

Reprint Compendium

Bio-protocol Selections 2022

Immunology

Foreword

We are pleased to launch the 2022 *Bio-protocol* series of reprint collections, comprising some of the most used protocols published in 2021 in several research areas. This collection focuses on Immunology.

Established in 2011 by a group of Stanford scientists, Bio-protocol aims to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. *Bio-protocol* invites contributions from authors who have published methods in brief, as part of other research articles, and who might want to provide more detailed versions to facilitate use by others.

A survey carried out in 2018 showed that, of more than 2300 users who had followed a protocol published in *Bio-protocol*, 91% (2166 users) were able to successfully reproduce the method they tried.

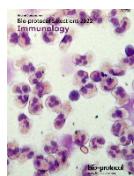
In this reprint collection, we have selected 17 of the most viewed, downloaded, and cited research protocols related to Immunology that were published in *Bio-protocol* in 2021.

Hopefully, you will find this collection intriguing and visit <http://www.bio-protocol.org> to check out the entire archive of protocols. Please feel free to email us (eb@bio-protocol.org) with feedback, and please consider contributing a protocol to Bio-protocol in the future.

The Bio-protocol Editorial Team

1. Isolation of Microvesicles from Human Circulating Neutrophils 1
Zhan, D. *et al.* 【Original Research Article: Int J Mol Sci, 22(13):7086】
2. *In vitro* STING Activation with the cGAMP-STING Δ TM Signaling Complex 9
He, Y. *et al.* 【Original Research Article: Sci Adv, 6(24):eaba7589】
3. Murine Monocyte and Macrophage Culture 20
Haag, S. and Murthy, A. 【Original Research Article: Elife, 8:e44452】
4. Liposomal Clodronate-mediated Macrophage Depletion in the Zebrafish Model 27
Yang, L. *et al.* 【Original Research Article: Elife, 9:e58191】
5. A Potent Vaccine Delivery System 37
Zhao, G. *et al.* 【Original Research Article: Sci Adv, 6(5):eaax2285】
6. *Ex vivo* Assessment of Mitochondrial Function in Human Peripheral Blood Mononuclear Cells Using XF Analyzer 49
Schöller-Mann, A. *et al.* 【Original Research Article: Sci Rep, 11(1):19905】
7. Analysis of Monocyte Cell Fate by Adoptive Transfer in a Murine Model of TLR7-induced Systemic Inflammation 59
Gamrekelashvili, J. *et al.* 【Original Research Article: Elife, 9:e57007】
8. Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer 71
Almeida, A. S. *et al.* 【Original Research Article: J Exp Med, 217(10):e20181551】
9. *In vitro* and *In vivo* CD8 $^{+}$ T Cell Suppression Assays 83
Xie *et al.* 【Original Research Article: BMC Immunol, 21(1):23】
10. Production of the Receptor-binding Domain of the Viral Spike Proteins from 2003 and 2019 SARS CoVs and the Four Common Human Coronaviruses for Serologic Assays and Inhibitor Screening 92
Segovia-Chumbez *et al.* 【Original Research Article: Sci Immunol, 5(48):eabc8413】
11. *In vivo* CD40 Silencing by siRNA Infusion in Rodents and Evaluation by Kidney Immunostaining 100
Hueso *et al.* 【Original Research Article: PLoS One, 8(6):e65068】
12. Isolation of Microglia and Analysis of Protein Expression by Flow Cytometry: Avoiding the Pitfall of Microglia Background Autofluorescence 127
Burns, J. C. *et al.* 【Original Research Article: Elife, 9:e57495】
13. One-step White Blood Cell Extracellular Staining Method for Flow Cytometry 142
Belkacem, I. A. *et al.* 【Original Research Article: Cytometry B Clin Cytom, 96(5):426-435.】

14.	Identification and Quantitation of Neutrophil Extracellular Traps in Human Tissue Sections	150
	Radermecker, C. <i>et al.</i> 【Original Research Article: J Exp Med, 217(12):e20201012】	
15.	Intact <i>in situ</i> Preparation of the <i>Drosophila melanogaster</i> Lymph Gland for a Comprehensive Analysis of Larval Hematopoiesis	161
	Rodrigues, D. <i>et al.</i> 【Original Research Article: Elife, 10:e61409】	
16.	Suppression of Human Dendritic Cells by Regulatory T Cells	177
	Huang, Q. <i>et al.</i> 【Original Research Article: Sci Transl Med, 12(557):eaaz3866.】	
17.	Production of Recombinant Replication-defective Lentiviruses Bearing the SARS-CoV or SARS-CoV-2 Attachment Spike Glycoprotein and Their Application in Receptor Tropism and Neutralisation Assays	189
	Thakur, N. <i>et al.</i> 【Original Research Article: PLoS Biol, 18(12):e3001016】	



On the Cover:

Image from protocol “**Isolation of Microvesicles from Human Circulating Neutrophils**”

Isolation of Microvesicles from Human Circulating Neutrophils

Dong Zhan^{1, #}, Ellie McConachie^{2, #}, Steven W. Edwards³, Helen L. Wright³, Robert J. Moots⁴ and Sittisak Honsawek^{1, *}

¹Department of Biochemistry, Osteoarthritis and Musculoskeleton Research Unit, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand

²School of Life Sciences, University of Liverpool, Liverpool, United Kingdom

³Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom

⁴Institute of Ageing and Chronic Disease, University Hospital Aintree, University of Liverpool, Liverpool, United Kingdom

#Contributed equally to this work

*For correspondence: sittisak.h@chula.ac.th

Abstract

Neutrophil-derived microvesicles (NDMVs) are liberated by neutrophils upon cell activation by molecules. Once activated, neutrophils are primarily involved in acute inflammation; however, the microvesicles they produce are largely anti-inflammatory. NDMVs have been shown to protect cartilage during inflammatory arthritis. They exert these effects by inhibiting or affecting the function of target cells, including macrophages. NDMVs have the potential to act as disease-modifying agents, especially for inflammatory diseases. This protocol describes a method using differential centrifugation to separate neutrophils from whole human blood. Subsequently, neutrophils are identified by cytopsin and Wright's staining, and then the NDMVs are isolated using differential centrifugation.

Keywords: Microvesicles, Neutrophils, Isolation, Differential centrifugation, Cytopsin

This protocol was validated in: Int J Mol Sci (2021), DOI: 10.3390/ijms22137086

Background

Microvesicles (MVs) are a type of extracellular vesicle that can be between 100 to 1,000 nm in size (Leblanc *et al.*, 2017; Zhan *et al.*, 2021). They are produced by exocytic budding from the plasma membrane and can be shed from diverse cell types as a result of events such as cell activation by stimuli or apoptosis (Lima *et al.*, 2011). Stimuli activating these events increase intracellular calcium, leading to cytoskeletal rearrangement and eventually microvesicle budding (Distler *et al.*, 2005). MVs can carry specific sets of secreted molecules between cells, such as functional proteins, lipids, and nucleic acids including mRNAs and microRNAs, making them an important mode of intercellular communication (Leblanc *et al.*, 2017). Through horizontal transfer of these molecules, MVs have the ability to transcriptionally regulate target cells (Greening *et al.*, 2017). This ability, coupled with their association with a multitude of different diseases, including rheumatoid arthritis, vasculitis, and various cancers, makes them interesting targets for disease modification. MVs also exhibit superior potential as non-invasive biomarkers for certain diseases, as they are detectable in all biological fluids, including blood (both serum and plasma), urine, and saliva (Leblanc *et al.*, 2017). Moreover, surface proteins differ based on the type of cell they are shed from (Lima *et al.*, 2011), and both the inducer and severity of disease can alter what set of molecules MVs carry (Ridger *et al.*, 2017).

Neutrophil-derived microvesicles (NDMVs) are liberated by neutrophils upon cell activation by molecules. Activators include N-formyl peptides, which are proteins produced by bacteria or eukaryotic mitochondria containing N-formylmethionine, and tumor necrosis factor- α (TNF- α) (Mantovani *et al.*, 2011). When stimulated, neutrophils play a crucial role in acute inflammation; however, the microvesicles they generate are essentially anti-inflammatory (Trontorsak *et al.*, 2018; Zhan *et al.*, 2021). In fact, neutrophils are part of a small group of cells whose MVs are known to promote tissue regeneration and, in some cases, repair (Rhys *et al.*, 2018). For example, polymorphonuclear neutrophil-derived MVs (NDMVs) can protect cartilage during inflammatory arthritis (Headland *et al.*, 2015; Topping *et al.*, 2017). They exert these effects by inhibiting or affecting the function of target cells, including macrophages and fibroblast-like synoviocytes, which can be modified by NDMVs to display a more anti-inflammatory phenotype (Rhys *et al.*, 2018; Zhan *et al.*, 2021). These characteristics suggest NMDVs have potential disease-modifying effects, especially for inflammatory diseases.

In this protocol, we describe a method using differential centrifugation to separate polymorphonuclear neutrophils from whole blood. TNF- α is added to activate these neutrophils and stimulate MV release. Finally, the MVs are isolated using differential centrifugation, and their size distribution and concentration are characterized using Nanoparticle Tracking Analysis (NTA).

Materials and Reagents

1. Safety blood collection set with Luer adapter (Greiner Bio-One, Vacuette®, catalog number: 450081)
2. 4 mL NH sodium heparin blood collection tubes (Greiner Bio-One, Vacuette®, catalog number: 454051)
3. 50 mL polypropylene centrifuge tube (Labcon, PerformR™, catalog number: 3186-345)
4. 15 mL polypropylene centrifuge tubes (Labcon, PerformR™, catalog number: 3136-345)
5. 1.5 mL polypropylene microcentrifuge tubes (Labcon, SuperSpin®, catalog number: 3016-870)
6. Ultracentrifuge tube (Beckman Coulter, Quick-Seal Polypropylene Tube, bell-top, catalog number: 365470)
7. Foil
8. 1 mL syringe (Nipro, catalog number: JD+01D2238)
9. Syringe filter (Whatman, Anotop 10 syringe filter, pore size 0.02 μ m, catalog number: Z747637)
10. ddH₂O (purified by Thermo Fisher Scientific Water Purification System)
11. Whole human blood collected in accordance with institutional biosafety and ethics guidelines
12. Polymorphprep™ (Axis-Shield, catalog number: 1114683)
13. RPMI 1640 (Gibco, catalog number: 11530586)
14. Red Blood Cell Lysis Buffer (Roche, catalog number: 11 814 389 001)
15. Human AB serum (Thermo Fisher Scientific, Coring Human AB serum 100 mL, catalog number: MT35060CI)
16. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: 60004)

17. Glass slide (Tharmac, catalog number: JC311-100)
18. Filter cards (Tharmac, catalog number: JC305-200)
19. Cytospin funnel (Tharmac, Single Cellfunnel, catalog number: JC304-12)
20. Cytoclip (Tharmac, Accessories for Cellspin, catalog number: JC302-1)
21. Wright's Solution (Merck, Wright's eosin methylene blue solution, catalog number: 101383)
22. Recombinant Human Tumor Necrosis Factor- α (rhTNF- α) (Biologics, catalog number: 717904)
23. NaCl (Vivantis, catalog number: PC0914-1kg)
24. KH₂PO₄ (Sigma-Aldrich, catalog number: 7758-11-4)
25. KCl (Sigma-Aldrich, catalog number: 7447-40-7)
26. Na₂HPO₄ (Sigma-Aldrich, catalog number: 7558-79-4)
27. HCl (VWR International, catalog number: 7647-01-0)
28. Phosphate buffer saline (PBS) (see Recipes)

Equipment

1. Water Purification System (Thermo Scientific, Barnstead Pacific RO, catalog number: 50132389)
2. Pipettes: 0.5-10 μ L, 10-100 μ L, and 100-1,000 μ L (Eppendorf, Eppendorf Research plus, catalog numbers: 3120000020, 3120000046, 3120000060)
3. Balance (Fisher Scientific, model: SartoriusTM MSE125P100DU)
4. Universal 320R centrifuge equipped with a swing-out rotor (6-place) and fixed angle rotor (24-place) (Hettich, model: UNIVERSAL 320R), Centrifuge Rotor (Hettich, model: Rotor 1619); Centrifuge Accessory (Hettich, model: accessory 1462-A); Centrifuge Rotor (Hettich, model: Rotor 1420B)
5. Ultracentrifuge equipped with type 100Ti fixed-angle rotor (Beckman Coulter, model: OptimaTM XPN-100), Ultracentrifuge rotor (Beckman Coulter, model: Type 100Ti Fixed angle rotor, catalog number: 363013)
6. Water bath equipment (Memmert, Waterbath WNB 22)
7. Hemocytometer (Hausser Scientific, Bright-Line Hemacytometer, catalog number: 3120)
8. Cytospin device (Tharmac, Cellspin I)
9. Light Microscope (Nikon, Upright Microscope Eclip 50i)
10. NanoSight NS300 (Malvern Panalytical, O-ring top-plate, 405 nm laser)
11. Autoclave (Tomy, catalog number: LSX-500)
12. Refrigerator (4°C) (Samsung, 21 cu. ft. Capacity Top Freezer Refrigerator with FlexZone)
13. Refrigerator (-80°C) (Thermo, standard performance ultra-low freezers)

Software

1. Nanosight NTA 3.1 Software Build 3.1.54 (Malvern Panalytical)

Procedure

A. Isolation of neutrophils from whole blood

- A. Collect approximately 40 mL of whole human blood into 10 \times 4 mL tubes with heparin anticoagulant by phlebotomy in accordance with institutional biosafety and ethics guidelines.
- B. Add 6 mL of PolymorphprepTM to 6 \times 15-mL centrifuge tubes.
- C. Add 6 mL of whole human blood into these tubes (Polymorphprep:white blood v/v= 1:1). Be sure to slowly add so the blood sits on top of the PolymorphprepTM and the two layers do not mix.

- D. Centrifuge at $500 \times g$ (Hettich 320R) for 35 min (room temperature) to separate the layers (Figure 1).

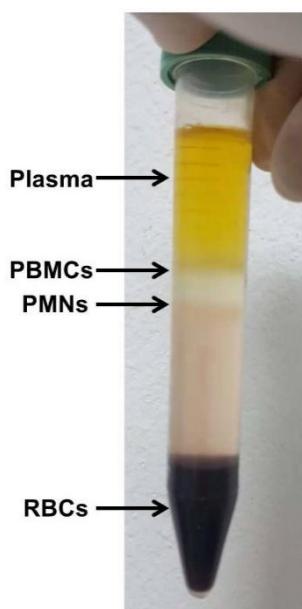


Figure 1. Separated layers from bottom to top.

Red blood cells (RBCs), polymorphonuclear layer (PMNs), peripheral blood mononuclear cells (PBMCs), plasma. Polymorphprep™ is used to isolate a pure PMN layer.

- E. Remove plasma and peripheral blood mononuclear cell layers from solution using a 1,000- μL pipette.
- F. Collect the lower PMN layer and place into $3 \times 15\text{-mL}$ centrifuge tubes. Avoid the red blood cell layer at the bottom.
- G. Add 1-2 mL RPMI 1640 to each tube.

Note: Add some extra RPMI 1640 as needed to equalize the volumes in all tubes. This will ensure the centrifuge is balanced.

- H. Centrifuge at $500 \times g$ (Hettich 320R) for 5 min (room temperature) to wash the PMN layer and remove any residual Polymorphprep™.
- I. Remove supernatant and add 9 mL of Red Blood Cell Lysis Buffer along with 1 mL of RPMI 1640 (v/v ratio 9:1) to resuspend pellet. The lysis buffer will remove any contaminating erythrocytes (RBCs) by hypotonic cell lysis. Incubate at room temperature for 3 min.
- J. Centrifuge at $400 \times g$ (Hettich 320R) for 3 min (room temperature). The pellet produced will contain neutrophils.
- K. Remove supernatant and resuspend the neutrophil pellet in 1 mL of RPMI 1640. Count the number of cells using a hemocytometer.
- L. Adjust neutrophil concentration to 20×10^6 cells/mL by adding the appropriate amount of RPMI 1640 (approximately 200-600 μL).

B. Microscopic observation of isolated neutrophils: cytopsin and Wright's staining

1. Add 5 μL of neutrophil solution containing 10^5 neutrophils from Step A12 into 200 μL of PBS with 10 mM EDTA and pipette mixture gently.
2. Align and assemble cytopsin funnel, filters card, glass slide, and cytoclip, then put them into cytopsin slots of cytopsin device.

3. Transfer all neutrophil mixture into cytopsin funnel rapidly and centrifuge at $32 \times g$ for 5 min at room temperature.
4. Unlock cytoclip and remove cytopsin funnel and filter card from glass slides to avoid touching the cell smear.
5. Air-dry the glass slides for 2 min.
6. Add 1 mL of Wright's solution to each glass slide and incubate for 3 min at room temperature.
7. Add 1 mL of purified water to the Wright's solution on each glass slide and incubate for 5 min.
8. Rinse glass slides with ddH₂O for 1 min and repeat this step twice.
9. Observe the glass slides with a light microscope and count three fields of view (approximately 300-400 cells in one view). A representative image is shown in Figure 2.

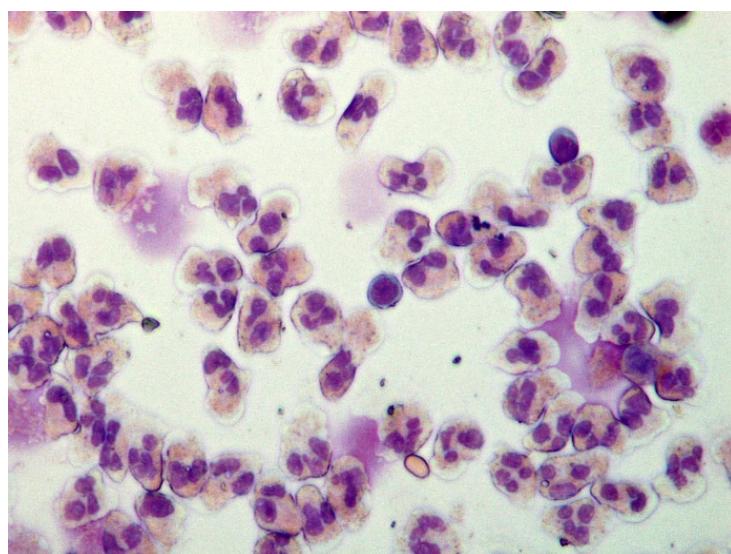


Figure 2. Isolated neutrophils stained with Wright's solution and visualized under a light microscopy.
Neutrophil nuclei stained with methylene blue are subdivided into 2-5 lobes, and their cytoplasms contain fine granules stained by eosin red. The purity of neutrophils isolated by Polymorphprep™ is approximately 95%-99%. 100× magnification.

C. Microvesicle release from treated neutrophils

1. Supplement the neutrophil solutions from Step A12 with human AB serum (approximately 50-150 µL) to be 5% of the volume.
2. Add the appropriate volume of rhTNF-α (400-600 µL) to achieve a final concentration of 50 ng/mL to activate neutrophils, promoting microvesicle release.
3. Incubate in a water bath at 37°C for 20 min to simulate body temperature and roll the tube gently and periodically. This prevents the neutrophils from dying.

D. Isolation of microvesicles

1. Move the solution from Step C3 to two freshly sterilized 1.5 mL microcentrifuge tubes.
2. Centrifuge at $4,400 \times g$ (Hettich 320R) for 15 min at 4°C to remove any large cell bodies.
3. Collect the supernatant, which contains the microvesicles, carefully to not disturb the pellet and transfer to two freshly sterilized 1.5 mL microcentrifuge tubes.
4. Centrifuge at $13,000 \times g$ (Hettich 320R) for 2 min at 4°C to remove platelets.
5. Collect supernatant and transfer into two freshly sterilized 1.5 mL microcentrifuge tubes.
6. Place the supernatant into an ultracentrifuge tube that is sealed to prevent evaporation of the solution.

Note: This will be ultracentrifuged; however, only one tube will be used, so a balance tube is needed. We used an equivalent tube weighing 5.76 g. To balance the tubes, add RPMI 1640 until the tube containing supernatant is of equal weight to your balance tube.

7. Ultracentrifuge at $100,000 \times g$ (Optima XPN-100) for 60 min at 4°C . The pellet produced will contain neutrophil-derived microvesicles.
8. Carefully remove the supernatant (Figure 3) and resuspend the pellet in 200 μL of PBS. Transfer the pellet to a freshly sterilized 1.5 mL microcentrifuge tube and cover it with foil to keep it dark.

Note: Freshly isolated microvesicles can be used immediately to test microvesicle uptake by other cells. Microvesicles stored at -80°C should be used within one month for analysis of protein, RNA, lipid, or other component contents.

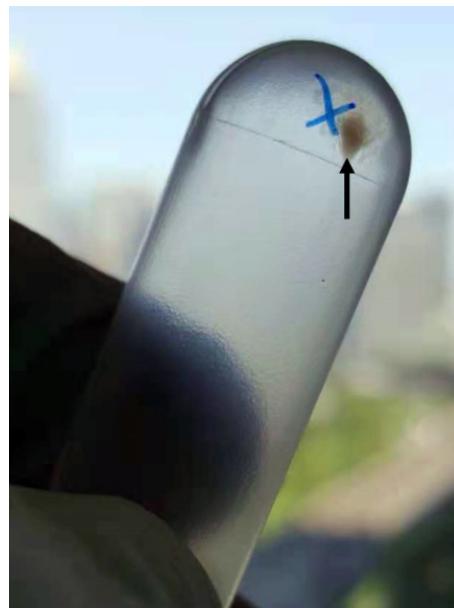


Figure 3. Isolated neutrophil-derived microvesicles (NDMVs) at the bottom of an ultracentrifugation tube.

The black arrow indicates the white microvesicle pellet.

E. Nanoparticle Tracking Analysis (NTA)

1. Set up NanoSight NS300 hardware and software using the following parameters: 60 s length videos for 5 times with a highly sensitive camera level, 15 at 25°C , detective threshold 3, “water” viscosity, “automatic” blur size, and “automatic” max jump distance.
2. Dilute fresh NDMVs from Step D8 $1,000\times$ in particle-free PBS to achieve a density range of $10^6\text{-}10^9$ particles/mL.
3. Aspirate the diluted NDMV solution into a 1 mL syringe, avoiding air bubbles.
4. Insert the syringe bore into the inlet port of the O-ring top-plate of the laser module and slowly inject 1 mL of NDMV solution over 15 to fill the chamber, avoiding air bubble production.
5. Mount the laser module into the main instrument housing and connect the power adaptor insider.
6. Start the NTA software to record NDMVs Brownian motion and analyze the nanoparticle motion automatically (Figure 4).

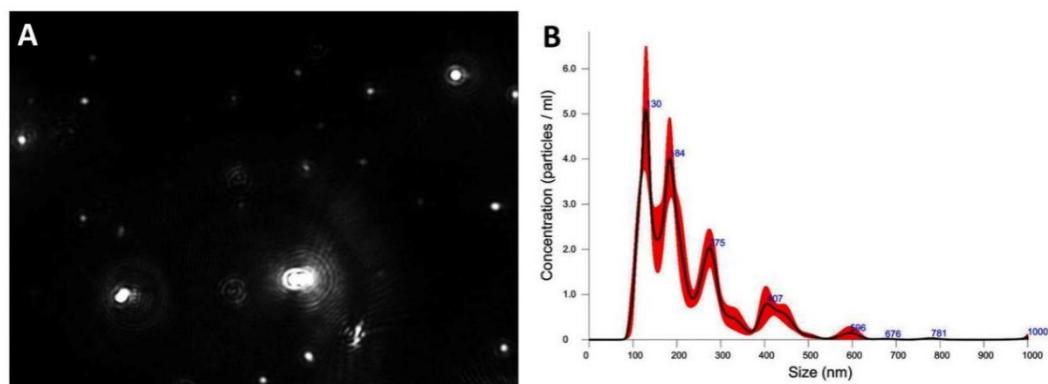


Figure 4. Nanoparticle tracking analysis (NTA) results for neutrophil-derived microvesicles (NDMVs).

A. Highlighted white particles are NDMVs captured in an image from a recorded video. B. The graph illustrates the distribution of NDMV sizes from 100 to 1,000 nm and their concentrations. NTA showed that NDMV concentration was $5.80 \times 10^8 \pm 2.34 \times 10^7$ particles/mL (mean \pm standard deviation).

Recipes

1. Phosphate-buffered saline (PBS)

9 g/L NaCl
0.24 g/L KH₂PO₄
0.2 g/L KCl
1.42 g/L Na₂HPO₄

Adjust the pH to 7.4 with HCl

Note: All PBS used for experiments should be passed through a syringe filter to obtain particle-free PBS.

Acknowledgments

This work was supported by the Ratchadapiseksompotch Fund, Chulalongkorn University, grant number CUGR63953002, and Osteoarthritis and Musculoskeleton Research Unit. Dong Zhan was supported by a grant from the 100th anniversary Chulalongkorn University Fund and China Scholarship Council for Doctoral Scholarship and Overseas Research Experience Scholarship for Graduate Student.

Competing interests

The authors declare no conflict of interest.

Ethics

The study protocol conformed to the ethical standards outlined in the Declaration of Helsinki and was approved by the Ethical Committee on Human Research of the Faculty of Medicine, Chulalongkorn University (No. 565/59). All study subjects were fully informed regarding the study protocol and procedures prior to participating in the study.

Written informed consent was obtained from all participants.

References

- Distler, J. H. W., Pisetsky, D. S., Huber, L. C., Kalden, J. R., Gay, S. and Distler, O. (2005). [Microparticles as regulators of inflammation: Novel players of cellular crosstalk in the rheumatic diseases](#). *Arthritis Rheum* 52(11): 3337-3348.
- Greening, D. W., Xu, R., Gopal, S. K., Rai, A. and Simpson, R. J. (2017). [Proteomic insights into extracellular vesicle biology – defining exosomes and shed microvesicles](#). *Expert Rev Proteomic* 14(1): 69-95.
- Headland, S. E., Jones, H. R., Norling, L. V., Kim, A., Souza, P. R., Corsiero, E., Gil, C. D., Nerviani, A., Dell'Accio, F., Pitzalis, C., et al. (2015). [Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis](#). *Sci Transl Med* 7(315): 315ra190.
- Leblanc, P., Arellano-Anaya, Z. E., Bernard, E., Gallay, L., Provansal, M., Lehmann, S., Schaeffer, L., Raposo, G. and Vilette, D. (2017). [Isolation of exosomes and microvesicles from cell culture systems to study prion transmission](#). *Methods Mol Biol* 1545: 153-176.
- Lima, L. G., Oliveira, A. S., Campos, L. C., Bonamino, M., Chammas, R., Werneck, C., Vicente, C. P., Barcinski, M. A., Petersen, L. C. and Monteiro, R. Q. (2011). [Malignant transformation in melanocytes is associated with increased production of procoagulant microvesicles](#). *Thromb Haemost* 106(4): 712-723.
- Mantovani, A., Cassatella, M. A., Costantini, C. and Jaillon, S. (2011). [Neutrophils in the activation and regulation of innate and adaptive immunity](#). *Nat Rev Immunol* 11(8): 519-531.
- Rhys, H. I., Dell'Accio, F., Pitzalis, C., Moore, A., Norling, L. V. and Perretti, M. (2018). [Neutrophil microvesicles from healthy control and rheumatoid arthritis patients prevent the inflammatory activation of macrophages](#). *EBioMedicine* 29: 60-69.
- Ridger, V. C., Boulanger, C. M., Angelillo-Scherrer, A., Badimon, L., Blanc-Brude, O., Bochaton-Piallat, M. L., Boilard, E., Buzas, E. I., Caporali, A., Dignat-George, F., et al. (2017). [Microvesicles in vascular homeostasis and diseases. Position Paper of the European Society of Cardiology \(ESC\) Working Group on Atherosclerosis and Vascular Biology](#). *Thromb Haemost* 117(7): 1296-1316.
- Topping, L., Rhys, H., Norling, L. and Nissim, A. (2017). [FRI0011 Targeting neutrophil microvesicles to damaged cartilage using antibodies to post translationally modified collagen ii](#). *Ann the Rheum Dis* 76(Suppl 2): 483-484.
- Trongtorsak, P., Olankijanunt, W., Trongtorsak, A., Intamaso, U. (2018). [Antidepressant and antiinflammatory effects of a combined fluoxetine and celecoxib treatment in a rat model of depression](#). *Chula Med J* 62(4): 653-665.
- Zhan, D., Cross, A., Wright, H. L., Moots, R. J., Edwards, S. W. and Honsawek, S. (2021). [Internalization of Neutrophil-Derived Microvesicles Modulates TNF \$\alpha\$ -Stimulated Proinflammatory Cytokine Production in Human Fibroblast-Like Synoviocytes](#). *Int J Mol Sci* 22(14):7409.

***In vitro* STING Activation with the cGAMP-STING Δ TM Signaling Complex**

Yanpu He^{1,2}, Celestine Hong^{1,2}, Darrell J. Irvine^{3,4,5}, Jiahe Li^{6,*} and Paula T. Hammond^{1,2,*}

¹Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA

²Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

³Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

⁴Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

⁵Howard Hughes Medical Institute, Chevy Chase, MD, USA; ⁶Department of Biological Engineering, Northeastern University, Boston, MA, USA

*For correspondence: jiah.li@northeastern.edu hammond@mit.edu

Abstract

Activating the STING (stimulator of interferon genes) signaling pathway via administration of STING agonist cyclic GMP-AMP (cGAMP) has shown great promise in cancer immunotherapy. While state-of-the-art approaches have predominantly focused on the encapsulation of cGAMP into liposomes or polymersomes for cellular delivery, we discovered that the recombinant STING protein lacking the transmembrane domain (STING Δ TM) could be used as a functional carrier for cGAMP delivery and elicit type I IFN expression in STING-deficient cell lines. Using this approach, we generated anti-tumoral immunity in mouse melanoma and colon cancer models, providing a potential translatable platform for STING agonist-based immunotherapy. Here, we report the detailed *in vitro* STING activation protocols with cGAMP-STING Δ TM complex to assist researchers in further development of this approach. This protocol can also be easily expanded to other applications related to STING activation, such as control of various types of infections.

Keywords: STING pathway, cGAMP delivery, Protein purification, Ribonucleoprotein complex, Interferon stimulation *in vitro*

This protocol was validated in: Sci Adv (2020), DOI: 10.1126/sciadv.aba7589

Background

Over the past two decades, the STING (stimulator of interferon genes) signaling pathway has emerged as a crucial feature of the immune system and a promising therapeutic target against viral and bacterial infections, autoimmune disorders, and cancers. As such, the delivery of STING agonists to boost the immune response has become an area of great interest in both academic institutions and pharmaceutical companies (Ohkuri *et al.*, 2017). While existing efforts have focused mostly on developing synthetic delivery vehicles (Shae *et al.*, 2019), this assumes the presence of fully functional STING in cells. STING signaling has not only been shown to be frequently impaired in cancer cells due to epigenetic silencing of the protein (Ahn *et al.*, 2015; Xia *et al.*, 2016); there is also an ongoing debate on whether the general population is responsive to agonist-only therapies, since 19% of humans carry a mutated STING variant (R71H-G230A-R293Q, HAQ STING) reported to exhibit impaired function (Jin *et al.*, 2011; Fu *et al.*, 2015; Patel *et al.*, 2017; Sivick *et al.*, 2017).

To address these concerns, we engineered a truncated portion of the original STING protein to pre-assemble with STING agonists, acting as a functional carrier that can effectively trigger STING signaling even in the absence of STING proteins in mammalian cells. Our *in vivo* vaccination studies with this platform has shown efficient activation of B cells, cytotoxic T cells and memory precursor T cells, as well as robust anti-tumoral immunity against melanoma and colon cancer mouse models (He *et al.*, 2020).

Here, we report the detailed protocols of our *in vitro* STING activation assays (Table 1) with cGAMP-STING Δ T ribonucleoprotein complex in three cell lines: human embryonic kidney (HEK293T) cell, mouse macrophage (RAW264.7) and mouse dendritic cell (DC2.4). The purification protocol of STING Δ T is also included to ensure the reproducibility of our work. We believe this protocol may assist further mechanistic discoveries in the signaling pathway and more engineering applications of this platform in vaccinology and cancer immunotherapy.

Table 1. Summary of *in vitro* STING activation assays

Cell line	Medium	Assay	Performed with
HEK293T-luc2p/ISRE/Hygro	DMEM + 10% FBS + 1% P/S	IFN-luciferase assay	Cells 24 h post treatment
DC2.4	RPMI + 10% FBS + 1% P/S	CXCL10 ELISA mIFN- β qPCR	Medium 48 h post treatment Cells 24 h post treatment
RAW264.7	DMEM + 10% FBS + 1% P/S	CXCL10 ELISA mIFN- β qPCR	Medium 48 h post treatment Cells 24 h post treatment
RAW-Blue ISG	DMEM + 10% heat inactivated FBS + 1% P/S	IFN-SEAP assay	Medium 12-48 h post treatment

Materials and Reagents

Protein Purification

1. Poly-Prep chromatography column (Bio-Rad, catalog number: 7311550)
2. ZebaTM Spin Desalting Columns 40k MWCO 10 mL (Thermo Fisher Scientific, catalog number: 87772)
3. BL21 (DE3) competent *E. coli* (NEB, catalog number: C2527I)
4. RosettaTM (DE3) competent *E. coli* (Millipore Sigma, catalog number: 70954)
5. Isopropyl- β -D-thiogalactopyranoside (IPTG, Millipore Sigma, catalog number: I6758-10G)
6. Phosphate Buffered Saline (PBS, Lonza, catalog number: 17-516F)
7. Imidazole (Millipore Sigma, catalog number: I5513)
8. Lysozyme from chicken egg white (Millipore Sigma, catalog number: 62971-10G-F)
9. TritonTM X-100 (Millipore Sigma, catalog number: T8787-250ml)

10. Triton™ X-114 (Millipore Sigma, catalog number: 93422-250ml)
11. Phenylmethylsulfonyl fluoride (PMSF, Millipore Sigma, catalog number: P7626-5G)
12. cComplete™ Mini, EDTA free (Roche, catalog number: 11836170001)
13. Bovine Serum Albumin (BSA, Millipore Sigma, catalog number: A3608)
14. HisPur™ Cobalt Resin (Thermo Fisher Scientific, catalog number: 89964)
15. 1,4-Dithiothreitol (DTT, Millipore Sigma, catalog number: 10197777001)
16. Sodium Phosphate Monobasic (Millipore Sigma, catalog number: S3139)
17. Sodium Phosphate Dibasic (Millipore Sigma, catalog number: S3264)
18. Sodium Chloride (Millipore Sigma, catalog number: S9888)
19. HEPES (Millipore Sigma, catalog number: H3375)
20. Glycerol (Millipore Sigma, catalog number: G5516)
21. Protein Binding Buffer (see Recipes)
22. Protein Elution Buffer (see Recipes)
23. Protein Storage Buffer (see Recipes)

Cells and cell culture media

1. HEK293T and RAW264.7 cells were obtained from the American Type Culture Collection (ATCC)
2. DC2.4 cells were obtained from Rock lab, University of Massachusetts Medical School, MA, USA
3. RAW-Blue ISG cells were obtained from Invivogen
4. Dulbecco's modified Eagle's medium (DMEM, Corning, catalog number: 10-041-CV)
5. Roswell Park Memorial Institute (RPMI) medium (Corning, catalog number: 10-013-CV)
6. 0.25% Trypsin-EDTA (Gibco, catalog number: 25200-056)
7. Fetal bovine serum (FBS, Gibco, catalog number: 10437-028)
8. Penicillin-Streptomycin Solution, 100× (Corning, catalog number: 30-002-CI)

IFN-luciferase assay

1. 96-well clear bottom white plate (Millipore Sigma, catalog number: CLS3610)
2. pGL4.45[luc2p/ISRE/Hygro] Vector (Promega)
3. Firefly Luciferase Assay Kit (Biotium, catalog number: 30075-2)
4. TransIT-X2® Transfection Reagent (Mirus, catalog number: MIR 6004)

RNA extraction, Reverse Transcription, and qPCR

1. LightCycler™ 480 Multiwell Plate 96 clear with Sealing Foils (Roche, catalog number: 05102413001)
2. RNeasy™ micro kit (Qiagen, catalog number: 74004)
3. Beta-Mercaptoethanol (β ME, Millipore Sigma, catalog number: M6250-10ML)
4. Reverse transcription kit (Thermo Fisher Scientific, catalog number: 4374966)
5. SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, catalog number: 4309155)
6. qPCR primers used for detection: mIFN- β -F: 5'-GCCTTGCCATCCAAGAGATGC-3', mIFN- β -R: 5'-ACACTGTCTGCTGGAGTTC-3', mActin-F: 5'-CATTGCTGACAGGATGCAGAAGG-3', and mActin-R: 5'-TGCTGGAAGGTGGACAGTGAGG-3' (ordered from IDT as custom oligo DNA)

mCXCL10 ELISA

1. Greiner Bio-One MICROLON™ 600 High Binding 96-Well ELISA Assay Microplates (Fisher Scientific, catalog number: 07-000-627)
2. Mouse CXCL10 ELISA kit (R&D, catalog number: DY466)
3. TMB Substrate Set (BioLegend, catalog number: 421101)

4. TWEEN 20 (Millipore Sigma, catalog number: P9416-100ml)

SEAP-IFN assay

1. QUANTI-Blue™ Solution (Invivogen, catalog code: rep-qbs)

Equipment

1. Misonix sonicator 3000
2. Real-time PCR system (Roche, model: LightCycler 480)
3. Nanodrop spectrophotometer (Thermo Fisher, model: ND-1000)
4. Thermal cycler (Bio-Rad, model: T100)
5. Plate reader (Tecan, model: Infinite M200)

Software

1. GraphPad Prism

Procedure

A. STING Δ TM protein purification

The DNA sequences (sources: Tmem173 NM_028261 Mouse Tagged ORF Clone, Origene Catalog number: MR227544, STING TMEM173 NM_198282 Human Tagged ORF Clone, Origene Catalog number: RC208418) of STING Δ TM protein (138 to 378 amino acids for mouse STING, 139 to 379 amino acids for human STING) were synthesized as gBlock DNA fragments (Integrated DNA Technologies) and cloned into the pSH200 expression vector (linearized via Nco I and Not I restriction enzymes) with a hexa-histidine-tag at the N-terminus (Figure 1). Plasmids for STING Δ TM mutants such as S365A were then generated via site-specific mutagenesis. DE3 *Escherichia coli* (*E. coli*) was used to express the proteins (mouse STING Δ TM in BL21 DE3, human STING Δ TM in Rosetta DE3). Lysogeny Broth (LB) containing the antibiotic ampicillin (100 mg/L) was used for bacteria culture, shaker conditions were 37°C 220 rpm for growth and 18°C 220 rpm for induction.

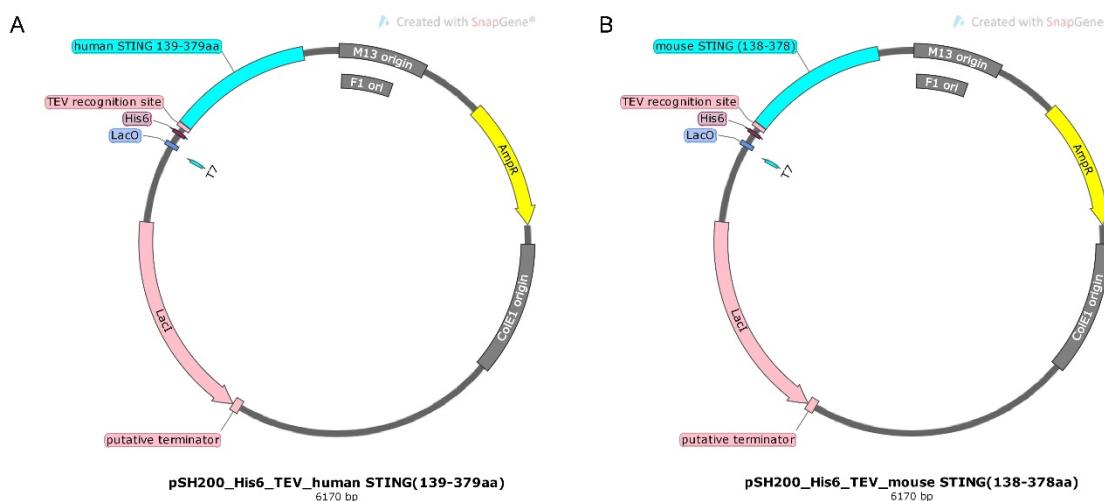


Figure 1. Plasmid maps of (A) pSH200_His6_TEV_human STING (139-379aa) and (B) pSH200_His6_TEV_mouse STING (138-378aa)

Day 1: Bacteria culture

- Pick DE3 *E. coli* from glycerol stock and culture in 50 mL of LB with ampicillin at 37°C overnight.

Day 2: Induction

- Transfer 50 mL of overnight culture into a flask containing 1 L LB with ampicillin and culture for approximately 2 h at 37°C until the culture's OD₆₀₀ reaches 0.4. This OD value is critical for expressing human STINGΔTM in Rosetta DE3, as we have found that higher concentration can compromise protein purity; for the expression of mouse STINGΔTM in BL21 DE3, the OD₆₀₀ can be in the range of 0.4-0.8 without compromising yield and purity.
- Cool the 1 L culture on ice and add 0.5 mL 1 M IPTG, then shake at 18 °C for approximately 20 h.

Day 3: Protein purification

- After induction, centrifuge bacteria cultures at 6,000 × g for 20 min.
- Collect pellets and wash once with 30 mL PBS, then lyse in 20 mL protein binding buffer (Recipe 1) with 20 mg lysozyme, 200 µL Triton X-100, 1 mM PMSF (replenished every 30 min until Cobalt binding) and one tablet of cOmplete protease inhibitor cocktail tablets at room temperature for 20 min with gentle rotation at 20 rpm. After cells are lysed, the proteins should be kept ice-cold throughout the purification to minimize degradation (using cold protein binding buffer, protein elution buffer, and protein storage buffer).
- Cool cell lysate on ice water and sonicate with Misonix sonicator 3000 at 18 W (with 3-s on and 5-s off intervals to prevent heat-deactivation of the protein) for a total of 5 min.
- After sonication, centrifuge cell lysate at 14,000 × g at 4°C for 30 min.
- Wash 0.5 mL HisPur Cobalt Resin with 5 mL protein binding buffer, then add to the bacteria supernatant separated from the centrifugation, along with 20 µL Triton X-114 (to eliminate endotoxin) for Cobalt-HisTag binding (4°C for 1 h with gentle rotation at 20 rpm).
- After binding, carefully aspirate the supernatant and wash the Cobalt resin twice (4°C for 30 min each time with gentle rotation at 20 rpm) with 5 mL protein binding buffer containing 5 µL Triton X-114.
- Transfer washed Cobalt resin into a Poly-Prep chromatography column with 5 mL of protein binding buffer. After all the protein binding buffer has drained out under gravity, add 1.5 mL protein elution buffer (Recipe 2) and collect the elution.
- Desalt the protein elution with Zeba™ Spin Desalting Columns (40k MWCO 10 mL). Add 10 mL of protein storage buffer (Recipe 3) into the column and allow it to completely drain under gravity (until no liquid remains above the resin surface). Add the 1.5 mL protein elution dropwise onto the middle of the

- resin and allow it to drain completely. Finally, collect five 1 mL fractions with protein storage buffer (add 1 mL of storage buffer then collect the flow-through as “Fraction 1”, then add another 1 mL of storage buffer and collect “Fraction 2” ... all the way till “Fraction 5”). Combine Fractions 3 and 4 (which contains the majority of the desalted protein) for SDS-PAGE characterization and BCA quantification of protein concentration. Fractions 1, 2 may contain minimal amounts of protein, fraction 5 may contain protein with imidazole. The concentrations of each fraction may also be quantified through the BCA assay.
9. For storage, 1 mM DTT was added and protein solution was aliquoted then stored in -80°C. Protein function can be maintained for years under this storage condition, but can be compromised by multiple freeze-thaw cycles.

B. Cell lines used for *in vitro* STING activation

Human embryonic kidney 293T (HEK293T) cells are deficient in cGAMP synthase (cGAS) and STING proteins, but express other essential proteins in downstream STING signaling, including TANK-binding kinase 1 (TBK1) and Interferon regulatory factor 3 (IRF3). Therefore, it provides a good model for studying the function of cGAMP-STING Δ TM without interactions with endogenous cGAMP or STING proteins (Figure 2). Additionally, it can be transfected to overexpress full-length mutant STING proteins (for example HAQ STING). To detect STING activation, we generated an interferon (IFN) reporter cell line by integrating an IFN-stimulated response element (ISRE) that drives luciferase expression through the stable transfection of pGL4.45[luc2p/ISRE/Hygro] plasmid selected in hygromycin (200 μ g/ml). HEK293T cells are not capable of uptaking cGAMP-STING Δ TM complex directly, so commercial transfection reagents (we primarily used TransIT-X2, though others like Lipofectamine also work) are required in order to deliver cGAMP-STING Δ TM complex into the cells. HEK293T cells are cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Mouse macrophage RAW264.7 and dendritic cell DC2.4 both express cGAS and STING, as well as TBK1 and IRF3 (Figure 2). They are also capable of internalizing cGAMP-STING Δ TM complex without the help of a transfection reagent. To detect STING activation, we can measure the cell secreted CXCL10 concentration in the medium with enzyme-linked immunosorbent assay (ELISA) or quantify the interferon- β mRNA level with qPCR. The aforementioned media contains no cells – medium that contains cells is denoted simply as ‘cells’. In addition, a reporter cell line derived from RAW264.7: RAW-BlueTM ISG cells can be used to study the kinetics of STING activation *in vitro*, and since the Secreted embryonic alkaline phosphatase (SEAP) – IFN assay only requires 20 μ L of media, it can be performed at multiple time points post treatment. RAW264.7 cells are cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For SEAP-IFN assay, RAW-BlueTM ISG cells are cultured in DMEM with 10% heat-inactivated (56°C, 30 min) FBS and 1% penicillin/streptomycin. DC2.4 cells are cultured in Roswell Park Memorial Institute (RPMI) medium with 10% FBS and 1% penicillin/streptomycin.

All cells are cultured in a 37°C, 5% CO₂ incubator, used at low passage number and tested negative for Mycoplasma contamination.



Figure 2. Immunoblotting of endogenous expression of cGAS, STING, TBK1, and IRF3 proteins from HEK293T, RAW264.7, and DC2.4 cells

C. IFN-luciferase assay with HEK293T-luc2p/ISRE/Hygro

Day 1: Preparation of Cells

1. Seed HEK293T-luc2p/ISRE/Hygro cells into 96-well plates (clear bottom flat white plates for luciferase assay) at a density of 3×10^5 cells/ml in 100 μL media per well.

Day 2: Cell treatment

1. For each well, mix 1 μg of STING ΔTM protein with 0.025 μg cGAMP, then add 1 μL of TransIT-X2 in OptiMEM media to a total volume of 20 μL and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
3. Incubate treated cells for 24 h.

