 1506–1514.
7. Dorfel, D., Appel, S., Grunebach, F., Weck, M. M., Muller, M. R., Heine, A., and Brossart, P. (2005) Processing and presentation of HLA class I and II epitopes by dendritic cells after transfection with in vitro transcribed MUC1 RNA. *Blood* **105,** 3199–3205.
8. Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E. M., Pardoll, D. M., Bornkamm, G. W., and Mautner, J. (2003) Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur. J. Immunol.* **33,** 1250–1259.
9. Paludan, C., Schmid, D., Landthaler, M., Vockerodt, M., Kube, D., Tuschl, T., and Münz, C. (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* **307,** 593–596.
10. Schmid, D., Pypaert, M., and Münz, C. (2007) MHC class II antigen loading compartments continuously receive input from autophagosomes. *Immunity* **26,** 79–92.
11. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *Embo J.* **19,** 5720–5728.
12. Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. (2004) In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol. Biol. Cell* **15,** 1101–1111.
13. Dengjel, J., Schoor, O., Fischer, R., Reich, M., Kraus, M., Muller, M., Kreymborg, K., Altenberend, F., Brandenburg, J., Kalbacher, H., Brock, R. R., Driessen, C., Rammensee, H. G., and Stevanovic, S. (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc. Natl. Acad. Sci. USA* **102,** 7922–7927.
14. Martin, K., and Helenius, A. (1991) Transport of incoming influenza virus nucleocapsids into the nucleus. *J. Virol.* **65,** 232–244.
15. Fonteneau, J. F., Larsson, M., Somersan, S., Sanders, C., Münz, C., Kwok, W. W., Bhardwaj, N., and Jotereau, F. (2001) Generation of high quantities of viral and tumor-specific human $CD4^+$ and $CD8^+$ T-cell clones using peptide pulsed mature dendritic cells. *J. Immunol. Methods* **258,** 111–126.