Day 3: Luminescence measurement

- a. Remove plates from the incubator, aspirate medium and add 25 μL lysis buffer (five-fold diluted with DI water from the provided 5 \times firefly luciferase lysis buffer) to each well, then incubate at room temperature for 15 min with orbital shaking.
- b. Add 50 μL of luciferase assay buffer to each well (with D-luciferin freshly added at final concentration of 0.2 mg/ml), read plate for bioluminescence on a Tecan microplate reader.

D. mCXCL10 ELISA with RAW264.7 and DC2.4

Day 1: Preparation of Cells

1. Seed RAW264.7 or DC2.4 cells into 96-well plates at a density of 2×10^5 cells/ml in 100 μL medium per well.

Day 2: Cell treatment

1. For each well, mix 1 μg of STING ΔTM protein with 0.025 μg cGAMP and 1 μL of TransIT-X2 in 20 μL OptiMEM medium, then incubate at room temperature for 15 min. Alternatively, for vehicle-free treatment, mix 5 μg of STING ΔTM protein with 0.125 μg cGAMP in 20 μL OptiMEM medium and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
3. Incubate treated cells for 48 h.

Day 3: Preparation of ELISA plates

ELISA was performed according to the manufacturer's protocol: Mouse CXCL10 ELISA kit (R&D, DY466)

1. Dilute CXCL-10 capture antibody in PBS to 2 µg/mL.
2. Add 100 µL capture antibody solution to each well of the ELISA assay plates. Incubate at 4 °C overnight.

Day 4: ELISA

1. Aspirate each well of the ELISA assay plates, wash three times with Wash Buffer (PBS with 0.05% Tween 20).
2. Add 300 µL Reagent Diluent (PBS with 1% BSA) to each well to block plates. Incubate at room temperature for 1 h with orbital shaking at 220 rpm.
3. Aspirate plates and wash three times with Wash Buffer.
4. Add 100 µL media of RAW264.7 or DC2.4 cells 48 h post-treatment without dilution. Incubate at room temperature for 2 h with orbital shaking at 220 rpm.
5. Aspirate plates and wash three times with Wash Buffer.
6. Add 100 µL of the Detection Antibody at 100 ng/ml (diluted in Reagent Diluent) to each well. Incubate at room temperature for 2 h with orbital shaking at 220 rpm.
7. Aspirate plates and wash three times with Wash Buffer.
8. Add 100 µL of the provided Streptavidin-HRP stock solution 40-fold diluted in Reagent Diluent to each well. Incubate at room temperature for 20 min avoiding light with orbital shaking at 220 rpm.
9. Aspirate plates and wash three times with Wash Buffer.
10. Add 100 µL of TMB Substrate Solution, freshly prepared from mixing equal volumes of TMB Substrate A with TMB Substrate B. Incubate at room temperature for 20 min with orbital shaking at 220 rpm, avoiding light.
11. Add 50 µL of Stop Solution (2 N H₂SO₄) to each well. Gently tap the plate for mixing. Measure absorbance at 450 nm with a spectrophotometer/plate reader.

E. Quantification of mIFN-β expression by qPCR with RAW264.7 and DC2.4**Day 1: Preparation of Cells**

1. Seed RAW264.7 or DC2.4 cells into 24-well plates at a density of 3×10^5 cells/ml in 400 µL media per well.

Day 2: Cell treatment

1. For each well, mix 5 µg of STINGΔTM protein with 0.125 µg cGAMP and 5 µL of TransIT-X2 in 50 µL OptiMEM medium. Incubate at room temperature for 15 min. Alternatively, for vehicle-free treatment, mix 25 µg of STINGΔTM protein with 0.625 µg cGAMP in 50 µL OptiMEM medium and incubate at room temperature for 15 min.
2. Add the mixture to the cell medium without pipetting up and down.
3. Incubate treated cells for 24 h.

Day 3: RNA extraction, reverse transcription and qPCR

Perform RNA extraction following protocol provided by RNeasy Micro Kit:

1. Prepare fresh lysis buffer by adding 10 µL β-mercaptoethanol into 1 mL RLT buffer.
2. Aspirate medium, wash cells once with PBS, then add 350 µL lysis buffer per well. Incubate at room temperature for 5 min.
3. Transfer the cell lysate into Eppendorf tubes, vortex for 20 s, then add 350 µL 70% ethanol, pipette up and down, transfer to a spin column provided in the kit, and centrifuge for 15 s at 8,000 × g.
4. Discard flowthrough, add 350 µL RW1 buffer, and centrifuge for 15 s at 8,000 × g.
5. Prepare DNase buffer from DNase supplied in lyophilized form in glass vials. Use syringe to inject 500 µL water into the glass vial to dissolve the powder. Then open the vial and add 10 µL DNase solution + 70 µL RDD buffer to the column. Incubate at room temperature for 15 min. Then add 350 µL RW1 buffer to the spin column, centrifuge for 15 s at 8,000 × g and discard the collection tube.

6. Place the spin column in a new 2 mL collection tube as supplied. Add 500 µL RPE buffer, centrifuge for 15 s at 8,000 × g, and discard the flow through.
7. Add 500 µL 80% ethanol to the spin column, centrifuge for 2 min at 8,000 × g and discard the collection tube.
8. Place the spin column in another new 2 mL collection tube as supplied. Centrifuge at 8,000 × g for 5 min, discard the collection tube.
9. Place the spin tube in a new 1.5 mL collection tube as supplied, add 14 µL RNase-free water to the center of the spin column, and centrifuge for 1 min at 8,000 × g to elute the RNA.
10. Measure RNA concentration of each cell sample with Nanodrop spectrophotometer.

Reverse transcription

1. Dilute 1 µg extracted RNA with water to a total volume of 10 µL.

2. For each RNA sample, prepare below mixture in a PCR tube:

Diluted RNA	10 µL
10× RT buffer	2 µL
dNTP	0.8 µL
Random primer	2 µL
RNase inhibitor	1 µL
Enzyme	1 µL
Water	3.2 µL
Total volume	20 µL

3. Treat the sample with thermal cycler with the program below:

25°C	10 min
37°C	2 h
85°C	5 min
4°C	Infinite hold

qPCR

1. Dilute qPCR primers to 10 µM working concentration.
2. Add 80 µL water to 20 µL reverse transcription product from the previous step (this 5-fold dilution is denoted as ‘template’ in the following step)
3. Add the following to the qPCR 96-well plate for each reaction (add primer first, then add master mix):

Template	1 µL
2x SYBR mixture	10 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Water	8 µL
Total volume	20 µL

F. IFN-SEAP assay with RAW-Blue ISG cells

Day 1: Preparation of Cells

1. Seed RAW264.7 or DC2.4 cells into 96-well plates at a density of 2×10^5 cells/ml in 100 µL media per well.

Day 2: Cell treatment

1. 2 h prior to treatment, remove the medium and replenish it with 100 µL DMEM with 10% heat-inactivated FBS and 1% penicillin/streptomycin pre-warmed to 37°C in order to reduce the noise level in subsequent QUANTI-Blue assay.
2. For each well, mix 1 µg of STINGΔTM protein with 0.025 µg cGAMP then 1 µL of TransIT-X2 in 20 µL OptiMEM medium, incubated at room temperature for 15 min. Alternatively, for vehicle-free treatment,

- mix 5 µg of STING Δ TM protein with 0.125 µg cGAMP in 20 µL OptiMEM medium and incubate at room temperature for 15 min.
3. Add the mixture to the cell medium without pipetting up and down. At least 3 replicates should be performed for each treatment.
 4. Incubate treated cells in 37 °C incubator.

Days 3-4: IFN-SEAP assay

1. Take 20 µL of medium from treated cell wells and mix with 180 µL of QUANTI-Blue assay buffer in a separate 96-well plate. (Multiple time points between 12 to 48 h can be taken to study the kinetics.)
2. Incubate the plate at 37 °C for 6 to 10 h until a visible color difference is observed (Figure 3).
3. Determine the IFN-SEAP activity by measuring the absorbance at 635 nm with a spectrophotometer/plate reader.

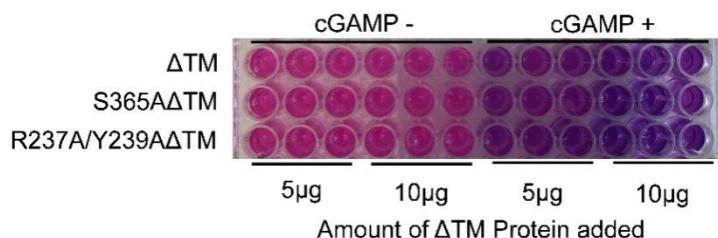


Figure 3. Example IFN-SEAP assay of mixing medium of treated RAW-Blue ISG cells with QUANTI-Blue assay buffer showing color differences due to STING signaling

Data analysis

Data can be analyzed with GraphPad Prism and statistical analyses performed with one-way analysis of variance (ANOVA) followed by Student's t-test for statistical significance.

Recipes

1. Protein Binding Buffer

50 mM sodium phosphate
0.5 M NaCl
10 mM imidazole
pH 7.4

2. Protein Elution Buffer

50 mM sodium phosphate
0.5 M NaCl
150 mM imidazole
pH 7.4

3. Protein Storage Buffer

20 mM HEPES
150 mM NaCl
10% glycerol
pH 7.4

Acknowledgments

This work was supported by the Department of Defense Congressionally Directed Medical Research Program's (CDMRP) Ovarian Cancer Research Program, Cancer Center Support Grant (CCSG) Pilot Awards at the David H. Koch Institute for Integrative Cancer Research at MIT, the Institute for Soldier Nanotechnologies (ISN) at MIT, the Marble Center for Cancer Nanomedicine, Northeastern University Faculty start-up funding, and the Peer Reviewed Medical Research Program from the Department of Defense's Congressionally Directed Medical Research Programs (W81XWH18PRMRPDA).

Competing interests

The authors declare that they have no competing interests.

References

- Ahn, J., Konno, H. and Barber, G. N. (2015). [Diverse roles of STING-dependent signaling on the development of cancer](#). *Oncogene* 34(41): 5302-5308.
- Fu, J., Kanne, D. B., Leong, M., Glickman, L. H., McWhirter, S. M., Lemmens, E., Mechette, K., Leong, J. J., Lauer, P. and Liu, W. (2015). [STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade](#). *Sci Transl Med* 7(283): 283ra252-283ra252.
- He, Y., Hong, C., Yan, E. Z., Fletcher, S. J., Zhu, G., Yang, M., Li, Y., Sun, X., Irvine, D. J. and Li, J. (2020). [Self-assembled cGAMP-STING \$\Delta\$ TM signaling complex as a bioinspired platform for cGAMP delivery](#). *Sci Adv* 6(24): eaba7589.
- Jin, L., Xu, L., Yang, I. V., Davidson, E. J., Schwartz, D. A., Wurfel, M. M. and Cambier, J. C. (2011). [Identification and characterization of a loss-of-function human MPYS variant](#). *Genes Immun* 12(4): 263-269.
- Ohkuri, T., Kosaka, A., Ishibashi, K., Kumai, T., Hirata, Y., Ohara, K., Nagato, T., Oikawa, K., Aoki, N. and Harabuchi, Y. (2017). [Intratumoral administration of cGAMP transiently accumulates potent macrophages for anti-tumor immunity at a mouse tumor site](#). *Cancer Immunol Immunother* 66(6): 705-716.
- Patel, S., Blaauboer, S. M., Tucker, H. R., Mansouri, S., Ruiz-Moreno, J. S., Hamann, L., Schumann, R. R., Opitz, B. and Jin, L. (2017). [The common R71H-G230A-R293Q human TMEM173 is a null allele](#). *J Immunol* 198(2): 776-787.
- Shae, D., Becker, K. W., Christov, P., Yun, D. S., Lytton-Jean, A. K., Sevimli, S., Ascano, M., Kelley, M., Johnson, D. B. and Balko, J. M. (2019). [Endosomolytic polymersomes increase the activity of cyclic dinucleotide STING agonists to enhance cancer immunotherapy](#). *Nat Nanotechnol*: 1.
- Sivick, K. E., Surh, N. H., Desbien, A. L., Grewal, E. P., Katibah, G. E., McWhirter, S. M. and Dubensky, T. W. (2017). [Comment on “The Common R71H-G230A-R293Q Human TMEM173 Is a Null Allele”](#). *J Immunol* 198(11): 4183-4185.
- Xia, T., Konno, H. and Barber, G. N. (2016). [Recurrent loss of STING signaling in melanoma correlates with susceptibility to viral oncolysis](#). *Cancer Res* 76(22): 6747-6759.

Murine Monocyte and Macrophage Culture

Simone M. Haag and Aditya Murthy*

Department of Cancer Immunology, Genentech, South San Francisco, CA, USA

*For correspondence: murthy.aditya@gene.com

Abstract

Myeloid progenitors in the bone marrow generate monocytes, macrophages, granulocytes and most dendritic cells. Even though these innate immune cells are part of the same lineage, each cell type plays a specific and critical role in tissue development, host defense and the generation of adaptive immunity. Protocols have been developed in the past to differentiate myeloid cell types from bone marrow cells, enabling functional investigation and furthering our understanding about their contribution to mammalian physiology. In this protocol, we describe a simple and rapid method to isolate monocytes from murine bone marrow, culture them for up to 5 days and lastly, differentiate them into bone marrow derived macrophages (**Figure 1**).

Keywords: Bone marrow isolation, Monocyte culture, BMDMs, Macrophage differentiation, Monocyte differentiation

This protocol was validated in: eLife (2019), DOI: 10.7554/eLife.44452

Graphical Abstract:

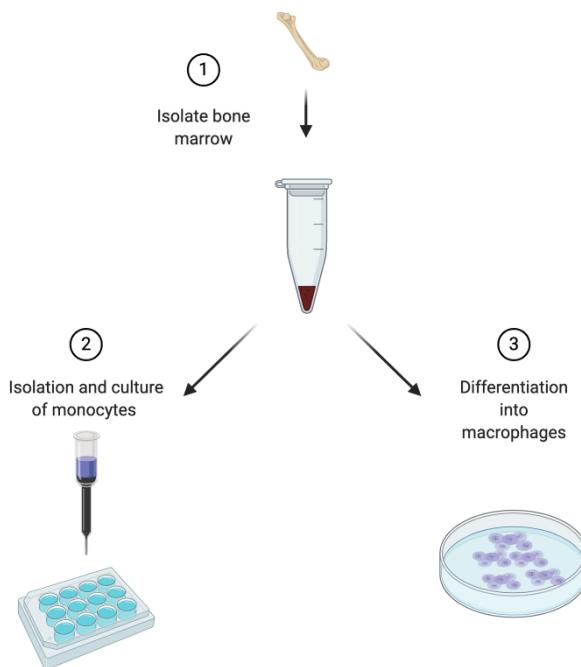


Figure 1. Experimental outline depicting steps for murine monocyte and macrophage culture

Background

Innate immune cells within the hematopoietic compartment constitutes myeloid cells such as monocytes, macrophages, granulocytes as well as most dendritic cells (DCs). These cells are primary sensors of infection or tissue injury and provide a bridge to adaptive immunity via antigen presentation, lymphocyte priming, and cytokine production.

Monocytes exhibit remarkable plasticity in their capacity to patrol tissue, remodel local microenvironments and present antigens under specific conditions. These cells can differentiate into DC or macrophage subsets upon sustained tissue residence (Davies and Gordon, 2005). Macrophages, on the other hand, develop from yolk sac-derived or hematopoietic progenitors or monocytes and are critical for organ development and homeostasis (Francke *et al.*, 2011).

To study myeloid cell biology in primary cells, researchers have developed several protocols to either isolate monocytes or macrophages directly from primary tissue or generate bone marrow derived macrophages (BMDMs) from hematopoietic progenitors. BMDMs can be maintained in culture for several days and can be generated by culture of isolated bone marrow in media containing M-CSF/CSF1 (Freund *et al.*, 2020; Geissmann *et al.*, 2010; Helft *et al.*, 2015). Dendritic cell (DC) subsets can be generated in culture by differentiation in media containing GM-CSF/CSF2 or Fms-like tyrosine kinase 3 ligand (FLT3L). While both systems generated a mixed population of myeloid and DC subsets, FLT3L allows for differentiation of multiple physiologically relevant subsets such as plasmacytoid DCs (pDCs), CD8⁺ and CD8⁻ DCs (Jakubzick *et al.*, 2017; Naik *et al.*, 2005).

Our protocol describes the isolation of monocytes from the bone marrow and provides methods for culture and maintenance of these cells for several days. Additionally, we describe a rapid method to isolate total bone marrow from isolated tibia and femur, allowing for maximum cell recovery and survival. Our techniques have the advantages of circumventing the need of generating specific conditioned media for BMDM culture (*e.g.*, from L929 cells) and shortcut the lengthy process of harvesting bone marrow through aspiration or flushing.

Taken together, these protocols allow for fast and simple isolation of bone marrow and provide the basis for growing primary monocytes and macrophages in high yields, which might be desired for downstream studies such

Cite as: Haag, S. and Murthy, A. (2021). Murine Monocyte and Macrophage Culture. Bio-protocol 11(6): e3928. DOI: 10.21769/BioProtoc.3928.

as gene editing, functional genomics or adoptive cell transfer (Zhang *et al.*, 2008).

Materials and Reagents

1. Cell scraper (Corning/VWR, catalog number: 15621-010)
2. 70 µm nylon sterile cell strainer (Corning, catalog number: 352350)
3. G18 needle (BD Bioscience, catalog number: 305196)
4. 0.5 mL microfuge tube (Eppendorf, catalog number: 022363611)
5. 1.5 mL microfuge tube (Eppendorf, catalog number: 022363204)
6. 50 mL Falcon tube, screw top (Eppendorf, catalog number: 0030122178)
7. LC columns (Miltenyi, catalog number: 130-042-401)
8. Non-tissue culture treated 6-well plates (Corning, catalog number: 351146)
9. Serological pipettes (*e.g.*, Fisher scientific catalog number: 10710810)
10. Low adherent plates (Petri-dish) (VWR, catalog number: 25384-326)
11. DMEM high glucose media (Gibco, catalog number: 11965092)
12. (Optional) EasySep™ (Stemcell Technologies, catalog number: 19861)
13. Penicillin/Streptomycin (Gibco, catalog number: 15070063)
14. Recombinant murine Macrophage Colony Stimulating Factor (rmM-CSF) (Genentech)
15. GlutaMAX (Gibco, catalog number: 35050061)
16. Fetal Calf Serum (FCS) (Thermo Fisher, catalog number: 26140)
17. Ammonium Chloride Potassium (ACK) lysis buffer (Thermo Fisher, catalog number: A1049201)
18. Sterile Phosphate Buffered Saline (PBS) (Genentech)
19. Monocyte isolation kit (Miltenyi, catalog number: 130-100-629)
20. Recombinant murine Granulocyte Macrophage Colony Stimulating Factor (rmGM-CSF) (R&D Systems, catalog number: 415-ML010)
21. Recombinant murine Interleukin 4 (rmIL-4) (R&D Systems, catalog number: 404-ML-010)
22. Macrophage media (see Recipes)

Equipment

1. Pipette boy (*e.g.*, Integra, catalog number: 155017)
2. Tabletop centrifuge (*e.g.*, Beckman, model: Allegra X14)
3. Microcentrifuge (*e.g.*, Thermo Fisher Scientific, catalog number: 75002404)
4. Water-bath at 37°C (Thermo Fisher Scientific, catalog number: TSCIP19)
5. Laminar Flow Hood (VWR)
6. Incubator at 37°C with 5% CO₂ (*e.g.*, Heracell 150 CO₂ incubator)
7. Vi-CELL XR Cell Viability Analyzer (Beckmann)
8. MACS Multi Stand magnet (Miltenyi, catalog number: 130-042-303)
9. Sterile Scissors (Thermo Fisher Scientific, catalog number: 08-950)
10. Sterile Forceps (Thermo Fisher Scientific, catalog number: 08-890)

Procedure

A. Bone marrow isolation (Figure 2)

1. Euthanize mouse using institutionally approved protocols.
2. Use sterile forceps and scissors to isolate femur and tibia.

3. Remove residual muscle or tissue attached to the bones.
4. Store isolated bones in a 6-well plated in PBS on ice until further processing.
5. Push a G 18 needle through the bottom of a 0.5 mL microcentrifuge tube to generate a small hole.
6. Place the 0.5 mL microcentrifuge tube in a larger 1.5 mL microcentrifuge tube.
7. Carefully place the isolated femur and tibia (maximum 1 femur and 2 tibia) in the 0.5 mL microcentrifuge tube nested in the 1.5 mL tube and close the lid.
8. Centrifuge the tubes at 10,000 × g for 30 s.
9. After centrifugation confirm complete transfer of marrow into the bottom tube. If transfer was not successful, cut off epiphysis and repeat centrifugation step.
10. Discard the 0.5 mL microcentrifuge tube containing the bones. They should be empty of marrow.
11. Resuspend the obtained bone marrow in 1 mL ACK lysis buffer and incubate at room temperature for 2 min.
12. Place a 0.70 µm sterile filter on a 50 mL Falcon tube.
13. Filter the bone marrow suspension into tube and wash the filter with 10 mL PBS.
14. Add an additional volume of 30 mL PBS through the filter; discard the filter.
15. Close the lid on the tube and centrifuge at 350 × g for 4 min.
16. Remove PBS by gently decanting; retain bone marrow pellet.
17. If desired, repeat PBS wash by gently resuspending the pellet.
18. Count isolated bone marrow cells using a Vi-CELL or other standard method.

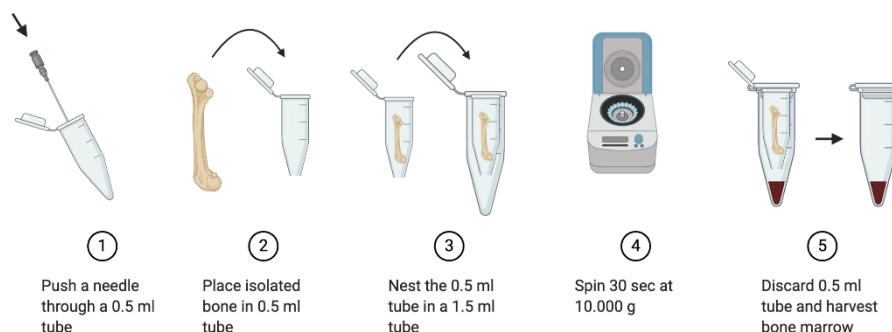


Figure 2. Experimental outline depicting bone marrow isolation steps

B. Isolation and culture of monocytes

1. Following bone marrow isolation, monocytes are enriched by negative selection using the monocyte isolation kit (Miltenyi) according to manufacturer's instructions. Please note that other mouse monocyte isolation kits can be used for this step (*e.g.*, EasySep™, Stemcell Technologies); however, these have not been evaluated for this specific protocol.
2. Isolated monocytes can be cultured in macrophage media supplemented with 5 ng/mL rmGM-CSF and 2.5 ng/mL rmIL-4 for up to 5 days. Monocytes should be plated at a density of 1×10^6 cells in 2 mL final volume in a non-tissue culture treated 6-well plate.
3. If desired, monocytes can be enriched from the day 5 culture by sorting for Ly6C positive, F4/80 negative, MHCII negative and CD11c negative cells at day 5 (Figure 3).

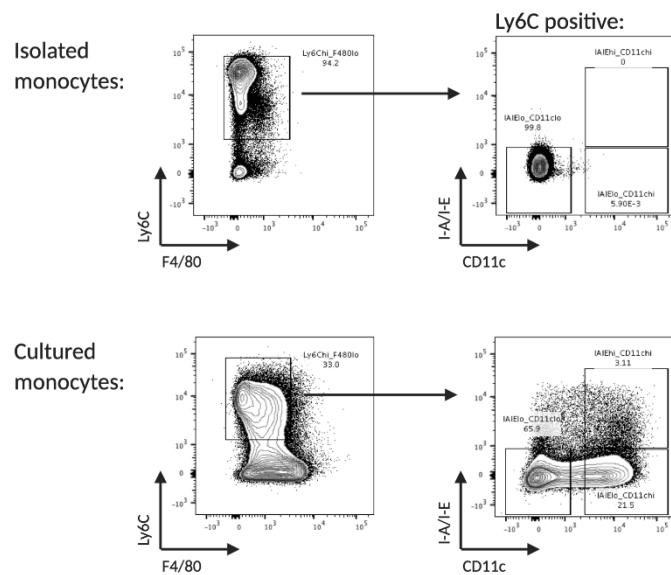


Figure 3. Flow cytometry analysis of freshly isolated and cultured monocytes.

Example of surface marker expression of Ly6C, F4/80, CD11b and I-A/I-E of freshly isolated and cultured monocytes. Monocytes are identified as Ly6C^{high}, F4/80^{low}, MHCII^{low} and CD11c^{low}.

C. Differentiation into bone marrow derived macrophages (BMDMs)

1. Supplement macrophage media with 50 ng/mL rmM-CSF and pre-warm in a water bath to 37°C.
2. Suspend 12 × 10⁶ isolated total bone marrow cells (from Procedure A) or enriched monocytes (Procedure B) in 20 mL supplemented macrophage media and plate cells in 15 cm non-tissue culture treated plates.
3. Place dish in cell culture incubator at 37 °C and 5 % CO₂ and culture for a total of 5 days.
4. At day 3 add 20 mL freshly supplemented macrophage media without removal of the original media.
5. At day 5 confirm adherence of differentiated BMDMs under a microscope. Assess differentiation by flow cytometry (Figure 4).

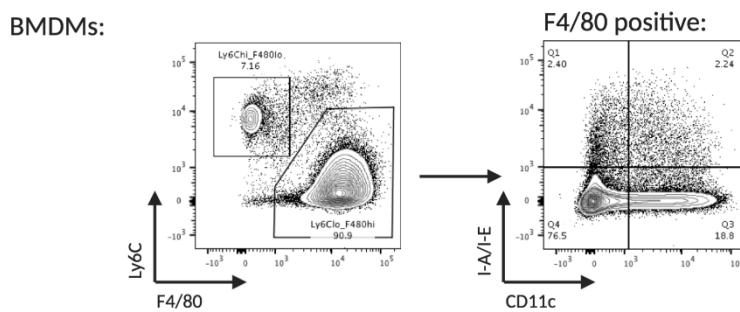


Figure 4. Flow cytometry analysis of monocytes differentiated into bone marrow derived macrophages (day 5).

Differentiated, resting macrophages can be identified as Ly6C^{low}, F4/80^{high}, CD11c^{low} and I-A/I-E^{low}.

6. For downstream assays, remove media and gently wash day 5 cells in 20 mL sterile PBS. Carefully scrape cells off the dish using a cell scraper in 5 mL macrophage media. If desired, wash the dish with an additional 5 mL of macrophage media to collect additional cells.
7. Count collected cells and re-suspend in macrophage media supplemented with 50 ng/ml rmM-CSF at

Cite as: Haag, S. and Murthy, A. (2021). Murine Monocyte and Macrophage Culture. Bio-protocol 11(6): e3928. DOI: 10.21769/BioProtoc.3928.

- desired cell density (typical density is 0.5×10^6 cells/ml). Plate cells onto tissue-culture treated dishes of desired formats.
8. If desired, resting macrophages can be enriched from the day 5 culture by sorting for Ly6C^{low}, F4/80^{high}, CD11c^{low} and I-A/I-E^{low}.

Recipes

1. Macrophage medium

DMEM high glucose media
10% FBS
1× GlutaMAX
1× Penicillin/Streptomycin

Acknowledgments

This study was funded by Genentech, Inc.

References 8 and 9 have utilized the procedure for murine BMDM isolation and culture.

Competing interests

SMH and AM are employees of Genentech, Inc. AM is a shareholder in Roche.

Ethics

All mice and experiments in this study were conducted following protocols approved by Genentech Institutional Animal Care and Use Committee (Protocol ID TH19-0370; valid until 02/14/2022).

References

- Jakubzick, C. V., Randolph, G. J. and Henson, P. M. (2017). [Monocyte differentiation and antigen-presenting functions](#). *Nat Rev Immunol* 17(6): 349-362.
- Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M. and Ley, K. (2010). [Development of monocytes, macrophages, and dendritic cells](#). *Science* 327(5966): 656-661.
- Davies, J. Q. and Gordon, S. (2005). [Isolation and culture of murine macrophages](#). *Methods Mol Biol* 290: 91-103.
- Francke, A., Herold, J., Weinert, S., Strasser, R. H. and Braun-Dullaeus, R. C. (2011). [Generation of mature murine monocytes from heterogeneous bone marrow and description of their properties](#). *J Histochem Cytochem* 59(9): 813-825.
- Zhang, X., Goncalves, R. and Mosser, D. M. (2008). [The isolation and characterization of murine macrophages](#). *Curr Protoc Immunol* Chapter 14: Unit 14 11.
- Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B. U., Goubau, D. and Reis e Sousa, C. (2015). [GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c⁺MHCII⁺ Macrophages and Dendritic Cells](#). *Immunity* 42(6): 1197-1211.
- Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K. and 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(11): 6592-6597.
- Freund, E. C., Lock, J. Y., Oh, J., Maculins, T., Delamarre, L., Bohlen, C. J., Haley, B. and Murthy, A. (2020). [Efficient gene knockout in primary human and murine myeloid cells by non-viral delivery of CRISPR-Cas9.](#) *J Exp Med* 217(7).
- Lim, J., Park, H., Heisler, J., Maculins, T., Roose-Girma, M., Xu, M., Mckenzie, B., van Lookeren Campagne, M., Newton, K., and Murthy, A. (2019). [Autophagy regulates inflammatory programmed cell death via turnover of RHIM-domain proteins.](#) *Elife* 8: e44452.

Liposomal Clodronate-mediated Macrophage Depletion in the Zebrafish Model

Linlin Yang^{1,§, #}, Alison M. Rojas^{1, #} and Celia E. Shiu^{1, 2, *}

¹Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, United States

²Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, United States

§Current address: Department of Immunology and Microbiology, Scripps Research, La Jolla, United States

*For correspondence: shiauce@unc.edu

#Contributed equally to this work

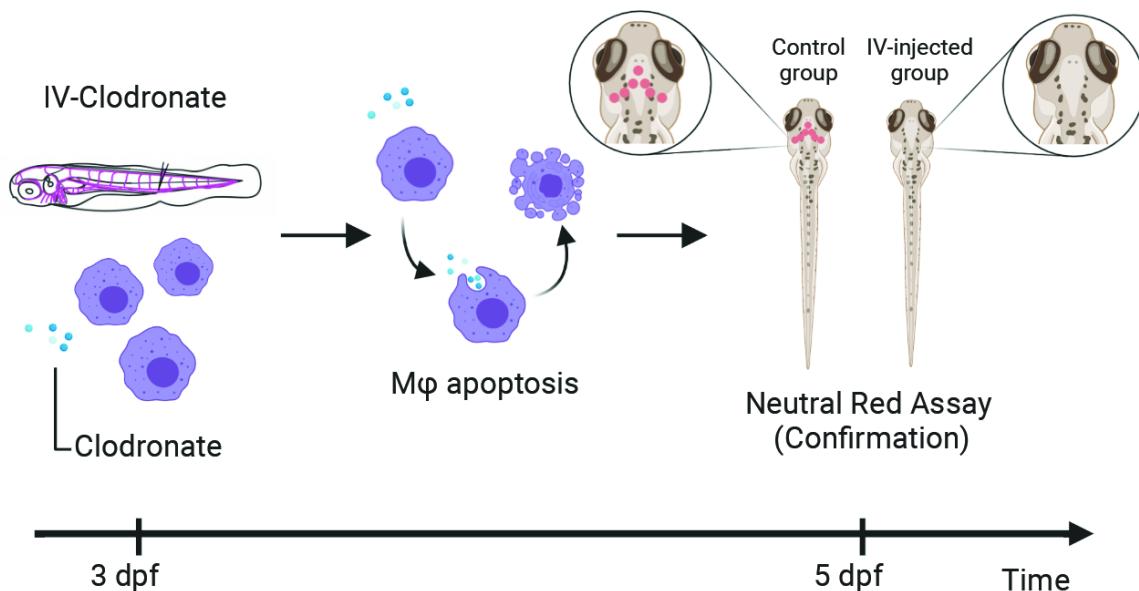
Abstract

The ability to conduct *in vivo* macrophage-specific depletion remains an effective means to uncover functions of macrophages in a wide range of physiological contexts. Compared to the murine model, zebrafish offer superior imaging capabilities due to their optical transparency starting from a single-cell stage to throughout larval development. These qualities become important for *in vivo* cell specific depletions so that the elimination of the targeted cells can be tracked and validated in real time through microscopy. Multiple methods to deplete macrophages in zebrafish are available, including genetic (such as an *irf8* knockout), chemogenetic (such as the nitroreductase/metronidazole system), and toxin-based depletions (such as using clodronate liposomes). The use of clodronate-containing liposomes to induce macrophage apoptosis after phagocytosing the liposomes is effective in depleting macrophages as well as testing their ability to phagocytose. Here we describe a detailed protocol for the systemic depletion of macrophages in zebrafish larvae by intravenous injection of liposomal clodronate supplemented with fluorescent dextran conjugates. Co-injection with the fluorescent dextran allows tracking of macrophage depletion in real time starting with verifying the successful intravenous injection to macrophage uptake of molecules and their eventual death. To verify a high degree of macrophage depletion, the level of brain macrophage (microglia) elimination can be determined by a rapid neutral red vital dye staining when clodronate injection is performed at early larval stages.

Keywords: Liposomes, Clodronate, Larva injection, Macrophage depletion, Zebrafish, Innate Immunity

This protocol was validated in: eLife (2020), DOI: 10.7554/eLife.58191

Graphical Abstract:



Experimental workflow for *in vivo* macrophage-specific depletion by liposomal clodronate in larval zebrafish

Background

Macrophages are key constituents of the innate immune system and play important roles in response to infections, sterile inflammation, and environmental changes. One of the most effective ways to uncouple the functions of macrophages from a complex mix of interacting cell types in different physiological contexts is to be able to specifically eliminate macrophages and analyze the phenotypic consequence. Such depletion experiments in mice have provided much insight into the role of macrophages (Hua *et al.*, 2018; Rosowski, 2020). However, our understanding of macrophage functions remains incomplete, and cell depletion experiments in the mouse model are difficult to track and validate in real time. For these reasons, the optical transparency and ease of manipulation of larval zebrafish offer clear advantages for highly traceable and tractable cell ablation *in vivo* by imaging the target cells in real time and in the whole intact organism. Zebrafish genes and immune system also share a high degree of orthology with those of the human (Yoder *et al.*, 2002; Santoriello *et al.*, 2012; Howe *et al.*, 2013). Furthermore, the adaptive immune system of zebrafish does not become functionally mature until juvenile adult stages (Lam *et al.*, 2004), making the larval zebrafish an excellent platform to study the innate immune system independent of the adaptive immune contributions.

Currently available macrophage depletion methods in zebrafish include genetic and chemogenetic manipulations, and toxin-based depletion. The development of macrophages requires early and continual function of the transcription factor Pu.1 (with the gene name *spil1b*), together with another transcription factor Irf8 (Li *et al.*, 2011; Shiau *et al.*, 2015; Tenor *et al.*, 2015). Either disruption of *pu.1* or *irf8* by gene knockout or knockdown by morpholino (MO) anti-sense oligomers, makes a reliable approach for macrophage depletion, while the former ablates myeloid cells, and the latter is more specific to macrophages but also causes an increase in neutrophil numbers (Shiau *et al.*, 2015; Yang *et al.*, 2020). These methods are not amenable to temporal control (Rhodes *et al.*, 2005; Li *et al.*, 2011; Shiau *et al.*, 2015; Rosowski, 2020), while clodronate-mediated depletion of macrophages based on localized microinjection enables some degree of spatial and temporal designation (Bernut *et al.*, 2014).

Clodronate (also known as dichloromethylene diphosphonate) can be metabolized by cells to block mitochondrial respiration due to the formation of a non-hydrolyzable ATP analog, which then causes cell death (apoptosis) (Rosowski, 2020). Once injected as encapsulated in liposomes, clodronate is easily ingested and eliminated by

macrophages as it accumulates within the cell (van Rooijen and Hendrikx, 2010). Since neither the clodronate nor the liposomal phospholipids utilized are toxic to other non-phagocytic cells (van Rooijen and Hendrikx, 2010), this approach allows specific depletion of already existing phagocytosing macrophages.

As part of the design for our protocol, we co-injected fluorescently labeled dextran with the liposomal clodronate to allow us to verify a precise and accurate injection and track the effect of the clodronate on macrophages in the entire larvae. To this end, after intravenous co-injection of clodronate liposome with fluorescently tagged dextran, we visually verified the successful injection of the substances into circulation and monitored the uptake of fluorescent dextran by macrophages and their ultimate demise over time. We designed the protocol to include a 48-hour post injection period to allow the effect of clodronate induced apoptosis in macrophages to materialize in light of previous work in chicken and mice showing that the efficacy of clodronate can take a few days depending on the tissue (Kameka *et al.*, 2014; Ponzoni *et al.*, 2018). We confirmed the efficacy of clodronate-mediated macrophage depletion at 48 hours post injection by assessing the remaining number of brain-resident macrophages (microglia), because a rapid analysis of microglia in the living larvae is available by the neutral red vital dye staining. We have chosen to inject at the early larval stage at 3 dpf (days post fertilization) because this is prior to maturation of the blood-brain-barrier (Jeong *et al.*, 2008; O'Brown *et al.*, 2019) at which time we found that our injected substances easily reach macrophages throughout the body including the brain. Using the 48-hour window for clodronate to take effect, we were able to achieve full ablation of microglia in most injected larval zebrafish (Yang *et al.*, 2020). Overall, we found that intravenous microinjection of liposomal clodronate at 3 dpf with a 48-hour incubation time is effective in eliminating macrophages.

Materials and Reagents

1. 1.5 mL microfuge tubes (Eppendorf, SafeLock, catalog number: 0030120086)
2. Polystyrene Petri dish (VWR, catalog number: 25384-342)
3. Thin wall borosilicate glass capillaries, 4 inches, OD 1.5 mm with filament (World Precision Instruments, catalog number: TW150F-4)
4. Glass bottle
5. 7.5 mL transfer pipettes (VWR, catalog number: 414004-005)
6. Low melt agarose (Fisher Scientific, IBI Scientific, catalog number: 50-550-455), store at room temperature
7. PTU (*N*-Phenylthiourea) (Sigma-Aldrich, catalog number: P7629), store at room temperature; made into PTU solution, store at -20°C
8. Clodronate Liposomes (Liposoma, catalog number: C41J0781, <https://clodronateliposomes.com>), store at 4-7°C
9. Control Liposomes (Liposoma, catalog number: B#P37J0718, <https://clodronateliposomes.com>), store at 4-7°C
10. Dextran, Alexa Fluor™ 568: 10,000 MW (Invitrogen, catalog number: D22912), store in freezer and protect from light
11. Neutral Red Dye (Sigma-Aldrich, catalog number: N4638), store at room temperature
12. Tricaine (3-amino benzoic acid ethylester) (Sigma-Aldrich, catalog number: A-5040) made into tricaine solution, store at -20°C
13. 50× PTU stock (see Recipes)
14. 25× Tricaine stock solution (100 mL) (see Recipes)
15. 3% Methyl cellulose (see Recipes)
16. 1,000× neutral red solution (see Recipes)
17. 1.5% low melt agarose (see Recipes)

Equipment

1. Stereomicroscope with LED illuminated base and articulating mirror (World Precision Instruments, model: 504941, PZMIII-MI)

2. Fluorescent stereomicroscope fully apochromatic corrected with 16:5:1 zoom optics (Leica, model: M165 MC)
3. Manual micromanipulator (World Precision Instruments, catalog number: M3301)
4. PicoNozzle Kit v2 (World Precision Instruments, catalog number: 5430-ALL)
5. Pneumatic PicoPump PV 820 (World Precision Instruments, catalog number: SYS-PV820)
6. Incubator (Benchmark Scientific, model: H2200-H)
7. Microelectrode Puller (World Precision Instruments, catalog number: PUL-1000)
8. Microwave

Software

1. Fiji (Schindelin *et al.*, 2012, <https://fiji.sc/>)

Procedure

A. 2 Larval zebrafish growth

1. Incubate zebrafish embryos in fish water in a 28.5°C incubator.
2. Conduct a daily water change, and starting at 24 h post-fertilization (hpf), the fish water is also supplemented with PTU at a final concentration of 0.003% to inhibit pigmentation. PTU (N-Phenylthiourea) inhibits melanogenesis (pigmentation) by blocking all tyrosinase-dependent steps in the melanin pathway (Karlsson *et al.*, 2001).
3. Check the health status of the zebrafish larvae before injection. By 3 dpf healthy larvae will have a consistent rhythmic heartbeat with apparent blood flow, intermittent bursts of movement, and a straight body.

B. Prepare liposome mix with fluorescent dextran

1. Use clodronate liposomes and control liposomes directly at 5 mg/mL at the concentration they were delivered in liquid form from the manufacturer. We recommend making aliquots of the liposomes for storage at 4°C to eliminate possible contamination from multiple usage; we use the aliquots within 12 months of storage. Supplement the liposome with Alexa 568 labeled dextran at 1:100 dilution of a 5 ng/nL stock for visualization of the injection.
For example: add 0.5 µL of a 1:10 dilution of Alexa 568 labeled dextran at 5 ng/nL to 4.5 µL of clodronate liposomes at its original concentration of 5 mg/mL.
2. Vortex to mix until a homogenous mixture for about 30-60 s.

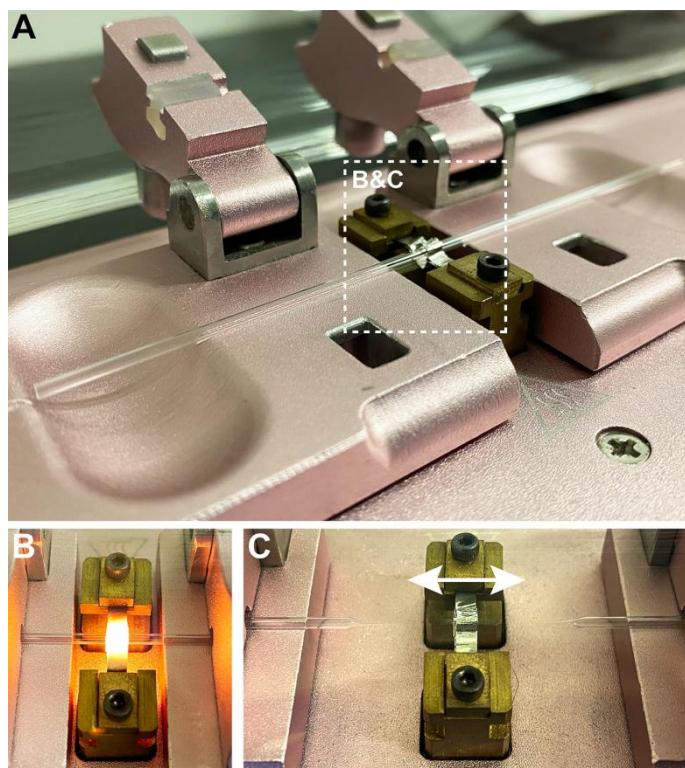
C. Needle and microinjector set-up

Review the manufacturer's recommendations and instructions for the micro-needle puller and the microinjector before operating.

1. Use a micropipette needle puller to make a fine microinjection needle (refer to Table 1 and Figure 1). Microelectrode puller program (see Table 1)

Table 1. 4-step protocol for glass capillary tube pulling

Step	Heat	Force	Distance	Delay
1	690	260	7.3	0
2	500	240	0.5	4
3	500	230	0.5	10
4	380	240	0.5	20

**Figure 1. Pulling microinjection needle.**

A. Capillary tube loaded into micro-needle puller. B. Magnified view of heated filament surrounding glass capillary. C. Creation of 2 microinjection needles from pulling (double-sided white arrow).

2. Load 3 μ L of injection material into the wide end of the microcapillary glass tube.
3. Shake the capillary tube to bring the injection material to the tip of the needle and to remove any air bubbles.
4. Turn on the air source and the microinjector, and transfer the needle into the microinjector.
5. Break the tip of the microinjection needle using forceps to an extent that allows the injection materials to release consistently and minimize the needle puncture on the target tissue.
6. Adjust the pressure on the pneumatic pump and the location of microinjection syringe pump to ensure consistent 0.5 nL per foot-pedal kick of injection so each injection consists of two kicks per larval fish for a total of 1 nL.
Size of each injection kick can be estimated by injecting into air which creates a spherical drop at the needle tip. The diameter of the liquid sphere of about 100 μ m is estimated using a stage micrometer to give 0.5 nL volume.
7. Adjust the micromanipulator so that the needle can reach the center of the stage and touch the base so that the needle can reach any mounted larvae in your field of view under microscope.

D. Mounting zebrafish for microinjections

1. Use a microwave to melt 1.5% low-melt agarose (for a short time, 10-20 s). See Recipes for low-melt agarose preparation.
2. Use a plastic transfer pipette to transfer multiple larvae at 3 dpf to the center of a 10 cm Petri dish lid. Many larvae can be transferred at once upwards of 10-15 larvae.
3. Remove as much fish water as possible to leave only minimal water around fish as to not dehydrate them, then slowly pipette in lukewarm low melt agarose and gently swirl the larvae to mix them into the agarose (or by pipetting them up and down 1-2 times).
 - a. The agarose will be very hot immediately after heating. To prevent scalding and killing the larvae, wait until the agarose is not hot to the touch, but still warm and fluid. The low-melt agarose will remain fluid at 37°C and set rapidly below 25°C. So you want to aim to use the agarose at the temperature between 30°C and 40°C. You can also keep the agarose on a heat block at 60°C so it remains in solution for a longer period of time as you work on mounting the larvae. You can expedite the cooling process by pipetting hot agarose up and down on a clean surface (such as in another dish).
 - b. The goal is to have the larvae embedded in a thin layer of agarose so that when it comes to using the fine glass needle for injection, the needle does not need to penetrate through much gel to reach your desired tissue target (see Figure 2). Too much agarose will make it hard to direct the needle to your target site without having it bend or swerve in the agarose.

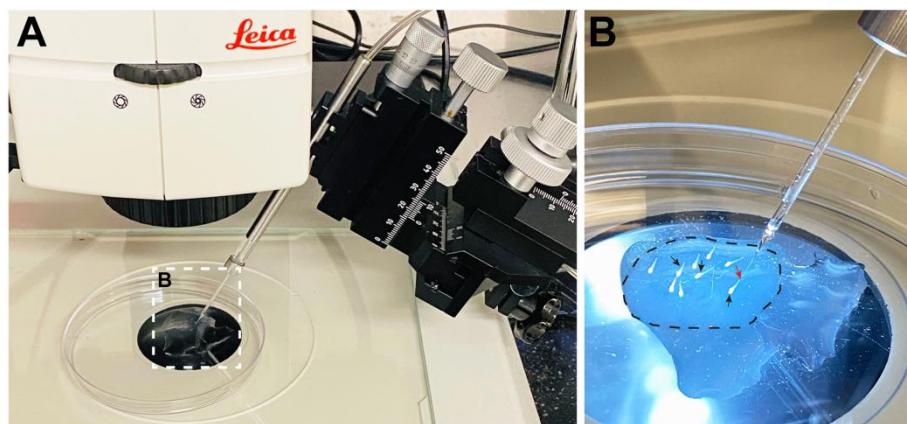


Figure 2. Set-up of zebrafish larval microinjections.

A. Mounted larvae are placed under a fluorescent stereomicroscope adjacent to a microinjection needle attached to a micro-manipulator. B. Higher magnification of mounted larvae. Dotted circle shows solidified thin layer of low-melt agarose covered with a small pool of system water supplemented with PTU. Black arrows point to larvae. Red arrow points to tip of needle (Note: This arrow is not pointing to the injection site.)

4. The most time-sensitive step is in orienting all the zebrafish larvae quickly in the agarose before it begins to re-solidify. You can expect to have less than 1-2 min for this step. Use fine forceps to quickly orient each one on their sides, but without concern of the exact orientation or body alignment, only that the tail region containing the caudal vein plexus is accessible to your needle. Take caution to not poke the larvae to cause injury but to use the agarose around the larvae to move them.
5. Wait for agarose around the larvae to cool down and solidify before starting injections.
6. Add a small amount of fish system water to cover the agarose to prevent dehydration and keep the larvae healthy during the operation (see Figure 2).

E. Intravenous microinjections

1. Use the foot pedal to inject 1 nL of clodronate liposomes or control liposomes into caudal vein plexus, which is located in the tail tissue right past the yolk extension (see Graphical abstract).
2. Validate each injection by seeing an immediate distribution of the injected fluorescent dextran mix into the heart and subsequent circulation throughout the body vasculature using a fluorescent stereomicroscope. Any larvae after injection that do not show the correct distribution of the fluorescent marker in the body vasculature is removed from the dish using forceps.
3. Gently recover the injected fish from the agarose into fresh fish water supplemented with 0.003% PTU and transfer them back into the 28.5°C incubator.
Use the side of the forceps to create a break in the agarose beginning underneath the head and dragging the forceps along the body until the tip of the tail is reached. Larvae should be able to squirm out easily and swim into the system water surrounding the agarose. Pipette released larval zebrafish into the dish with fresh fish water.
4. After microinjection, monitor all the fish for normal health and behavior. Remove any sickly or abnormal fish, and only continue processing the healthy and normal larvae.

F. Neutral red staining

1. In a Petri dish, stain a subset of your injected larvae at 48 hpi (hours post injection) with neutral red at 2.5 µg/mL in fish water supplemented with 0.003% PTU at room temperature for 45 min to 1 h (see Figure 3A).
2. Remove the majority of the neutral red supplemented fish water by exchanging it for fresh new fish water supplemented with PTU (see Figure 3B).
3. Leave the larvae in incubator for another 2-3 h before analyzing under a stereomicroscope.
4. Before analyzing, anesthetize larvae with tricaine at 0.5× strength, which the larvae can stay in for up to 45 min with the ability to fully recover back to normal and moving again. For the anesthetics to be working, larvae should not be moving so you can use them for your analysis.

Note: Please follow your institutional guidelines on the appropriate protocol for anesthetizing zebrafish.

5. Mount larvae in 3% methyl cellulose to position the larvae for analyzing and imaging under a stereomicroscope with a color camera. The larvae should be mounted brain side up to image the microglia under an upright stereomicroscope (see Figure 3C-3E).

Notes:

- a. You can mount larvae on a dish or any flat platform to visualize them under a stereoscope.
- b. Because methyl cellulose is highly viscous and these larvae were temporarily anesthetized, they remain sufficiently still for quick imaging without further anesthesia in only methyl cellulose for at least up to 10 min.
- c. Depending on the endpoint of the experiment, these larvae can be directly processed after imaging for genotyping, or recovered by washing them 1-2 times in clean fish water to remove the methyl cellulose at which point they can return to their growing chamber (an incubator) or fixed for further analysis. Fish larvae should remain well and alive during this entire period up to the endpoint processing.

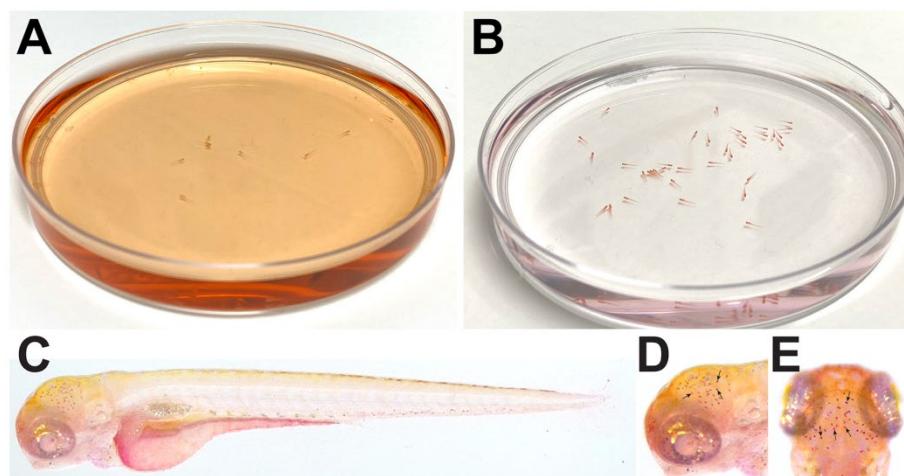


Figure 3. Neutral red analysis.

A. Color of fish water after addition of neutral red. B. Color of fish and water after removal of neutral red. C. Full body image of control uninjected 4 dpf larvae after neutral red staining. D and E. Lateral (D) and dorsal (E) view of neutral red staining. C-E. Larvae imaged in 3% methyl cellulose. Black arrows point to individual microglial cells.

Data analysis

The intravenous administration of clodronate liposomes offer the ability to deplete macrophages throughout the body, including microglia and peripheral macrophages, without the maintenance of a transgenic line. Analyze the efficiency of clodronate-mediated macrophage depletion by comparing the number of microglia in the brains of liposomal clodronate injected group with the control liposomal vehicle or control uninjected group using the neutral red vital dye assay at 48 hpi (see Figure 4–Figure Supplement 2 in Yang *et al.*, 2020).

Recipes

1. 50× PTU stock (1 L)

- Dissolve 1.5 g N-Phenylthiourea (PTU) in 1 L of distilled water by stirring overnight at room temperature.
- Aliquot and store concentrated stock at -20°C.
- To inhibit pigmentation in developing embryos, use at a 1× concentration in fish water and replace daily.
- 1× is 0.003% [weight (g) by volume (per 100 mL)] in fish water.

2. 25× Tricaine stock solution (100 mL)

Add 400 mg of Tricaine powder (3-amino benzoic acid ethyl ester) to 100 mL of distilled water. Store concentrated stock at -20°C. To use tricaine as an anesthetic, use at a 0.5-1.0× concentration.

3. 3% Methyl cellulose

Note: You will need 3 g of methyl cellulose powder in 100 mL of distilled water.

- To dissolve methyl cellulose powder in water, bring half of the distilled water to 60°C. The remaining volume of water should be stored at 4°C and left to cool for 30 min.

- b. Add 3 g of methyl cellulose powder to boiling water and stir. The mixture will become extremely viscous quickly.
- c. Remove the solution from the heat as soon as stirring becomes difficult.
- d. Add the chilled water and continue to stir for 30 s.
- e. To fully dissolve particles and remove bubbles the solution must be placed at -20°C overnight. To prevent glass from breaking, gradually lower the temperature by placing glass at 4°C for 1-2 h before placing at -20°C overnight.

4. 1,000× neutral red dye solution

Dissolve neutral red powder in distilled water at a concentration of 2.5 mg/mL for a 1,000× stock. For live staining of microglia in zebrafish, use neutral red at 1× strength.

5. 1.5% low melt agarose

- a. Dissolve 1.5 g of low melt agarose in 100 mL distilled water using the microwave for less than 30 s.
- b. Monitor the heating process as low melt agarose can easily overheat and bubble over.
- c. Once this solution is made, it can be stored in a glass bottle with a lid at room temperature and be repeatedly re-used by re-melting the agarose in the microwave.

Acknowledgments

The graphical abstract was created with BioRender.com. This protocol accompanies the publication (Yang *et al.*, 2020). The work was funded by NIH NIGMS grant 1R35GM124719 to C.E.S.

Competing interests

The authors declare no competing interests.

Ethics

Animal experimentation: This study was performed in strict accordance with the approved institutional animal care and use committee (IACUC) protocols (#16-160 and #19-132) of the UNC Chapel Hill.

References

- Bernut, A., Herrmann, J. L., Kiss, K., Dubremetz, J. F., Gaillard, J. L., Lutfalla, G. and Kremer, L. (2014). [Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation](#). *Proc Natl Acad Sci U S A* 111(10): E943-952.
- Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K. and Matthews, L. *et al.* (2013). [The zebrafish reference genome sequence and its relationship to the human genome](#). *Nature* 496(7446): 498-503.
- Hua, L., Shi, J., Shultz, L. D. and Ren, G. (2018). [Genetic models of macrophage depletion](#). *Methods Mol Biol* 1784: 243-258.
- Jeong, J. Y., Kwon, H. B., Ahn, J. C., Kang, D., Kwon, S. H., Park, J. A. and Kim, K. W. (2008). [Functional and developmental analysis of the blood-brain barrier in zebrafish](#). *Brain Res Bull* 75(5): 619-628.
- Kameka, A. M., Haddadi, S., Jamaldeen, F. J., Moinul, P., He, X. T., Nawazdeen, F. H., Bonfield, S., Sharif, S., van Rooijen, N. and Abdul-Careem, M. F. (2014). [Clodronate treatment significantly depletes macrophages in](#)

- [chickens](#). *Can J Vet Res* 78(4): 274-282.
- Karlsson, J., von Hofsten, J. and Olsson, P. E. (2001). [Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development](#). *Mar Biotechnol (NY)* 3(6): 522-527.
- Lam, S. H., Chua, H. L., Gong, Z., Lam, T. J. and Sin, Y. M. (2004). [Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study](#). *Dev Comp Immunol* 28(1): 9-28.
- Li, L., Jin, H., Xu, J., Shi, Y. and Wen, Z. (2011). [Irf8 regulates macrophage versus neutrophil fate during zebrafish primitive myelopoiesis](#). *Blood* 117(4): 1359-1369.
- O'Brown, N. M., Megason, S. G. and Gu, C. (2019). [Suppression of transeytosis regulates zebrafish blood-brain barrier function](#). *Elife* 8: e47326.
- Ponzoni, M., Pastorino, F., Di Paolo, D., Perri, P. and Brignole, C. (2018). [Targeting macrophages as a potential therapeutic intervention: impact on inflammatory diseases and cancer](#). *Int J Mol Sci* 19(7).
- Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T. X., Look, A. T. and Kanki, J. P. (2005). [Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish](#). *Dev Cell* 8(1): 97-108.
- Rosowski, E. E. (2020). [Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish](#). *Dis Model Mech* 13(1): dmm041889.
- Santoriello, C. and Zon, L. I. (2012). [Hooked! Modeling human disease in zebrafish](#). *J Clin Invest* 122(7): 2337-2343.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis](#). *Nat Methods* 9(7): 676-682.
- Shiau, C. E., Kaufman, Z., Meireles, A. M. and Talbot, W. S. (2015). [Differential requirement for irf8 in formation of embryonic and adult macrophages in zebrafish](#). *PLoS One* 10(1): e0117513.
- Tenor, J. L., Oehlers, S. H., Yang, J. L., Tobin, D. M. and Perfect, J. R. (2015). [Live imaging of host-parasite interactions in a zebrafish infection model reveals cryptococcal determinants of virulence and central nervous system invasion](#). *mBio* 6(5): e01425-01415.
- van Rooijen, N. and Hendrikx, E. (2010). [Liposomes for specific depletion of macrophages from organs and tissues](#). *Methods Mol Biol* 605: 189-203.
- Yang, L., Jimenez, J. A., Earley, A. M., HamLin, V., Kwon, V., Dixon, C. T. and Shiau, C. E. (2020). [Drainage of inflammatory macromolecules from the brain to periphery targets the liver for macrophage infiltration](#). *Elife* 9: e58191.
- Yoder, J. A., Nielsen, M. E., Amemiya, C. T. and Litman, G. W. (2002). [Zebrafish as an immunological model system](#). *Microbes Infect* 4(14): 1469-1478.

A Potent Vaccine Delivery System

Guangzu Zhao¹, Armira Azuar¹, Istvan Toth^{1, 2, 3, *} and Mariusz Skwarczynski^{1, *}

¹School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Australia

²Institute for Molecular Bioscience, The University of Queensland, St Lucia, Australia; ³School of Pharmacy, The University of Queensland, Woolloongabba, Australia

*For correspondence: i.toth@uq.edu.au; m.skwarczynski@uq.edu.au

Abstract

Most vaccines require co-delivery of an adjuvant in order to generate the desired immune responses. However, many currently available adjuvants are non-biodegradable, have limited efficacy, and/or poor safety profile. Thus, new adjuvants, or self-adjuvanting vaccine delivery systems, are required. Here, we proposed a self-adjuvanting delivery system that is fully defined, biodegradable, and non-toxic. The system is produced by conjugation of polyleucine to peptide antigen, followed by self-assembly of the conjugate into nanoparticles. The protocol includes solid-phase peptide synthesis of the vaccine conjugate, purification, self-assembly and physicochemical characterization of the product. Overall, this protocol describes, in detail, the production of a well-defined and effective self-adjuvanting delivery system for peptide antigens, along with tips for troubleshooting.

Keywords: Poly (hydrophobic amino acid), Delivery system, Subunit vaccine, Peptide synthesis, Particle self-assembly, Self-adjuvanting nanoparticles, Polyleucine, Secondary structure

This protocol was validated in: Sci Adv (2020), DOI: 10.1126/sciadv.aax2285

Background

Peptide subunit vaccines, which use the small antigen fragments (epitopes) to trigger protective immune responses against infectious diseases, are one of the most promising vaccine technologies to have emerged in recent decades (Skwarczynski and Toth, 2016; Malonis *et al.*, 2020). However, as peptides, themselves, are always poorly immunogenic, they need to be co-administered with an adjuvant (immune stimulator) and/or delivery system (Azmi *et al.*, 2014; Nevagi *et al.*, 2018). Currently, only a few options exist when it comes to adjuvants that are safe enough to be administered to humans. While more numerous in options, experimental adjuvants are often poorly defined, toxic, or have limited efficacy (Shi *et al.*, 2019). One of the most recent strategies developed to deliver vaccines utilizes nanostructures with self-adjuvanting properties (Skwarczynski and Toth, 2014). Self-assembling polymers, in particular, have been widely investigated (Zhao *et al.*, 2017; Nevagi *et al.*, 2019). However, the structures of these polymers are not fully defined (number of units, stereochemistry) and, therefore, batch variability may affect vaccine activity and safety profile.

We have conceptualized, designed, and developed a new vaccine adjuvant/delivery system to overcome the disadvantages outlined above. This system is based on fully-defined and biodegradable polymers built from our own natural hydrophobic amino acids. The lead vaccine candidate produced based on this system was able to stimulate the production of highly opsonic antibodies against six clinical isolate strains of group A streptococcus in mice (Skwarczynski *et al.*, 2020). The compound was more efficient than the powerful, but toxic, “gold standard” Complete Freund’s Adjuvant and did not induce undesired inflammatory responses. The strategy to deliver antigenic epitopes attached to self-adjuvanting amino acid-based polymer described here offers an attractive, safe alternative to conventional vaccine adjuvants. Importantly, this approach can be fully customized to match the properties of the antigen of choice. The procedure on how to produce this vaccine candidate (Figure 1) is presented here, with reference to the published vaccine study (Skwarczynski *et al.*, 2020). Notes provide additional helpful information.

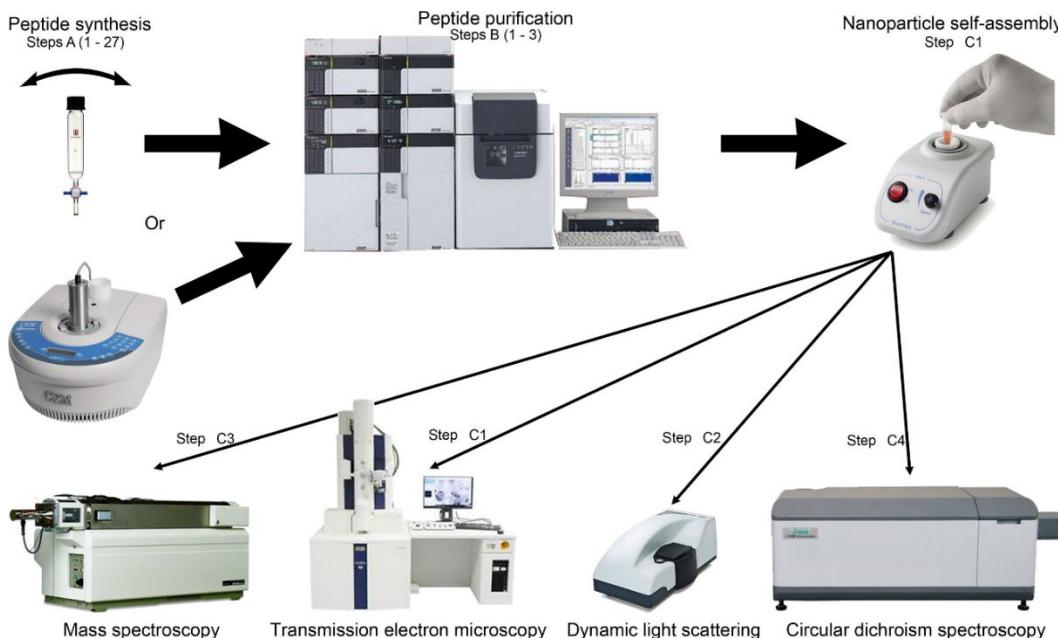


Figure 1. Flowchart of vaccine candidate synthesis, purification and characterization steps

Materials and Reagents

Note: All chemicals should be analytical grade, unless stated otherwise.

A. Vaccine candidate synthesis

1. Chemical resistance gloves (Ansell, catalog number: 02-100)
2. Rink amide p-methyl-benzhydrylamine hydrochloride (pMBHA·HCl) resin (substitution: 0.59 mmol/g; 100-200 mesh; Peptides International, catalog number: RMB-1045-PI)
3. N,N-dimethylformamide (DMF; Merck, catalog number: 227056) (see Note 1)
4. N,N-diisopropylethylamine (DIPEA; 6.2 equivalent; Merck, catalog number: 387649)
5. Trifluoroacetic acid (TFA; Merck, catalog number: 302031)
6. Butyloxycarbonyl (Boc)-protected L-amino acids (0.84 mmol/g; 4.2 equivalent; Novabiochem Merck Chemicals and Mimotopes)
7. 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 0.5 M; 4 equivalent; Mimotopes, catalog number: 148893-10-1) solution: 9.5 g HATU in 50 mL DMF (store solution at 0 °C for no longer than 1 week after preparation) (see Note 2)
8. Capping solution: 5% acetic anhydride (Sigma-Aldrich, catalog number: 320102), 5% DIPEA, and 90% DMF (v/v/v)
9. Dichloromethane (DCM; Merck, catalog number: 270997)
10. Piperidine deprotection solution: 20% piperidine (Sigma-Aldrich, catalog number: 8.22299) and 80% DMF (v/v)
11. Methanol (Merck, catalog number: 34860)
12. p-Cresol (Sigma-Aldrich, catalog number: C85751)
13. Hydrofluoric acid (HF; Ghc Gerling, Holz & Co. Handels gmbh, catalog number: 3100, Hydrogen Fluoride [99.95%])
14. Diethyl ether (Sigma-Aldrich, catalog number: 91238)
15. n-hexane (Merck, catalog number: 1.04367)
16. Acetonitrile (Merck, catalog number: 271004)
17. Endotoxin-free Milli-Q water (sensitivity of 18.2 MΩ.cm at 25 °C and total organic content below 5 parts per billion)
18. Solvent A: 100% Milli-Q water and 0.1% TFA (v/v; solution can be stored at room temperature for up to 3 months)
19. Solvent B: 90% acetonitrile, 10% Milli-Q water, and 0.1% TFA (v/v/v; solution can be stored at room temperature for up to 3 months)

B. Vaccine candidate purification

1. Phenex syringe filter (0.45 µm; Phenomenex, catalog number: AF3-3107-52)
2. Reagents listed previously (Solvent A and B)

C. Vaccine candidate characterization

1. Disposable capillary cuvettes (Malvern Analytical, model: DTS1070)
2. Whatman filter paper (Merck, catalog number: WHA1005090)
3. Phosphate-buffered saline (PBS; ThermoFisher Scientific, catalog number: 10010031)
4. Phosphotungstic acid (2%): 2 mg phosphotungstic acid hydrate (Sigma-Aldrich, catalog number: P4006-25G) in 100 mL Milli-Q water (stir for 1 h, then filter; solution can be stored at 2-8 °C for up to 3 months)

Equipment

A. Vaccine candidate synthesis

1. Laboratory glassware
2. CEM Discover Solid Phase Synthesis (SPS) reactor (CEM Corporation, model: Discover SPS; see Software 1) (see Note 3)
3. Peptide synthesis vessel (CEM Corporation, catalog number: 170897) (see Note 4)
4. Glass peptide synthesis vessel (Sigma-Aldrich, catalog number: Z41,850-1)
5. CEM Discovery SPS vacuum manifold filtration apparatus (CME Corporation, catalog number: 167993) (see Note 4)
6. Scintillation vials (Merck, catalog number: DWK986568)
7. Vortex mixer (Phoenix Instruments, model: RS-VA 1) or sonicator (Baranson Ultrasonicator Corporation, catalog number: 2510E-MTH)
8. Rotary mixer (Ratek Instruments, catalog number: RSM7DC)
9. Desiccator
10. Hydrofluoric acid (HF)-reaction apparatus (including HF reaction vessel) for peptide cleavage from the resin (refer to Jadhav *et al.*, 2020)
11. Alpha 2-4 LD freeze dryer (John Morris Scientific, catalog number: 101521)

B. Vaccine candidate purification

1. Shimadzu preparative reverse-phase HPLC (RP-HPLC) instrument (Shimadzu, models: LC-20AP × 2, CBM-20A, SPD-20A, FRC-10A) with a 20.0 mL/min flow rate (see Software 2)
2. Vydac C4 (Hichrom, catalog number: 214TP54, 5 µm, 4.6 × 250 mm; and 214TP1022, 10 µm, 22 × 250 mm) or C8 columns (Hichrom, catalog number: 208TP54; 5 µm, 4.6 × 250 mm)
3. Perkin-Elmer-Sciex API3000 electrospray ionization mass spectrometry (ESI-MS) instrument (Applied Biosystems/MDS Sciex, model: Sciex API3000; see Software 3)
4. Shimadzu analytical RP-HPLC instrument (Shimadzu, models: DGU-20A5, LC-20AB, SIL-20ACHT, SPD-M10AVP) with a 1.0 mL/min flow rate (see Software 2)

C. Vaccine candidate characterization

1. Malvern Zetasizer dynamic light scattering (DLS; Malvern Instruments, model: Nano ZS; see Software 4)
2. JEM-1010 transmission electron microscope (TEM; JEOL, see Software 5)
3. Carbon-coated copper grids (Pure Carbon Film 200 mesh, Ted Pella, catalog number: 01840-F)
4. Jasco J710 circular dichroism (CD) spectrometer (JASCO Corporation, model: J710; see Software 6)
5. CD 1.0 mm cell (Starna, catalog number: 21/Q/1/CD)

Software

1. SynergyTM (CME Corporation, North Carolina, USA, www.cemsynthesis.com)
2. LabSolutions (Shimadzu, Kyoto, Japan, www.shimadzu.com)
3. Analyst[®] 1.6 (Applied Biosystems/MDS Sciex, Toronto, Canada, www.scix.com)
4. Malvern Zetasizer Analyzer 6.2 (Malvern Instruments, Worcestershire, UK, www.malvernpanalytical.com)
5. Olympus Soft Imaging Solutions (Olympus Corporation, Tokyo, Japan, www.olympus-global.com)
6. Spectra ManagerTM (JASCO Corporation, Tokyo, Japan, www.jascoinc.com)

Procedure

A. Vaccine candidate synthesis (see Note 5)

- Resin swelling:** Weight out 339 mg pMBHA•HCl resin (0.2 mmol equivalent) into a peptide synthesis vessel and add 10 mL of DMF and 0.216 mL of DIPEA (see Note 6). Let the resin swell for at least 2.5 h (see Note 7).
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times using DMF (~5 mL per wash; see Notes 8-9).
- Boc deprotection** (see Note 9): Drain all of the solvent before adding ~5 mL of neat TFA. Stir the resin gently for 2 min with a stirring rod (see Note 10).
- Drain the TFA, and repeat Step A3.
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 mL per wash; see Notes 8-9).
- Amino acid activation:** Weigh out each amino acid in scintillation vials. Add 1.6 mL of HATU and 0.181 mL of DIPEA (see Note 11).
- Amino acid coupling:** Drain all of the solvent off before adding the preactivated amino acid to the washed resin and stir gently (see Note 12). Insert the vessel, with temperature probe, into the microwave and heat for 5 min at 70°C (see Note 13).
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times with DMF (~5 mL per wash; see Note 8).
- Repeat Steps A6-A7 for the second coupling (10 min at 70°C).
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 mL per wash; see Note 8).
- Acetylation:** Drain the solvent completely, then add 5 mL of capping solution to the resin. Stir gently, then insert the vessel, with temperature probe, into the microwave and heat the mixture for 10 min at 70°C (see Note 14).
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times with DMF (~5 mL per wash; see Note 8).
- Repeat Step A11 for the second acetylation.
- Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 mL per wash; see Note 8).
- Repeat Steps A2-A10 for the following amino acid sequence, until the **Peptide 1** (Figure 2) sequence is finished (Table 1, entry 1-42).

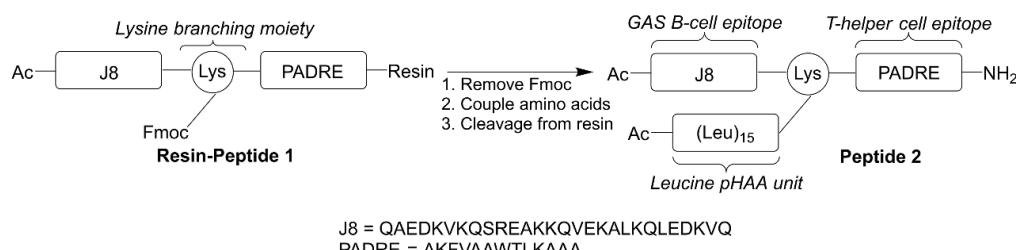


Figure 2. Synthesis of the vaccine candidate Peptide 2, which is comprised of (i) GAS J8 B-cell epitope, and (ii) PADRE T-helper cell epitope, branched with (iii) a leucine poly(hydrophobic amino acid) (pHAA) unit via lysine linker

Table 1. List of amino acids used for the synthesis of vaccine candidate Peptide 2

PADRE	1	Boc-Ala-OH	A	J8 (continue)	30	Boc-Lys(2Cl-Z)-OH	K
	2	Boc-Ala-OH	A		31	Boc-Ala-OH	A
	3	Boc-Ala-OH	A		32	Boc-Glu(OcHx)-OH	E
	4	Boc-Lys(2Cl-Z)-OH	K		33	Boc-Arg(Tos)-OH	R

	5	Boc-Leu-OH H ₂ O	L		34	Boc-Ser(Bzl)-OH	S
	6	Boc-Thr(Bzl)-OH	T		35	Boc-Gln-OH	Q
	7	Boc-Trp(For)-OH	W		36	Boc-Lys(2Cl-Z)-OH	K
	8	Boc-Ala-OH	A		37	Boc-Val-OH	V
	9	Boc-Ala-OH	A		38	Boc-Lys(2Cl-Z)-OH	K
	10	Boc-Val-OH	V		39	Boc-Asp(OcHx)-OH	D
	11	Boc-Phe-OH	F		40	Boc-Glu(OcHx)-OH	E
	12	Boc-Lys(2Cl-Z)-OH	K		41	Boc-Ala-OH	A
	13	Boc-Ala-OH	A		42	Boc-Gln-OH	Q
Branching moiety	14	Boc-Lys(Fmoc)-OH	K		43	Boc-Leu-OH H ₂ O	L
J8	15	Boc-Gln-OH	Q		44	Boc-Leu-OH H ₂ O	L
	16	Boc-Val-OH	V		45	Boc-Leu-OH H ₂ O	L
	17	Boc-Lys(2Cl-Z)-OH	K		46	Boc-Leu-OH H ₂ O	L
	18	Boc-Asp(OcHx)-OH	D		47	Boc-Leu-OH H ₂ O	L
	19	Boc-Glu(OcHx)-OH	E		48	Boc-Leu-OH H ₂ O	L
	20	Boc-Leu-OH H ₂ O	L		49	Boc-Leu-OH H ₂ O	L
	21	Boc-Gln-OH	Q		50	Boc-Leu-OH H ₂ O	L
	22	Boc-Lys(2Cl-Z)-OH	K		51	Boc-Leu-OH H ₂ O	L
	23	Boc-Leu-OH H ₂ O	L		52	Boc-Leu-OH H ₂ O	L
	24	Boc-Ala-OH	A		53	Boc-Leu-OH H ₂ O	L
	25	Boc-Lys(2Cl-Z)-OH	K		54	Boc-Leu-OH H ₂ O	L
	26	Boc-Glu(OcHx)-OH	E		55	Boc-Leu-OH H ₂ O	L
	27	Boc-Val-OH	V		56	Boc-Leu-OH H ₂ O	L
	28	Boc-Gln-OH	Q		57	Boc-Leu-OH H ₂ O	L
	29	Boc-Lys(2Cl-Z)-OH	K				

After coupling the final amino acid of **Peptide 1**, repeat Steps A2-A5 (washing and Boc deprotection), then proceed with Steps A11-A14 (**Peptide 1** acetylation at the N-terminus; see Note 15).

16. **Fmoc group deprotection:** Drain all of the solvent, then add 5 mL of piperidine deprotection solution to the resin and stir gently. Insert the vessel and temperature probe into the microwave. Heat to 70°C for 2 min.
17. **Resin washing:** Drain the solvent using vacuum filtration, then wash the resin three times with DMF (~5 mL per wash; see Note 8).
18. Repeat Step A16 for the second Fmoc group deprotection (5 min at 70°C; see Note 16).
19. **Resin washing:** Drain the solvent using vacuum filtration, then wash the resin five times with DMF (~5 mL per wash; see Note 8).
20. Repeat Steps A6-A10 for the first leucine in the pHAs.
21. Repeat Steps A2-A10 for the remaining leucine's, until the **Peptide 2** (Figure 2) sequence is complete (Table 1, entry 43-57).
22. After the final amino acid coupling of **Peptide 2**, repeat Steps A2-A5 (washing and Boc deprotection), then proceed with Steps A11-A14 (**Peptide 2** acetylation at the N-terminus; see Note 15).
23. **Resin drying:** Drain the solvent using vacuum filtration, then wash the resin using ~5 mL of DMF (three times), DCM (three times), then methanol (once). Remove all solvent and leave the resin under vacuum filtration for 5 min.

24. Leave the vessel with the resin in a desiccator under reduced pressure overnight to fully remove the solvents.
25. Measure the finished resin-**Peptide 2**, then weigh out 500 mg of the resin into a 15 mL Falcon tube (see Note 17).
26. **Peptide cleavage:** Transfer the 500 mg of resin into a HF reaction vessel, then add 0.25 mL of p-cresol scavenger (0.5 mL/g of resin) and 5 mL of HF (10 mL/g of resin). Cleave the peptide from the resin following the HF-cleavage protocol (see Note 18 and Jadhav *et al.*, 2020).
27. **Reaction work up:** Wash the resin with 30 mL of cold diethyl ether:n-hexane (4:1; v/v) twice, then remove the solution through filtration. Dissolve the white precipitate using 50% Solvent A and 50% Solvent B (v/v), then filter the solution (from the resin) into a round-bottom flask. Wash the resin with Solvent B and collect the filtrate into a round-bottom flask. Freeze-dry the filtrate to obtain a yellowish-white powder (crude **Peptide 2**).

B. Vaccine candidate purification

1. **Purification** (see Note 19): Dissolve 30 mg crude **Peptide 2** in 2 mL of 50% Solvent A and 50% Solvent B (v/v). Load the solution into a 5 mL syringe and filter the solution through syringe-filter into a scintillation vial. Wash the filter with 2 mL of 50% Solvent A and 50% Solvent B (v/v). Run the filtered crude **Peptide 2** using preparative RP-HPLC on a C4 column with solvent B gradients (65-85%) for 25 min, with compound detection at 214 nm.
2. Analyze the fractions of the purified **Peptide 2** using ESI-MS and analytical RP-HPLC on a C4 column with a 0-100% gradient of solvent B for 40 min and compound detection at 214 nm (see Note 20).
3. Combine and freeze-dry the fraction based on its purity (as analyzed by ESI-MS and analytical RP-HPLC; Figure 3; Note 21). The final product should be a white solid. Molecular weight: 6521.03. ESI-MS $[M + 4H]^{4+}$ m/z 1631.8 (calc. 1631.3), $[M + 5H]^{5+}$ m/z 1305.6 (calc. 1305.2), $[M + 6H]^{6+}$ m/z 1088.0 (calc. 1087.8), $[M + 7H]^{7+}$ m/z 932.9 (calc. 932.6), $[M + 8H]^{8+}$ m/z 816.3 (calc. 816.1), $[M + 9H]^{9+}$ m/z 725.6 (calc. 725.6). $t_R = 30.9$ min (0 to 100% solvent B; C4 column); purity $\geq 99\%$.

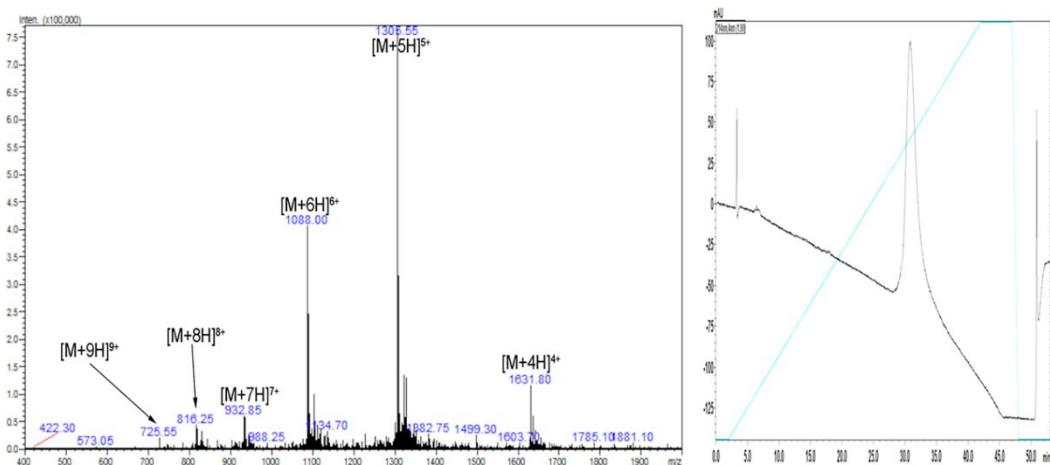


Figure 3. Analysis of the purified vaccine candidate Peptide 2 by ESI-MS (left) and analytical RP-HPLC (right)

C. Vaccine candidate characterization

1. Measure 1.5 mg of pure **Peptide 2** into a 2 mL microcentrifuge tube. Add 1 mL of PBS to prepare a 1.5 mg/ml solution (concentration of the vaccine candidate for the *in vivo* study). Vortex the mixture for 2-30 min (with 1-min intervals) until none of the solid peptide is visible (see Note 22).

2. Size and PDI:
 - a. Transfer 0.5 mL of self-assembled vaccine candidate (from Step C1) into a new 2 mL microcentrifuge tube, then add 1 mL of PBS to prepare a 0.5 mg/ml solution (1:3 dilution) for DLS analysis.
 - b. Turn on the Malvern Zetasizer DLS, open the Analyzer software, and connect with the instrument before starting measurements.
 - c. Fill the disposable cell with diluted solution (~800 μ L) and insert the cell into the instrument.
 - d. Perform measurements (size, PDI) at 25°C with non-invasive backscatter at a backscattering angle of 173°. Correlation times are based on 10 s per run; at least 10 consecutive runs should be made per measurement. Take at least five measurements per sample, and measure PBS as a blank (negative control).
 - e. Calculate the mean \pm standard deviation for each sample using the five measurements.
 - f. In general, compounds should self-assemble into a mixture of small nanoparticles (10-30 nm) and larger aggregates with high polydispersity indexes (PDI > 0.3, according to DLS).
3. Morphology:
 - a. Pipette 5 μ L of the 1:3 diluted solution (the same as what was used for DLS) onto a glow-discharged carbon-coated copper grid and leave for 2 min to dry (see Note 23).
 - b. Gently drain the excess liquid with a piece of filter paper and allow the grid to dry.
 - c. Add one drop (~5 μ L) of 2% phosphotungstic acid to the dried grid for 20 s to negatively-stain the sample, then drain the excess stain solution off with a piece of filter paper.
 - d. Air-dry the grid for 5 min before observing it under TEM. Take images at an accelerating voltage of 100 kV (Figure 4).
 - e. Distinct nanoparticles and chain-like aggregates of nanoparticles (CLAN) should be visible in **Peptide 2** when viewed under TEM (Figure 4).

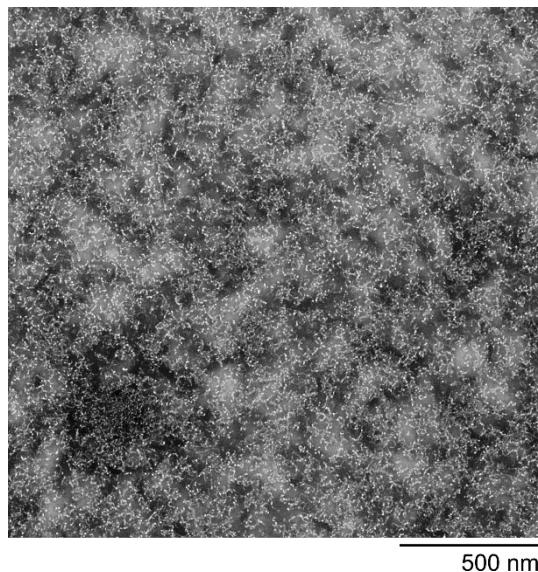


Figure 4. Particle image of vaccine candidate Peptide 2 captured by TEM [bar 500 nm; the dark areas are a result of the negative stain (2% phosphotungstic acid)]

4. Secondary structure:
 - a. Transfer 0.1 mL of the self-assembled vaccine candidate (non-diluted sample from Step C1) into a new 2 mL microcentrifuge tube and add 1.4 mL PBS to prepare 0.1 mg/ml solution (1:15 dilution) for CD analysis (see Note 24).
 - b. Set up the Spectra Manager software with the following parameters: bandwidth, 5 nm; scan rate, 50 nm/min; response time, 2 s; interval, 1 nm over the wavelength range of 195 to 260 nm.

- c. Insert the cell into the instrument and run a quick scan on an empty CD cell to make sure the cell is clean (the flat, horizontal line should be visible without substantial deviation from zero mdeg; see Note 25). Wash the cell if needed (see Note 26).
- d. Fill the CD cell with diluted solution (~200 μ L) and insert the cell into the instrument. Take at least six accumulations per measurement. Run PBS or water as a blank (negative control). After all measurements, clean the cell with water or methanol, only.
- e. Using Spectra Manager, subtract the baseline spectra (PBS blank reading) from the vaccine candidate spectra before converting millidegrees (mdeg) to molar ellipticity ($[\theta]$; deg \cdot cm 2 \cdot dmol $^{-1}$).
- f. The conversion is done via the following formula:

$$[\theta] = \text{mdeg}/(l \times c \times n) \times 1000$$

where:

l = path length (0.1 cm),

c = peptide concentration (mM), and

n = number of amino acids in the peptide.

- g. The vaccine candidate **Peptide 2** should adopt a helical conformation with a minimum at 222 nm and a lower-intensity minimum at 208 nm (Figure 5).

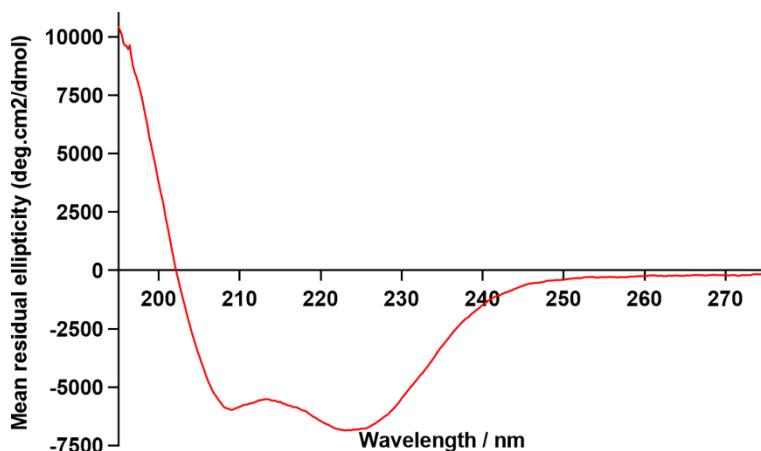


Figure 5. Circular dichroism (CD) spectra of vaccine candidate Peptide 2

Notes

1. Normal nitrile or latex gloves provide poor protection against DMF. Chemical resistance gloves should instead be worn when dealing with DMF.
2. HATU/HBTU/HCTU can induce an allergic response if it comes in contact with skin; caution should be taken when handling these chemicals.
3. The reactor is equipped with a vacuum manifold for liquid transfers and a fiber optic temperature probe. Each microwave system usually has its own complementary peptide synthesis vessels and vacuum filtration apparatus. Consult with your microwave provider for further information.
4. This peptide synthesis vessel is a 20 mL, microwave-safe, open-vessel apparatus equipped with porous frit (to allow filtration of the peptide from the resin) and a cap at the bottom. Alternatively, synthesis can be done without the assistance of a microwave (heat); a glass peptide synthesis vessel may be used instead. Draining and washing of this glass vessel need to be done through filtration under vacuum. A rotary mixer is also required to provide continuous mixing.

5. Synthesis goes from the C- to N-terminus of the peptide amino acid sequence. Make sure the sequence is correct before starting. The information provided here is calculated for a 0.2 mmol synthesis scale.
6. During resin swelling and peptide synthesis, the resin should remain submerged in DMF solvent, with solvent level at least 2 cm higher than the resin. A rotary mixer can be used to improve swelling by continuous mixing.
7. The resin can be left to swell for up to 24 h. Alternatively, insert the resin-containing vessel into the microwave, together with the temperature probe, and heat to 70°C for 10 min for fast swelling.
8. Perform each wash carefully to ensure complete removal of all reagents. Inadequate washing could result in the formation of side products during synthesis. If the microwave was used, remove the vessel from the microwave, then wash the temperature probe along with the resin and stirring rod (used in Boc deprotection).
9. Wash the resin five times with DCM (~5 mL for each wash; instead of DMF) before and after TFA deprotection of Boc from Boc-Gln(Xan)-OH. This prevents the cyclization of glutamine.
10. Alternatively, if the synthesis is done in a glass peptide synthesis vessel (for synthesis without microwave (heat) assistance), stirring can be done by placing the TFA/resin mixture on a rotary mixer for 2 min. Make sure the vessel is tightly closed on both ends after adding the TFA.
11. Each amino acid should be preactivated 2-5 min before reaction. Dissolve the amino acid fully before coupling to the resin. Use a sonicator or vortex to speed up the process.
12. Add additional DMF to the vessel to make sure the resin stays submerged.
13. Boc-amino acids are commonly heated at 70°C for 10 min during peptide synthesis. The double-couplings are done for 5 min, then 10 min. However, cystine, histidine, and arginine must be coupled for 15 min, twice, at 50°C. Alternatively, for coupling without microwave (heat) assistance, place the glass vessel onto a rotary mixer for 10 min (first coupling), then 20 min (second coupling), or 20 min each for cystine, histidine, and arginine. Make sure the vessel is tightly closed on both ends.
14. Double-acetylations are done for 10 min, each. The acetylation is performed after the first amino acid coupling to cap the unreacted site to the resin to avoid the formation of side products, which affect the purity of the final product. Do not remove Boc prior to this acetylation (non-N terminus acetylation), as it can impede any additional amino acid coupling. Alternatively, for acetylation without microwave (heat) assistance, place the glass vessel on a rotary mixer for 20 min. Make sure the vessel is tightly closed on both ends.
15. After all amino acids have been coupled, remove the Boc protective group of the final amino acid before proceeding with acetylation at the N-terminus of the peptide.
16. Double-deprotections are done for 2 min, then 5 min. Make sure that these deprotection steps are done after the acetylation of the N-terminus. The Fmoc protective group on the lysine (branching moiety) is removed using 20% piperidine. Piperidine also removes the formyl group of tryptophan (Boc-Trp(For)-OH; PADRE sequence). According to our experience, this does not trigger the production of side products. Alternatively, if the synthesis is done in a glass peptide synthesis vessel (for synthesis without microwave (heat) assistance), stirring can be done by placing the piperidine/resin mixture on a rotary mixer for 5 min (first deprotection), then 10 min (second deprotection). Make sure the vessel is tightly closed on both ends.
17. 500 mg is the maximum amount of resin for efficient HF cleavage. A 15 mL Falcon tube allows for easy transfer of the resin into a HF reaction vessel.
18. HF is highly toxic and corrosive. Follow the protocol attached to the HF apparatus precisely. If there is cysteine or methionine in the peptide sequence, add additional p-thiocresol scavenger (0.25 mL; 0.5 mL/g of resin) to the p-cresol/resin mixture. Make sure the HF is completely evaporated before proceeding to workup.
19. Make sure the peptide is fully soluble before filtration. Add a few drops of Solvent B and use a sonicator or vortex to aid solubility. Always filter the solution before running it through ESI-MS and RP-HPLC machines. **Peptide 2** is hydrophobic and needs to be run through a C4 column. More hydrophilic peptides may be purified using a C8 column for better separation. The crude compound can degrade easily and is not stable; therefore, it must be purified for long-term storage.
20. Analytical RP-HPLC graphs show pure compounds in a single peak. The mass from this peak must match the mass of the desired peptide in ESI-MS. **Peptide 2** is hydrophobic and needs to be run through a C4 column. Run more hydrophilic peptides through C8 columns (as described above).
21. Analyze the combined fraction through analytical RP-HPLC and ESI-MS to confirm purity. Keep the pure compound in powder form at -20°C to ease storage and prolong shelf-life.
22. The incorporation of a polyleucine tail increases the hydrophobicity of the compound and makes it difficult to

- dissolve. Using both sonication and vortex can help with the process. The end product should be a white, semi-cloudy or clear solution, which contains the self-assembled vaccine candidate. Vortex the solution before use.
- 23. The appropriate dilution is necessary for visualization; sample that contains too high concentration of nanoparticles will result in overlapping particles, which affects the visibility of particle morphology. Dilution ratio variability depends on the particular sample, but a concentration of 0.5 mg/ml is a good place to start.
 - 24. Dilution is required to avoid measurement with high tension (HT) > 600 V.
 - 25. A significant deviation from zero (> or < 1 mdeg) indicates that the CD cell is contaminated.
 - 26. Clean the cell by filling the cell with 5M nitric acid (nitric acid doesn't damage the quartz) and leave it for a few hours or overnight. A sonicator can be used to speed up the process. Rinse the cell with water before a final wash with methanol. Remove the contents and dry the cell completely before use or storage. Clean the outside of the cell with Kimwipes, only.

Acknowledgments

This work was supported by the National Health and Medical Research Council (Program Grant APP1132975) and an Australian Government Research Training Program (RTP) Scholarship. We acknowledge the original research paper “Poly (amino acids) as a potent self-adjuvanting delivery system for peptide-based nanovaccines”, from which this protocol was derived. We also acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland.

Competing interests

M.S., G.Z., and I.T. are co-inventors in a patent application entitled “Self assembling, self adjuvanting system for delivery of vaccines” filed by The University of Queensland (application number AU 2019900328). The remaining authors declare that they have no competing interests.

References

- Azmi, F., Ahmad Fuad, A. A., Skwarczynski, M. and Toth, I. (2014). [Recent progress in adjuvant discovery for peptide-based subunit vaccines](#). *Hum Vaccin Immunother* 10: 778-796.
- Jadhav, K. B., Woolcock, K. J. and Muttenthaler, M. (2020). [Anhydrous Hydrogen Fluoride Cleavage in Boc Solid Phase Peptide Synthesis](#). *Methods Mol Biol* 2103: 41-57
- Malonis, R., Lai, J. R. and Vergnolle, O. (2020). [Peptide-Based Vaccines: Current Progress and Future Challenges](#). *Chem Rev* 120: 3210-3229.
- Nevagi, R. J., Skwarczynski, M. and Toth, I. (2019). [Polymers for subunit vaccine delivery](#). *Eur Polym J* 114: 397-410.
- Nevagi, R. J., Toth, I. and Skwarczynski, M. (2018). [Peptide Applications in Biomedicine, Biotechnology and Bioengineering](#). Woodhead Publishing 327-358.
- Shi, S., Zhu, H., Xia, X., Liang, Z., Ma, X. and Sun, B. (2019). [Vaccine adjuvants: Understanding the structure and mechanism of adjuvanticity](#). *Vaccine* 37: 3167-3178.
- Skwarczynski, M. and Toth, I. (2014). [Recent advances in peptide-based subunit nanovaccines](#). *Nanomedicine (Lond)* 9: 2657-2669.
- Skwarczynski, M. and Toth, I. (2016). [Peptide-based synthetic vaccines](#). *Chem Sci* 7: 842-854.
- Skwarczynski, M., Zhao, G., Boer, J., Ozberk, V., Azuar, A., Cruz, J. G., Giddam, A. K., Khalil, Z., Pandey, M., Shibu, M., et al. (2020). [Poly\(amino acids\) as a potent self-adjuvanting delivery system for peptide-based nanovaccines](#). *Sci Adv* 6: eaax2285.

Zhao, G., Chandrudu, S., Skwarczynski, M. and Toth, I. (2017). [The application of self-assembled nanostructures in peptide-based subunit vaccine development](#). *Eur Polym J* 93: 670-681.

Ex vivo Assessment of Mitochondrial Function in Human Peripheral Blood Mononuclear Cells Using XF Analyzer

Alica Schöller-Mann*, Katja Matt, Barbara Hochecker, Jörg Bergemann

Department of Life Sciences, Albstadt-Sigmaringen University of Applied Sciences, Sigmaringen, Germany

*For correspondence: schoeller-mann@hs-albsig.de

Abstract

Cellular health and function, as we know today, depend on a large extent on mitochondrial function. The essential function of mitochondria is the energy production, more precisely ATP production, via oxidative phosphorylation. Mitochondrial energy production parameters therefore represent important biomarkers. Studies on human cells have mainly been performed on *in vitro* cell cultures. However, peripheral blood mononuclear cells (PBMCs) are particularly suitable for such examinations. That's why this protocol describes a method to measure key parameters of mitochondrial function in freshly isolated PBMCs with the latest technology, the XF Analyzer. For this *ex vivo* approach PBMCs are first isolated out of human anticoagulated blood. Next, they are attached to the surface of special microplates pre-coated with Poly-D-Lysine. During the subsequent measurement of oxygen consumption rate (OCR) as well as extracellular acidification rate (ECAR) the stress reagents oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone and antimycin A are injected. Several mitochondrial parameters can be calculated from the results obtained. The application of this protocol allows the analysis of various influences, such as pharmaceuticals or environmental factors, on human cells.

Keywords: Human peripheral blood mononuclear cells (PBMCs), Mitochondria, XF Analyzer, XF Cell Mito Stress Test, Oxygen consumption rate, Mitochondrial respiration, *Ex vivo*

This protocol was validated in: Sci Rep (2021), DOI: 10.1038/s41598-021-99152

Background

Mitochondria play a critical role in maintaining normal cellular function. It is now common knowledge that they not only produce ATP via oxidative phosphorylation but, for example, are also involved in the metabolism of amino acids, lipids and nucleotides, diverse signaling and redox processes as well as quality control and degradation processes including mitophagy and apoptosis (Pfanner *et al.*, 2019). However, mitochondria represent the major site of ATP synthesis in normal cells (Akbari *et al.*, 2019). For this purpose, an electrochemical proton gradient is generated across the mitochondrial inner membrane through the multi-subunit enzyme complexes I–IV. This proton gradient is used by the ATP synthase, also known as complex V, to turn ADP into ATP (Chaban *et al.*, 2014).

The process of oxidative phosphorylation is associated with the reduction of oxygen to water. Accordingly, the oxygen consumption rate of cells can be used for assessing mitochondrial function (Smolina *et al.*, 2017). This principle is the basis of Seahorse XF Analyzers (Agilent Technologies). They provide the possibility to measure not only oxygen consumption rate (OCR), but also the rate of extracellular acidification (ECAR), which is a key indicator of glycolysis. The realtime measurements are carried out in multi-well plates which are provided with solid-state sensors consisting of two fluorophores. One is quenched by oxygen (O_2) and the other one is sensitive to pH-value changes. The fluorophores are excited via light-emitting fiber optic bundles, which subsequently detect the fluorescence changes as a result of oxygen consumption or extracellular acidification (Plitzko and Loesgen, 2018). Furthermore, XF Analyzers enable up to four different injections per well during the measurement. All the properties mentioned constitute a significant advantage over the conventionally used clark-type oxygen electrodes for determining oxygen consumption.

Peripheral blood mononuclear cells (PBMCs) as sample in a XF Analyzer implies that the cells have to be attached to the surface of the microplates. Most commonly, this immobilization is done by means of protein solutions such as Cell-TakTM (Jones *et al.*, 2015; Traba *et al.*, 2016; Lee *et al.*, 2019) or Poly-D-Lysine (Hartman *et al.*, 2014; Nicholas *et al.*, 2017; Thaventhiran *et al.*, 2019). We examined both Cell-TakTM and Poly-D-Lysine and considered Poly-D-Lysine as most suitable coating method. Since we conducted the Agilent Seahorse XF Cell Mito Stress Test, optimal concentrations of the injected compounds oligomycin, FCCP, antimycin A and rotenone had to be tested as well. A typical curve of a Mito Stress Test is shown in Figure 1. Since oligomycin is an inhibitor of ATP synthase, OCR decreases after its injection. In contrast, OCR increases sharply after FCCP injection, which is an uncoupler of oxidative phosphorylation. The last injection of antimycin A and rotenone, again leads to a decline of OCR, as these two compounds inhibit complex III respectively I of the electron transport chain. The resulting curve is used to calculate various parameters of mitochondrial function (see Figure 1).

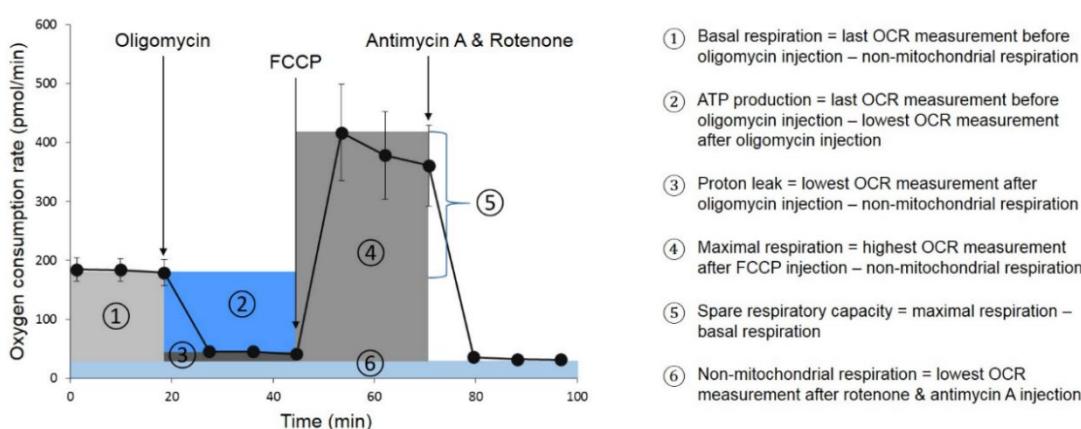


Figure 1. Assessment of mitochondrial respiration parameters by means of Agilent Seahorse XF Cell Mito Stress Test.

On the left side a typical course of an OCR measurement with injections of oligomycin after the third, FCCP after the sixth and antimycin A together with rotenone after the ninth measuring point is shown. From this curve, various parameters can be calculated, which are marked in different colors. The calculations of the parameters are shown

Cite as: Schöller-Mann, A. et al. (2021). Ex vivo Assessment of Mitochondrial Function in Human Peripheral Blood Mononuclear Cells Using XF Analyzer. Bio-protocol 11(7): e3980. DOI: 10.21769/BioProtoc.3980.

on the right side.

The protocol described in detail hereafter has been applied to examine the influence of 12 days of *in vivo* caloric reduction in humans. A significant increase of mitochondrial respiratory parameters could be detected in PBMCs of a subgroup of the test persons (Schöller-Mann *et al.*, 2020). Besides such *ex vivo* studies the protocol can be used to screen any soluble substance, including pharmaceuticals, dietary supplements or contaminants, due to their *in vitro* effects on PBMCs.

Materials and Reagents

1. 15 mL, 50 mL screw cap tubes (SARSTEDT, catalog numbers: 62.554.502; 62.547.254)
2. 1.5 mL, 2 mL reaction tubes (SARSTEDT, catalog numbers: 72.706; 72.695.500)
3. 20 µL, 200 µL, 1,000 µL pipette tips (SARSTEDT, catalog numbers: 70.1116; 70.760.002; 70.762)
4. 5 mL, 10 mL, 25 mL serological pipettes (SARSTEDT, catalog numbers: 86.1253.001; 86.1254.001; 86.1685.001)
5. 50 mL Leucosep™ tubes (Greiner Bio-One GmbH, catalog number: 227290)
6. Seahorse XF24 FluxPak containing XF24 sensor cartridges, XF24 cell culture microplates and XF Calibrant Solution (Agilent Technologies, catalog number: 100850-001)
7. Human venous blood, EDTA-anticoagulated – S-Monovette® 7.5 mL K3E (SARSTEDT, catalog number: 01.1605.001)
8. Ficoll-Paque™ PLUS (GE Healthcare, catalog number: 17-1440-03)
9. Poly-D-Lysine solution, 1.0 mg/mL (Merck Millipore, catalog number: A-003-E, storage temp. -20 °C)
10. Trypan Blue solution 0.4% (Sigma-Aldrich, catalog number: 93595)
11. Dulbecco's Modified Eagle's Medium (DMEM), high glucose (Sigma-Aldrich, catalog number: D7777, storage temp. 4°C)
12. Dimethyl sulfoxide (DMSO) (Carl Roth, catalog number: A994)
13. Oligomycin from *Streptomyces diastatochromogenes* (Sigma-Aldrich, catalog number: O4876, storage temp. -20 °C; mixture of isomers A, B, and C), 2.5 mM in DMSO
14. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich, catalog number: C2920, storage temp. 4°C), 2.5 mM in DMSO
15. Rotenone (Sigma-Aldrich, catalog number: R8875), 2.5 mM in DMSO
16. Antimycin A from *Streptomyces* sp. (Sigma-Aldrich, catalog number: A8674, storage temp. -20 °C), 2.5 mM in DMSO
17. NaCl (Carl Roth, catalog number: 3957)
18. NaOH (Carl Roth, catalog number: 6771)
19. KCl (Carl Roth, catalog number: 6781)
20. Na₂HPO₄·12H₂O (Carl Roth, catalog number: N350)
21. KH₂PO₄ (Carl Roth, catalog number: 3904)
22. 1× PBS (see Recipes)
23. 0.9% NaCl solution (see Recipes)
24. 10 M NaOH solution (see Recipes)
25. Poly-D-Lysine working solution (50 µg/mL) (see Recipes)
26. 2.5 mM oligomycin solution (see Recipes)
27. 2.5 mM FCCP solution (see Recipes)
28. 0.1 M rotenone solution (see Recipes)
29. 2.5 mM rotenone solution (see Recipes)
30. 2.5 mM antimycin A solution (see Recipes)
31. Assay medium (see Recipes)

Equipment

1. Pipettes: Eppendorf Research® Plus 10 µL, 20 µL, 200 µL, 1,000 µL (Eppendorf, catalog numbers: 3123000020; 3123000039; 3123000055; 3123000063)
2. Pipetting aid: PipetBoy acu 2 (Integra Biosciences, catalog number: 155 000)
3. Neubauer counting chamber improved (Carl Roth, catalog number: PC72.1)
4. Inverted microscope: Primovert (Carl Zeiss, catalog number: 415510-1100-000)
5. Water bath (GFL, catalog number: 1003)
6. Incubator without CO₂ (GFL, catalog number: 4010)
7. Swing out rotor centrifuge: 5804 R (Eppendorf, catalog numbers: 5805000010; 5804709004)
8. Water purification system: ELGA® PURELAB flex 3 (Veolia, catalog number: PF3XXXXM1)
9. pH meter: FE20 FiveEasy™ (Mettler Toledo, catalog number: 30266626)
10. Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, catalog number: 100737-100)

Software

1. Wave Controller Software (Agilent Technologies, version 1.8.1.1)
2. Optional: Wave Desktop Software (Agilent Technologies)

Procedure

The experimental procedure for the detection of mitochondrial function in human PBMCs using a XF Analyzer is shown schematically in Figure 2. This is followed by a detailed description of the individual work steps.

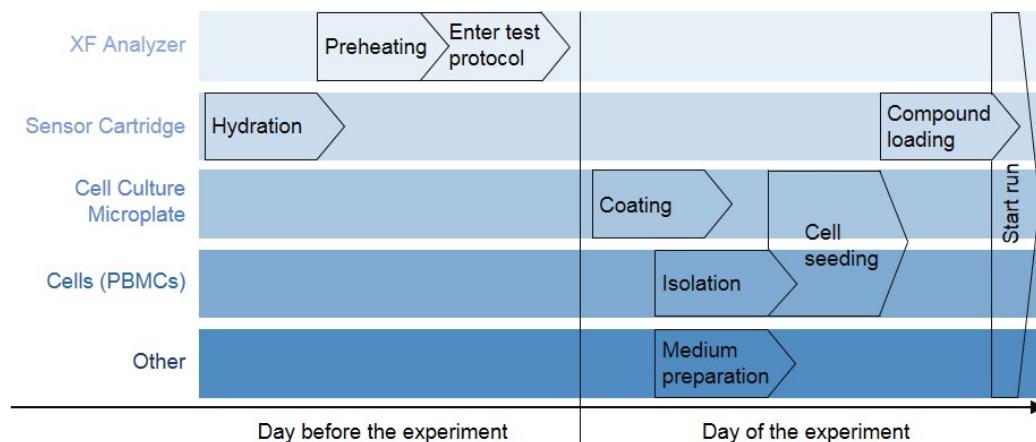


Figure 2. Workflow diagram of mitochondrial function measurement in human PBMCs using a XF Analyzer

A. Day before the experiment

1. Hydration of XF24 sensor cartridge (consisting of the cartridge lid, the sensor cartridge itself and the utility plate; see Figure 3B and 3C):
 - a. Add 1 mL XF Calibrant Solution into each well of the utility plate.
 - b. Remove air bubbles that might arise at the sensors with a pipette tip.
 - c. Incubate the assembled plate in a non-CO₂ 37°C incubator overnight.
2. Switch on the XF24 Analyzer (see Figure 3A) and let it warm up to 37°C overnight.

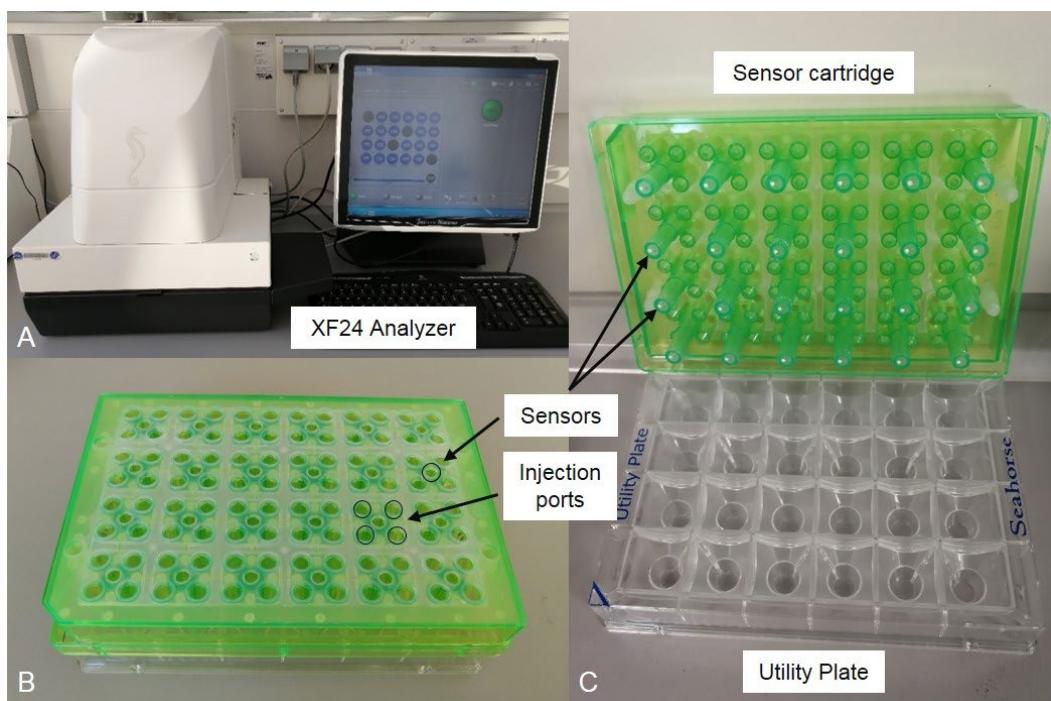


Figure 3. XF24 Analyzer (A) and XF24 sensor cartridge (B and C).

The sensor cartridge, including markings of the sensors, is shown from above (B) as well as from below (C). In addition, the injection ports are marked (B) and the utility plate is depicted (C).

3. Programming of measurement protocol on the XF Analyzer:
 - a. Equilibration: 30 min
 - b. Measurement period 1:
 - i. Mixing: 4 min
 - ii. Waiting: 2 min
 - iii. Measuring: 3 min→ 3 repetitions
 - c. Injection of port A (50 µL of 7.5 µM oligomycin; final concentration 0.75 µM)
 - d. Measurement period 2:
 - i. Mixing: 4 min
 - ii. Waiting: 2 min
 - iii. Measuring: 3 min→ 3 repetitions
 - e. Injection of port B (55 µL of 10 µM FCCP; final concentration 1 µM)
 - f. Measurement period 3:
 - i. Mixing: 4 min
 - ii. Waiting: 2 min
 - iii. Measuring: 3 min→ 3 repetitions
 - g. Injection of port C (60 µL of 16.7 µM rotenone/antimycin A; final concentration 1.67 µM)
 - h. Measurement period 4:
 - i. Mixing: 4 min
 - ii. Waiting: 2 min
 - iii. Measuring: 3 min→ 3 repetitions

B. Day of the experiment

1. Coating of XF24 cell culture microplate:
 - b. Add 30 μ L of Poly-D-Lysine working solution (50 μ g/mL) into each well and incubate the plate for 1 h at RT.
 - c. Discard the supernatant, wash each well with 300 μ L ddH₂O and let the plate air dry under sterile conditions (approximately 30 min).
2. Isolation of PBMCs (continue with this step during microplate coating; see Figure 4):
 - b. Add 15 mL of Ficoll-Paque™ PLUS (RT) into a 50 mL Leucosep™ tube and centrifuge at 1,000 \times g for 30 s at RT.
 - c. Dilute an appropriate volume of anticoagulated human blood at a ratio of 1:2 with 0.9% NaCl solution (at least 7.5 mL and at most 15 mL).
 - d. Transfer the resulting volume (at least 15 mL and at most 30 mL) into the prepared Leucosep™ tube and centrifuge at 1,000 \times g for 10 min at RT in a swing out rotor with brakes switched off.
 - e. Harvest the PBMC fraction which appears as white layer between the plasma and the Ficoll above the porous barrier of the Leucosep™ tube [for detailed information see manufacturer's instructions or refer to Matt and Bergemann, (2019)]. To do this use a 1,000 μ L Eppendorf pipette and collect the PBMCs in a 50 mL screw cap tube.
 - f. Wash the cells once with 10 mL PBS and centrifuge at 250 \times g for 10 min.
 - g. Repeat the washing step twice with 5 mL PBS each.
 - h. Determine the number of cells using a Neubauer counting chamber improved.

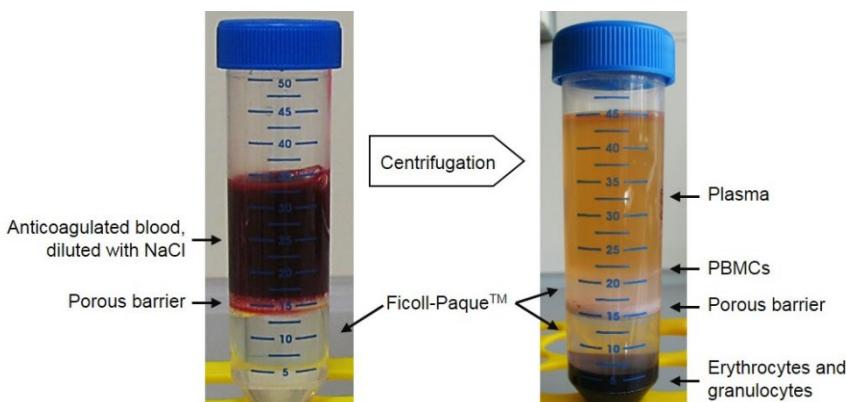


Figure 4. Isolation of PBMCs with a Leucosep™ tube.

On the left side, the tube is filled with Ficoll-Paque™ below and anticoagulated and diluted blood above the porous barrier. On the right side, after density gradient centrifugation, the tube shows the following layers from top to bottom: plasma – PBMCs – Ficoll-Paque™ – porous barrier – Ficoll-Paque™ – erythrocytes and granulocytes.

3. Prepare the assay medium and preheat at 37°C before use.
4. Seeding PBMCs onto pre-coated XF24 cell culture microplate:

Note: 4 to 5 wells per condition are recommended. The wells A1, B4, C3 and D6 are normally used as background wells (without cells). An exemplary plate layout as well as an image of PBMCs seeded on Poly-D-Lysine is shown in Figure 5.

- a. Take 5 \times 10⁵ of the isolated PBMCs per well and centrifuge at 250 \times g for 5 min.
- b. Resuspend the cell pellet in 100 μ L assay medium per well.
- c. Add 100 μ L of cell suspension per well.
- d. Fill the background wells with 450 μ L assay medium.

- e. Centrifuge the plate at $250 \times g$ for 1 min in a swing out rotor with brakes switched off.
- f. Add 350 μL assay medium to the wells with cells (final volume in each well: 450 μL).
- g. Incubate the plate at 37 °C without CO₂ until measurement (on average about 1 h).

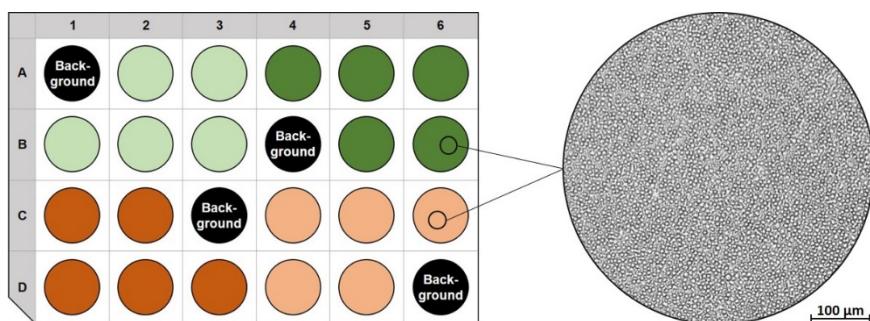


Figure 5. Exemplary plate layout of an XF24 cell culture microplate (left side) and a bright field microscopy image of PBMCs seeded on a Poly-D-Lysine coated XF24 cell culture microplate (right side).

Left side: The wells A1, B4, C3 and D6 are used as background wells (without cells). One color (light green, dark green, dark orange, light orange) represents one condition each. There are 5 wells per condition.
Right side: The image shows PBMCs in 100 \times magnification.

5. Loading of XF24 sensor cartridge with compounds:
 - a. Prepare compounds as follows:
 - i. Dilute 4.86 μL of 2.5 mM oligomycin solution in 1,615 μL assay medium (results in 7.5 μM).
 - ii. Dilute 6.48 μL of 2.5 mM FCCP solution in 1,614 μL assay medium (results in 10 μM).
 - iii. Dilute 10.82 μL of 2.5 mM rotenone solution and 10.82 μL of 2.5 mM antimycin A solution in 1,598 μL assay medium (results in 16.7 μM each).
 - b. Load the injection ports of the XF24 sensor cartridge as follows:

Note: Avoid the formation of air bubbles in the injection ports and handle the XF24 sensor cartridge with care after loading in order to prevent dripping down of the compounds.

- i. Fill all ports A with 50 μL of 7.5 μM oligomycin.
 - ii. Fill all ports B with 55 μL of 10 μM FCCP.
 - iii. Fill all ports C with 60 μL of 16.7 μM rotenone/antimycin A.
6. Start the run in the XF Analyzer (follow the instructions of the software):
 - a. Place the XF24 sensor cartridge (without lid) in the XF Analyzer and start the calibration.
 - b. After completion of the calibration, replace the utility plate by the XF24 cell culture microplate and start measurement.
7. Normalization of measurement results to the number of cells:
 - a. Resuspend PBMCs in the final volume of each well (615 μL) by repeatedly pipetting up and down.
 - b. Determine the number of cells per well using a Neubauer counting chamber improved.

Data analysis

1. Divide the counted number of cells per well by 10,000 and enter the results in the Wave software under 'Normalize' in order to normalize the data (see Figure 6).

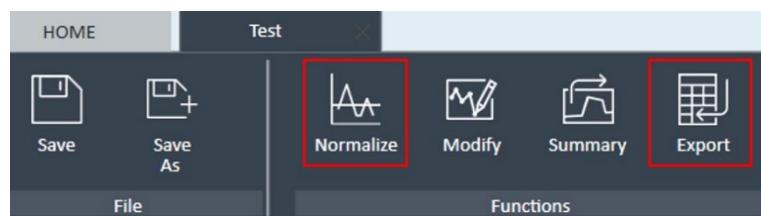


Figure 6. Screenshot of the Wave software with markings for normalization and data export

2. Define outliers based on comparing the course of the OCR of single wells of one condition and exclude them from analysis.

Note: At least three replicates should be used for analysis.

3. Export the results to the Seahorse XF Cell Mito Stress Test Report Generator, which is linked to the Wave software (see Figure 6). This software tool automatically calculates and reports the parameters, which are analyzed by the Mito Stress Test.
4. Take the calculated values from the Report Generator and analyze them with an appropriate statistical software.

Representative Data

A typical result of an oxygen consumption rate measurement using a XF Analyzer and the stress reagents oligomycin, FCCP, antimycin A and rotenone (Mito Stress Test) is shown in Figure 7.

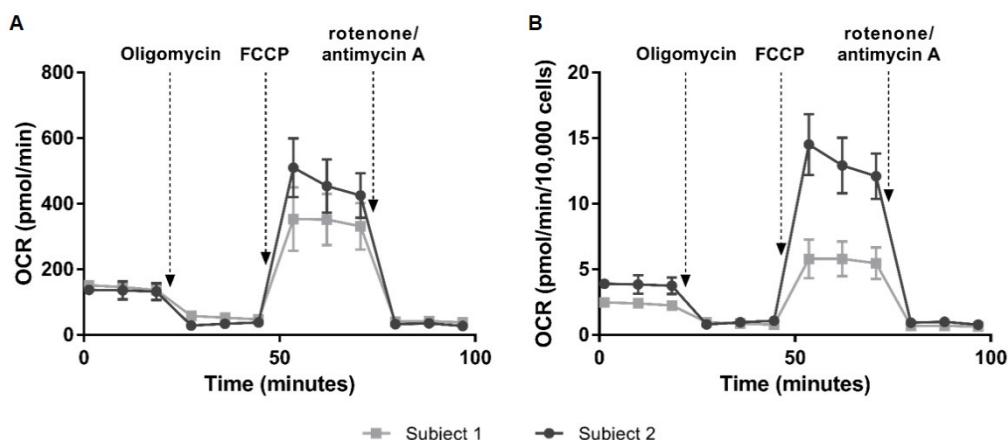


Figure 7. Oxygen consumption rate (OCR) of a Mito Stress Test of PBMCs measured with a XF Analyzer.
The results of two different subjects are depicted not normalized (A) and normalized (B).

Notes

1. Since human blood samples are often available only once per condition, the measurement of a standard PBMC sample, which is stored deep-frozen, is recommended. This can be used for an additional normalization step.
2. Port D of the XF24 sensor cartridge is not used in the described experiment and therefore all ports D can be left blank. Of the ports A, B and C all 24 wells have to be filled in order to ensure an equal injection of the compounds.

Recipes

1. 1× PBS

8 g NaCl
0.20 g KCl
2.88 g Na₂HPO₄·12H₂O
1.24 g KH₂PO₄
ddH₂O to 1 L, pH 7.4

2. 0.9% NaCl solution

9 g NaCl
ddH₂O to 1 L

3. 10 M NaOH solution

40 g NaOH
ddH₂O to 100 mL

4. Poly-D-Lysine working solution (50 µg/mL)

50 µL Poly-D-Lysine solution, 1.0 mg/mL
950 µL ddH₂O

5. 2.5 mM oligomycin solution

5 mg oligomycin
2.528 mL DMSO

6. 2.5 mM FCCP solution

10 mg FCCP
15.737 mL DMSO

7. 0.1 M rotenone solution

1 g rotenone
25.353 mL DMSO

8. 2.5 mM rotenone solution

25 µL of 0.1 M rotenone solution
975 µL DMSO

9. 2.5 mM antimycin A solution

25 mg antimycin A
18.529 mL DMSO

10. Assay medium

0.675 g Dulbecco's Modified Eagle's Medium, high glucose
ddH₂O to 50 mL, pH 7.4

Acknowledgments

This work was funded by the Baden-Württemberg Ministry of Science, Research and Art via the “Cooperative

Graduate School InViTe". We like to thank Dr. med. Adrian Schulte and his team of the F. X. Mayr Bodensee Centre in Überlingen-Hödingen for providing the blood samples.

Competing interests

The authors declare no competing interests.

Ethics

The experiments were conducted in accordance with the declaration of Helsinki and approved by the ethics committee of the Landesärztekammer Baden-Württemberg, Germany. Furthermore, all volunteers were informed in advance and gave their written consent to the use of their blood samples.

References

- Akbari, M., Kirkwood, T. B. L. and Bohr, V. A. (2019). [Mitochondria in the signaling pathways that control longevity and health span](#). *Ageing Res Rev* 54: 100940.
- Chaban, Y., Boekema, E. J. and Dudkina, N. V. (2014). [Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation](#). *Biochim Biophys Acta* 1837(4): 418-426.
- Hartman, M. L., Shirihi, O. S., Holbrook, M., Xu, G., Kocherla, M., Shah, A., Fetterman, J. L., Kluge, M. A., Frame, A. A., Hamburg, N. M. and Vita, J. A. (2014). [Relation of mitochondrial oxygen consumption in peripheral blood mononuclear cells to vascular function in type 2 diabetes mellitus](#). *Vasc Med* 19(1): 67-74.
- Jones, N., Piasecka, J., Bryant, A. H., Jones, R. H., Skibinski, D. O., Francis, N. J. and Thornton, C. A. (2015). [Bioenergetic analysis of human peripheral blood mononuclear cells](#). *Clin Exp Immunol* 182(1): 69-80.
- Lee, H. T., Lin, C. S., Pan, S. C., Wu, T. H., Lee, C. S., Chang, D. M., Tsai, C. Y. and Wei, Y. H. (2019). [Alterations of oxygen consumption and extracellular acidification rates by glutamine in PBMCs of SLE patients](#). *Mitochondrion* 44: 65-74.
- Matt, K. and Bergemann, J. (2019). [Ex vivo Analysis of DNA Repair Capacity of Human Peripheral Blood Mononuclear Cells by a Modified Host Cell Reactivation Assay](#). *Bio-protocol* 9 (15): e3325.
- Nicholas, D., Proctor, E. A., Raval, F. M., Ip, B. C., Habib, C., Ritou, E., Grammatopoulos, T. N., Steenkamp, D., Dooms, H., Apovian, C. M., Lauffenburger, D. A. and Nikolajczyk, B. S. (2017). [Advances in the quantification of mitochondrial function in primary human immune cells through extracellular flux analysis](#). *PLoS One* 12(2): e0170975.
- Pfanner, N., Warscheid, B. and Wiedemann, N. (2019). [Mitochondrial proteins: from biogenesis to functional networks](#). *Nat Rev Mol Cell Biol* 20(5): 267-284.
- Plitzko, B. and Loesgen, S. (2018). [Measurement of Oxygen Consumption Rate \(OCR\) and Extracellular Acidification Rate \(ECAR\) in Culture Cells for Assessment of the Energy Metabolism](#). *Bio-protocol* 8(10): e2850.
- Schöller-Mann, A., Matt, K., Schniertshauer, D., Hochecker, B. and Bergemann, J. (2020). [12 days of in vivo caloric reduction can improve important parameters of aging in humans](#). *Mech Ageing Dev* 188: 111238.
- Smolina, N., Bruton, J., Kostareva, A. and Sejersen, T. (2017). [Assaying Mitochondrial Respiration as an Indicator of Cellular Metabolism and Fitness](#). *Methods Mol Biol* 1601: 79-87.
- Thaventhiran, T., Wong, W., Alghanem, A. F., Alhumeed, N., Aljasir, M. A., Ramsey, S., Sethu, S., Yeang, H. X. A., Chadwick, A. E., Cross, M., Webb, S. D., Djouhri, L., Ball, C., Stebbings, R. and Sathish, J. G. (2019). [CD28 Superagonistic Activation of T Cells Induces a Tumor Cell-Like Metabolic Program](#). *Monoclon Antib Immunodiagn Immunother* 38(2): 60-69.
- Traba, J., Miozzo, P., Akkaya, B., Pierce, S. K. and Akkaya, M. (2016). [An Optimized Protocol to Analyze Glycolysis and Mitochondrial Respiration in Lymphocytes](#). *J Vis Exp*(117). DOI: 10.3791/54918.

Analysis of Monocyte Cell Fate by Adoptive Transfer in a Murine Model of TLR7-induced Systemic Inflammation

Jaba Gamrekelashvili^{1, 2, *}, Hermann Haller¹ and Florian P. Limbourg^{1, 2, *}

¹Vascular Medicine Research, Hannover Medical School, 30625 Hannover, Germany

²Department of Nephrology and Hypertension, Hannover Medical School, 30625 Hannover, Germany

*For correspondence: Limbourg.florian@mh-hannover.de; Gamrekelashvili.jaba@mh-hannover.de

Abstract

Myeloid plasticity is a hallmark of the innate immune response to Toll-like receptor (TLR) activation. Here, we provide a protocol for monocyte cell fate tracking by adoptive transfer in the context of systemic inflammation induced by TLR7 activation, the principal innate immune receptor sensing viral RNA in mice. Defined monocyte subsets are isolated from the bone marrow of donor mice by cell sorting and adoptively transferred into the systemic circulation of congenic hosts, with or without concurrent activation of TLR7 via the topical application of the small molecule agonist, imiquimod, in a cream formulation that induces a systemic inflammatory response. Advantages are the precise definition of donor cell populations and resulting cell fate without the need for host conditioning in a model that recapitulates key aspects of the systemic inflammatory response to TLR7 stimulation.

Keywords: Aldara/Imiquimod, TLR7, Inflammation, Monocyte differentiation, Adoptive transfer, Flow cytometry.

This protocol was validated in: eLife (2020), DOI: 10.7554/eLife.57007

Background

The diversity of myeloid differentiation responses is an integral part of the adaptive immune response and is critical for tissue restoration and maintenance during inflammation and inflammatory dysregulation. Infectious agents, such as viruses, or tissue injury trigger an inflammatory response involving monocytes of the major (classic) subtype in mice, Ly6C^{hi} monocytes (Bonnardel and Guilliams, 2018). These monocytes originate from progenitor cells in the bone marrow (BM), circulate in peripheral blood (PB), and respond to tissue signals by differentiating into a spectrum of effector phagocytes: macrophages (MF), dendritic cells (DC), and monocytes with patrolling behavior (Hettinger *et al.*, 2013; Gamrekelashvili *et al.*, 2016; Bonnardel and Guilliams, 2018; Arazi *et al.*, 2019; Chakarov *et al.*, 2019). Monocyte lineage studies are facilitated by *Cx3cr1*^{GFP/+} reporter mice, in which monocytes and macrophages, but not granulocytes, express distinct GFP signals (Jung *et al.*, 2000).

Toll-like receptor 7 (TLR7) is a member of the family of pathogen sensors expressed on myeloid cells. TLR7 is the principal innate immune receptor sensing viral RNA in mice, a function homologous to TLR7/8 in humans (Diebold *et al.*, 2004). It is highly expressed in endosomes of myeloid cells, where viral RNA triggers the production of pro-inflammatory cytokines and type I interferons, causing local and systemic inflammation and the induction of an antiviral state (Iwasaki and Pillai, 2014). Importantly, myeloid TLR7 signaling is required for the efficient protection of mice against virulent RNA viruses (Kaminski *et al.*, 2012), and it is involved in immune responses to the influenza A virus and SARS-CoV2. The activation of TLR7 can also be induced by the small molecule TLR7 agonist, imiquimod (IMQ), an imidazoquinoline derivative used clinically in a cream formulation as antiviral and antitumor therapies (van der Fits *et al.*, 2009). TLR7 stimulation induces cytokine production in both mouse and human patrolling monocytes and mediates the sensing and disposal of damaged endothelial cells by Ly6C^{lo} monocytes (Cros *et al.*, 2010; Carlin *et al.*, 2013); however, chronic TLR7 stimulation drives the differentiation of Ly6C^{hi} monocytes into specialized macrophages and causes the development of anemia (Akilesh *et al.*, 2019). Furthermore, systemic stimulation with TLR7 agonists induces the conversion of Ly6C^{hi} monocytes into patrolling Ly6C^{lo} monocytes in wildtype mice (Santiago-Raber *et al.*, 2011; Gamrekelashvili *et al.*, 2020).

Here, we provide a protocol to study monocyte cell fate under inflammatory conditions using adoptive transfer of monocyte subsets from genetically defined mouse strains, with the concurrent stimulation of host TLR7 via the topical application of IMQ in a cream formulation (Aldara). Ly6C^{hi} monocytes from the bone marrow of *Cx3cr1*^{GFP/+} CD45.2⁺ mice are FACS-sorted and transferred into congenic CD45.1⁺ mice by tail-vein injection, and donor-derived cells are identified by GFP and CD45.2 expression. The main distinguishing features in comparison with conventional bone-marrow reconstitution experiments are the precise definition of donor cell populations and resulting cell fate without the need for host conditioning. The protocol is transferrable to other progenitor or mature cell populations if suitable numbers can be retrieved from the donors. Furthermore, key aspects of the systemic response to TLR7 stimulation seen in viral models are recapitulated in a virus-free model.

Materials and Reagents

1. LS columns (Miltenyi Biotec, catalog number: 130-042-401)
2. 0.5 mL and 1.5 mL microfuge tubes (Sarstedt, catalog numbers: 72.699 and 72.690.001)
3. 15 mL tubes (Greiner Bio-One, CELLSTAR, catalog number: 188261)
4. 1 mL syringes with 25 G and 27 G needles (B. Braun, Injekt-F 9166033V, Sterican 4657705)
5. K₃EDTA-containing blood collection tubes (Microvette 100 μL; Sarstedt, catalog number: 20.1278)
6. 96-well V-bottomed plates (Greiner Bio-One, catalog number: 651101)
7. 6-well plates (Merck, Biochrom, catalog number: TPP92406)
8. Cell strainers (Greiner Bio-One, catalog numbers: 542040 [40 μm], 542000 [100 μm])
9. Nylon mesh (Heidland, catalog number: 03-70/41)
10. Anti-biotin microbeads (Miltenyi Biotec, catalog number: 130-090-485)
11. Propidium iodide (Merck, Sigma-Aldrich, catalog number: 81854-25MG, Stock 1 mg/mL in PBS, 1:12,000 final)
12. Histopaque 1083 (Merck, Sigma-Aldrich, catalog number: 10831-100ML)

13. Red blood cell lysis buffer (RBC lysis buffer; BioLegend, catalog number: 420301)
14. Imiquimod (Meda, Aldara, 5% cream, catalog number: PZN-00111981)
15. Vaseline (Winthrop GmbH, catalog number: PZN-02726847)
16. Depilating cream (Silk & Fresh depilating cream)
17. Bepanthen eye ointment (Bayer vital GmbH, catalog number: PZN-01578675)
18. Ketamine (Ketamine, 100 mg/mL; CP-Pharma, catalog number: 1202)
19. Rompun (Xylavet, 20 mg/mL; CP-Pharma, catalog number: 1205)
20. Midazolam (Midazolam-Actavis 1 mg/mL; Actavis, catalog number: PZN-03831807)
21. Isoflurane (Isoflurane CP 1 mL/mL; CP-Pharma, catalog number: 1214)
22. Heparin 5000 U/mL (Heparin-Natrium, 25000; Ratiopharm, catalog number: PZN-03029843)
23. Cell counting slides (Thermo Fisher Scientific, catalog number: C10228)
24. PBS (Merck, Sigma-Aldrich, catalog number: D8537)
25. Antibodies (Tables 1 and 2)

Table 1. Antibodies and fluorescent dyes for the sorting of inflammatory monocytes

Antibody/Dye	Clone	Company	Cat. No.	Final Dilution
CD16/32 (TruStain FcX)	93	BioLegend	101319	1:200
CD3 PE	17A2	BioLegend	100205	1:400
Ter119 PE	Ter119	BioLegend	116208	1:800
CD45R/B220 PE	RA3-6B2	BD Pharmingen	553089	1:800
Ly6G PE	1A8	BioLegend	127608	1:800
CD19 PE	6D5	BioLegend	115507	1:400
CD49b PE	DX5	BD Pharmingen	553858	1:200
NK1.1 PE	PK136	BioLegend	108707	1:200
CD90.2 PE	Thy1.2	BD Pharmingen	553005	1:800
CD11b Pacific Blue	M1/70	BioLegend	101224	1:200
Ly6C PE-Cy7	HK1.4	BioLegend	128018	1:1400

Table 2. Antibodies and fluorescent dyes for donor cell enrichment and flow cytometry

Antibody/Dye	Clone	Company	Cat. No.	Final Dilution
CD16/32 (TruStain FcX)	93	BioLegend	101319	1:200
CD3 Biotin	17A2	BioLegend	100243	1:400
Ter119 Biotin	Ter119	BioLegend	116203	1:400
CD45R/B220 Biotin	RA3-6B2	BioLegend	103203	1:400
Ly6G Biotin	1A8	BioLegend	127603	1:400
CD19 Biotin	6D5	BioLegend	115504	1:400
CD45.1-Biotin	A20	BioLegend	110703	1:200
CD117 APC-Cy7	2B8	BioLegend	105825	1:100
CD11b Pacific Blue	M1/70	BioLegend	101224	1:200
Ly6C PE-Cy7	HK1.4	BioLegend	128018	1:2800
F4/80 BV650	BM8	BioLegend	123149	1:200
CD11c BV605	N418	BioLegend	117334	1:400
I-A/I-E BV510	M5/114.15.2	BioLegend	107635	1:400
NK1.1 Biotin	PK136	BioLegend	108704	1:400
CD45.2 Alexa700	104	BioLegend	109821	1:200
CD43 PerCP-Cy5.5	S7	BD Pharmingen	562865	1:400
Streptavidin-PE-Dazzled594		BioLegend	405247	1:400

26. Flow cytometry buffer (FCS-EDTA-PBS, see Recipes)

27. Cell collection buffer (20% FCS-PBS, see Recipes)
28. Heparin-PBS (250 U/mL) for collection of peripheral blood (see Recipes)
29. RBC lysis buffer (see Recipes)

Equipment

1. Hair shaver (Aesculap Isis GT420, Type HS61)
2. Caliper (Pariere, 8030/R)
3. Mouse heat pad (Beurer, HK25, Type P10)
4. Centrifuge (HermLe, Z383K)
5. Table-top centrifuge (VWR, Micro Star 17R)
6. Flow cytometer (Beckton Dikinson, LSR-II)
7. Cell sorter (Beckton Dikinson, FACSAria Fusion)
8. Quadro MACS separation unit (Miltenyi Biotec, catalog number: 130-090-976)
9. Automated cell counter (Thermo-Fisher Scientific, Invitrogen, Countess II FL, AMQAF1000)
10. Automated blood counter (Scil Animal Care Company, Scil Vet ABC)

Software

1. FlowJo (version 10.6.2, FLOWJO LLC, <https://www.flowjo.com>)
2. Prism (version 7.04, GraphPad, <https://www.graphpad.com>)
3. Adobe Illustrator (version 25.0, <https://www.adobe.com>)

Procedure

A. Induction of systemic inflammation using imiquimod

Notes:

- a. Experimental procedures can only be performed after approval by the local Animal Care Committee.
- b. Perform experiments with 8-12-week-old mice. Reporter mice are B6.129P-Cx3cr1^{tm1Lit}/J, obtained from the Jackson Laboratory (Jung et al., 2000; Gamrekelashvili et al., 2016 and 2020).
- c. IMQ-treated mice must be kept separately from the Vaseline (Sham) group.
- d. Mix blood thoroughly with Heparin-PBS or K₃EDTA to avoid coagulation.
- e. Pre-warm RBC lysis buffer at room temperature to achieve efficient lysis.

1. Induction of systemic inflammation using IMQ (Figure 1)
 - a. On Day -1, weigh the mice, record the weight, and intraperitoneally inject Ketanest (Esketamine 80 mg/kg), Rompun (Xylazine 2.5 mg/kg), and Dormicum (Midazolam 2.5 mg/kg) in 0.9% NaCl solution to anesthetize the mice (injection volume 10 µL/g mouse weight, using a 1-mL syringe and 27 G needle).
 - b. Apply a drop of Bepanthen eye ointment from the tube to the eye to avoid dry eyes, and test paw reflexes to make sure the mouse is sleeping.
 - c. Shave the back (appr. 3 cm²) of the mice with an electric shaver and apply the depilating cream.
 - d. Keep the mice for appr. 2 min on a heat pad and wipe away the depilating cream with a spadel.
 - e. Wash and rinse the back of the mice with warm water to remove the remaining depilating cream.
 - f. Keep the mice on the heat pad until awake and then transfer to cages with *ad libitum* access to food and water.

- g. Two days later (Day 1), weigh the mice, record the weight, and place each mouse separately in a glass jar with appr. 200-300 µL isofluran for 10-15 s.
- h. Place each dizzy/sleepy mouse on the heat pad, measure the thickness of the ear using a caliper, and quickly apply 50 mg IMQ (Aldara) cream or an equal amount of Vaseline to the back and the right ear using a stainless-steel spadel.
- i. Repeat the same procedure for 3-4 consecutive days (Figure 1).

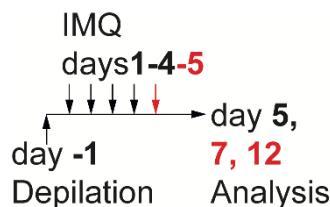


Figure 1. Experimental set-up for the induction of inflammation

2. Mouse preparation and analysis of systemic inflammation
 - a. On Day 5, 7, or 12, anesthetize the mice by intraperitoneal injection with Ketanest (Esketamin 80 mg/kg), Rompun (Xylazin 2.5 mg/kg), and Dormicum (Midazolam 2.5 mg/kg) in 0.9% NaCl solution (injection volume 10 µL/g mouse weight, using a 1-mL syringe and 27 G needle).
 - b. Check the footpad reflex to confirm that the mouse is sleeping.
 - c. Fix the sleeping mice to the pad, open the abdomen, and collect the blood from the inferior vena cava using a 1-mL syringe with a 25 G ×5/8" needle (Braun Melsungen) (Figure 2):
 - i. 300 µL blood (PB) in 100 µL Heparin-PBS (250 U/mL) for cell analysis.
 - ii. 50 µL blood in K₃EDTA-containing tubes for blood counts.
 - iii. The remaining blood without heparin for serum collection and analysis.

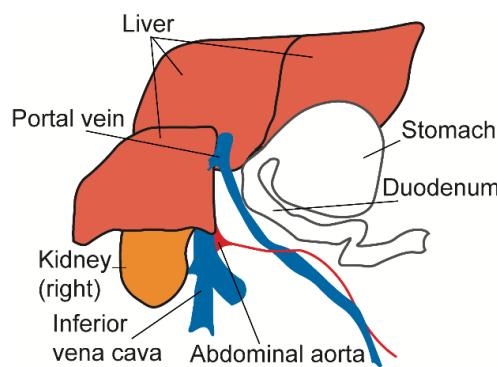


Figure 2. Schematic representation of the mouse abdominal splanchnic structures, ventral view.

After opening the abdominal cavity of deeply anesthetized mice, collect blood from the inferior vena cava.

- d. Euthanize the mice, open the abdomen, excise the spleen (Spl), and place in PBS on ice.
- e. Cut and remove the skin from the hind limb and collect the hind limb with the muscles on a paper towel.
- f. Carefully remove the muscles using scissors. Clean the rest of the muscles and soft tissue with a paper towel and isolate the bones [one tibial (Tibia) and one femoral (Femur) bone (Figure 3)].

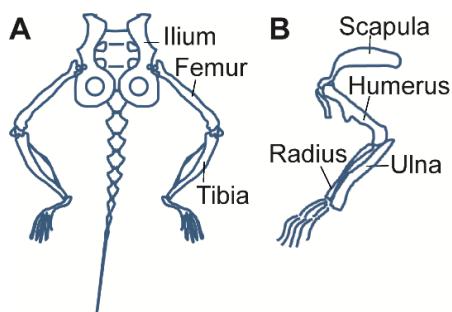


Figure 3. Schematic representation of the bones of the mouse hindlimb (A) and forelimb (B).

The Ilium, Femur, Tibia, and Humerus are collected for bone-marrow isolation and analysis.

- g. Pre-weigh the 1.5-mL Eppendorf collection tube of the custom-made bone-marrow (BM) isolating device (Figure 4). Cut one end of the bone, place in a 0.5-mL tube with a hole in the bottom, and collect the BM by centrifugation of the isolating device in a table-top centrifuge at $5,000 \times g$ for 20 s.

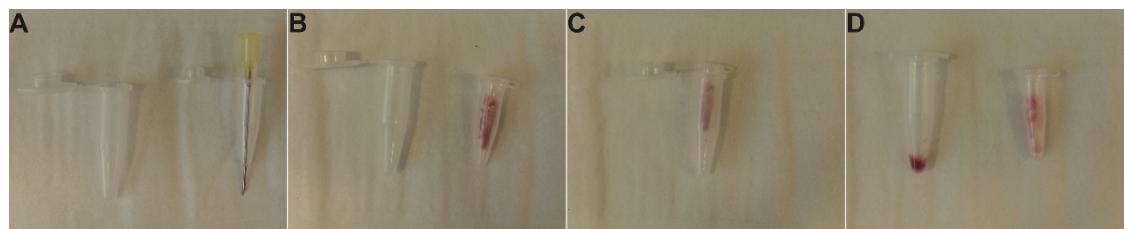


Figure 4. Assembly and use of the custom-made BM isolating device.

A. Perforation of a 0.5-mL tube with a 21 G needle for BM isolation. B. Bones cleaned from muscles and placed in a 0.5-mL tube. C. Assembly for centrifugation. D. Isolated BM in a 1.5-mL collection tube after centrifugation.

- h. Weigh the collection tube again, record the difference in weight to define the BM weight and place on ice.
- i. Resuspend BM in 10 mL PBS, filter through a 70- μ m nylon mesh, and wash by centrifugation at $400 \times g$ for 7 min.
- j. Aspirate the supernatant, resuspend the cell pellet in 10 mL PBS, and repeat the filtration and washing step once more.
- k. Weigh the spleen, prepare a single-cell suspension by passing through a syringe plunger in a 6-well plate and filtering through a nylon mesh, and centrifuge at $400 \times g$ for 7 min.
- l. To remove the erythrocytes, resuspend the PB and Spl pellets in 5 mL or 1 mL room-temperature RBC lysis buffer and incubate for 17 min or 1 min, respectively.
- m. Stop the lysis procedure by adding 10 mL PBS, filter the suspension through a nylon mesh, and centrifuge at $400 \times g$ for 7 min.
- n. Repeat the washing-filtration-centrifugation step once more.
- o. Resuspend the Spl, BM, and PB in PBS (10 mL, 10 mL, or 5 mL, respectively) and count the cells in an automated cell counter to define the absolute cell number.
- p. Resuspend the cells in FCS-EDTA-PBS buffer and place $\leq 1 \times 10^6$ cells per sample in a 96-well V-bottomed plate for staining and subsequent flow cytometry analysis.

B. Adoptive transfer of monocytes into IMQ-treated mice and monocyte differentiation analysis

Notes:

- a. Experimental procedures can only be performed after approval by the local Animal Care Committee.
 - b. Perform experiments with 8-12-week-old mice. Use B6.SJL-Ptprc^aPepc^b/BoyJ (CD45.1⁺) mice as recipients (Jackson Laboratory).
 - c. Collect no more than 2×10^6 sorted monocytes in 2 mL collection buffer and carefully mix the collection tube every 20-30 min to avoid excess stress to the cells. This step enhances cell viability and ensures a high yield of donor cells for analysis after adoptive transfer.
 - d. For successful flow cytometry analysis of transferred cells, it is important to deplete recipient cells from recipient Spl and BM but not from peripheral blood.
 - e. It is critical to remove erythrocytes using density centrifugation with Histopaque 1083 and not RBC lysis for successful analysis of transferred donor cells.
 - f. Cell enrichment on magnetic columns must be performed with ice-cold FCS-EDTA-PBS buffer. Avoid excess air bubble and foam formation.
1. Induction of systemic inflammation using IMQ
 - a. Depilate and treat recipient CD45.1⁺ mice on days 1-4 with IMQ as described in A and Figure 1.
 2. Isolation and adoptive transfer of inflammatory monocytes on day 2
 - a. Euthanize 4-5 donor CX₃CR1-GFP⁺ CD45.2⁺ mice and isolate the bones (hips (Ilium), femoral (Femur), tibial (Tibia), and brachial (Humerus) bones from both sides, 8 bones/mouse total, Figure 3). Clean the muscles from the bones using scissors and a paper towel. Collect the cleaned bones in PBS and keep on ice.
 - b. Dip the bones in 70% EtOH for 3-5 s and dry on EtOH-sterilized dry paper towel. Cut one end of the bone and put 4 bones/tube in 0.5-mL Eppendorf tubes with a hole in the bottom created using a 21 G needle (Figure 4).
 - c. Close the tube and place in another 1.5-mL collection tube. Centrifuge this device in a table-top centrifuge with a fixed-angle rotor at 5,000 $\times g$ for 20 s.
 - d. Collect the bone marrow, resuspend in 50 mL PBS, filter through a 100- μ m sterile cell strainer, and centrifuge in a swinging-bucket rotor at 400 $\times g$ for 7-8 min to collect the pellet.
 - e. Resuspend the cell pellet from 4-5 mice in 6-8 mL PBS, overlay 3-4 mL suspension on 3 mL Histopaque 1083 in 15-mL tubes, and centrifuge in a swinging-bucket rotor at 420 $\times g$ for 20 min without the brake.
 - f. Collect the interphase (appr. 5 mL) containing the cells in a 15-mL tube, fill the tube with PBS, and wash by centrifugation at 400 $\times g$ for 7 min.
 - g. Resuspend the pellet in 600 μ L FCS-EDTA-PBS and add 3 μ L Fc block (TruStain FcX). Incubate the tube at 4°C for 12-15 min.
 - h. Without washing, add PE-labeled anti-mouse CD3- (1.5 μ L), CD19- (1.5 μ L), B220- (0.75 μ L), Ly6G- (0.75 μ L), Ter119- (0.75 μ L), CD90.2- (0.75 μ L), NK1.1- (3 μ L), CD49b- (3 μ L); anti-mouse CD11b-Pacific Blue (3 μ L), and anti-mouse Ly6C-PE-Cy7 (0.43 μ L) antibodies (Table 1) to the 600 μ L cell suspension and incubate for an additional 15 min.
 - i. Wash the cells twice in 10 mL buffer, filter through a 40- μ m cell strainer, resuspend in 2 mL FCS-EDTA-PBS buffer, and sort Lin⁻CD11b⁺CX₃CR1-GFP^{lo}Ly6C^{hi} cells. Collect $\sim 2 \times 10^6$ sorted cells in 2 mL ice-cold 20% FCS-PBS and mix the collection tube every 20-30 min during sorting.
 - j. Immediately after sorting, wash the sorted cells twice with 10 mL PBS each, resuspend in PBS, and intravenously inject 0.7×10^6 - 2×10^6 cells per mouse in 100 μ L volume into the tail vein using a 1-mL syringe and 27 G needle. To dilate the tail vein and facilitate injection, prewarm the tail in warm water.
 3. Isolation and analysis of donor-derived CD45.2⁺ monocytes from CD45.1⁺ recipients 3 days after adoptive transfer.
 - a. Euthanize recipient mice in deep narcosis, collect blood in 200 μ L Heparin-PBS, collect the spleen and 8 bones (hips, femoral, tibial, and brachial bones).
 - b. Prepare a single-cell suspension from Spl by passing through a 5-mL syringe into a 6-well plate, resuspending in 4 mL PBS, and filtering through a 70- μ m nylon mesh.

- c. Isolate BM as described in Figure 4, resuspend in 4 mL PBS, and filter through a 70- μm nylon mesh.
- d. Dilute the blood (PB) with PBS to 7 mL.
- e. Load Spl, BM, and PB separately onto 2.5 mL Histopaque 1083 in 15-mL tubes and centrifuge in a swinging-bucket rotor at $420 \times g$ for 20 min without the brake.
- f. Collect the interphase (appr. 5mL) containing the cells in a clean 15-mL tube, fill up the tube with PBS, and wash once by centrifugation at $400 \times g$ for 7 min.
- g. Resuspend the cell pellet in 100 μL (BM, Spl) or 50 μL (PB) FCS-PBS-EDTA buffer supplemented with Fc block (1:200 dilution) and incubate at 4°C for 15 min. Without washing the Fc block, add biotin-labeled antibodies consisting of anti-mouse CD45.1, CD3, CD19, B220, Ly6G, and Ter119 to the final dilution indicated in Table 2, but do not add the biotinylated anti-mouse NK1.1 at this stage.
- h. Quickly vortex the tube and incubate at 4°C for 15 min.
- i. Fill the tubes with 5 mL FCS-EDTA-PBS buffer and centrifuge at $400 \times g$ for 8 min.
- j. Wash PB once more with 5 mL FCS-EDTA-PBS buffer and transfer the pellet to a 96-well V-bottomed plate for further staining.
- k. Resuspend the Spl and BM pellets in 240 μL FCS-EDTA-PBS buffer, add 60 μL anti-biotin microbeads, and incubate at 4°C for 15 min. Fill the tubes again with FCS-EDTA-PBS buffer to wash the excess beads and centrifuge at $400 \times g$ for 7-8 min.
- l. Place the LS columns in the Quadro MACS separation unit and equilibrate with ice-cold FCS-EDTA-PBS buffer (1 mL buffer per column).
- m. Resuspend the Spl and BM pellets in 2 mL FCS-EDTA-PBS buffer and load onto the pre-equilibrated LS columns.
- n. Wash the columns twice with 2 mL FCS-EDTA-PBS buffer, collect the flowthrough, pellet by centrifugation at $400 \times g$ for 7 min, transfer to a 96-well V-bottomed plate, and stain in 30 μL volume with the appropriate antibody cocktail (Table 2).
- o. Resuspend the cells after staining and wash in FCS-EDTA-PBS buffer. Add propidium iodide (PI 1 mg/mL stock in PBS, 1:12,000 final dilution) prior to acquisition and acquire flow cytometry data for further analysis.

Data analysis

A. Analysis of IMQ-induced systemic inflammation

1. Measure the mouse weight, ear thickness, blood count, coagulation, inflammatory cytokine levels, liver and kidney function, and myeloid cell composition of the BM, PB, and Spl, and plot the data as described in Figures 5A-5J in this manuscript and in Figure 3E, Figure 4G, Figure 3 – figure supplement 1A, Figure 4 – figure supplement 1C in Gamrekelashvili *et al.* (2020).

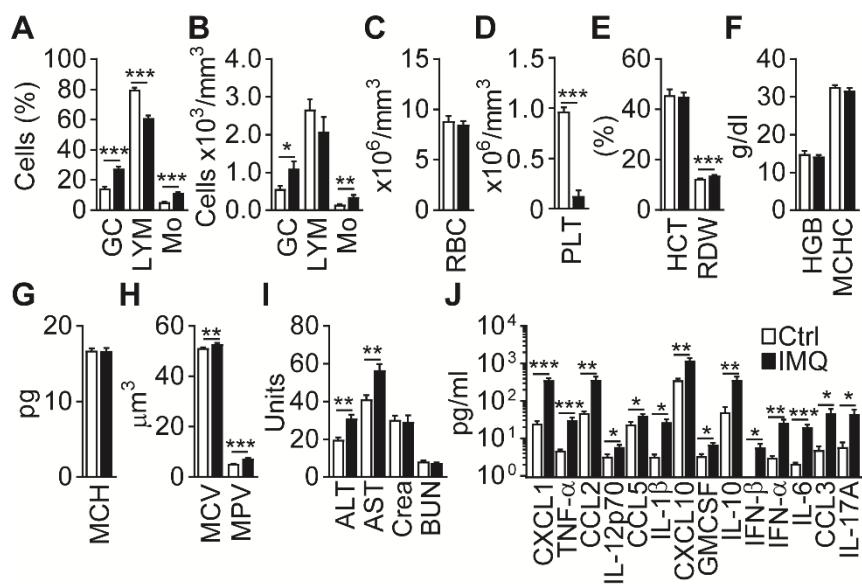


Figure 5. IMQ induces systemic inflammation.

(A-H) Blood parameters in Ctrl (Vaseline) or IMQ-treated mice (GC – granulocytes, LYM – lymphocytes, Mo – monocytes, RBC – red blood cells, PLT – platelets, HCT – hematocrit, RDW – red blood cell distribution width, HGB – Hemoglobin, MCHC – mean corpuscular hemoglobin concentration, MCH – mean corpuscular hemoglobin, MCV – Mean corpuscular volume, MPV – mean platelet volume). (I) ALT/AST, creatinine (Crea) and blood urea nitrogen (BUN) in IMQ-treated mice. (J) Cytokine and chemokine concentrations in Ctrl or IMQ-treated mouse sera. (A-J) Data are pooled from 3-4 experiments, $n = 4-9$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed unpaired Student's *t*-test.

- Analyze the flow cytometry data using the Flowjo software. Calculate the relative frequency of myeloid cell subsets [for a definition of the subsets, see Figure 3E, Figure 3 – figure supplement 2, Supplementary File 1 in Gamrekelashvili *et al.* (2020)] in live cells after the exclusion of doublets, export the numerical data to Excel, and calculate the absolute cell numbers per mg BM, mg Spl, or μL blood.
- Transfer the data to GraphPad Prism, plot the values as the mean and standard error of the mean, and calculate the statistical significance using an unpaired two-tailed Student's *t*-test. Define $P < 0.05$ as statistically significant and indicate on the graphs.

B. Analysis of adoptively transferred monocytes using flow cytometry

- Analyze the flow cytometry data using the Flowjo software. Calculate the relative frequency of myeloid cell subsets in live CD45.2 $^+$ CD11b $^+$ CX3CR1-GFP $^+$ donor cells after the exclusion of doublets and CD45.1 $^+$ recipient cells, export the numerical data into GraphPad Prism, and plot the values as the mean and standard error of the mean, as shown in Figures 5F and 5G in Gamrekelashvili *et al.* (2020). Calculate the statistical significance using an unpaired two-tailed Student's *t*-test. Define $P < 0.05$ as statistically significant and indicate on the graphs.
- Create representative flow cytometry plots, as shown in Figure 6, depicting the gating strategy, surface phenotype, and frequency of donor-derived cells. Initially, define the cells according to size and granularity (FSC-A vs. SSC-A plot), exclude doublets (SSC-W vs. SSC-A plot) and dead (PI $^+$) cells (FSC-A vs. PI plot), and gate donor cells (CD45.2 $^+$ GFP $^+$). As a next step, define monocytes (CD11b $^+$ GFP $^+$ population) in donor cells and create subsequent plots showing the expression of Ly6C, F4/80, CD11c, CD43, and I-A/I-E as markers of monocyte subsets.

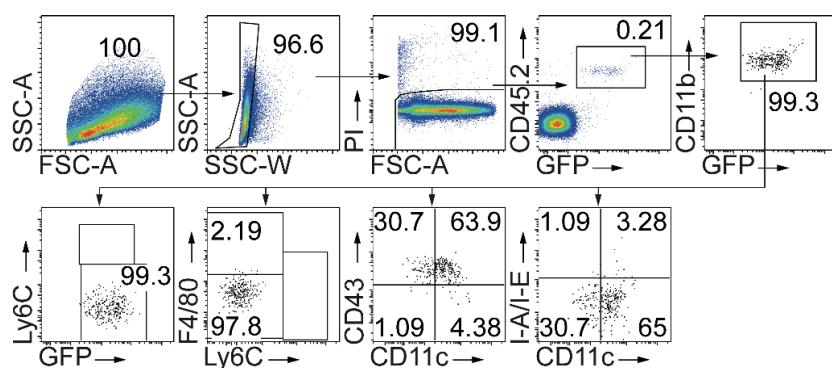


Figure 6. Adoptively transferred classic monocytes differentiate into patrolling monocytes.

Flow cytometry plots showing the gating strategy and analysis of donor cells 3 days after adoptive transfer of CD45.2⁺ classic monocytes in the spleen of IMQ-treated CD45.1⁺ recipients. Donor-derived monocytes (CD45.2⁺CD11b⁺CX₃CR1-GFP⁺Ly6C^{hi}F4/80^{lo/-}CD11c⁻CD43^{lo/-}I-A/I-E^{lo/-} cells) no longer show a classic monocyte phenotype in recipient mice but instead show a phenotype consistent with patrolling monocytes (CD45.2⁺CD11b⁺CX₃CR1-GFP⁺Ly6C^{lo/-}F4/80^{lo}CD11c^{lo}CD43^{hi}I-A/I-E^{lo/-}).

Recipes

1. FCS-EDTA-PBS buffer

FCS 20 mL (2% final)
EDTA (0.5 M stock in PBS, pH 7.2) 4 mL (2 mM final)
PBS 1,000 mL

2. Sorted cell-collection buffer (20% FCS-PBS buffer)

FCS 10 mL
PBS 40 mL
Filter through a 0.22-μm filter and keep sterile

3. Heparin-PBS (250 U/mL) for collection of peripheral blood

Heparin stock 5,000 U/mL 50 μL
PBS 950 μL
Take 100 μL heparin-PBS for ≤ 500 μL blood.

4. RBC lysis buffer

RBC lysis buffer (10×) 1 mL
Deionized water 9 mL

Acknowledgments

We thank the Central Animal Facility and the Research Core Facility Cell Sorting of Hannover Medical School for their support. We thank Stefan Sablotny and Herle Chlebusch for their excellent technical help. This work was funded by grants from Deutsche Forschungsgemeinschaft to JG (GA 2443/2-1) and FPL (Li948-7/1). This protocol describes experiments published in Gamrekelashvili *et al.* (2020).

Competing interests

The authors declare no competing financial or non-financial interests.

Ethics

All animal experiments described in this protocol were approved by the local Animal Welfare Board (LAVES, Lower Saxony, Animal Studies Committee, TVA 16/2251, 18/2777, 2018/221).

References

- Akilesh, H. M., Buechler, M. B., Duggan, J. M., Hahn, W. O., Matta, B., Sun, X., Gessay, G., Whalen, E., Mason, M., Presnell, S. R., Elkon, K. B., Lacy-Hulbert, A., Barnes, B. J., Pepper, M. and Hamerman, J. A. (2019). [Chronic TLR7 and TLR9 signaling drives anemia via differentiation of specialized hemophagocytes](#). *Science* 363(6423): eaao5213-5213.
- Arazi, A., Rao, D. A., Berthier, C. C., Davidson, A., Liu, Y., Hoover, P. J., Chicoine, A., Eisenhaure, T. M., Jonsson, A. H., Li, S., Lieb, D. J., Zhang, F., Slowikowski, K., Browne, E. P., Noma, A., Sutherby, D., Steelman, S., Smilek, D. E., Tosta, P., Apruzzese, W., Massarotti, E., Dall'Era, M., Park, M., Kamen, D. L., Furie, R. A., Payan-Schober, F., Pendergraft, W. F., 3rd, McInnis, E. A., Buyon, J. P., Petri, M. A., Putterman, C., Kalunian, K. C., Woodle, E. S., Lederer, J. A., Hildeman, D. A., Nusbaum, C., Raychaudhuri, S., Kretzler, M., Anolik, J. H., Brenner, M. B., Wofsy, D., Hacohen, N., Diamond, B. and Accelerating Medicines Partnership in, S. L. E. n. (2019). [The immune cell landscape in kidneys of patients with lupus nephritis](#). *Nat Immunol* 20(7): 902-914.
- Bonnardel, J. and Guilliams, M. (2018). [Developmental control of macrophage function](#). *Curr Opin Immunol* 50: 64-74.
- Carlin, L. M., Stamatiades, E. G., Auffray, C., Hanna, R. N., Glover, L., Vizcay-Barrena, G., Hedrick, C. C., Cook, H. T., Diebold, S. and Geissmann, F. (2013). [Nr4a1-dependent Ly6C^{low} monocytes monitor endothelial cells and orchestrate their disposal](#). *Cell* 153(2): 362-375.
- Chakarov, S., Lim, H. Y., Tan, L., Lim, S. Y., See, P., Lum, J., Zhang, X. M., Foo, S., Nakamizo, S., Duan, K., Kong, W. T., Gentek, R., Balachander, A., Carbajo, D., Bleriot, C., Malleret, B., Tam, J. K. C., Baig, S., Shabeer, M., Toh, S. E. S., Schlitzer, A., Larbi, A., Marichal, T., Malissen, B., Chen, J., Poidinger, M., Kabashima, K., Bajenoff, M., Ng, L. G., Angeli, V. and Ginhoux, F. (2019). [Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches](#). *Science* 363(6432): eaau0964.
- Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S. Y., Senechal, B., Puel, A., Biswas, S. K., Moshous, D., Picard, C., Jais, J. P., D'Cruz, D., Casanova, J. L., Trouillet, C. and Geissmann, F. (2010). [Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors](#). *Immunity* 33(3): 375-386.
- Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S. and Reis e Sousa, C. (2004). [Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA](#). *Science* 303(5663): 1529-1531.
- Gamrekelashvili, J., Giagnorio, R., Jussofie, J., Soehnlein, O., Duchene, J., Briseno, C. G., Ramasamy, S. K., Krishnasamy, K., Limbourg, A., Kapanadze, T., Ishifune, C., Hinkel, R., Radtke, F., Strobl, L. J., Zimber-Strobl, U., Napp, L. C., Bauersachs, J., Haller, H., Yasutomo, K., Kupatt, C., Murphy, K. M., Adams, R. H., Weber, C. and Limbourg, F. P. (2016). [Regulation of monocyte cell fate by blood vessels mediated by Notch signalling](#). *Nat Commun* 7: 12597.
- Gamrekelashvili, J., Kapanadze, T., Sablotny, S., Ratiu, C., Dastagir, K., Lochner, M., Karbach, S., Wenzel, P., Sitnow, A., Fleig, S., Sparwasser, T., Kalinke, U., Holzmann, B., Haller, H. and Limbourg, F. P. (2020). [Notch and TLR signaling coordinate monocyte cell fate and inflammation](#). *Elife* 9: e57007.
- Hettinger, J., Richards, D. M., Hansson, J., Barra, M. M., Joschko, A. C., Krijgsveld, J. and Feuerer, M. (2013). [Origin of monocytes and macrophages in a committed progenitor](#). *Nat Immunol* 14(8): 821-830.
- Iwasaki, A. and Pillai, P. S. (2014). [Innate immunity to influenza virus infection](#). *Nat Rev Immunol* 14(5): 315-328.

- Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A. and Littman, D. R. (2000). [Analysis of fractalkine receptor CX₃CR1 function by targeted deletion and green fluorescent protein reporter gene insertion](#). *Mol Cell Biol* 20(11): 4106-4114.
- Kaminski, M. M., Ohnemus, A., Cornitescu, M. and Staeheli, P. (2012). [Plasmacytoid dendritic cells and Toll-like receptor 7-dependent signalling promote efficient protection of mice against highly virulent influenza A virus](#). *J Gen Virol* 93(Pt 3): 555-559.
- Santiago-Raber, M. L., Baudino, L., Alvarez, M., van Rooijen, N., Nimmerjahn, F. and Izui, S. (2011). [TLR7/9-mediated monocytosis and maturation of Gr-1^{hi} inflammatory monocytes towards Gr-1^{lo} resting monocytes implicated in murine lupus](#). *J Autoimmun* 37(3): 171-179.
- van der Fits, L., Mourits, S., Voerman, J. S., Kant, M., Boon, L., Laman, J. D., Cornelissen, F., Mus, A. M., Florencia, E., Prens, E. P. and Lubberts, E. (2009). [Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis](#). *J Immunol* 182(9): 5836-5845.

Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer

Ana S. Almeida^{*, \$a}, Miriam R. Fein^{\$b} and Mikala Egeblad^{*}

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

^{\$a}Present address: APC Microbiome Ireland and School of Microbiology, University College Cork, Cork, Ireland;

^{\$b}Present address: Hospital for Special Surgery, New York, USA

*For correspondence: a.almeida@ucc.ie; egeblad@cshl.edu

Abstract

Flow cytometry is a popular laser-based technology that allows the phenotypic and functional characterization of individual cells in a high-throughput manner. Here, we describe a detailed procedure for preparing a single-cell suspension from mammary tumors of the mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) and analyzing these cells by multi-color flow cytometry. This protocol can be used to study the following tumor-infiltrating immune cell populations, defined by the expression of cell surface molecules: total leukocytes, tumor-associated macrophages (TAMs), conventional dendritic cells (DCs), CD103-expressing DCs, tumor-associated neutrophils, inflammatory monocytes, natural killer (NK) cells, CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, and regulatory T cells.

Keywords: Flow cytometry, Immune cell subsets, Breast cancer, Tumor microenvironment, Mouse model, Immunophenotyping, Antibodies

This protocol was validated in: J Exp Med (2020), DOI: 10.1084/jem.20181551

Background

Tumor-infiltrating immune cells comprise a major part of the tumor microenvironment and play a crucial role in controlling cancer, with both anti- and pro-tumorigenic effects (Dunn *et al.*, 2002; Grivennikov *et al.*, 2010). Inflammation and infiltration of innate immune cells, including macrophages and neutrophils, are necessary to fight infections but, in the case of cancer, often promote the progression of the disease. Cytotoxic T cells and natural killer (NK) cells can destroy tumors, but cancer cells have developed several mechanisms to evade immune destruction (Dunn *et al.*, 2002; DeNardo *et al.*, 2010). For instance, cancer cells can secrete cytokines that directly inhibit cytotoxic CD8⁺ T cells and recruit regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Beatty and Gladney, 2015).

Tumoral immune cell infiltration can be prognostic in some breast cancer subtypes (DeNardo *et al.*, 2011). For instance, high lymphocyte infiltration is associated with increased survival in breast cancer patients and a favorable prognosis (Ménard *et al.*, 1997). In addition, clinical studies have shown that the accumulation of tumor-associated macrophages (TAMs) is strongly correlated with poor prognosis in breast cancer (Noy and Pollard, 2014).

Over the past two decades, advances in multi-color flow cytometry have allowed researchers to gain insights into the role of immune cells within the tumor microenvironment. Flow cytometry is widely used to characterize and quantify different cell types in heterogeneous cell populations, such as tumors, by detecting cell surface and intracellular molecules (Perfetto *et al.*, 2004).

Here, to investigate immune cell infiltration during tumor development, we utilized the mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) model of luminal B breast cancer, where the polyoma virus middle T antigen is expressed under the direction of the mouse mammary tumor virus promoter (Lin *et al.*, 2003; Fein *et al.*, 2020). This protocol was optimized for use with mouse mammary tumors, where immune cells may represent 20–40% of total cells. This protocol has two major steps: first, we prepare a single-cell suspension of the mouse mammary tumors, and second, we use multi-color flow cytometry to identify different immune cell subsets. We used this protocol to identify and characterize the following tumor-infiltrating immune cell populations: TAMs, conventional dendritic cells (DCs), CD103-expressing DCs, tumor-associated neutrophils, inflammatory monocytes, NK cells, CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, and Tregs. Other immune cell types can be studied (*e.g.*, B cells), depending on the aim of the experiment. This protocol can also be used with other mouse models of breast cancer (*i.e.*, orthotopic transplantation models based on cell lines, such as the 4T1) and can be easily adapted to other murine cancer models.

Materials and Reagents

1. Multi-channel pipette 200 μ L + 200 μ L tips
2. Multi-dispenser pipette 1,000 μ L + 1,000 μ L tips
3. 60 mm \times 15 mm Petri dish (Sigma, catalog number: P5481)
4. Cell strainers 70 μ m or 100 μ m nylon (Falcon, catalog number: 352350 or 352360)
5. Falcon conical tubes (15 mL, 50 mL) (Falcon, catalog numbers: 352196 and 352070)
6. 1 mL sterile syringes (Fisher Scientific, catalog number: 15889142)
7. 5 mL, 10 mL, and 25 mL pipettes (VWR, catalog numbers: 612-3702, 612-3700, and 612-3698)
8. Eppendorf tubes 1.5 mL
9. Scalpels
10. Parafilm
11. Fluorescence-activated cell sorting (FACS) tubes with cell strainer (Corning, catalog number: 352235)
12. FACS tubes polystyrene 5 mL round bottom 12 \times 75 mm (Corning, catalog number: 352052)
13. UltraComp eBeads compensation beads (eBiosciences, catalog number: 01-2222-42), store at 4°C
14. 96-wells V-/conical-bottom plates (Sarstedt, catalog number: 82.1583.001)

Note: Alternatively, we have used round-bottom plates with similar results.

15. 1× Dulbecco's Phosphate-Buffered Saline (PBS) (sterile, without Ca^{++} and Mg^{++}) (Gibco, catalog number: 14190144), store at room temperature
16. Hank's balanced salt solution (HBSS) (Gibco, catalog number: 14170112), store at 4°C
17. 0.02% Sodium Azide (Sigma, catalog number: S2002), store at 4°C
18. Bovine serum albumin (BSA) lyophilized powder, suitable for cell culture (Sigma, catalog number: A9418), store at 4°C
19. RPMI 1640 (Gibco, catalog number: 21875034), store at 4°C
20. Red Blood Cell Lysing (RBCL) buffer Hybri-Max (Sigma, catalog number: R7757), store at room temperature
21. Collagenase IV (Sigma, catalog number: C5138), store at -20°C
22. DNase I recombinant, RNase-free, 10 U/ μl (Roche, catalog number: 4716728001), store at -20°C
23. Trypan blue solution (Gibco, catalog number: 15250061), store at room temperature
24. Purified anti-mouse CD16/CD32 (Fc block) (Biolegend, catalog number: 101302), store at 4°C
25. Zombie Red Fixable Viability Kit (Biolegend, catalog number: 423109), store at 4°C
26. True-Nuclear Transcription Factor Buffer Set (Biolegend, catalog number: 424401), store at -20°C
27. Dissociation buffer (see Recipes)
28. DPBS (or HBSS) with 0.5% BSA (see Recipes)
29. 10% Sodium azide stock solution (see Recipes)
30. Ice-cold FACS buffer (see Recipes)
31. FACS antibodies (see Table 1)

Table 1. Flow antibodies information

Antibody	Fluorophore	Host	Clone	IgG subtype	Catalog no.	Producer
CD45	eFluor450	Rat	30-F11	IgG2b, kappa	48-0451-82	eBiosciences
MHC II	APC-eFluor780	Rat	M5/114.15.2	IgG2b, kappa	47-5321-82	eBiosciences
CD103	PE	Armenian Hamster	2E7	IgG	121405	Biolegend
CD3	FITC	Rat	17A2	IgG2b, kappa	100203	Biolegend
CD4	PerCP/Cy5.5	Rat	RM4-4	IgG2b, kappa	116011	Biolegend
CD11b	FITC	Rat	M1/70	IgG2b, kappa	101205	Biolegend
CD11c	APC	Armenian Hamster	N418	IgG	117309	Biolegend
F4/80	PerCP/Cy5.5	Rat	BM8	IgG2a, kappa	123127	Biolegend
CD8	AF700	Rat	53-6.7	IgG2a, kappa	100729	Biolegend
NKp46/C D335	PE	Rat	29A1.4	IgG2a, kappa	137603	Biolegend
$\gamma\delta$TCR	APC	Armenian Hamster	GL3	IgG	118115	Biolegend
FOXP3	PE	Rat	NRRF-30	IgG2a, kappa	14-4771-80	eBiosciences

Equipment

1. Pipettor (*e.g.*, Pipet-Aid)
2. Counting chamber slides or a hemacytometer
3. Dissection tools (Fine Science Tools)
4. Caliper (to measure tumor size) (Fine Science Tools, catalog number: 30087-00)
5. BD LSRII flow cytometer (BD, Franklin Lakes, NJ)
6. Four-laser setup: Violet (403 nm), Blue (488 nm), Yellow/Green (561 nm), and Red (640 nm). This protocol is

Cite as: Almeida, A. S. et al. (2021). Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer. Bio-protocol 11(11): e4012. DOI: 10.21769/BioProtoc.4012.

73

written for analysis on a BD LSRII flow cytometer, but it can be easily adapted for use with any 4-laser cytometer. The availability of the lasers and the configuration of the mirrors in the user's cytometer will determine which fluorochromes can be used.

7. Shaker incubator at 37°C
8. Table-top centrifuge (with plates adaptor) at room temperature and 4°C
9. Automated cell counter (Invitrogen) or a light microscope
10. Bench-top vortex with a 96-well plate adaptor (optional)

Software

1. FlowJo (BD, version 10, <https://www.flowjo.com/>)
2. GraphPad Prism (GraphPad, version 8, <https://www.graphpad.com/>)

Procedure

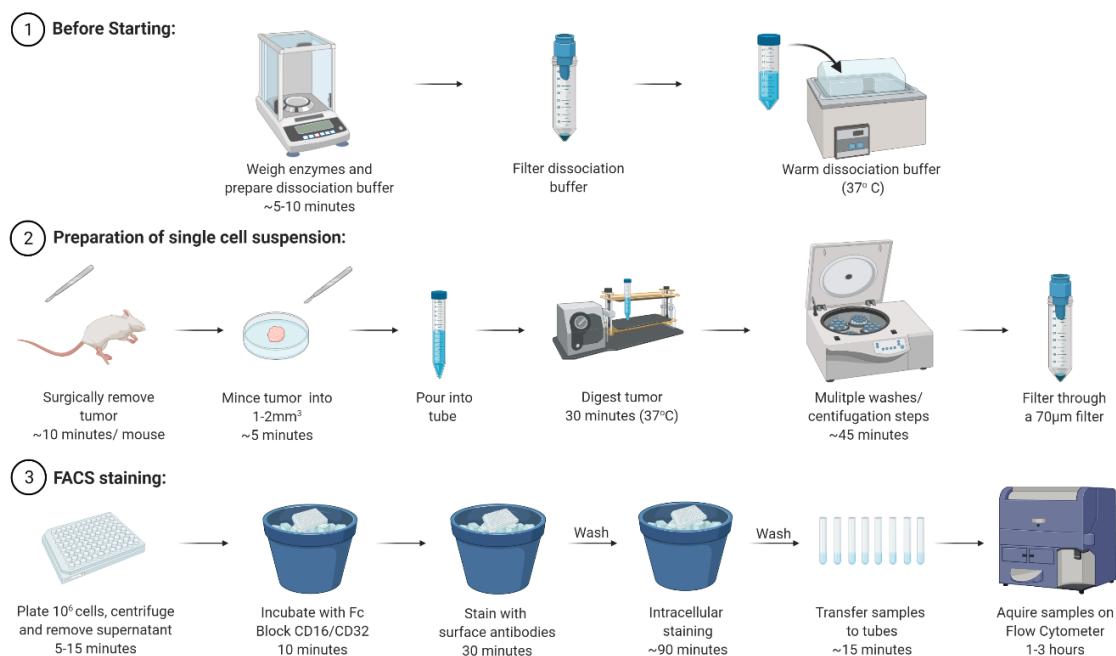


Figure 1. Schematic of the main steps of the protocol

Before starting:

Prepare the Dissociation buffer (see Recipes and Notes below). Use 10 mL per tumor (for diameters of 0.6-1.2 cm), filter it, and warm it at 37 °C (*e.g.*, in a water bath). Turn on the shaker incubator at 37 °C. The dissociation buffer should be prepared fresh before each experiment.

Animal euthanasia must be performed according to the instructions of the local Institutional Animal Care and Use Committee (IACUC).

A. Preparation of a single cell suspension from mammary tumors (Figure 1)

Cite as: Almeida, A. S. et al. (2021). Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer. Bio-protocol 11(11): e4012. DOI: 10.21769/BioProtoc.4012.

74

1. Measure the tumor with a caliper and take note of the tumor size.
2. Surgically remove the tumor from the mouse and place it on a sterile 60 mm × 15 mm Petri dish containing 3 mL of ice-cold RPMI or ice-cold DPBS/0.5% BSA. Take care to avoid taking the lymph node embedded within the mammary gland/tumor tissue.
3. Mince the tumor into 1-2 mm³ pieces using two scalpels (5-10 min, depending on the tumor size) and pour the minced tumor in the buffer into a 15 mL conical tube.
4. Add 10 mL of pre-warmed Dissociation buffer (Recipe 1).
5. Incubate for 30 min at 37°C in a shaker incubator. Tighten the 15 mL conical tube cover and wrap it with parafilm. Disrupt tissue every 10 min by vigorously pipetting up and down with a 5 mL pipette.

Note: The sample should be cloudy but not stringy. If it is stringy at this step, the DNase I concentration in the dissociation buffer can be increased by adding more DNase I solution.

6. Pipette cell suspension up and down for 2 min using a 5 mL pipette; it is very important to resuspend the cells completely as red blood cells tend to attach to myeloid cells.
7. Centrifuge at 300 × g (1,200 rpm) for 10 min at room temperature.

Note: Do not centrifuge at speeds higher than 300 × g if working with lymphocytes as they are sensitive to high g-forces.

8. Aspirate the supernatant and gently resuspend the pellet in 10 mL of DPBS (or HBSS) supplemented with 0.5% BSA.
9. Place a 100 µm cell strainer directly on top of a fresh 50 mL conical tube. Filter the resuspended cell suspension through the cell strainer. Lift the cell strainer from the tube to allow the content to go through. If necessary, rinse the cell strainer once with 2-3 mL of DPBS (or HBSS) supplemented with 0.5% BSA.
10. Centrifuge at 300 × g (1,200 rpm) for 10 min at room temperature. Aspirate and discard the supernatant.
11. [Skip Steps A10-A14 if tumor cell suspension is not bloody]. Resuspend the pellet in 2 mL of Red Blood Cell Lysing (RBCL) buffer. Gently mix for 1 min at room temperature.

Note: Do not place cells on ice during this incubation step.

12. Add 20 mL of DPBS (or HBSS) supplemented with 0.5% BSA.
13. Centrifuge at 300 × g (1,200 rpm) for 7 min at room temperature. Discard the supernatant.

Note: If red blood cell lysis is incomplete, which will be evident macroscopically as a red-colored cell pellet, repeat Steps A10-A12.

14. Resuspend the pellet in 20 mL of DPBS (or HBSS) supplemented with 0.5% BSA.
 15. Centrifuge at 300 × g (1,200 rpm) for 7 min at room temperature. Discard the supernatant.
- Note: This washing step is to ensure that no RBCL buffer remains in the sample.*
16. Resuspend the pellet in 1 mL of DPBS (or HBSS) supplemented with 0.5% BSA.
 17. Place a 70 µm cell strainer directly on top of a fresh 50 mL conical tube. Filter the resuspended cell suspension through the cell strainer.

Note: The cells tend to clump; therefore, ensure you resuspend the cells carefully and filter them before counting.

18. Count the cells to calculate the concentration of live cells using Trypan blue. Keep the cells on ice or at 4°C.

B. Stain cell populations for FACS analysis (Figure 1)

Cite as: Almeida, A. S. et al. (2021). Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer. Bio-protocol 11(11): e4012. DOI: 10.21769/BioProtoc.4012.

75

Note: Use ice-cold reagents/solutions and perform all steps at 4 °C (on ice); low temperatures prevent the modulation and internalization of surface antigens, which can reduce fluorescence intensity.

1. Plate cells in a 96-well V-conical-bottom plate. Each well should contain approximately 1×10^6 cells. Be sure to include wells for single-color controls for compensation (see Figure 2 for example).
2. Centrifuge the plate of cells at $300 \times g$ (1,200 rpm) for 4 min at 4°C.
3. Discard the supernatant by flicking the plate.

Note: Ensure there are cells collected at the bottom of each inoculated well as a visible pellet.

4. Resuspend the cells in 50 µL of ice-cold FACS Buffer with Fc Block CD16/CD32 (1:50 dilution for a concentration of 0.5 µg per well).

Note: This blocking step is important to prevent non-specific binding and background fluorescence.

5. Incubate on ice for 10 min.

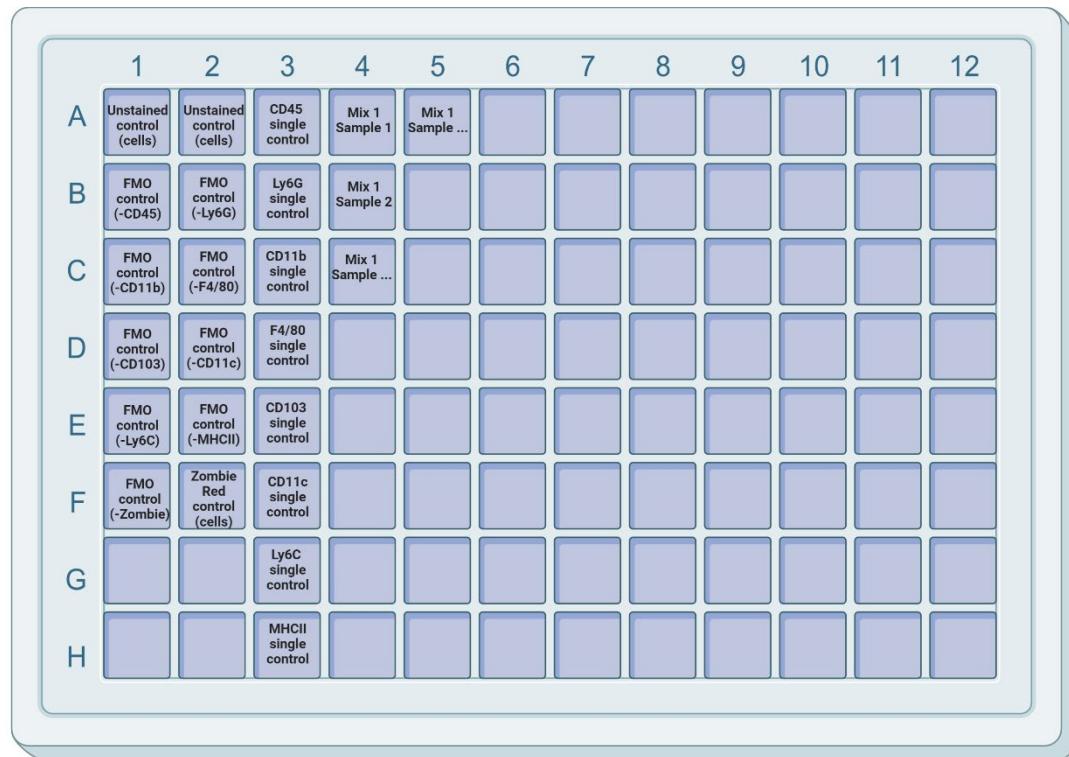


Figure 2. Ninety-six-well plate layout.

Example of a suggested layout for Mix 1, including unstained, single stain, and FMO controls. For single-color controls, compensation beads (*e.g.*, UltraComp eBeads) can be used. Unstained and FMO controls must be done with the sample of interest to account for auto-fluorescence. Single-color controls must be included for each antibody/fluorophore used.

Cell-Surface Staining:

6. While cells are incubating in Fc block, prepare the antibody solutions (see Table 1) with the FACS buffer. Suggested antibody combinations and dilutions (based on a total volume of 100 µL/well, 50 µL of Fc block + 50 µL of antibody mix) are shown in Table 2. Prepare 50 µL antibody mixtures per sample.

Table 2. Suggestion of three different antibody panels

Laser	Filter	Fluorochrome	Panel 1 - Myeloid cells	Final Recommended Dilution (100 μL/well)	Panel 2 - Lymphocytes	Final Recommended Dilution (100 μL/well)	Panel 3 - Lymphocytes (intracellular)	Final Recommended Dilution (100 μL/well)
403-nm violet laser	403_450/40	BV421	CD45	1/100	CD45	1/100	CD45	1/100
	403_780/60	BV786	Ly6G	1/100				
488-nm blue laser	488_530/30	FITC	CD11b	1/100	CD3	1/100	CD3	1/100
	488_695/40	PerCP-Cy5.5	F4/80	1/80	CD4	1/200	CD4	1/200
561-nm yellow/green laser	561_582/15	PE	CD103	1/200	NKp46/CD335	1/50	FOXP3*	1/100
	561_610/20	610/620	Zombie Red	1/300	Zombie Red	1/300	Zombie Red	1/300
640-nm red laser	640_670/30	APC	CD11c	1/200	γδTCR	1/200		
	640_730/45	AF700	Ly6C	1/200	CD8	1/200	CD8	1/200
	640_670/30	APC-eFluor780	MHC II	1/200				

Note: Antibodies are added as 50 μL solutions to final total volumes of 100 μL. Therefore, the final dilution represents half of the dilutions prepared. * Intracellular antibody.

From this step on, keep the plate in the dark and on ice.

7. Add 50 µL of antibody solution (Table 2) to the appropriate wells (it is unnecessary to wash the FC block off the cells; the total volume of the well will be 100 µL, with final dilution factors indicated in Table 2) and mix by pipetting up and down. Incubate the cells in the dark and on ice for 30 min.
8. For flow cytometer compensation, prepare a sample of unstained cells (control well) and samples with cells (or beads) stained with each antibody-fluorophore combination used in the experiment. In control wells that do not receive antibody, it is critical to add the 50 µL of FACS buffer to avoid letting the cells dry out. Compensation beads can also be used for single-cell staining. We suggest the use of UltraComp eBeads (follow the suggested protocol from the manufacturer).
9. Wash the cells by adding 150 µL of ice-cold FACS buffer to each well.
10. Centrifuge the plate with cells at 300 × g (1,200 rpm) for 4 min at 4 °C.
11. Discard the supernatant by flicking the plate. Wash 2× with 200 µL of ice-cold FACS buffer.

Intracellular (nuclear) staining:

(If no intracellular staining is to be performed, go directly to Step B23)

To stain for FOXP3, an intracellular antigen, we used the True-Nuclear™ Transcription Factor Staining Buffer from Biolegend, following the exact instructions provided by the manufacturer. Other kits might be used instead of the protocol is adapted for staining of intracellular cytokines (e.g., IL-6).

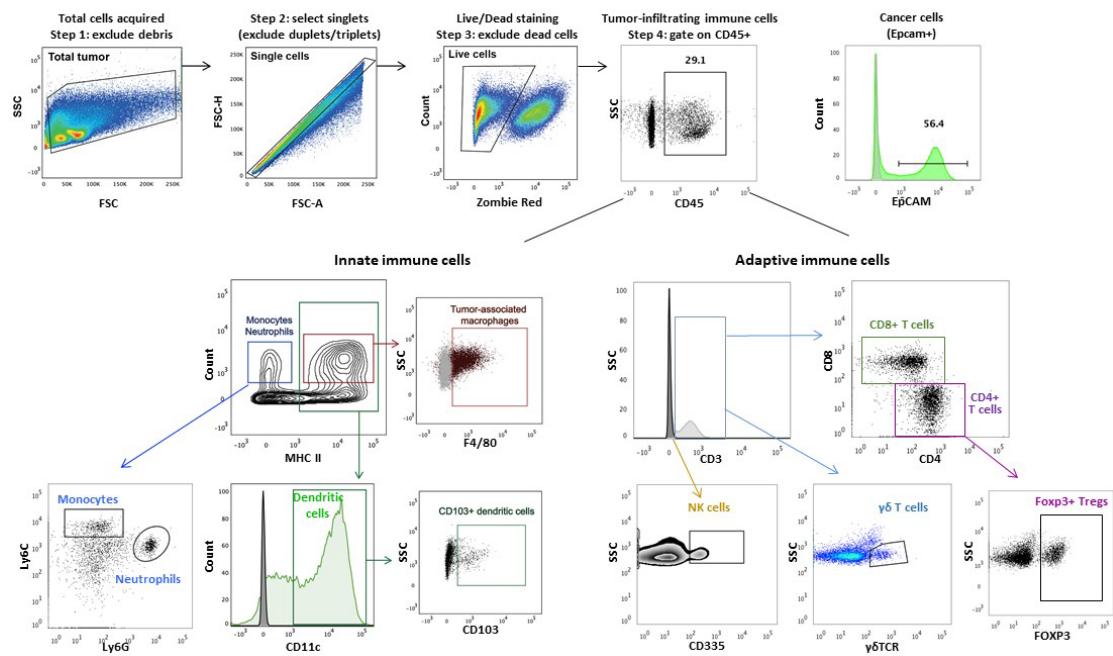
12. After the last wash, discard the supernatant and gently vortex the samples (or pipette up and down) to dissociate the cell pellet.
13. Prepare fresh True-Nuclear Fix working solution by diluting the 4× Fix Concentrate (1 part) with the Fix Diluent (3 parts)
14. Add 200 µL of the True-Nuclear 1× Fix working solution to each well. Gently pipette up and down to ensure cells are fully resuspended. Incubate at room temperature in the dark for 45–60 min.

Note: A longer fixation period can help reduce high background.

15. Centrifuge the plate at 300 × g (1,200 rpm) for 4 min at room temperature. Discard the supernatant.
16. Prepare a 1× working solution of the Perm Buffer by diluting the 10× Perm Buffer with distilled water.
17. Add 200 µL of the True-Nuclear 1× working solution Perm Buffer to each well.
18. Centrifuge the plate at 300 × g (1,200 rpm) for 4 min at room temperature. Discard the supernatant.
19. Repeat Steps B14-B15 for two additional times, for a total of three washes using the True-Nuclear 1× Perm Buffer.
20. Add the appropriate amount of FOXP3 antibody diluted in True-Nuclear 1× Perm Buffer to each well and incubate in the dark at room temperature for at least 30 min.
21. Add 200 µL of the True-Nuclear 1× Perm Buffer to each well. Repeat Steps B15-B16.
22. Resuspend the cells in 150 µL of ice-cold FACS Buffer.
23. Transfer the cells to labeled 12 × 75 mm polystyrene test tubes and add 350 µL of FACS Buffer (so that the final volume is 500 µL).
24. Acquire the samples on a flow cytometer as fast as possible (keep the cells in the dark and on ice or at 4 °C). We typically acquire 500,000–750,000 events or cells per sample.
25. Analyze data using software such as FlowJo or FACSDiva. Immune cell populations are identified based on the expression of cell surface molecules (see Table 3); an illustrative gating scheme is shown in Figure 3.

Table 3. Definition of immune cell populations based on the expression of cell surface markers

Immune cell Population	Cell Surface Markers
Total leukocytes	CD45 ⁺
Conventional Dendritic Cells (myeloid lineage)	CD45 ⁺ CD11b ⁺ MHCII ⁺ CD11c ⁺
CD103 ⁺ Dendritic cells	CD45 ⁺ CD11b ⁻ MHCII ⁺ CD11c ⁺ CD103 ⁺
Tumor-associated macrophages	CD45 ⁺ CD11b ⁺ MHCII ⁺ F4/80 ⁺
Inflammatory Monocytes	CD45 ⁺ CD11b ⁺ MHCII ⁺ F4/80 ⁻ Ly6C ^{high}
Myeloid-derived suppressor cells (monocytic)	CD45 ⁺ CD11b ⁺ MHCII ⁻ Ly6C ^{high} Ly6G ⁻
Neutrophils/Myeloid-derived Suppressor cells (granulocytic)	CD45 ⁺ CD11b ⁺ MHCII ⁻ Ly6C ^{low} Ly6G ⁺
T lymphocytes	CD45 ⁺ CD3 ⁺
CD8 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD8 ⁺
CD4 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺
FOXP3 ⁺ T regulatory cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ FOXP3 ⁺
NK cells	CD45 ⁺ CD3 ⁻ CD335/NKp46 ⁺
NK T cells	CD45 ⁺ CD3 ⁺ CD335/NKp46 ⁺
Gamma-delta T cells	CD45 ⁺ gdTCR ⁺

**Figure 3. Illustrative gating strategy for immune cells.**

First, cells from tumors are plotted on a Forward Scatter (FSC) versus Side Scatter (SSC) plot to discriminate intact cells from debris. Then, single cells are selected by displaying a plot of FSC area (FSC-A) versus FSC height (FSC-H). Live/dead cells are discriminated by selecting the Zombie Red low (live cells) or high (dead cells) population. Live single cells are then evaluated for the expression of selected

cell surface markers (Table 3). Epithelial-derived cancer cells can be further discriminated from CD45⁺ cells by expression of EpCAM. The plots in this figure are representative of data presented in Fein *et al.* (2020) and adapted with permission.

Data analysis

Raw data output files of a flow cytometry experiment are generated as .fcs. These can be easily opened in the FlowJo software (<https://www.flowjo.com/>).

The gating strategy used is depicted in Figure 3 (adapted from Fein *et al.*, 2020).

Statistical analyses can be performed using GraphPad Prism Version 8 software (<https://www.graphpad.com/>).

Figures 1 and 2 were created with BioRender.com.

Notes

1. Proper compensation using single-color controls is necessary to account for bleed-through fluorescence being measured in a detection channel other than the primary channel. Compensation parameters can be automatically calculated “live” after signal detection by the cytometer or after the data have been collected (“offline”). In this protocol, we performed “live” compensation using the FACSDiva software.
2. We strongly recommend the use of fluorescent-minus-one (FMO) controls. This is a sample that has been stained with all the reagents except one; the analysis of these samples allows for the precise definition of cells that have fluorescence above background levels. For example, in the myeloid panel 1 (Table 2), the FMO controls would be 1) all antibodies minus CD45, 2) all antibodies minus Ly6G, 3) all antibodies minus CD11b, and so on (see Figure 2).
3. Isotype controls should also be used when first using a new antibody. Isotype controls are primary antibodies that lack specificity to the target but match the class and type of the primary antibody. They are used as negative controls to help differentiate non-specific background signals from specific antibody signals. However, isotype controls do not replace FMO controls (Maecker and Trotter, 2006; Cossarizza *et al.*, 2017).
4. For inexperienced users, we highly recommend discussing with the institution’s flow cytometry core manager before planning the experiment.

Recipes

1. Dissociation buffer

Collagenase IV (final concentration 2 mg/mL)
DNase I 10U/mL (final concentration 4 U/mL) in RPMI
For 10 mL of RPMI, use 20 mg of collagenase IV + 4 µL of 10 U/µL DNase I

2. DPBS (or HBSS) with 0.5% BSA

- a. Dissolve 2.5 g of BSA in 500 mL of DPBS
- b. Filter the solution and keep the buffer at 4°C

3. 10% Sodium azide stock solution

- a. Dissolve 10 g of sodium azide in 100 mL of distilled H₂O
- b. Prepare 1 mL aliquots and store at -20°C

4. Ice-cold FACS buffer (filtered)

DPBS/0.5%BSA/Sodium Azide 0.02% w/v

Keep at 4°C for up to 2 months.

Notes:

- a. Use Ca/Mg^{2+} free PBS. The absence of these ions reduces cation-dependent cell-to-cell adhesion and prevents clumping.
- b. Use 0.1 to 1% BSA. Serum proteins protect cells from apoptosis, prevent non-specific staining and prevent cells from sticking.
- c. EDTA prevents cation-based cell-to-cell adhesion and should be included in the buffer if dealing with sticky and adherent cells, like macrophages, and if these cells are to be sorted for functional cell culture assays. In that case, we recommend including 0.5-5 mM EDTA (the optimum concentration should be determined in pilot experiments to avoid cell toxicity). For characterization of immune cell infiltration into tumors using this protocol, the use of EDTA is optional.
- d. Sodium azide (0.01-1%) at low concentrations reduces bacterial contamination, prevents photobleaching, and blocks antibody shedding. The optimum concentration should be determined to avoid cell toxicity. If cells are to be collected for functional assays, do not use sodium azide because it inhibits metabolic activity.

Acknowledgments

The authors would like to acknowledge support from the Cold Spring Harbor Cancer Center Support Grant (CCSG, P30-CA045508) shared resources, the Animal Facility, and P. Moody in the Flow Cytometry Facility. This work was supported by funds from the Simons Foundation to CSHL. This protocol was adapted from previous work (Fein *et al.*, 2020).

Competing interests

M. Egeblad holds stocks in Agios Pharmaceutical and has received personal fees from MPM, CytomX, and Insmed for consulting services, outside the submitted work.

Ethics

All animal procedures and studies were approved by the Institutional Animal Care and Use Committee at CSHL and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (ID 18-15-12-09-6, valid from July 2018 to July 2021). This protocol was modified from Fein *et al.* (2020).

References

- Beatty, G. L. and Gladney, W. L. (2015). [Immune escape mechanisms as a guide for cancer immunotherapy](#). *Clin Cancer Res* 21(4): 687-692.
- DeNardo, D. G., Andreu, P. and Coussens, L. M. (2010). [Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity](#). *Cancer Metastasis Rev* 29(2): 309-316.
- DeNardo, D. G., Brennan, D. J., Rexhepaj, E., Ruffell, B., Shiao, S. L., Madden, S. F., Gallagher, W. M., Wadhwan, N., Keil, S. D., Junaid, S. A., Rugo, H. S., Hwang, E. S., Jirstrom, K., West, B. L. and Coussens, L. M. (2011). [Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy](#). *Cancer Discov* 1(1): 54-67.
- Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. and Schreiber, R. D. (2002). [Cancer immunoediting: from immunosurveillance to tumor escape](#). *Nat Immunol* 3(11): 991-998.
- Fein, M. R., He, X. Y., Almeida, A. S., Bružas, E., Pommier, A., Yan, R., Eberhardt, A., Fearon, D. T., Van Aelst,

Cite as: Almeida, A. S. et al. (2021). Multi-color Flow Cytometry for Comprehensive Analysis of the Tumor Immune Infiltrate in a Murine Model of Breast Cancer. Bio-protocol 11(11): e4012. DOI: 10.21769/BioProtoc.4012.

- L., Wilkinson, J. E., Dos Santos, C. O. and Egeblad, M. (2020). [Cancer cell CCR2 orchestrates suppression of the adaptive immune response](#). *J Exp Med* 217(10).
- Grivennikov, S. I., Greten, F. R. and Karin, M. (2010). [Immunity, inflammation, and cancer](#). *Cell* 140(6): 883-899.
- Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J. and Pollard, J. W. (2003). [Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases](#). *Am J Pathol* 163(5): 2113-2126.
- Ménard, S., Tomasic, G., Casalini, P., Balsari, A., Pilotti, S., Cascinelli, N., Salvadori, B., Colnaghi, M. I. and Rilke, F. (1997). [Lymphoid infiltration as a prognostic variable for early-onset breast carcinomas](#). *Clin Cancer Res* 3(5): 817-819.
- Noy, R. and Pollard, J. W. (2014). [Tumor-associated macrophages: from mechanisms to therapy](#). *Immunity* 41(1): 49-61.
- Perfetto, S. P., Chattopadhyay, P. K. and Roederer, M. (2004). [Seventeen-colour flow cytometry: unravelling the immune system](#). *Nat Rev Immunol* 4(8): 648-655.
- Cossarizza, A., Chang, H., Radbruch, A., Akdis, M., Andrä, I., Annunziato, F., Bacher, P., Barnaba, V., Battistini, L., Bauer, W.M., et al. (2017). [Guidelines for the use of flow cytometry and cell sorting in immunological studies](#). *Eur J Immunol* 47: 1584-1797.
- Maecker, H.T., and Trotter, J. (2006). [Flow cytometry controls, instrument setup, and the determination of positivity](#). *Cytometry A* 69(9): 1037-1042.

***In vitro* and *In vivo* CD8⁺ T Cell Suppression Assays**

Lu Xie^{1,2, #}, Guihuan Liu^{3, #}, Yanjun Liu³ and Yuming Yu^{1,2, *}

¹Department of Urology, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Guangzhou, China

²School of Medicine, South China University of Technology, Guangzhou, China

³Department of Immunology, School of Basic Medical Science, Southern Medical University, Guangzhou, China

*For correspondence: yuym72@163.Com

#Contributed equally to this work

Abstract

CD8⁺CD28⁻ T suppressor cells (Ts) have been documented to promote immune tolerance by suppressing effector T cell responses to alloantigens following transplantation. The suppressive function of T cells has been defined as the inhibitory effect of Ts on the proliferation rate of effector T cells. ³H-thymidine is a classical immunological technique for assaying T cell proliferation but this approach has drawbacks such as the inconvenience of working with radioactive materials. Labeling T cells with CFSE allows relatively easy tracking of generations of proliferated cells. In this report, we utilized antigen presenting cells (APCs) and T cells matched for human leukocyte antigen (HLA) class I or class II to study CD8⁺CD28⁻ T cell suppression generated *in vitro* by this novel approach of combining allogeneic APCs and γc cytokines. The expanded CD8⁺CD28⁻ T cells were isolated (purity 95%) and evaluated for their suppressive capacity in mixed lymphocyte reactions using CD4⁺ T cells as responders. Here, we present our adapted protocol for assaying the Ts allospecific suppression of CFSE-labeled responder T cells.

Keywords: CD8⁺CD28⁻ T cells, Suppression, Immune tolerance, Alloantigen specific tolerance, Common gamma chain cytokines

This protocol was validated in: BMC Immunol (2020), DOI: 10.1186/s12865-020-00354-z.

Background

T regulatory cells (Tregs) with dedicated suppressor function play a crucial role in the homeostatic control of immunity in the periphery. Regulatory CD8⁺ T cells have also been demonstrated to play an important role in neonatal tolerance and autoimmune diseases (Tang *et al.*, 2005). There are two broad categories of immune regulation by Tregs: non-specific and antigen-specific. Non-specific immunosuppression potentially causes general immunosuppression and produces undesirable side effects, such as infectious diseases. These Tregs include CD8⁺CD25⁺, CD8⁺CD122⁺, CD45RC^{low}, and IL-2/GM-CSF-induced CD8⁺ Tregs. On the contrary, antigen-specific Tregs are primed during the immune response to foreign or self-antigens and subsequently specifically downregulate that immune response. These Tregs include CD8⁺CD28⁻, CD8⁺CD75s⁺, plasmacytoid dendritic cell (DC2)-induced CD8⁺, CD8⁺CD45RC^{high} Tc1, and TCR peptide-specific CD8^{αα} Tregs. CD8⁺CD28⁻ Tregs have been recently documented to play an important role in alloimmunity. In our previous studies, we have expanded large numbers of human CD8⁺CD28⁻ Tregs in a relatively short period of time by stimulating CD8⁺ T cells with APCs following supplementation with the triple common gamma chain cytokines IL-2, IL-7, and IL-15 *in vitro*; however, the detailed characteristics of the expanded CD8⁺CD28⁻ Tregs were unclear. Moreover, the principal function of this population when transferred *in vivo* was yet to be examined. Measurement of suppression has been achieved through the co-culture of Tregs and T effector cells. Methods include the detection of cell proliferation, cytokine production, and activation markers (CD25 or CD134) (Long *et al.*, 2017). CFSE-based co-culture has become the gold standard for proliferation assays and has been used successfully to assess the function of Tregs. Here, based on CFSE co-culture assays, we show that the *in vitro*-expanded CD8⁺CD28⁻ Tregs maintain allospecific suppressive capacity both *in vitro* (Figure 1) and *in vivo*.

The main steps of *In vitro* CD8⁺ T Cells Suppression Assays protocol

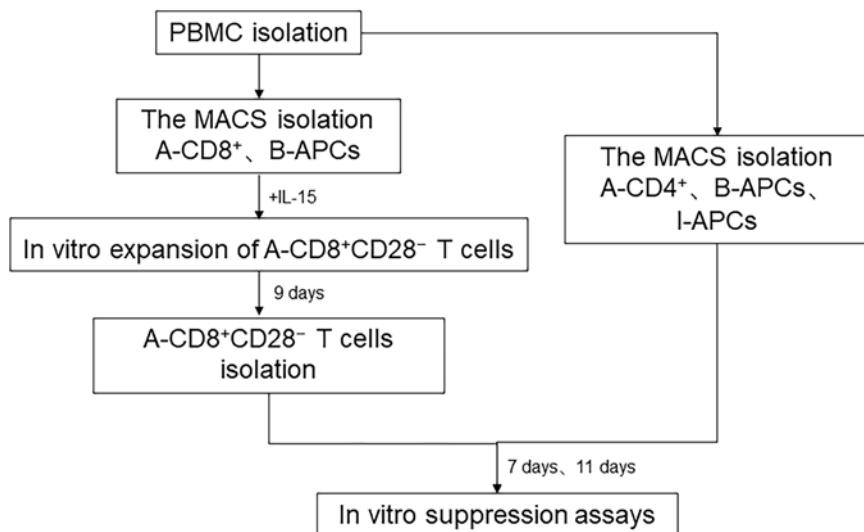


Figure 1. The main steps of the *in vitro* CD8⁺ T cell suppression assay protocol

Materials and Reagents

1. Heparin sodium anticoagulation tubes, 5 mL (JiangSu, YuLi)
2. Centrifuge tubes, 50 mL (Corning, catalog number: 430829)
3. Centrifuge tubes, 15 mL (Corning, catalog number: 430791)
4. 24-well round-bottomed plates (Corning, catalog number: 3524)
5. 96-well round-bottomed plates (Corning, catalog number: 3799)

6. MidiMACS separator (Miltenyi, catalog number: 130-042-302)
7. LS column (Miltenyi, catalog number: 130-042-401)
8. Disposable syringe with a needle (ShuangGe, China)
9. 70- μ m cell strainer (Biologix, catalog number: 15-1070)
10. NOG mice (Beijing Vital River Laboratory Animal Technology Co Charles River Laboratories)
11. Ficoll-Hypaque solution (Haoyang Biologiacal, TBD sciences, catalog number: LTS1077)
12. RPMI 1640, 500 mL (ThermoFisher, Gibco, catalog number: C11875500BT)
13. FBS Qualified Australia Origin (ThermoFisher, Gibco, catalog number: 10099141 C)
14. Bovine serum albumin, BSA (SIJIA, catalog number: N0008-1)
15. Phosphate-buffered saline, PBS (ThermoFisher, Gibco, catalog number: C10010500BT)
16. CD8 MicroBeads, human (Miltenyi, catalog number: 130-045-201)
17. CD28 MicroBeads, human (Miltenyi, catalog number: 130-093-247)
18. CD2 MicroBeads, human (Miltenyi, catalog number: 130-091-114)
19. CD4 MicroBeads, human (Miltenyi, catalog number: 130-045-101)
20. IL-2 (PeproTech, catalog number: AF-200-02-50)
21. IL-7 (PeproTech, catalog number: AF-200-07-50)
22. IL-15 (PeproTech, catalog number: AF-200-15-50)
23. 10 \times RBC lysis buffer (ThermoFisher, Invitrogen, catalog number: 00-4300-54)
24. Trypan Blue solution 0.4%, liquid (MERCK,Sigma-Aldrich, catalog number: T8154-100ML)
25. 7-AAD viability stain solution (ThermoFisher, Invitrogen, catalog number: 00-6993-50)
26. CFDA, SE (ThermoFisher, Invitrogen, catalog number: C1157)
27. Flow cytometry antibodies
 - a. AlexaFluorTM 700 mouse anti-human CD3 monoclonal antibody, OKT3 (ThermoFisher, eBioscience, catalog number: 56-0037-42)
 - b. efluor 450 mouse anti-human CD8a monoclonal antibody, SK1 (ThermoFisher, eBioscience, catalog number: 48-0088-41)
 - c. APC-mouse anti-human CD28 monoclonal antibody, CD28.2 (ThermoFisher, eBioscience, catalog number: 17-0289-42)
 - d. APC-mouse anti-human CD4 monoclonal antibody, OKT4 (ThermoFisher,eBioscience, catalog number: 17-0048-42)
 - e. FITC mouse anti-human CD2 monoclonal antibody, RPA-2.10 (ThermoFisher, eBioscience, catalog number: 11-0029-42)
 - f. PE-mouse anti-human CD45 monoclonal antibody, HI30 (ThermoFisher,eBioscience, catalog number: 12-0459-42)
28. Fixable viability stain (FVS) 620 100 μ g (BD Pharmingen, catalog number: 564996)
29. NaCl
30. KCl
31. Na₂HPO₄
32. KH₂PO₄
33. EDTA
34. 1 \times PBS (pH 7.4) (see Recipes)
35. D-PBS (pH 7.4) (see Recipes)
36. 1% BSA-PBS (see Recipes)
37. 0.5% BSA-PBS (see Recipes)

Equipment

1. Centrifuge (Eppendorf, model: 5810R)
2. Electronic balance (Sartorius, model: BP61)
3. Microelectronic balance (OHAUS, model: AX124ZH)
4. Hemocytometer

5. Constant temperature water box
6. Incubator (Thermo, model: Thermo3111)
7. FACS LSRLFortessa (BD)
8. Finnpipette (Eppendorf)
9. Magnetic stirrer (BG-stirrelDB)
10. Optical microscope (CONIC, XDS-1B)
11. Clean bench

Software

1. Flowjo vX.0.7
2. SPSS 20.0
3. GraphPad Prism 5.01

Procedure

A. Peripheral blood mononuclear cell (PBMC) isolation

Isolate PBMCs from samples acquired in heparinized tubes from healthy volunteers. PBMCs are isolated by density gradient centrifugation using Ficoll-Hypaque solution.

1. Dilute the blood sample 1:2 with the same volume of RPMI 1640.
2. Add a volume of Ficoll-Hypaque solution equal to that of the blood sample in a 50-mL centrifuge tube.
3. Add the diluted blood sample carefully and slowly to the surface of the separation fluid. Centrifuge at $800 \times g$ for 20-30 min at room temperature.

Note: The blood sample volume determines the centrifugal conditions; read the separation solution instructions.

4. Carefully absorb the second annular opalescent lymphocyte layer into another centrifuge tube, add 10-20 mL RPMI 1640, mix well, and centrifuge the cell suspension at $580 \times g$ for 10 min at room temperature.
5. Aspirate the supernatant completely. Wash the cell pellet by adding 10 ml RPMI 1640 and centrifuging at $290 \times g$ for 10 min at room temperature.
6. Repeat Step A5.

B. MACS separation

CD8⁺ cells (A-CD8⁺) and CD4⁺ cells (A-CD4⁺) are isolated from PBMCs of individual A by positive selection using the MACS system. APCs are obtained by depletion of CD2⁺ cells from PBMCs of individual B (B-APCs) or individual I (I-APCs). Donors were selected according to their HLA-A, -B, and -DR compatibility or incompatibility based on the specific requirements of individual experiments. Several groups of individuals designated as A, B, and I, which are fully HLA-A, -B, and -DR mismatched were screened out from 130 volunteers and used for independent experiments.

Note: Keep the cells and the buffer cold. Centrifuge at 4°C.

1. Add 10 mL 0.5% BSA-PBS buffer to resuspend PBMCs (from Step A6) and determine the cell number.
2. Centrifuge the cell suspension at $300 \times g$ for 10 min. Aspirate the supernatant completely.
3. Resuspend the cell pellet in 80 μ L 0.5% BSA-PBS buffer per 10^7 cells.

4. Add 20 μ L appropriate Microbeads per 10^7 cells.
5. Mix well and incubate for 20 min in the refrigerator (2–8°C).
6. Wash the cells by adding 1–2 mL 0.5% BSA-PBS buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 min. Aspirate the supernatant completely.
7. Resuspend up to 10^8 cells in 500 μ L 0.5% BSA-PBS buffer.

Note: For higher cell numbers, scale up the buffer volume accordingly.

8. Place the LS column in the magnetic field of a MidiMACS separator.

Note: Choose an appropriate MACS column and MACS separator according to the numbers of total and positive cells. For details, see the Microbead instructions.

9. Prepare the column by rinsing with 3 mL 0.5% BSA-PBS buffer.
10. Apply the cell suspension to the LS column.
11. Collect the unlabeled cells that pass through and wash the column with 3 mL 0.5% BSA-PBS buffer. Collect the total effluent; this is the unlabeled cell fraction (such as CD2⁻ cells).
12. Wash the column by adding 3 mL 0.5% BSA-PBS buffer twice. Only add fresh 0.5% BSA-PBS buffer when the column reservoir is empty.
13. Remove the column from the separator and place on a suitable collection tube.
14. Pipette 5 ml buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column (such as CD8⁺ cells, CD4⁺ cells).
15. Perform flow cytometry analysis to evaluate the purity of the sorted cell suspensions; the purity should be ≥97%.

C. In vitro expansion of CD8⁺CD28⁻ T cells

A-CD8⁺ cells are isolated by positive selection using the MACS system according to Procedure B. B-APCs (CD2⁻ cells) from HLA-A, -B, -DR mismatched individual B are isolated by depletion of CD2⁺ cells using CD2 microbeads.

1. Centrifuge the A-CD8⁺ cells and B-APCs obtained above at $300 \times g$ for 10 min. Aspirate the supernatant completely.
2. Resuspend in 5 mL RPMI1640 and determine the cell number.
3. Centrifuge the A-CD8⁺ cells and B-APCs at $300 \times g$ for 10 min. Aspirate the supernatant completely.
4. Resuspend the cells in cell culture medium (RPMI 1640 supplemented with 15% FBS) to achieve A-CD8⁺ at 2×10^6 cells/ml and B-APCs at 1×10^6 cells/mL.
5. Seed the A-CD8⁺ cells (2×10^6 /well) and B-APCs (1×10^6 /well) onto 24-well flat-bottomed plates at a ratio of 2:1 in a total volume of 2 mL, in the presence of IL-2 at 20 U/ml, IL-7 at 50 ng/ml, and IL-15 at 50 ng/ml, in an incubator at 37°C and a humidified 5.5% CO₂ atmosphere.
6. Change semi-culture medium (1 mL) after 3 days. After 5 days and 7 days of coculture, mix well and divide one well into two.
7. On day 9, harvest the cells into 50-ml centrifuge tubes. Add 10 mL 0.5% BSA-PBS buffer to wash the cells and determine the cell number.
8. Centrifuge the cell suspension at $300 \times g$ for 10 min at 4°C. Aspirate the supernatant completely.
9. Resuspend the cell pellet in 40 μ L 0.5% BSA-PBS buffer per 10^7 cells.
10. Add 10 μ L CD28-PE per 10^7 cells.
11. Mix well and incubate for 10 min in the refrigerator (2–8°C).
12. Wash the cells by adding 1–2 mL 0.5% BSA-PBS buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 min at 4°C. Aspirate the supernatant completely.
13. Resuspend the cell pellet in 80 μ L 0.5% BSA-PBS buffer per 10^7 cells.
14. Add 20 μ L anti-PE microbeads per 10^7 cells.
15. Mix well and incubate for an additional 15 min in the refrigerator (2–8°C).

16. Wash the cells by adding 1-2 mL 0.5% BSA-PBS buffer per 10^7 cells and centrifuging at $300 \times g$ for 10 min at 4°C . Aspirate the supernatant completely.
17. Resuspend up to 10^8 cells in 500 μL 0.5% BSA-PBS buffer.

Note: For higher cell numbers, scale up the buffer volume accordingly.

18. Place the LS column in the magnetic field of a MidiMACS separator.

Note: Choose an appropriate MACS column and MACS separator according to the numbers of total and positive cells. For details, see the microbead instructions.

19. Prepare the column by rinsing with 3 mL 0.5% BSA-PBS buffer.
20. Apply the cell suspension to the column.
21. Collect the unlabeled cells that pass through and wash the column with 3 mL 0.5% BSA-PBS buffer. Collect the total effluent; this is the unlabeled cell fraction (CD28^- cells).
22. Perform flow cytometry analysis to evaluate the purity of CD28^- cell suspensions; the purity should be $\geq 95\%$.

D. In vitro suppression assays

Responder CD4^+ cells are isolated from PBMCs of individual A (A- CD4^+ T cells) by positive selection using CD4 microbeads. As stimulators, APCs cells from individual B (B-APCs) or from HLA-A, -B, -DR fully mismatched indifferent individual I (I-APCs) are isolated by depletion of CD2^+ cells using CD2 microbeads.

1. Centrifuge the A- CD4^+ cell suspension (from Step B14) at $300 \times g$ for 10 min at 4°C . Aspirate the supernatant completely.
2. Wash the freshly isolated CD4^+ cells with 10 mL PBS to remove any proteins. Centrifuge at $400 \times g$ for 10 min at 4°C .
3. Resuspend the cells in 1 mL PBS.
4. Add CFSE working dilution to a final concentration of 0.5 μM at room temperature.
5. Incubate for 7 min in the incubator (37°C); work gently and protect from light.
6. Add 1 mL FBS and 9 mL RPMI 1640 to terminate the reaction, then centrifuge at $300 \times g$ for 10 min. Aspirate the supernatant completely.
7. Wash the cells once again with RPMI 1640. Aspirate the supernatant completely.
8. Resuspend in 5 mL RPMI 1640 and count the cells.

Note: After labeling with CFSE, perform flow cytometry analysis to ensure that the cells are stained.

9. Centrifuge the A- $\text{CD8}^+\text{CD28}^-$ cells, B-APCs, and I-APCs obtained above at $300 \times g$ for 10 min. Aspirate the supernatant completely.
10. Resuspend in 5 mL RPMI 1640 and count the corresponding cells.
11. Centrifuge the A- $\text{CD8}^+\text{CD28}^-$ cells, B-APCs, I-APCs, and A- $\text{CD4}^{+\text{CFSE}}$ at $300 \times g$ for 10 min. Aspirate the supernatant completely.
12. Resuspend the cell pellet in culture medium (RPMI 1640 supplemented with 15% FBS) to achieve A- $\text{CD4}^{+\text{CFSE}}$ at 1×10^6 cells/mL, B-APCs or I-APCs at 1×10^6 cells/mL, and A- $\text{CD8}^+\text{CD28}^-$ cells at 2.5×10^5 cells/mL.
13. Seed the A- $\text{CD4}^{+\text{CFSE}}$ cells (5×10^4 /well), B-APCs or I-APCs (5×10^4 /well), and CD8 $^+\text{CD28}^-$ cells (2.5×10^4 /well) onto 96-well round-bottomed plates in a total volume of 200 μL cell culture medium containing 15% FBS.

Note: Responder CD4^+ T cells, stimulated with B-APCs or I-APCs, only served as positive controls (B-APCs were used as priming cells in vitro to expand $\text{CD8}^+\text{CD28}^-$ T cells from individual A, whereas I-

APCs had never had immune recognition by CD8⁺CD28⁻ T cells from individual A during the *in vitro* expansion period).

14. Change semi-culture medium (100 µl) after 3 days, 5 days, and 9 days of coculture.
15. After 7 or 11 days of coculture, harvest, stain, and detect the CD4⁺ cells for CFSE dilution by flow cytometry.

E. In vivo suppression assays

1. Isolate the responder CD4⁺ T cells and APCs (Procedure A and B).
2. Mix a total of 4×10^6 human CD4⁺ T cells with an equal number of APCs, either B-APCs or I-APCs, and combine with 2×10^6 *in vitro*-expanded human CD8⁺CD28⁻ T cells in a total volume of 1.5 mL PBS. The responder CD4⁺ T cells alone plus B-APCs are used in the same fashion as a positive control.
3. Administer the cell mixture for each group to NOG mice by intraperitoneal injection.
4. Sacrifice the mice on day 11 post-injection.
5. Isolate the spleen and homogenize to generate a single-cell suspension.
6. Centrifuge the cell suspension at $500 \times g$ for 5 min at 4°C. Aspirate the supernatant completely.
7. Add 3 mL 1× RBC lysis buffer to lyse the red cells, mix well, and incubate for 5 min at room temperature.
8. Add 2 mL PBS, centrifuge at $350 \times g$ for 10 min at room temperature. Aspirate the supernatant completely.
9. Stain the cells and detect those expressing CD4⁺ by flow cytometry.

Note: You can also detect human CD8⁺ and CD4⁺ cells in mouse spleen tissue by immunohistochemical techniques.

F. Staining cells for analysis by flow cytometry

Flow cytometry analysis is performed on cells harvested from the culture under different conditions (Procedures D and E).

1. Place the cells to be analyzed into 12 × 75 mm polystyrene tubes.
2. Wash the cells in 3 mL 1% BSA-PBS buffer at $350 \times g$ for 10 min at 4°C.
3. Discard the supernatant and quickly vortex to loosen the pelleted cells.
4. Add 2 µL antibodies (such as anti-CD4), mix well, and incubate for 30 min at 4°C in the dark.
5. Wash the cells in 3 mL 1% BSA-PBS buffer at $350 \times g$ for 10 min at 4°C.
6. Discard the supernatant and quickly vortex to loosen the pelleted cells.
7. Add 3 µL 7-AAD Viability Staining Solution and incubate for 10 min at room temperature in the dark.
8. Resuspend the cells in an appropriate volume of 1% BSA-PBS and detect by flow cytometry.

Data analysis

1. Data analysis is performed using offline analysis software such as Flowjo. As shown in Figure 2, the identification of CD4⁺ T cells begins with the creation of gates to isolate the lymphocyte population (FSC-A; SSC-A), followed by rigorous doublet exclusion (FSC-W vs. FSC-H and SSC-W vs. SSC-A). Exclude cells that are positive for dead staining and then gate on the target population of cells.

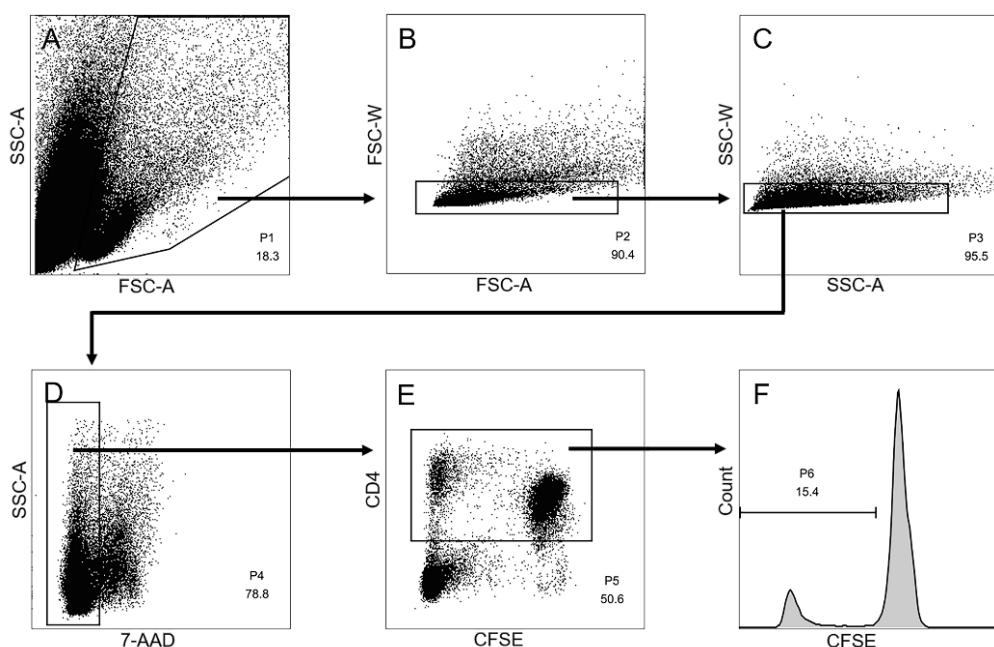


Figure 2. Gating strategy to identify CD4⁺ cells in suppression assays.

A. Lymphocyte population identified in FSC-A vs. SSC-A plot. B-C. This is followed by doublet discrimination using FSC and SSC area and width plots. D. After staining with 7-AAD, live cells are then analyzed. E. Live CD4⁺ T cells are gated. F. The proliferation of CD4⁺ T cells was measured by CFSE dilution.

2. The suppressive percentage is calculated as follows: % suppression = [1 - (% CD4⁺ T cell proliferation in the presence of CD8⁺CD28⁻ T cells) / (% CD4⁺ T cell proliferation in the absence of CD8⁺CD28⁻ T cells)] × 100.
3. Statistical analysis is carried out using the SPSS version 22 software. For comparison of different groups, where appropriate, an independent-samples t-test and nonparametric test are used to determine statistical significance. Graphs are created using the GraphPad Prism version 5.01 software.

Recipes

1. 1× PBS (pH 7.4)

NaCl 8 g

KCl 0.2 g

Add ultrapure H₂O to a total volume of 1 L

2. D-PBS (pH 7.4)

NaCl 8 g

KCl 0.2 g

Na₂HPO₄ 1.15 g

KH₂PO₄ 0.2 g

Add ultrapure H₂O to a total volume of 1 L

3. 1% BSA-PBS

BSA 10 g

NaCl 8 g

KCl 0.2 g

Na₂HPO₄ 1.44 g

KH₂PO₄ 0.24 g
Add ultrapure H₂O to a total volume of 1 L

4. 0.5% BSA-PBS

EDTA 2 mM
BSA 5 g
NaCl 8 g
KCl 0.2 g
Na₂HPO₄ 1.44 g
KH₂PO₄ 0.24 g
Add ultrapure H₂O to a total volume of 1 L

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China [No. 81270839, 81428007] and the Natural Science Foundation of Guangdong Province [2017A030313524]. The protocols outlined here were originally described in several papers from the Yu lab (Yu *et al.*, 2011; Feng *et al.*, 2018; Liu *et al.*, 2020).

Competing interests

The authors declare no competing interests, financial or non-financial.

Ethics

All experiments using human cells were carried out in accordance with the recommendations of the Ethical Review Board of Southern Medical University (Guangzhou, China); all subjects gave written informed consent in accordance with the Declaration of Helsinki. All animal experiments were approved by the Guidelines for the Care and Use of Animals established by the Animal Care and Use Committee of Southern Medical University.

References

- Feng, F., Liu, Y., Liu, G., Zhu, P., Zhu, M., Zhang, H., Lu, X., Liu, J., Luo, X. and Yu, Y. (2018). [Human CD8⁺CD28⁻ T suppressor cells expanded by IL-15 *in vitro* suppress in an allospecific and programmed cell death protein 1-dependent manner](#). *Front Immunol* 9: 1442.
- Liu, G., Yu, Y., Feng, F., Zhu, P. and Liu, Y. (2020). [Human CD8⁺CD28⁻ T suppressor cells expanded by common gamma chain \(gammac\) cytokines retain steady allospecific suppressive capacity *in vivo*](#). *BMC Immunol* 21(1): 23.
- Tang, X. L., Smith, T. R. and Kumar, V. (2005). [Specific Control of Immunity by Regulatory CD8 T Cells](#). *Cell Mol Immunol* 2(1):11-19.
- Long, A. E., Tatum, M., Mikacenic, C. and Buckner, J. H. (2017). [A novel and rapid method to quantify Treg mediated suppression of CD4 T cells](#). *J Immunol Methods* 449: 15-22.
- Yu, Y., Zitzner, J. R., Houlihan, J., Herrera, N., Xu, L., Miller, J., Mathew, J. M., Tambur, A. R. and Luo, X. (2011). [Common gamma chain cytokines promote rapid *in vitro* expansion of allo-specific human CD8⁺ suppressor T cells](#). *PLoS ONE* 6(12): e28948.

Production of the Receptor-binding Domain of the Viral Spike Proteins from 2003 and 2019 SARS CoVs and the Four Common Human Coronaviruses for Serologic Assays and Inhibitor Screening

Bruno Segovia-Chumbez, Stephen D. Graham, Ramesh Jadi, Aravinda M. de Silva* and Lakshmanane Premkumar*

Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill, Chapel Hill, United States

*For correspondence: aravinda_desilva@med.unc.edu; prem@med.unc.edu

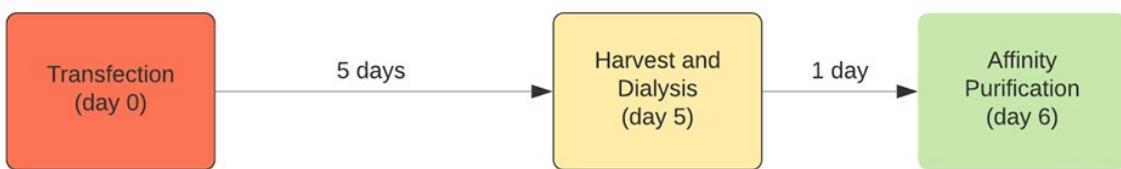
Abstract

The recombinant receptor-binding domain (RBD) of the viral spike protein from SARS-CoV-1 and 2 are reliable antigens for detecting viral-specific antibodies in humans. We and others have shown that the levels of RBD-binding antibodies and SARS-CoV-2 neutralizing antibodies in patients are correlated. Here, we report the expression and purification of properly folded RBD proteins from SARS and common-cold HCoVs in mammalian cells. RBD proteins were produced with cleavable tags for affinity purification from the cell culture medium and to support multiple immunoassay platforms and drug discovery efforts.

Keywords: SARS-CoV-2, SARS-CoV, Coronavirus, COVID-19, Antigen, Immunoassay, Halo-tag, Spike protein, Antibody, Inhibitor screening, Drug discovery

This protocol was validated in: Sci Immunol (2020) DOI: 10.1126/sciimmunol.abc8413

Graphical Abstract:



High-Yield Production of Viral Spike RBDs for Diagnostics and Drug Discovery

Background

The receptor-binding domain (RBD) of the coronavirus spike protein is critical for viral attachment, fusion, and entry. It is also the primary target for antibody response and the development of entry inhibitors and vaccines. The RBDs of 2003 and 2019 SARS CoVs and the four common endemic human CoVs are poorly conserved, representing a promising antigen for detecting viral-specific antibodies in humans. We have recently shown that the RBD of SARS-CoV-2 is highly sensitive and specific for detecting antibodies nine days after the onset of symptoms (Premkumar *et al.*, 2020). Levels of RBD-binding antibodies in human sera are strongly correlated with the SARS-CoV-2 neutralizing titer in patients. Thus, RBD-based serologic assays are attractive to identify individual and environmental risk factors for severe illness and to monitor SARS-CoV-2 transmission in the community. Prior immunity to common human endemic coronaviruses (229E, NL63, OC43, and HKU1) has been reported to enhance the inflammatory response to SARS-CoV-2 (Grifoni *et al.*, 2020; Mateus *et al.*, 2020). Here, we present a detailed step-by-step method for expressing and purifying the RBD of 2003 and 2019 SAR CoVs and the four common endemic human CoVs for serologic assays and inhibitor screening (Premkumar *et al.*, 2020 and Puhl *et al.*, 2021). The technique allows the production of RBDs fused to a TEV protease cleavable self-labeling protein (HaloTag) at the N-terminus and a Twin-Strep-tag and a His-tag at the C-terminus. The tags were designed to aid affinity purification and oriented capture of antigens on solid supports incorporating streptactin, streptavidin, or nickel-nitrilotriacetic acid. The current protocol utilizes a mammalian expression system (Expi293) to produce milligram quantities of recombinant RBDs from a small cell culture volume within 5-7 days, using a single affinity purification step.

Materials and Reagents

1. Cryopreservation Tubes (Thermo Scientific, catalog number: 374081)
2. Poly-Prep Chromatography Columns (Bio-Rad, catalog number: 7311550)
3. Econo-Pac Chromatography Columns (Bio-Rad, catalog number: 7321010)
4. SnakeSkin Dialysis Tubing (Thermo Fisher Scientific, catalog number: 68700)
5. SnakeSkin Dialysis Clips (Thermo Fisher Scientific, catalog number: 68011)
6. Staples 2" Binder Clips, Large (Staples, catalog number: 10669)
7. 5 mL Serological pipette (Thermo Fisher Scientific, catalog number: 13-678-11D)
8. 10 mL Serological pipettes (Thermo Fisher Scientific, catalog number: 13-678-11E)
9. 50 mL Falcon tube (Cell star, catalog number: 22761)
10. Precision Plus Protein Kaleidoscope 500 mL (Bio-Rad, catalog number: 161-0375)
11. Any kD Mini-PROTEAN TGX Stain-Free Protein Gels, 12-well, 20 µL (Bio-Rad, catalog number: 4568125)
12. 2× Laemmli Sample Buffer (Bio-Rad, catalog number: 1610737)
13. Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels, 2-gel (Bio-Rad, catalog number: 1658005)
14. 2-Mercaptoethanol, 10 ml (Sigma-Aldrich, catalog number: M6250)

15. Coomassie Brilliant Blue R-250 Dye (Thermo Fisher Scientific, catalog number: 20278)
16. Expi293 Expression System Kit (Thermo Fisher Scientific, catalog number: A14635)

Note: Store cells in liquid nitrogen and other reagents at 2°C to 8°C.

17. Mr. Frosty (Thermo Scientific, catalog number: 5100-0001)
18. Ni-NTA Agarose (QIAGEN, catalog number: 30230). Store at 2°C to 8°C
19. DMSO (Millipore Sigma, catalog number: 41640)
20. Opti-MEM Medium (Thermo Fisher Scientific, catalog number: 11-058-021)
21. Tris (MP, catalog number: 103133)
22. NaCl (Fisher, catalog number: S271-10)
23. Glycerol (VWR, catalog number: BHD 1172-1LP)
24. Sucrose (Fisher, catalog number: BP-220-1)
25. Imidazole (Thermo Fisher Scientific, catalog number: 03196-500)
26. Liquid nitrogen (Arc gases)
27. Purify Buffer (see Recipes)
28. Elution Buffer (see Recipes)
29. Dialysis Buffer (see Recipes)
30. Stain Buffer for SDS PAGE (see Recipes)
31. Destain Buffer for SDS PAGE (see Recipes)

Equipment

1. Fisherbrand Shaker Flasks, Plain Bottom, Vented (Thermo Fisher Scientific, catalog number: PBV12-5). Store at room temperature
2. 4 L Beaker (Thermo Fisher Scientific, catalog number: 02-555-25K)
3. Forma Steri-Cycle i160 CO₂ Incubator (Thermo Fisher Scientific, Forma, catalog number: 51030301)
4. CO₂ Resistant Shaker (Thermo Fisher Scientific, catalog number: 88881101)
5. Biological safety cabinet (Labguard, Class II, Type A2)
6. Precision Water Bath GP 15D–5 L and 10 L (Thermo Scientific, catalog number: TSGP15D)
7. Magnetic stir bar (Thermo Fisher Scientific, catalog number: 14-512-136)
8. Centrifuge (Sorvall, model: RC-5B)
9. Centrifuge (Eppendorf, model: 5810 R)
10. Centrifuge (Thermo, model: Sorvall Legend Micro 21R)
11. Freezer (Thermo Scientific Revco RLE60086A -86°C)
12. Cryogenic dewar (Cole Parmer)
13. Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (Bio-Rad, Catalogue number: 1658004)

Procedure

A. Establishment of the Expi293 Cell Line (Thermo Fisher Scientific)

1. Remove a cell aliquot from liquid nitrogen.
2. Immediately hand thaw the cells and place them in a 37°C water bath. Once thawed, swirl the tube gently without submerging completely until only a small amount of ice remains.
3. Spray hands with 70% ethanol and gently rub the cell vial to decontaminate before transferring into the laminar flow hood.
4. Use a serological pipette to transfer all tube contents into a plain bottom, vented Fisherbrand Shaker

- Flask, prewarmed with 30 ml of Expi293 Expression Medium.
- Incubate cells at 37°C with ≥ 80% relative humidity and 8% CO₂. Set shaking speed to 125 RPM for a 125 ml shaker flask.
- Passage cells when the cell density reaches 1 × 10⁶-3 × 10⁶ cell/ml.

Note: This usually occurs 4-6 days post-thaw.

- Proceed to transfection once the cell density reaches approximately 3 × 10⁶-5 × 10⁶ viable cells/ml and cell viability is ≥ 95%.

Note: To cryopreserve cells for future use, grow the cell culture to 3 × 10⁶-5 × 10⁶ viable cells/ml and centrifuge them at 300 × g for 5 min.

- a. Discard the supernatant, add Expi293 Expression medium with 10% DMSO, and gently resuspend the cells by pipetting.
- b. Dilute the cells to 1 × 10⁷ viable cells/ml and pipet 1 ml aliquots into cryopreservation tubes and freeze with a controlled-rate freezing apparatus at -80°C freezer. After 24 h, transfer to cryogenic dewar for long-term storage and future use. Allow cells to recover in culture for two more passages post-thaw before transfecting.

Note: For general cell maintenance, passage cells at 0.5 × 10⁶ cell/ml when they reach a density of 3 × 10⁶-5 × 10⁶ cell/ml. Growing past 5 × 10⁶ cell/ml is not recommended.

B. Transfection (30 mL)

1. Dilute a total of 75 × 10⁶ cells to a final density of 3 × 10⁶ cell/mL with 25 mL of prewarmed Expi293 Expression Medium in a 125 mL shaker flask.
2. Dilute 25 µg of RBD expression plasmid DNA in 1.5 mL of Opti-MEM Medium.
3. Dilute 80 µL of ExpiFectamine 293 Reagent in 1.4 mL of Opti-MEM Medium. Incubate the solution at room temperature for 5 min.
4. Add diluted plasmid DNA to the solution containing ExpiFectamine 293 Reagent and incubate at room temperature 10-20 min. The volume should be approximately 3 mL.
5. Transfer 3 mL of the solution into a shaker flask and incubate cells at 37°C with ≥ 80% relative humidity and 8% CO₂.
6. Eighteen to twenty-two hours post-transfection, add 150 µL of ExpiFectamine 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine 293 Enhancer 2 to the shaker flask. Incubate cells for up to 5 days post-transfection.

Note: The procedure can be scaled up proportionally for larger transfections. Cell viability should be above 50% on day 5.

C. Harvest and Dialysis

1. Transfer the cell culture to a 50 ml Falcon tube and centrifuge at 3,000-5,000 × g for 5 min at 25°C (Figure 1).

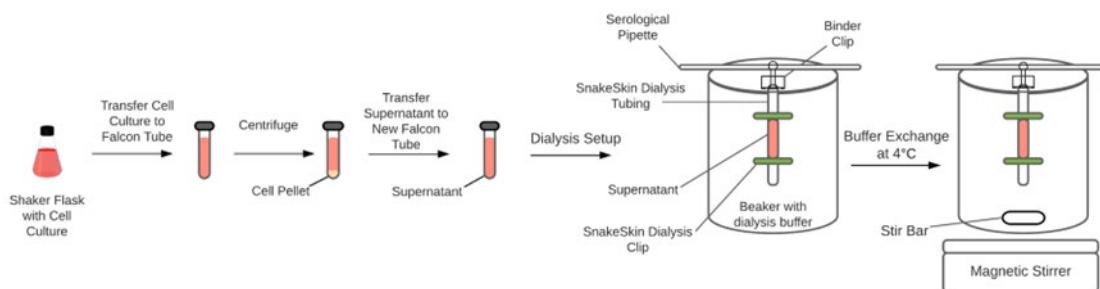


Figure 1. Schematics of harvesting cell culture and dialysis of supernatant

2. Transfer the supernatant into a fresh 50 mL Falcon tube and keep it on ice.
3. Prepare 3 L of dialysis buffer.
4. Use about 7-8 inches of SnakeSkin Dialysis Tubing and SnakeSkin Dialysis Clips to transfer 30 mL of harvested supernatant into the dialysis buffer.

Note: Hydrate the membrane with the dialysis buffer before transferring the supernatant.

5. Place the sealed snakeskin tubing and a magnetic stir bar in a 4-L beaker.
6. Place the beaker on a magnetic stirrer and allow buffer exchange at 4°C overnight.
7. Transfer the buffer-exchanged supernatant into a 50 mL Falcon tube.

Notes:

Upon harvesting, it is possible to evaluate the success of transfection and protein expression before the affinity chromatography step by SDS-PAGE with the cell culture supernatant (optional). To perform this:

- a. Mix 50 μ L of 2 \times Laemmli Sample Buffer with 2-mercaptoethanol and 50 μ L of cell culture supernatant and boil the sample at 95°C for 5 min.
- b. Load 8-15 μ L of the reduced sample onto the SDS-PAGE.
- c. Run sample at 170 v in the Mini-PROTEAN Tetra Vertical Electrophoresis Cell for 35 min and visualize the bands after Coomassie staining using the manufacturer's protocol.

D. Immobilized metal affinity chromatography

1. Take 0.5 mL of Ni-NTA resin in a poly-prep chromatography column and equilibrate with 5 mL of purifying buffer in 1 mL increments. Close the column and resuspend the resin in 1 mL of purifying buffer.

Note: Proportionally adjust the amount of resin needed for larger transfections.

2. Transfer the equilibrated resin into a 50 mL Falcon tube containing the buffer exchanged supernatant.
3. Incubate the resin with the supernatant on a rocking shaker for 1 h at 4°C.
4. Remove the 50 mL Falcon tube from the rocker and place it on a stand. Allow the resin to settle for 20 min.
5. Transfer the supernatant into a new 50 mL Falcon tube without disturbing the resin.
6. Transfer the resin directly onto the bed of the poly-prep chromatography column.
7. Wash the resin with 6 mL of purifying buffer in 1 mL increments by pipetting on the chromatography column wall.

Note: Allow the buffer to flow through the column completely before adding another ml of wash buffer.

8. Elute the protein by adding 200 μ L of elution buffer onto the column wall for a total of 7 fractions.

9. Quantify the protein by measuring the absorbance of the fraction at 280 nm.
10. Assess the protein purity by SDS PAGE run under reducing conditions (Figure 2).

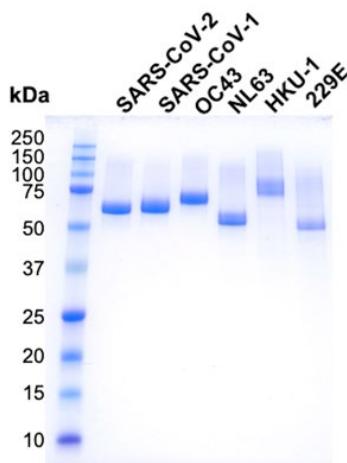


Figure 2. SDS-PAGE analysis of purified spike RBD proteins

E. Storage

Purified proteins can be stored at 4°C for a few weeks. For long-term storage, protein samples can be aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C. Frozen protein samples can be quickly thawed before use.

Recipes

1. Purify buffer

50 mM Tris pH 8
105 mM NaCl
10% glycerol
10% sucrose

2. Dialysis buffer

50 mM Tris-HCl, pH 8
100 mM NaCl

3. Elution buffer

50 mM Tris pH 8
105 mM NaCl
10% glycerol
10% sucrose
300 mM imidazole

4. Stain Buffer for SDS PAGE (1 L)

500 mL deionized water
100 mL methanol
100 mL glacial acetic acid

3 g brilliant blue

5. Destain Buffer for SDS PAGE (1 L)

500 mL deionized water

400 mL methanol

100 mL glacial acetic acid

Notes

Codon-optimized nucleotide sequences encoding the RBDs of SARS-CoV-1 (318-514 aa, P59594), SARS-CoV-2 (331-528 aa, QIS60558.1), OC43 (329-613 aa, P36334.1), HKU-1 (310-611 aa, Q0ZME7.1), 229E (295-433 aa, P15423.1), and NL63 (480-617 aa, Q6Q1S2.1) are available in GenBank under the accession codes MT649401, MT649402, MT649403, MT649404, MT649405, and MT649406. The genes encoding the proteins above were cloned between KpnI and XhoI sites of the mammalian expression plasmid p α H. The mammalian expression plasmids will also be made available by the authors from the plasmid repository (Addgene).

Acknowledgments

This work was funded by the University of North Carolina School of Medicine and NCI U54 CA260543-01 (L.P. and A.D.). This protocol was adapted with minor modification from previous study published by Premkumar *et al.* (2020).

Competing interests

The authors have declared no competing interest.

References

- Grifoni, A., Weiskopf, D., Ramirez, S. I., Mateus, J., Dan, J. M., Moderbacher, C. R., Rawlings, S. A., Sutherland, A., Premkumar, L., Jadi, R. S., Marrama, D., de Silva, A. M., Frazier, A., Carlin, A. F., Greenbaum, J. A., Peters, B., Krammer, F., Smith, D. M., Crotty, S. and Sette, A. (2020). [Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals](#). *Cell* 181(7): 1489-1501 e1415.
- Mateus, J., Grifoni, A., Tarke, A., Sidney, J., Ramirez, S. I., Dan, J. M., Burger, Z. C., Rawlings, S. A., Smith, D. M., Phillips, E., Mallal, S., Lammers, M., Rubiro, P., Quiambao, L., Sutherland, A., Yu, E. D., da Silva Antunes, R., Greenbaum, J., Frazier, A., Markmann, A. J., Premkumar, L., de Silva, A., Peters, B., Crotty, S., Sette, A. and Weiskopf, D. (2020). [Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans](#). *Science* 370(6512): 89-94.
- Premkumar, L., Segovia-Chumbez, B., Jadi, R., Martinez, D. R., Raut, R., Markmann, A., Cornaby, C., Bartelt, L., Weiss, S., Park, Y., Edwards, C. E., Weimer, E., Scherer, E. M., Roush, N., Edupuganti, S., Weiskopf, D., Tse, L. V., Hou, Y. J., Margolis, D., Sette, A., Collins, M. H., Schmitz, J., Baric, R. S. and de Silva, A. M. (2020). [The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients](#). *Sci Immunol* 5(48): eabc8513.
- Puhl, A. C., Fritch, E. J., Lane, T. R., Tse, L. V., Yount, B. L., Sacramento, C. Q., Fintelman-Rodrigues, N., Tavella, T. A., Maranhao Costa, F. T., Weston, S., Logue, J., Frieman, M., Premkumar, L., Pearce, K. H., Hurst, B. L., Andrade, C. H., Levi, J. A., Johnson, N. J., Kisthardt, S. C., Scholle, F., Souza, T. M. L., Moorman, N. J., Baric, R. S., Madrid, P. B. and Ekins, S. (2021). [Repurposing the Ebola and Marburg Virus Inhibitors Tilorone](#).

[Quinacrine, and Pyronaridine: *In Vitro* Activity against SARS-CoV-2 and Potential Mechanisms.](#) *ACS Omega* 6(11): 7454-7468.

In vivo CD40 Silencing by siRNA Infusion in Rodents and Evaluation by Kidney Immunostaining

Miguel Hueso¹, Adrián Mallén², Elia Ripoll², Laura de Ramón², Núria Bolaños², Cristian Varela², Jordi Guiteras², Javier Checa³, Estanislao Navarro⁴, Josep Maria Grinyo⁵, Josep Maria Cruzado^{1,5}, Josep Maria Aran³ and Joan Torras^{1,5,*}

¹Department of Nephrology, Hospital Universitari Bellvitge, and Institut d'Investigació Biomèdica de Bellvitge-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain

²Experimental Nephrology Laboratory, Institut d'Investigació Biomèdicas de Bellvitge-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain

³Immunoinflammatory Processes and Gene Therapeutics Lab, Institut d'Investigació Biomèdica de Bellvitge-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain

⁴Independent Researcher, Barcelona, Spain

⁵University of Barcelona, Department of Clinical Sciences, Bellvitge Campus, L'Hospitalet de Llobregat, Barcelona, Spain

*For correspondence: 15268jta@comb.cat

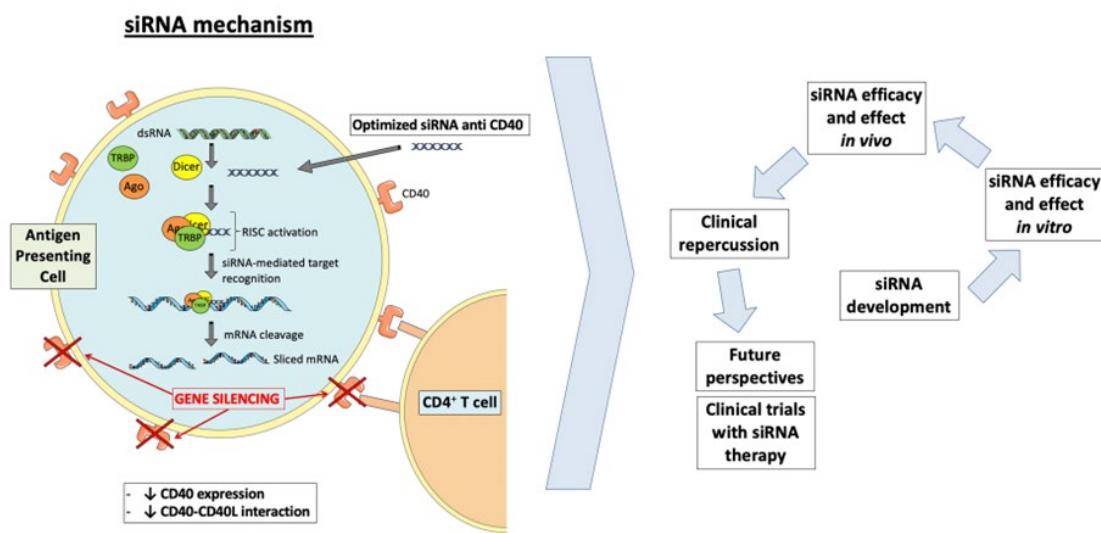
Abstract

The co-stimulatory molecule CD40 and its ligand CD40L play a key role in the regulation of immunological processes and are involved in the pathophysiology of autoimmune and inflammatory diseases. Inhibition of the CD40-CD40L axis is a promising therapy, and a number of strategies and techniques have been designed to hinder its functionality. Our group has broad experience in silencing CD40 using RNAi technology, and here we summarize protocols for the systemic administration of a specific anti-CD40 siRNA in different rodents models, in addition to the subsequent quantification of CD40 expression in murine kidneys by immunostaining. The use of RNAi technology with specific siRNAs to silence genes is becoming an essential method to investigate gene functions and is rapidly emerging as a therapeutic tool.

Keywords: siRNA, CD40, CD40L, Cholesterol-derived oligonucleotide, Kidney, siRNA therapy, Off-target effects

This protocol was validated in: PLoS One (2013), DOI: 10.1371/journal.pone.0065068. Print 2013.

Graphical Abstract:



CD40 siRNA mechanism

Background

The co-stimulatory molecule CD40 and its ligand CD40L are one of the best characterized immune checkpoints involved in the pathophysiology of autoimmune and inflammatory diseases, including cancer, Graft-versus-Host-Disease, inflammatory bowel diseases, systemic lupus erythematosus (SLE), rheumatoid arthritis, type 1 diabetes mellitus, allograft rejection, and atherosclerosis (Lutgens *et al.*; 2010; Ripoll *et al.*, 2013; deRamón *et al.*, 2015; Hueso *et al.*, 2016; Hueso *et al.*, 2019; Karnell *et al.*, 2019). CD40 is a 43–50 kDa transmembrane protein belonging to the tumor necrosis factor (TNF) receptor superfamily and is expressed on the surface of many immune cells. The interaction of CD40 with its ligand CD40L (CD154) induces the trimerization of CD40 and stimulates downstream signaling, including the NF- κ B pathway that upregulates proinflammatory genes (Elgueta *et al.*, 2009). Thus, inhibition of the CD40-CD40L dyad is a promising therapy, and a number of strategies and techniques have been designed to hinder its functionality, such as the administration of small molecule inhibitors of the interaction of CD40 with TRAF6 (Bosmans *et al.*, 2020), liposome-loaded anti-CD40 antisense oligonucleotides (ASO) (Arranz *et al.*, 2013), anti-CD40 siRNAs (Pluvinet *et al.*, 2004; deRamón *et al.*, 2015; Hueso *et al.*, 2016), or monoclonal antibodies (Remer *et al.*, 2017). Currently, there are three clinical trials testing the safety and efficacy of the anti-CD40 monoclonal antibody, CFZ533, to prevent acute rejection in renal (NCT03663335) or liver (NCT03781414) transplant patients and to evaluate its effects on the kidney in patients with lupus nephritis (NCT03610516). Other clinical trials under development use an anti-CD40L antibody (NCT03605927) to prevent acute Graft-Versus-Host-Disease (NCT03605927), or an oncolytic adenoviral vector that expresses an anti-CD40 antibody (NCT03852511) to treat advanced or metastatic tumors. Our group has developed a chemically stabilized, cholesterol-conjugated small inhibitory RNA molecule against murine CD40 and reported the evaluation of its potency, distribution, and durability of effects following systemic administration (Ripoll *et al.*, 2013; deRamón *et al.*, 2015, Hueso *et al.*, 2016, Hueso *et al.*, 2019). Here, we summarize a protocol describing the systemic administration of this specific anti-CD40 siRNA in different mouse models.

Materials and Reagents

A. Consumables

1. Disposable gloves
2. RNAase-free 1.5 ml polypropylene tubes
3. 96-well plates
4. 0.5 mL polypropylene tubes
5. 50 mL Falcon conical tubes (Corning, catalog number: 45352054)
6. 70 µm nylon cell strainers (BD Biosciences, catalog number: 45352350)
7. Cell culture plates
8. Syringes
9. Towels
10. Sample collection tubes
11. 23-25 G needles
12. 2 mL RNAase-free Eppendorf tubes (Merck KGaA, catalog number: T2795)
13. Poly-L-lysine-coated slides (Merck KGaA, catalog number: P0425)
14. Whatman No. 1 filter paper

B. Reagents and Kits

1. Nuclease-free water (DEPC-treated water)
2. Absolute isopropanol (Merck KGaA, catalog number: I9516)
3. Tris base (Tris-hydroxymethyl-aminomethane; Bio-Rad Lab, catalog number: 161-0719)
4. 3 M sodium acetate (Merck KGaA, catalog number: S7899)
5. Bromophenol Blue-Xylene Cyanole Dye Solution (Merck KGaA, catalog number: B3269-5mL)
6. N,N,N',N'-tetramethylethylenediamine, TEMED (Bio-Rad, Hercules, catalog number: 161-0800)
7. Amonium persulfate, APS (Bio-Rad, Hercules, catalog number: 161-0700)
8. Methanol (Merck KGaA, catalog number: 82762)
9. Absolute ethanol (Obtained from general chemical providers)
10. Hydrogen peroxide (Obtained from general chemical providers)
11. D+ sucrose (AppliChem GmbH, catalog number: 200-334-9)
12. Ethylenediaminetetraacetic (EDTA, Merck KGaA, catalog number: E6758)
13. RNase-ZAP (Merck KGaA, catalog number: R2020)
14. BHT (Butylated hydroxytoluene; Merck KGaA, catalog number: W218405)
15. FACS lysing solution (Becton Dickinson, catalog number: 349202)
16. Xylene (mixture of isomers, VWR International, catalog number: 28975.360)
17. DPX mounting medium (VWR International, catalog number: 360294H)
18. Jelly from porcine skin (Sigma-Aldrich, catalog number: G1890)
19. Oil-Red O (Merck KGaA, catalog number: O0625)
20. RPMI 1640 medium (Biological Industries)

Note: Reagents 1-20 were stored at room temperature.

21. Opti-MEM (Themo Fisher, catalog number: 51985034)
22. Fetal bovine serum (Lonza Pharma&Biotech, catalog number: 14-802F)
23. Albumin ELISA kit (Active Motif)
24. DEPC (Diethyl Pyrocarbonate; Merck KGaA, catalog number: D5758).
25. 40% acrylamide/Bis 29:1 (Bio-Rad, Hercules, catalog number: 161-0146)
26. Propidium iodine (StemCell Technologies, catalog number: 75002)
27. TRIzol (ThermoFisher Scientific, catalog number: 15596026)

28. DAB substrate (3,3'Diaminobenzidine tetrahydrochloride hydrate, Sigma-Aldrich, catalog number: D5637-5G)
29. Flow cytometry staining buffer (ThermoFisher Scientific, catalog number: 00-4222)
30. 100 mM Penicillin/Streptomycin (ThermoFisher Scientific, catalog number: 15070-063)

Note: Reagents 21-30 were stored at 4°C.

31. 200 mM L-glutamine (ThermoFisher Scientific, catalog number: 25030-024)
32. High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, catalog number: 4368814)
33. PureLinkTM RNA Mini kit (ThermoFisher Scientific, catalog number: 12183020)
34. 5' RACE system for rapid amplification of cDNA ends kit (ThermoFisher Scientific)
35. Oligofectamine 2000 (ThermoFisher Scientific, catalog number: 12252011)
36. GM-CSF (R&D systems, catalog number: 215-GM)
37. Lipopolysaccharides (LPS) from *E. coli* Serotype O111:B4 (Sigma-Aldrich, catalog number: L4391)
38. GeneEraser Luciferase Suppression-Test System (Stratagene/Agilent, catalog number: 240192)
39. Luciferase Assay System (Promega, catalog number: E1531)
40. Polyfect transfection reagent (Qiagen, catalog number: 301105)
41. Serum normal goat (Merck KGaA, catalog number: G9023) or horse 20% (Merck KGaA, catalog number: H0146)

Note: Reagents 31-41 are stored at -20°C.

42. Taqman gene expression assays (ABI/ThermoFisher Scientific) (Table 1)

Table 1. Commercial TaqMan probes used in this study

Name of the gene tested	Probe code ¹	Gene unique identifier ²	Position ³
<i>CD40</i>	Mm00441891_m1	NM_011611	121
<i>CD40L</i>	Mm00441911_m1	NM_011616	348
<i>IL1b</i>	Mm01336189_m1	NM_008361	55
<i>NLRP3</i>	Mm00840904_m1	NM_145827	499
<i>Apelin (Apln)</i>	Mm00443562_m1	NM_013912	414
<i>C3</i>	Mm01232779_m1	BC029976	517
<i>CD55</i>	Mm00438377_m1	NM_010016	1154
<i>FOXP3</i>	Mm00475165_m1	NM_001199347	1307
<i>IL6</i>	Mm99999064_m1	NM_031168	233
<i>IL10</i>	Mm00439614_m1	NM_010548	232
<i>MCP1 (Ccl2)</i>	Mm00441242_m1	NM_011333	165
<i>RANTES (Ccl5)</i>	Mm01302428_m1	NM_013653	245
<i>TLR3</i>	Mm00628112_m1	NM_126166	280
<i>TLR4</i>	Mm00445273_m1	NM_021297	112
<i>TLR9</i>	Mm00446193_m1	NM_031178	106
<i>AIM2</i>	Mm01295719_m1	NM_001013779	869
<i>18S rRNA</i>	Hs99999901_s1	X03205	604

(1) Commercial code of the Taqman probes used (ThermoFisher Scientific).

(2) Refseq/Genbank unique identifiers of the genes tested.

(3) Base position contained within the probe.

43. 0.1 M citrate buffer, pH 6 (see Recipes), stored at room temperature
44. 0.01 M citrate buffer (see Recipes), stored at room temperature
45. Oil-Red working solution (see Recipes), stored at room temperature
46. Phosphate-buffered saline (PBS), pH 7.5 (see Recipes), stored at room temperature

47. PBS-Triton (PBST) (see Recipes), stored at room temperature
48. PS buffer (see Recipes), stored at room temperature
49. Complete culture medium (see Recipes), stored at 4°C
50. Secondary antibodies (for a 1:200 dilution) (see Recipes), keep at -20°C
51. Annealing buffer 10× (see Recipes)
52. DEPC-treated water (see Recipes)
53. 1 M Tris-HCl, pH 7.5 (see Recipes)
54. 12% acrylamide gel (see Recipes)
55. Non-denaturing gel loading buffer (see Recipes)
56. 4% PFA solution in PBS (see Recipes)
57. 0.3% Oil-Red O stock solution (see Recipes)
58. Serum normal goat or horse 20% (see Recipes)

C. Primary antibodies

In this work we used the following specific primary antibodies:

1. Rabbit polyclonal anti-CD40 antibody (C-20, Santa Cruz Biotech, catalog number: sc-975)
2. Rabbit polyclonal anti-CD154 antibody (H215, Santa Cruz Biotech, catalog number: sc-9097)
3. Mouse monoclonal anti-DC-SIGN antibody (1B10, Santa Cruz Biotech, catalog number: sc-23926)
4. Rabbit polyclonal anti-NF-κB p65 antibody (anti-phospho-S536, Abcam, catalog number: Ab86299)
5. Anti-F4/80 antibody (Hycult Biotech, catalog number: HM1066)
6. Rabbit anti-mouse collagen-IV antibody (Chemicon international, catalog number: AB756p)
7. Goat polyclonal anti-human C3c antibody conjugated to FITC (Nordic-MuBio, catalog number: GAHu/C3c/FITC)

Furthermore, the following antibodies from BD Biosciences (San Jose, CA, USA) were used for flow cytometry:

8. Anti-CD11c antibody (clone HL3)
9. Anti-CD11b antibody (clone M1/70)
10. Anti-CD40 antibody (clone HM40-3)
11. Anti-CD80 antibody (clone 16-10A1)
12. Anti-CD86 antibody (clone GL1)

Note: All primary antibodies were stored at -20°C, unless another temperature was specifically stated by the manufacturer.

D. Secondary antibodies and histological reagents

The following secondary antibodies were used in this work:

1. Alexa 488-labeled chicken anti-goat (ThermoFisher Scientific, catalog number: AB_2535870)
2. Goat anti-rabbit (ThermoFisher Scientific, catalog number: AB_2633280)
3. Alexa 555-labeled goat anti-mouse (ThermoFisher Scientific, catalog number: AB_2535844)
4. Alexa 546-labeled goat anti-rabbit (ThermoFisher Scientific, catalog number: AB_2633280)
5. Unconjugated goat anti-rat (Novus Biologicals)
6. Biotinylated horse anti-goat (Vector Laboratories, catalog number: BA-9500)
7. FITC-conjugated goat anti-mouse (Merck KGaA, catalog number: F0257)
8. VECTASTAIN Elite ABC HRP kit (Vector Laboratories, catalog number: PK-6100)
9. Avidin/Biotin blocking kit (Vector Laboratories, catalog number: PK-4001-NB)

Note: All secondary antibodies were stored at 4°C, unless another temperature was specifically stated by the manufacturer.

The following histological reagents were used:

10. Harris hematoxylin solution (Sigma-Aldrich, catalog number: HHS32-1L)
11. Eosin Y solution, alcoholic (Sigma-Aldrich, catalog number: HT110132-1L)
12. Periodic acid (Sigma-Aldrich, catalog number: P0430-25G)
13. Schiff reagent (Sigma-Aldrich, catalog number: 3952016-500ML)
14. PAS staining kit (Sigma-Aldrich, catalog number: 101646)
15. Oil Red-O reagent (Sigma-Aldrich, catalog number: O0625-100G)
16. Tissue Tec OCT inclusion compound (Sakura FinetekEurope)
17. UltraCruz Tm mounting medium (Santa Cruz Biotech, catalog number: sc-24941)
18. DRAQ5 (ThermoFisher Scientific, catalog number: 65-0880-92)

Notes:

- a. Used for nuclear counterstaining.
- b. All histological reagents were stored at room temperature.

E. Animals

1. Six-month-old NZB/NZW (F1) female mice (The Jackson Lab, Charles River, Wilmington, MA, USA)
2. Six to eight-week-old male ICR mice (The Jackson Lab, Charles River, Wilmington, MA, USA)
3. Eight-week-old ApoE-/- female mice (The Jackson Lab, Charles River, Wilmington, MA, USA)

Note: Animals were housed in a room maintained at a constant temperature and given free access to water and a standard laboratory diet. To accelerate atherosclerosis, ApoE-/- mice were fed a Western diet that contained 0.2% cholesterol and provided 42% of the energy as fat (TD.88137; Harlan-Tekland, Madison, WI, USA). Animals were euthanized by inhalation of isoflurane. Protocols were approved by the Ethics Committee for Animal Research of UB-Bellvitge, and experiments were performed in accordance with the European legislation on Laboratory Animal Experiments.

F. siRNA oligonucleotides

In this work, we used a CD40-specific ds-siRNA homologous to both the mouse and rat CD40 mRNA sequence (herein antiCD40-siRNACHol) and a scrambled sequence (s/s) ds-siRNA as the control (herein s/s-control siRNACHol). Both were modified by conjugating a cholesterol (chol) molecule to the 3' end of the sense strand by means of a pyrrolidine linker.

The siRNA sequences were as follows:

Anti-CD40 sense strand: 5'-GUGUGUUACGUGCAGUGACUU-3'
Anti-CD40 antisense strand: 5'-GUCACUGCACGUACACACTG-3'
(s/s), control siRNA sense strand: 5'-ACUACAAGACUCGUGACCAUU-3'
(s/s), control siRNA antisense strand: 5'-UGGUCACGAGUCUUGUAGUUU-3'

To determine the transfection efficiencies and organ distributions of the siRNAs, a cholesterol-conjugated and an unmodified anti-CD40 siRNA were labeled with Cy5.5.

Note: All oligonucleotides were obtained from Microsynth AG (Balgach, Switzerland) and stored at -20°C.

G. Cell lines

1. The highly transfecable human embryonic kidney 293FT cell line (ThermoFisher Scientific, catalog number: R70007)
2. Primary dendritic cells (obtained from the bone marrow of ICR mice)

Equipment

1. Cell scrapers (Sarstedt AG & CO, Nümbrecht, GE, catalog number: 83.3950)
2. Scalpels
3. Forceps: Straight, serrated-tip forceps; straight or curved, serrated-tip fine forceps; and straight fine-tip forceps
4. Scissors: Straight, blunt scissors; straight, sharp, fine scissors; and micro-dissecting spring scissors
5. Automatic pipettes (1-10 µL, 20-200 µL)
6. Hemocytometer
7. Water bath
8. Jasco V-650 spectrophotometer (Easton, MD, USA, used to determine the melting point of siRNA duplexes)
9. Olympus autoanalyzer AU400 (Hamburg, Germany, used to determine urinary protein and creatinine concentrations)
10. TaqMan real-time PCR ABI Prism® 7700 (ThermoFisher Scientific, used for the qPCR experiments)
11. BD FACS Canto II cytometer (BD Biosciences, used in the flow cytometry experiments)
12. Zeiss SteREOLumar V12 microscope (Carl Zeiss AG, used for microscopy experiments)
13. Leica TCS-SL spectral confocal microscope (Leica Camera AG, used for microscopy experiments)
14. TD-20/20 luminometer (Turner Designs, used for luciferase assays)
15. T-25 ULTRA-TURRAX™ homogenizer (IKA®-Werke GmbH & Co)
16. NanoDrop 2000c spectrophotometer (ThermoFisher Scientific)
17. Tabletop centrifuge (Eppendorf Cooled Centrifuge, mode: 5424R)
18. Cell culture CO₂ incubator (NuAire Autoflow NU 8700)

Software

1. Leica confocal software (Leica Camera AG, Wetzlar, Germany)
2. Image analysis software ProgResCapturePro 2.7.7 (JenoptiK AG, Jena, GE)
3. ImageJ v1.48 (NIH, Bethesda, MD, USA)
4. ExpressionSuite software v1.0 or later (ABI, ThermoFisher Scientific, Waltham, MA, USA)
5. SDS software v2.4 (ABI, ThermoFisher Scientific, Waltham, MA, USA)
6. FACS DIVA software (BD Biosciences, San Jose, CA, USA)

Procedure

A. Synthesis and preparation of siRNAs

Requirements: Disposable gloves, heated water bath, microcentrifuge, automatic pipettes (1-10 µL, 20-200 µL), Jasco V-650 spectrophotometer, RNase-free 1.5-mL polypropylene tubes, ice, nuclease-free water, annealing buffer (10 mM Tris pH 7.5, 20 mM NaCl), 3 M sodium acetate (pH 5.2), isopropanol, 70% ethanol, oligonucleotides.

1. Design of anti-CD40 oligonucleotides.

Analyze the target mRNA sequence (herein murine CD40, NCBI accession X60592.1) to select a number of sequences that comply with the following structure, **5'-GN17C-3'**. Nine different sequences were generated to select an optimal target site (Pluvinet *et al.*, 2004). General rules to improve the effectiveness of siRNA silencing are: 1) presence of G/C at the 5' end of the sense strand; 2) presence of A/U at the 5' end of the antisense strand; 3) presence of at least 5 A/U residues in the first 7 bases of the 5' end of the antisense strand; 4) no runs of more than 9 G/C residues should be allowed in the sequence (Ui-Tei *et al.*, 2004); 5) secondary structure at the target site should be kept to a minimum (Bohula *et al.*, 2003; Kretschmer-Kazemi Far and Sczakiel., 2003).

2. Annealing of oligonucleotides to generate ds-siRNAs.
For oligonucleotide annealing, mix equimolar amounts of complementary sense and antisense strands of the different anti-CD40 and control siRNAs by mixing:

Oligonucleotide 1/sense strand (100 pmol/μL).....	10 μL
Oligonucleotide 2/antisense strand (100 pmol/μL).....	10 μL
2x oligo annealing buffer (10 mM Tris, pH 7.5, 20 mM NaCl).....	50 μL
Nuclease-free water (up to)	100 μL

 - a. Heat for 3 min at 90°C in a water bath and switch it off.
 - b. Let the tubes cool down slowly in the water bath to room temperature (<60 min). The final concentration should be 100 pmol/μL ds-siRNA.
 - c. Store the annealed ds-siRNA oligonucleotides at 4°C for immediate use or at -20°C for longer storage.
3. Determination of the melting temperature (Tm) of the ds-siRNAs.
Heat samples in the Jasco V-650 spectrophotometer using a linear temperature ramp of 0.5°C/min. Melting temperature is obtained as the maximum of the first derivative. The melting point of the unmodified CD40 ds-siRNA is 79°C and that of the cholesterol-derived CD40 ds-siRNA (antiCD40-siRNA^{Chol}) is 71°C.
4. Alcohol precipitation of ds-siRNAs.
 - a. Add a 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol, mix.
 - b. Keep on ice for 5 min and spin down at the top speed in a microcentrifuge for 10 min.
 - c. Carefully aspirate the supernatant, wash the pellet with 0.5 mL cold 70% ethanol, and carefully remove all ethanol.
 - d. Air-dry the pellet for no longer than 15 min at room temperature and resuspend the ds-siRNA in nuclease-free water in 2-5-times the original volume.
 - e. Quantitate the siRNA concentration using a NanoDrop or equivalent. The final concentration should be 20-50 pmol/μL.
 - f. Analyze the siRNA on a non-denaturing 12% polyacrylamide gel (see Recipes) for size and integrity, and store at -20°C or -70°C.
 - i. Mix up to 5 μL oligonucleotides with 2 μL loading buffer (see Recipes).
 - ii. Load the sample on a non-denaturing 12% polyacrylamide gel and subject to electrophoresis at 200-250 V.
 - iii. Stop electrophoresis when the Bromophenol Blue dye front has migrated two-thirds of the way down the gel.
 - iv. Stain the gel for 2-5 min in a 1 μg/mL solution of ethidium bromide.
 - v. Soak the gel for 2-5 min in water.
 - vi. Visualize the siRNA using a UV transilluminator.

The siRNA should migrate as a 21-22 bp band that runs slightly behind the Bromophenol Blue dye front. A second, less intense band running behind the primary siRNA band may be apparent and represents one partially digested strand of siRNA. The underdigested RNA strand is 27 nt in length and does not create any non-specific effects when used to transfect cells.

B. Determination of the silencing ability of anti-CD40-siRNA^{Chol} using the GeneEraser Luciferase Suppression-test system

Requirements: Disposable gloves, 96-well plates, automatic pipettes (1-10 μL, 20-200 μL), cell scrapers (Sarstedt AG & CO, Nümbrecht, GE, catalog number: 83.3950), microcentrifuge, ice, 0.5-ml polypropylene tubes, pTarget-luc-rCD40, Oligofectamine, HEK-293 cells, Polyfect transfection reagent, 70% ethanol, 1× phosphate-buffered saline (PBS), 1× lysis reagent, luciferase assay reagent, TD-20/20 luminometer.

1. Generate the apTarget-luc-CD40 plasmid.

A blunt-end 506 bp fragment corresponding to the partial rat CD40 cDNA sequence (coding sequence of nucleotides 41-547; GenBank Acc. No AF241231) was blunt-end cloned into the plasmid pTarget-luc at

- the 3'UTR of the luciferase gene.
2. Transfect HEK-293 cells with siRNAs (100 nM).
 - a. Prepare a 100 nM solution of the ds-siRNAs, transfect confluent HEK-293 cells using Oligofectamine according to the manufacturer's instructions.
 - b. After 6 h, transfet the above cells with 400 ng pTarget-luc-CD40 using Polyfect transfection reagent according to the manufacturer's instructions. Normalize the transfection efficiency by co-transfeting 500 ng pCMV-bGal.
 - c. Incubate the cells for 48 h at 37°C in a humidified atmosphere with 5% CO₂.
 3. Prepare cell lysates for analysis.
 - a. Remove the growth medium from the cultured cells.
 - b. Rinse the cells in 1× PBS and remove as much as possible.
 - c. In a 96-well plate, dispense 20 µl/well 1× lysis reagent (1× lysis reagent is prepared by adding 4 vol. water to 1 vol. 5× lysis reagent from the kit).
 - d. Scrape the cells from the dish and transfer the solution to a microcentrifuge tube.
 - e. Pellet the cell debris by brief centrifugation and transfer the supernatant to a new tube.
 - f. Mix 20 µL cell lysate with 100 µL luciferase assay reagent and measure the light in the TD-20/20 luminometer.

C. Bone marrow-derived dendritic cell (DC) extraction, culture, activation, and transfection with siRNAs *in vitro*

Requirements: Disposable gloves, 50-ml Falcon conical tubes (Corning, catalog number: 45352054), 70-µm nylon cell strainers (BD Biosciences, catalog number: 45352350), autoclaved materials (forceps, scalpels, scissors), cell culture hood, ice, mice femurs and tibiae, hemocytometer, cell culture plates, sample collection tubes, Oligofectamine 2000, BD FACS lysing solution, complete RPMI 1640 medium (RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 µM L-glutamine, 10% heat-inactivated FBS, and 20 ng/ml GM-CSF), Opti-MEM medium, propidium iodine (stock solution: 1.5 mM), siRNAs, LPS, cytometer.

1. Euthanize ICR mice according to the institutional guidelines and extract the femurs and tibiae.
 - a. Place each mouse onto a sterile surgical pad in a sterile hood.
 - b. Spray the mouse with 70% ethanol to avoid contamination of the samples.
 - c. Soak the femurs and tibiae in RPMI 1640 medium.
2. Harvest the bone marrow (BMC).

Note: All work should be performed under sterile conditions in a cell culture hood.

- a. Cut the femurs at both ends with sterile scissors.
 - b. Transfer the bones to RPMI 1640 in a sterile Petri dish.
 - c. Vigorously flush the inside of the bones with 1 ml ice-cold RPMI 1640 medium using a 1-ml pipette. Flush 2-3 times until the bones are completely white.
 - d. Flush the bone marrow out onto a 70-µm nylon cell strainer placed in a 50-mL Falcon conical tube.
 - e. Smash the bone marrow through the cell strainer using a 5-mL plunger, and wash the strainer with 5 mL RPMI.
 - f. Centrifuge the cells at 250 × g for 8 min at 4°C and discard the supernatant.
 - g. Resuspend the cell pellet in 1 mL RBC lysis buffer and incubate for 5 min at room temperature.
 - h. Neutralize the lysis buffer by adding 5 mL FBS.
 - i. Centrifuge the cells at 250 × g for 8 min at 4°C, discard the supernatant, and resuspend in 5 mL RPMI 1640 medium. Take out an aliquot of cell suspension to count.
3. Determine the total number of cells obtained.
 - a. Dilute 10 µL bone marrow suspension in 90 µL 1× BD FACS lysing solution (see above).
 - b. Count 10 µL cell suspension using a hemocytometer.
 4. Grow 5 × 10⁶ cells.

Grow cells in 5 mL complete RPMI 1640 medium (see above) supplemented with 10 ng/mL IL-4 and 20 ng/ml GM-CSF in T25 flasks at 37°C in a humidified atmosphere with 5% CO₂.

After 24 h, some of the cells adhere to the plate but many others are suspended in the culture medium. Colonies appear after 72 h. The number of adherent cells gradually decreases over time and on day 5, the suspended cells with dendritic protrusions gradually increase. On day 7, the suspended cells begin to aggregate and the protrusions elongate.

5. Change the culture medium on days 3 and 5.
 - a. Remove the medium carefully so as not to disturb the growing cells.
 - b. Briefly centrifuge (250 × g for 8 min) the removed volume of media since DCs are only loosely adherent. Resuspend the cell pellet in the same flask with 10 ml fresh RPMI 1640 supplemented with 20 ng/mL GM-CSF.
6. Harvest BMDCs (day 8)
 - a. Collect non-adherent cells by gently pipetting the culture medium, and transfer into sterile 50-mL centrifuge tubes. Discard the adherent cells that include macrophages.
 - b. Loosely adherent BMDCs become easily dislodged into suspension by this process, while macrophages remain adhered to the Petri dish.
 - c. Count the total number of cells obtained.
7. Transfect with siRNAs on day 8.
Incubate 1 × 10⁶ immature BMDCs/ml with 2 μM unmodified siRNA-Cy5.5 or Chol-siRNA-Cy5.5 (see Section E of the Materials and Reagents section, above) using the cationic lipid Oligofectamine 2000 in Opti-MEM medium on 6-cm dishes.
8. Treat immature BMDCs with 100 ng/mL LPS on day 9 for 12 h.
9. Harvest BMDCs
 - a. Collect all the cells (detach cells with a scraper).
 - b. Centrifuge the cells at 250 × g for 8 min.
 - c. Remove the supernatant and resuspend the cell pellet in 5 mL PBS (wash 1)
 - d. Centrifuge the cells at 250 × g for 8 min.
 - e. Remove the supernatant and resuspend the cell pellet in 5 mL PBS (wash 2)
 - f. Centrifuge the cells at 250 × g for 8 min.
 - g. Resuspend the cell pellet in 1 mL RPMI 1640 supplemented with 10% FBS and 20 mM penicillin/streptomycin.
 - h. Count the cells.
10. Stain the DCs with a final concentration of ≤1 μg/mL propidium iodide (PI).
 - a. Prepare a 1 mg/mL (1.5 M) stock solution by dissolving solid PI in PBS. Protect from prolonged exposure to light.
 - b. Whenever possible, prepare and use the stock solution on the same day. If the stock solution must be made in advance, aliquot and store in tightly sealed vials at -20°C (generally stable for up to 1 month).
 - c. Resuspend 50 μL cell suspension in 50 μL flow cytometry staining buffer.
 - d. Add 5 μL PI staining solution per 100 μL cell suspension. Do not wash the cells after the addition of PI.
 - e. Incubate for 5–15 min on ice in the dark.
 - f. Analyze the samples immediately by flow cytometry.
11. Characterize the cell phenotype by flow cytometry.
 - a. Block non-specific Fc-mediated interactions with 0.5 μg anti-mouse CD16/CD32 per 100 μL for 15 min at 4°C before staining.
Antibody-binding kinetics are temperature dependent. Staining on ice may require longer incubation times.
 - b. Aliquot 50 μL cell suspension to each tube (one per antibody).
 - c. Combine the cell suspension with primary antibodies (the isotype control and the following antibodies: anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-CD40 (clone HM40-3), anti-CD80 (clone 16-10A1), and anti-CD86 (clone GL1)) and the appropriate volume of flow cytometry

- staining buffer to reach a final staining volume of 100 µL. Vortex.
- d. Incubate for at least 30 min on ice. Protect from light.
 - e. Wash the cells by adding 2 mL flow cytometry staining buffer to each tube.
 - f. Repeat the wash step twice by centrifuging the cells at 250 × g for 8 min at room temperature.
 - g. Remove the supernatant and resuspend the pellet in 100 µL flow cytometry staining buffer.
 - h. Transfer the cell suspension to polypropylene tubes for flow cytometry.
 - i. For storage of the samples before analysis, add 100 µL IC fixation buffer.
 - j. Analyze the cells according to fluorescence intensity of the above markers and acquire representative images for subsequent analysis/quantitation.

D. Evaluation of the silencing activity of antiCD40-siRNA^{Chol} in in vivo mouse models

Requirements: Gloves, scalpels, scissors, syringes, towels, cotton, sample collection tubes, 23–25 G needles, mice, siRNAs, PS buffer (see Recipes), isoflurane, isoflurane chamber, portable liquid nitrogen container, liquid nitrogen, and blood collection tubes.

1. Separate the mice into treatment and control groups.
 - a. Study the *in vivo* effect of the systemic administration of chol-siRNAs: Use 6-8-week old male ICR mice (4 mice per group). Administer 50 µg antiCD40-siRNA^{Chol} or s/s-control siRNA^{Chol} via i.p. injection (10 mL/kg in 0.2-µm-filtered PBS). At predefined time points (days 0, 1, 3, 5, 7, and 9), administer single doses of 5 µg LPS from *E. coli* via i.p. injection. Euthanize animals 4 h post-administration of LPS.
 - b. For the lupus nephritis study with 5-month-old NZB/NZW F1, set up the following groups:
 - i. CYP: Administer 50 mg/kg i.p. every 10 days (n = 9).
 - ii. CTLA4: Administer 50 µg abatacept (Orencia, Bristol Myers Squibb) three times weekly (n = 9).
 - iii. siCD40-1w: Administer 50 µg anti-CD40-siRNA^{Chol} i.p. once weekly (n = 9).
 - iv. siCD40-2w: Administer 50 µg anti-CD40-siRNA^{Chol} i.p. twice weekly (n = 9).
 - v. Control: Administer 50 µg s/s-control siRNA^{Chol} i.p. twice weekly (n = 15).
 - c. For the atherosclerosis study with 8-week-old female ApoE^{-/-} mice. Treat the mice twice weekly i.p. with 50 µg anti-CD40-siRNA^{Chol}, s/s-control siRNA^{Chol}, or vehicle (5 mice per group). Euthanize the animals at 8 weeks (basal group), 10 weeks, 14 weeks, or 24 weeks.
2. Tissue collection: urine and venous blood.
 - a. Place the animals in individual metabolic cages with water and the usual diet, and collect 24 h urine specimens before the onset of treatment and thereafter at monthly intervals.

Note: If mice do not produce urine in the given time, repeat the procedure on the following day in a warmer room.

- b. Centrifuge the urine samples at 500 × g for 10 min. Collect the urine and retain the sediment for further studies; store the urine and sediment at -20°C.
- c. Determine the weight of the mice twice monthly.
- d. On a monthly basis, perform a non-terminal venous blood collection from the tail vein (without replacement of fluids) using short-term anesthesia by isoflurane inhalation.

Important points to remember for animal comfort:

- i. Make the animal comfortable by maintaining the temperature at 24-27°C.
- ii. DO NOT rub the tails from the base of the tip as this will result in leukocytosis. If the vein is not visible, dip the tail into warm water (40°C).
- iii. Insert a 23-25 G needle into the blood vessel and collect blood using a capillary tube or syringe with a needle. In case of difficulties, cut a minimal surface of the skin, prick the vein with a bleeding lancet or needle and collect blood with a capillary tube or a syringe with a needle.
- iv. DO NOT try to collect blood more than three times. The maximum collection volume should be 0.2 mL blood per mouse. The estimated blood volume in an adult animal is 55-70 mL/kg

- body weight (Parasuraman *et al.*, 2010).
- v. After completing blood collection, stop the bleeding using pressure.
 - vi. Wash the restraint frequently to avoid pheromonal-induced stress or cross infection.
- e. At the end of the study, extract arterial blood by cardiac puncture.
 - i. Perform terminal anesthesia of animals in an isoflurane chamber.
 - ii. Open the chest (thoracotomy) and obtain a blood sample directly from the ventricle, taking care not to collapse the heart.
- 3. Tissue collection: liver and kidneys
 - a. Euthanize all mice by inhalation of isoflurane.
 - b. Dissect out the kidneys and liver through the abdomen and wash them by perfusing ice-cold 1× PBS via the left ventricle.
 - c. Resect the kidneys. One kidney will be used for histological evaluation and the other for total RNA extraction.
 - d. For histological analysis, remove the upper third of the kidney to ensure that both cortical and juxtamedullary glomeruli are present and fix the piece in 5 mL PS buffer at 4°C for 24-48 h.
 - e. For immunofluorescence analysis, place a third of the kidney piece (to ensure that both cortical and medullary glomeruli are present) into the tissue mold and coat in OCT. Place it onto dry ice to freeze and store at -80°C.
 - f. For protein and RNA extraction, place 3 × 2 mm³ pieces of kidney cortex and liver into 0.5 ml plastic tubes and snap freeze them in liquid N₂. Store at -80°C. For long-term tissue storage for RNA extraction, place the tissue in five volumes of RNA stabilization reagent and store at -80°C.
- 4. RNA extraction and purification from kidney tissue.
Requirements: Disposable gloves, laboratory fume hood, RNase-free pipettes and pipette tips, sterile plasticware, cold TRIzol™, rotor-stator homogenizer (ULTRA-TURRAX™), ice, round-bottomed RNase-free tube for tissue homogenization, RNase-free water (water treated with DEPC, see Receips), tabletop centrifuge, 2 ml RNAase-free Eppendorf tubes, NanoDrop, PureLink RNA mini kit, chloroform, 70% ethanol (100% ethanol mixed with RNase free water), vortex.
 - a. Add 1 ml cold TRIzol to 50-100 mg renal tissue in a round-bottomed RNase-free tube, homogenize on ice. Avoid foaming by keeping the tip of the probe submerged in the lysis solution while holding the tip against the tube wall.
 - b. Incubate for 5 min at room temperature to allow complete dissociation of the nucleoprotein complex.
 - c. Add 0.2 mL chloroform and vortex.
 - d. Incubate for 2-3 min at room temperature.
 - e. Centrifuge the sample for 15 min at 12,000 × g, 4°C.
 - f. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Carefully transfer the aqueous phase to a 2-mL Eppendorf tube (important: avoid transferring any of the interphase or organic layer).
 - g. Add one volume 70% ethanol and vortex.
 - h. Transfer ≤700 µL sample to the spin cartridge and purify total RNA according to the kit instructions (in this case the PureLink RNA mini kit).
 - i. Quantify the total RNA using the NanoDrop.
- 5. First strand DNA synthesis (reverse transcription).
Requirements: Ice, RNase-free water, tabletop centrifuge, Eppendorf tubes, High-Capacity cDNA Reverse Transcription kit, thermal cycler, 70% ethanol, vortex.
The reverse transcription reaction is performed according to the instructions of the kit used (in this case the High-Capacity cDNA Reverse Transcription) using 500 ng RNA.
 - 6. Determine CD40 mRNA expression in the kidney and liver by qPCR.
Set up reactions with CD40 primers (Mn_00441891_m1; Taqman gene expression assays from ABI/ThermoFisher Scientific, Waltham, MA, USA).
 - 7. Analysis of anti-CD40-siRNA^{Chol}-directed cleavage products by 5'RACE.
Requirements: Gloves, total RNA (from section C1), thermocycler, cDNA synthesis kit, 5'RACE kit.

- a. Synthesize first strand cDNA from 5 µg total RNA (kidney) using the High-Capacity cDNA RT Kit with the gene-specific primer #1 (GSP1): 5'-GCCGACTGGGCAGGGATGACAGACG.
- b. Ligate the adaptor oligonucleotide from the 5'RACE kit to the 5' end of the cDNA.
- c. Specifically amplify cleavage products using a primer complementary to the adaptor (5'RACE kit) and the nested gene-specific primer #2 (GSP2): GSP2: 5'-AGCCAGGGATACAGGGCGTGTGC. Use the following PCR program: 1 m, 94°C × 60 s and 35 cycles of 94°C × 30 s, 55°C × 30 s and 72°C × 60 s, followed by a final extension of 72°C × 5 min
- d. Check for the presence of a 310 bp amplification product, corresponding to CD40 mRNA specifically cleaved by the anti-CD40 siRNA.
Use 20 µL PCR product for analysis on a 1% ethidium bromide-stained agarose gel by electrophoresis with a corresponding DNA molecular weight marker.

E. Histological evaluation of renal lesions in siRNA-treated animals.

In this section, we will prepare kidney samples for standard histological analysis (embedded in paraffin), immunofluorescence analysis (in OCT), and protein and RNA analysis (stored in RNA stabilization solution).

Requirements: One of the kidneys obtained in section D2, gloves, ice-cold 1× PBS, tissue molds and cassettes, forceps, paraffin, paraffin dispenser, cold plate, PS buffer (see Recipes), 15% sucrose, 4% paraformaldehyde (PFA), OCT, dry ice, RNA stabilization reagent, high-density polyethylene (HDPE), 50% ethanol, 70% ethanol, 96% ethanol, absolute ethanol, deionized water (MilliQ or similar), microtome.

1. For standard histological analysis.
Remove the upper third of a kidney to ensure that both cortical and juxtamedullary glomeruli are present and fix the piece in 5 ml PS buffer at 4°C for 24-48 h. Place the tissue in a labeled cassette (use a pencil, as solvents will dissolve the ink) and prepare for paraffin embedding.
 - a. Dip cassettes in a wide-mouth high-density polyethylene (HDPE) 1 L jar filled with **15% sucrose** for 24 h before embedding in paraffin blocks.
 - b. Dip in **50% ethanol** for 2-3 days.
 - c. Dip in **70% ethanol** for a minimum of 3 h (but can be maintained for up to 7 days).
 - d. Dip in **96% ethanol** overnight.
 - e. Dip in **absolute ethanol** for a minimum of 3 h.
 - f. Wash with clearing agent (**xylene**) for 1 h at room temperature.
 - g. Perform a **first paraffin wax** at 60°C overnight.
 - h. Perform a **second paraffin wax** at 60°C for a minimum of 3 h.
 - i. Place a small amount of molten paraffin in the mold (dispense from a paraffin reservoir). Use warm forceps, **transfer the tissue into the mold** cut side down, as it was placed in the cassette.
 - j. Transfer the mold to a **cold plate** and gently press the tissue flat. Paraffin will solidify in a thin layer that holds the tissue position.
 - k. When the tissue is in the desired orientation, add the labeled tissue cassette to the top of the mold as a backing. Press firmly.
 - l. Hot paraffin is added to the mold from the paraffin dispenser. Be sure that there is enough paraffin to cover the face of the plastic cassette. If necessary, fill the cassette with paraffin while cooling, keeping the mold full until solid.
 - m. Paraffin solidifies in 30 min. The paraffin block can then be removed and sectioned. If the wax cracks or the tissues are not well aligned, melt them again and start over. Tissue blocks can be stored at room temperature for years.
 - n. Tissues are sectioned using a microtome (Leica RM 2155).
 - i. Turn on the water bath and check that the temperature is 45°C. Use 1 L fresh deionized water and add 1 g jelly from porcine skin (G1890, Sigma-Aldrich, Saint Louis, MO, USA). Place a fresh blade on the microtome (blades may be used to section up to 10 blocks, but replace if sectioning becomes problematic). The blade should be angled at 5°. Blocks to be sectioned are placed face down for about 15 min on an ice block (cold wax allows thinner sections). After cutting, use forceps to pick up the ribbons of sections and float them on the surface of

- the water in the water bath to allow for expansion of the sample.
 - ii. Insert the block into the microtome with the wax block facing the blade, and align the vertical plane. Set the dial to cut 10- μ m sections in order to plane the block; once cutting smoothly, set to 2–3 μ m-thick sections. Face the block by cutting it down to the desired tissue plane and discard the paraffin ribbon.
 - iii. If the block is ribboning well, cut another four sections, pick them up with forceps or a fine paint brush, and float them on the surface of the water in the 45°C water bath. Float the sections onto the surface of clean glass slides.
 - iv. If the block is not ribboning well, place it back on the ice block to cool for a longer period to harden the wax.
 - v. If the specimens are fragmented when placed in the water bath, then it may be too hot.
 - vi. Place the slides on the warming block in a 60°C oven for 10 min (so the wax starts to melt) to bond the tissue to the glass. Slides can be stored overnight at room temperature.
 2. For immunofluorescence analysis.

Place the other pole of the kidney into a tissue mold, coat in OCT, and place it onto dry ice to freeze. Store at -80°C.

 - a. Place a small amount of molten OCT (tissue freezing medium, Leica Ref = 14020108926, Leica Biosystems, Richmond, IL) in the mold.
 - b. Using forceps, transfer the tissue to the mold, cut side down.
 - c. Pour liquid N₂ into a polystyrene box containing a 50-mL tube rack (do not submerge the tube rack in the liquid N₂). Place the OCT mold on the tube rack and freeze using the vapor.
 - d. Wrap with foil and snap freeze in liquid N₂ until storage at -80°C.
 3. For protein and RNA extraction.

Place 3 × 2 mm³ pieces of kidney cortex into 0.5-mL plastic tubes and snap freeze in liquid N₂. Store at -80°C. For long-term tissue storage for RNA extraction, place the tissue in five volumes of RNA stabilization reagent and store at -80°C.

F. Direct immunofluorescence analysis of IgG and C3 deposits in the kidney.

Requirements: OCT-embedded kidney samples, gloves, ice-cold 1× PBS, cryostat, poly-L-lysine-coated slides (Merck KGaA, catalog number: P0425) or FLEX IHC microscope slides (Agilent, Ref. K8020, Santa Clara, CA, USA), 4% PFA (see Recipes), acetone, blocking solution, conjugated primary antibodies (FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-mouse C3), UltraCruz Tm mounting medium, and DRAQ5.

1. Prepare the kidney slides for immunofluorescence analysis.
 - a. Place the OCT compound mold containing frozen kidney (section D3) at -20°C for 2 h prior to sectioning. Ensure that the [cut] surface of the kidney is carefully placed against the bottom of the OCT mold to enable well-oriented tissue sections.
 - b. Use a cryostat to cut 5 µm-thick sections and place them on the surface of poly-L-lysine-coated slides or FLEX IHC microscope slides. Ensure that there are no folds or holes in the tissue section, which can distort tissue morphology. As positive IF controls, use samples of human parotid glands or ganglions; and as negative controls, omit the primary antibodies from the staining process. For the isotypic control, use an irrelevant immunoglobulin of the same isotype, species, and concentration as the primary antibody.
 - c. Upon removal from the cryostat, keep at room temperature for 20 min and then fix the slides by submerging into ice-cold pure acetone for 20 min.
 - d. Wash the slides 3 times with 100 µL PBS for 10 min each.
 - e. Wash the slides twice with distilled water for 5 min each.
 - f. Incubate the slides in blocking solution (20% normal goat serum in 1× PBST + 0.2% jelly from porcine skin) overnight at 4°C.
 - g. Wash the slides 3 times with 100 µL PBS for 10 min each.
 - h. Incubate the sections with primary antibodies (FITC-conjugated goat anti-mouse IgG at 1:300, and

FITC-conjugated goat anti-mouse C3 at 1:50) in PBST-jelly buffer containing 1% normal serum. Incubate the slides for 1 h at room temperature in a humidified chamber to avoid the tissue drying out, which would lead to non-specific binding and high background staining.

- i. Wash the slides 3 times in PBS for 5 min each. Primary antibodies can be saved for subsequent experiments.
- j. Mount the samples with a drop of mounting medium containing 1 µg/ml DRAQ5.

G. Analysis of CD40 expression in the kidney by immunostaining with horseradish peroxidase (HRP).

Requirements: Gloves, paraffin blocks, pressure cooker, timer, 1× PBS, 0.01 M citrate buffer pH 6, 1% Triton X-100 in PBS, 0.1% fish jelly, bovine serum albumin (BSA), xylene, absolute ethanol, primary antibodies (optimal dilutions and incubation times should be determined for each primary antibody prior to use), biotinylated secondary antibody (135 µL normal serum + 45 µL biotinylated secondary antibody from the ABC staining kit in 10 mL PBS), humidified chamber, ABC (avidin-biotin complex) peroxidase standard staining kit (prepare reagent 30 min before use), DPX mounting medium, DAB substrate.

Controls: Prepare a negative control with diluent alone (without antibodies) and a positive control with a tissue known to contain the antigen of interest.

1. Prepare kidney slides for HRP analysis.
 - a. Deparaffinize the samples from section D5 by performing standard xylol-ethanol washes.
 - b. Wash the slides 3 times in 1% PBS for 5 min each.
 - c. Block endogenous peroxidase activity with a methanol wash (30% methanol in PBS + 1% peroxide hydrogen) for 10 min.

Note: Place the slides on a flat surface. Do not allow the slides to touch each other. Do not allow the sections to dry out.

- d. Wash three times in 1% PBS for 5 min each.
- e. Facilitate antigen retrieval by heating the samples for 5 min in a pressure cooker (on a plastic rack for slides) containing 10 mM citrate buffer, pH 6. This will break protein cross-links after formalin fixation.
- f. Allow to cool slowly at room temperature for 30 min.
- g. Wash twice in 1% Triton X-100 in PBS (PBST) for 5 min each.
- h. To minimize cross-reactivity and reduce non-specific binding caused by hydrophobic interactions, pre-incubate with 20% normal goat (NGS) or horse serum in PBS-Triton + 0.2% jelly at 4°C for 2 h. Remove excess fluid from the slides using a brisk motion and carefully wipe each slide around the sections.
- i. Incubate with primary antibody (anti-CD40 at 1/100, anti-DC-SIGN at 1/50; anti-NF-κB at 1/1,000) in 1% normal goat serum. Apply 100 µL to each slide, covering the tissue sections; tilt each slide from side to side. Incubate in a humidity chamber overnight at 4°C.
- j. Keep at room temperature for 30 min.
- k. Wash 3 times in 1× PBST for 5 min each.
- l. Add 100 µL biotinylated secondary antibodies (at 1/200 + 1% NGS from the Vectastain ABC kit in PBST-jelly). Incubate in the humidified chamber for at least 45 min at room temperature. During this step, it is recommended to prepare the substrate mixture (reagent A = avidin + Reagent B = biotinylated HRP).
- m. Wash 3 times in PBST for 5 min each. Wash well to remove traces of sodium azide as this will inhibit peroxidase activity when developing.
- n. Add the substrate mixture at 1/100 and incubate for 45 min.
- o. Wash 3 times in PBST for 5 min each.
- p. Wash 3 times in PBS for 5 min each.
- q. Incubate the tissue sections with 50 µL Vector DAB substrate in the dark for 3 min or until the

desired color reaction is observed when monitored under the microscope. Terminate the reaction before background staining appears in the negative controls by rinsing gently with distilled water from a wash bottle. Discharge DAB in the corresponding recipient. All plasticware that come into contact with DAB must be treated with diluted bleach.

- r. Wash in running tap water for 5 min.
- s. Wash in distilled water.
2. Counterstain with hematoxylin:
 - a. Add hematoxylin (1 vol. hematoxylin solution + 2 vol. distilled water).
 - b. Wash with tap water for 5 min.
 - c. Wash in distilled water.
 - d. Wash with 70% ethanol for 5 min.
 - e. Perform three washes with 96% ethanol for 5 min each.
 - f. Perform three washes with absolute ethanol for 5 min each.
 - g. Perform three washes with xylene for 5 min each.
 - h. Mount with DPX.
3. Microscope examination:
Sections should be independently examined by two blinded pathologists, and at least 10 fields from each kidney section should be studied at high magnification.
 - a. Determine the extent of renal damage. Assess typical glomerular active lesions of lupus nephritis: mesangial expansion, endocapillary proliferation, glomerular deposits, extracapillary proliferation, and interstitial infiltrates; as well as tubule-interstitial chronic lesions: tubular atrophy and interstitial fibrosis using a Zeiss SteREOLumar V12 microscope from Carl Zeiss AG (Oberkochen, Germany). Grade the lesions semi-quantitatively using a scoring system from 0 to 3 (0 = no changes, 1 = mild, 2 = moderate, and 3 = severe changes).
 - b. Quantitate the number of positive cells for each of the markers studied using semi-quantitative evaluation of expression from 0 to 4 (0 = no staining, 1 = staining in <25% of the sample, 2 = staining in 25–50%, 3 = staining in 50–75%, and 4 = staining in 75–100%) in the different compartments of the kidney (glomeruli, vessels, and interstitium). NF κ B p65 immunostaining was considered positive when located inside the nuclei.

H. Evaluation of atherosclerotic lesions in mouse aortas.

Requirements: ApoE^{-/-} mice, isoflurane chamber, dissection tools (scissors: straight blunt-ended scissors, straight sharp fine scissors, and micro-dissecting spring scissors; tissue forceps: straight serrated-tip forceps, straight or curved serrated-tip fine forceps, and straight fine-tip forceps), syringes, 23 G and 27 G needles, tubes (one EDTA-coated tube, a serum tube, and two lithium heparin-coated tubes), ice-cold PBS, deionized water, RNazap, 70% ethanol, ice, liquid N₂, microscope, cork bed, pinning bed, needles, scalpel, Oil Red O, minutien pins 0.1-mm, Petri dishes, 6 \times magnification microscope.

1. Check the extension of ATH lesions in the entire aorta length.
 - a. Euthanize mice with isoflurane.
 - b. Spray each mouse with 70% ethanol to avoid contamination of the samples.
 - c. Excise whole aortas (as modified from Centa *et al.*, 2019) and prepare samples for microscope analysis:
 - i. Make a midline incision with scissors from the jugular notch to the pubic bone.
 - ii. Exsanguinate the mouse by cardiac puncture through the thorax wall (use a 23 G needle). This procedure usually yields 500 μ L blood from a 20-week-old mouse. Collect 4 tubes (one EDTA-coated tube, a serum tube, and two lithium heparin-coated tubes). Keep them at room temperature.
 - iii. Open the abdominal cavity. Cut the parietal peritoneum with scissors in the midline and laterally on both sides.
 - iv. Open the diaphragm and the chest cavity (cut the rib cage as laterally as possible).
 - v. Make an incision in the right auricle for perfusion fluid drainage. Insert a 27 G needle through

- the apex of the heart in the cranial direction. Keep the needle fixed in the left ventricle while slowly perfusing with 10 ml ice-cold PBS over a minimum of 2 min.
- vi. Dissect the liver, spleen, and kidneys.
 - vii. Cut the trachea and oesophagus on the right side of the heart without damaging the aortic arch. Cut the diaphragm and structures attaching the viscera to the retroperitoneum. Leave the heart, aorta, and kidneys *in situ*. Fold away the lungs and viscera caudally and cover with a napkin to begin retroperitoneal microdissection of the abdominal aorta (perform microdissection under a microscope at 6× magnification).
 - viii. Dissect the aortic bifurcation (lift the surrounding tissue with forceps and cut under tension with scissors). Dissect the abdominal aorta cranially. Cut the abdominal branches from the aorta and free the aorta proximally through the aortic hiatus in the diaphragm.
 - ix. Remove the adipose tissue covering the thoracic aorta. Dissect dorsally of the thymus (carefully) to free the aortic arch with branches. Continue dissecting the carotid arteries as distally as possible in the thoracic cavity. Neck dissection should include carotid bifurcation.
 - x. Clean the instruments by sequential rinses in deionized water, RNase decontamination solution, 70% ethanol, and PBS before cutting the aorta.
 - xi. Lift the heart by the apex with the forceps. Cut the aorta close to the heart and place the whole heart in a tube with PBS. The heart may be stored on ice for a couple of hours before continuing with processing and cryo-mounting of the aortic root.
 - xii. Cut the aortic arch and place half in a tube containing 1 ml 4% formaldehyde overnight at 4°C.
 - xiii. Dissect the remaining descending aorta, place in a tube, and snap freeze in liquid N₂ for RNA analysis.
 - xiv. Place the heart on a cork bed with the ventral side facing up. Fix the heart to the cork with a needle through the apex. Hold the base of the heart with anatomical forceps. Cut away the apical 2/3 of the heart between the two auricles with a scalpel (angled 20° caudally in the sagittal plane and 20° cranially in the transversal plane).
 - xv. Embed the aortic root in OCT compound (see section F). Store the specimens in zip lock bags at 80°C until cryo-sectioning.
2. “En face” analysis of the aortic arch and brachiocephalic artery.
 - a. Clean the aorta from the remaining periadventitial adipose tissue under a microscope (since Oil Red O stains most hydrophobic and neutral lipids such as triglycerides, diacylglycerols, and cholesterol esters, it is crucial to remove all such tissue at this point). Use scissors and forceps without manipulating or damaging the aorta. Always keep the aorta moist by applying additional PBS when needed.
 - b. Place the cleaned and fixed aorta in a 1.5-mL tube (one aorta per tube).
 - c. Add 1 mL 78% methanol to each tube and place on a tilted roller with gentle movement for 5 min. Replace the methanol solution and repeat this step twice.
 - d. Discard the methanol and add 1 ml fresh Oil Red O.
 - e. Incubate the tube on the tilted roller for 50-60 min.
 - f. Transfer the aorta to a clean tube and wash twice with 1 ml 78% methanol for 5 min each on the tilt roller.
 - g. Discard the methanol and refill the tube with 1 ml PBS.
- Note: If necessary, at this step, aortas can be stored at 4°C.*
- h. Prepare a pinning bed: Place a sheet of 25 × 25 mm paraffin-wax film, wrapped with black electrical insulation tape, on a cork bed to make a dark background for the aorta. Place a label on the backside of the pinning bed and use a lead pencil to write the mouse identification number (normal pen ink will disappear in the staining process).
 - i. Transfer the fixed aortic arch to the pinning bed and place a drop of PBS on top.
 - j. Remove any small stained remnants of adventitial fat (carefully by microscope examination).

- k. Cut open the aorta longitudinally to expose the intimal surface. Introduce the tips of a pair of microdissection spring scissors into the artery lumen and cut the outer curvature of the aortic arch from the ascending arch to the left subclavian artery. Begin to cut the outer curvature of the ascending arc in the distal direction and continue to cut open the branches, including the brachiocephalic artery. Continue to cut along the length of the thoracic aorta.
 - l. Cut open the lesser curvature and fold open the aorta to display the intimal surface.
 - m. Pin the open arch to the pinning bed using the blunt end of minutien insect pins. Gently bend the pins away from the specimen when in place. Pin the aorta flat on the bed without stretching the specimen. Store the pinned arch facing downwards in a Petri dish filled with PBS at 4°C.
 - n. Acquire images with a Zeiss steREOLumar V12 microscope connected to a RGB camera (ProgRes C F scan). Place a ruler next to the aorta for calibration of the image.
3. Quantitate the extension of atherosclerotic plaques.
Use image analysis software (ImageJ) to determine the lesion area and total intima surface. Lesion quantitation should be performed in a blinded fashion, and it is advisable that a second investigator confirms the results.
 - a. Calculate the total arch area: In ImageJ, select the “polygon selection” tool and encircle the total arch area by repetitive clicks. Select “measure” in the analyze menu to display the total arch area in the result window.
 - b. Calculate the lesion-free area: In ImageJ, select the “freehand selection” tool and encircle all plaques (stained in an orange-red color) in the arch area while pressing the Alt key. Click “measure” in the analyze menu to display the lesion-free area in the result window.
 - c. Calculate the relative lesion area by subtracting the lesion-free from the total arch area and dividing the result by the total arch area.
 4. Cryo-sectioning of the aortic root.
 - a. Set the cryostat temperature at -20°C and section thickness to 10 µm. Mount the OCT block containing the aortic root on the specimen holder with the ventricular tissue facing outward. While starting to cut, fine-tune the alignment of the section surface to be parallel to the specimen holder.
 - b. Remove the surrounding excess OCT to make it easier to collect the sections without folds. The aortic root should be positioned perpendicularly to the knife blade.
 - c. Collect initial control sections on ordinary microscope slides and discard. The first sections should only contain heart muscle tissue. Progress the sectioning by 200 µm. Collect a section and check the progress under a light microscope.
 - d. When getting closer to the left ventricle outflow tract, check every 100 µm under the microscope. When initial indications of a vessel wall are observed, slow down the pace to 50 µm. When the first aortic valve appears, this will be point zero for collecting sections. It can be difficult to see exactly when the valves appear, but an exact localization is crucial to perform comparations of lesions in the same region.
 - e. Tilt the specimen toward the point zero valve to align the section plane with the two other valves. This is crucial for obtaining true cross-sections of the aorta. Make a drawing of the aortic root, indicating the valves as they appear, and count every 10-µm section that is cut from the point zero onward. When a second aortic valve appears, slightly tilt the specimen again from the valve to align the specimen with the third valve. The distance from where the first aortic valve leaflet appeared to where all 3 of the aortic valve leaflets appeared together did not exceed 80 µm. The first 5-µm thick section with the 3 aortic valve leaflets was mounted onto a glass slide. The adjoining 8 sections, including the first, were collected one by one onto 8 glass slides and marked in order from 1 to 8. The 9th section was collected onto the first glass slide, the 10th onto the second glass slide, and so forth, until each of the 8 glass slides held 8 sections. The 8 frozen sections collected on each glass slide covered a distance of 400 µm.
 - f. Fix the sections collected for Oil Red O staining in 4% formaldehyde for 10 min and for immunohistochemistry or immunofluorescence with ice-cold pure acetone for 10 min. Dry at room temperature for 30 min. Store the sections at -20°C.
 - g. Capture the images directly using an RGB camera (ProgRes C F scan) attached to a light

microscope (NIKON E800). Save high resolution images, preferably in tagged image file (TIFF) format.

5. Morphometric image analysis using ImageJ.

The lesion quantitation should be performed in a blinded fashion, and it is advisable that a second investigator confirms the obtained results.

- a. Use the area quantitation feature in the image analysis software to define the total vessel area by encircling the external elastic lamina of the aortic vessel. In ImageJ, select the “polygon selection” tool and encircle the area by repetitive clicks. Then select “measure” in the analyze menu. The total vessel area is displayed in the result window.
- b. Continue to quantitate the ATH lesions in the intimal layer of the vessel, defined by the internal elastic lamina and the luminal boundary. Usually, lesions on the aortic valves are excluded from measurement. In ImageJ, select the “freehand selection” tool and encircle all plaques while pressing the Alt key. Select “measure” in the analyze menu to display the lesion-free vessel area in the result window.
- c. Calculate the relative lesion area by subtracting the lesion-free area from the total vessel area and dividing the result by the total vessel area. Calibrate the results in the image analysis software according to the magnification used in order to obtain the absolute lesion area in μm^2 .

Data analysis

The cholesterol-conjugated, specific anti-CD40 siRNA demonstrated efficient transfection efficiency in dendritic cells (DCs) without decreasing cell viability (evaluated by propidium iodide). Here, we demonstrate the inhibition of CD40 expression and the subcellular localization of the anti-CD40-siRNAChol in the cytoplasm of DCs at 45 min post-transfection (Ripoll *et al.*, 2013). The specificity of siRNA cleavage of CD40 mRNA was confirmed by 5'RACE.

In the silencing experiments (Ripoll *et al.*, 2013), we firstly matured DCs with LPS (confirmed by an increase in CD40, CD80, and CD86 cell surface expression as measured by flow cytometry) and transfected them with anti-CD40-siRNAChol, which caused a 35% decrease in CD40 expression as compared with the scrambled controls, as well as a significant reduction in the release of TNF α , MCP1, and IL6. In addition, LPS increased CD40 mRNA expression by almost 20-fold (in the kidney) and 35-fold (in the liver) as compared with control levels in a mouse model. CD40 mRNA expression returned to baseline after 24 h in the kidney and after 48 h in the liver. A single anti-CD40-siRNAChol administration reduced renal and hepatic CD40 mRNA expression by 65% and 60%, respectively, for 3 days as compared with control values, with the effects persisting for up to 5 days.

In the lupus nephritis model (Ripoll *et al.*, 2013), Cy5.5-labeled anti-CD40-siRNAChol was localized in tubular cells from the kidney (Figure 1). In this disease model, we studied the inhibitory effects of anti-CD40-siRNAChol on animal survival, renal function, inflammatory mediators, anti-DNA antibody levels, and renal lesions. For these experiments, mice were distributed into one of five groups subjected to different treatments:

1. CYP group (n = 9): 50 mg/kg i.p. CYP every 10 days
2. CTLA4 group (n = 9): 50 μg i.p. CTLA4 (ORENCIA, Abatacept, Bristol Myers Squibb, Uxbridge, UK) three times a week
3. Anti-CD40-siRNAChol-1w group (n = 9): 50 μg i.p. anti-CD40-siRNAChol once a week
4. Anti-CD40-siRNAChol-2w group (n = 8): 50 μg i.p. anti-CD40-siRNAChol twice a week
5. Control group (n = 15): 50 μg i.p. ss-control siRNAChol twice a week

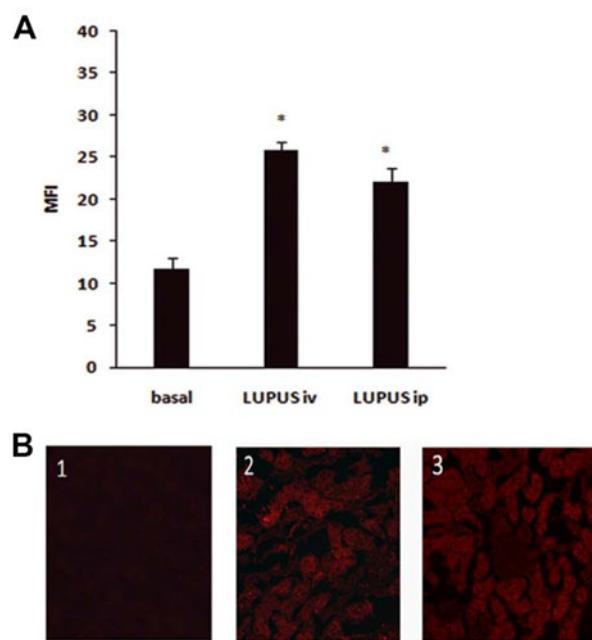


Figure 1. In the lupus nephritis model, Cy5.5-labeled anti-CD40-siRNA^{Chol} was localized in tubular cells from the kidney.

A. Quantitation of renal internalization of Chol-siRNA administered i.v./i.p. in lupus mice. B. Representative kidney photomicrographs ($\times 400$) of: 1) basal autofluorescence; 2) i.v. administration; and 3) i.p. administration. Data are expressed as the mean \pm SEM of four separate experiments. * $P < 0.05$ vs. basal.
doi:10.1371/journal.pone.0065068.g005.

Animal survival (by the Kaplan-Meier method) was 100% for the CYP, CTLA4, and CD40-2w groups; 88% for the CD40-siRNA-1w group, and 73% for the untreated group at the end of follow-up. Mice treated with siCD40-2w showed no increase in proteinuria or albuminuria and displayed a dose-dependent reduction in total IgG, anti-dsDNA antibodies, and all IgG fractions. The levels of IgM, IgA, and IgE, as well as pro-inflammatory cytokines such IL2, TNF, IFN γ , MCP1, and IL6, were also reduced in the anti-CD40-siRNA^{Chol}-2w group. Histological lesions were graded semi-quantitatively using a scoring system from 0 to 3 (Control = 8 ± 1.5 ; CYP = 4.3 ± 1.1 ; CTLA4 = 1.6 ± 0.7 ; anti-CD40-siRNA^{Chol}-1w = 3.8 ± 1.5 ; anti-CD40-siRNA^{Chol}-2w = 1.6 ± 0.6), and IgG and C3 glomerular deposits were reduced in all treated groups (Figures 2 and 3). The anti-CD40-siRNA^{Chol}-2w group showed an absence of extra-capillary proliferation, interstitial infiltrates, tubular atrophy, and interstitial fibrosis. Infiltrating CD3 $^+$ cells in the tubule-interstitial space were significantly reduced in all treatment groups except siCD40-1w. Furthermore, kidney expression levels of CD40, C3 (a manifestation of local complement synthesis), and pro-inflammatory cytokines were reduced in anti-CD40-siRNA^{Chol}-2w mice (Figure 4). In addition, a significant reduction in CD40 protein expression was observed in the interstitial, glomerular, and vascular compartments of the kidneys and in the circulation in the antiCD40-siRNA^{Chol}-2w group.

Finally, we used the CD40 silencing strategy to reduce the progression of atherosclerotic (ATH) lesions in ApoE $^{-/-}$ mice (Hueso *et al.*, 2016). We distributed the mice into 8 treatment groups:

1. Basal B/8w (n = 5)
2. ss-control siRNACHol/10 w (n = 5)
3. ss-control siRNACHol/24w (n = 5)
4. Scrambled oligonucleotide control SC/14w (n = 5)
5. Anti-CD40-siRNACHol/10w (n = 5)
6. Anti-CD40-siRNACHol/14w (n = 5)
7. Anti-CD40-siRNACHol/24w (n = 10)
8. Vehicle (n = 5)

The “en face” analysis of whole aorta stained with Oil Red O confirmed that the number and extension of ATH plaque areas decreased in anti-CD40-siRNA^{Chol}/24w as compared with control values. Less F4/80 infiltrating macrophages were detected in the vessel walls from anti-CD40-siRNA^{Chol}/24w animals, suggesting a role for CD40 in the recruitment of macrophages to the plaque. Finally, less NF-κB⁺ cells were detected in the intima of anti-CD40-siRNA^{Chol}/24w mice, indicating that the protective effect of CD40 silencing may be mediated by NF-κB signaling. Since the strategy aimed to systemically silence CD40, a reduction in the splenic populations of CD3⁺CD40⁺ (T-lymphocytic) and CD11b⁺CD40⁺ (monocytic) cells was observed in the anti-CD40-siRNA^{Chol}/24w animals, suggesting that the reduction in atherosclerotic lesions may be associated with anti-inflammatory mechanisms in the vessel wall via systemic effects.

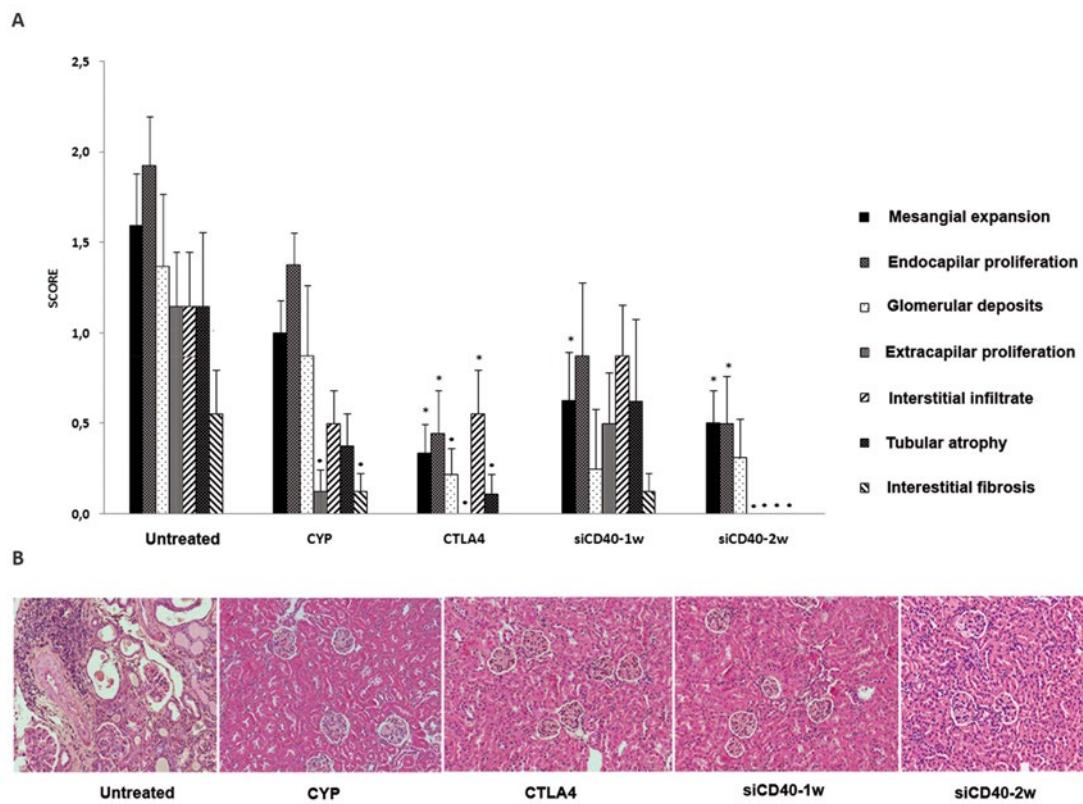


Figure 2. Histopathological lesions in the lupus nephritis model silenced by CD40 siRNA.

A. Costimulatory blockade reduced the lesions in lupus nephritis. B. Representative photomicrograph ($\times 200$) of the renal histology from each group. Data are expressed as the mean \pm SEM. * $P < 0.05$ vs. untreated, $P < 0.01$ vs. untreated. doi: 10.1371/journal.pone

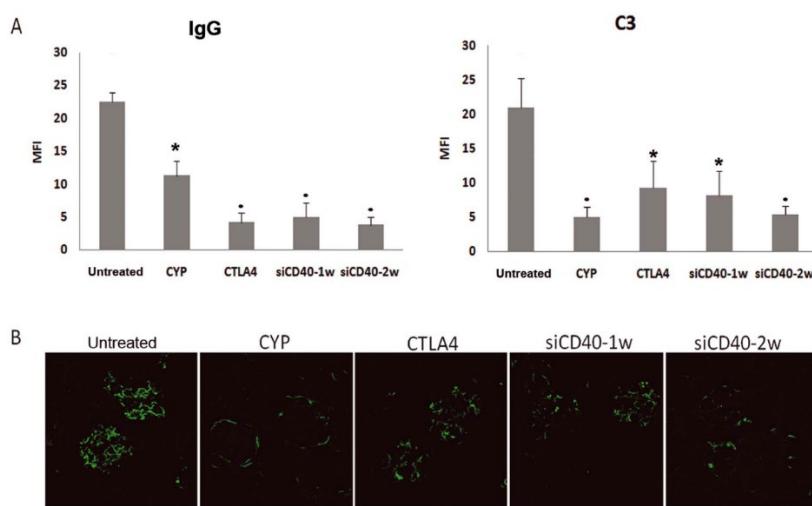


Figure 3. Immunohistochemical analysis of renal IgG and C3 in the lupus nephritis model silenced by CD40 siRNA.

A. Deposits of renal IgG and C3 were quantitated by confocal microscopy (MFI). All treatments reduced glomerular deposits. B. Representative photomicrographs of C3 deposits ($\times 630$) for each group. Data are expressed as the mean \pm SEM. * $P < 0.05$ vs. untreated, $P < 0.01$ vs. untreated.
doi:10.1371/journal.pone.0065068.g006.

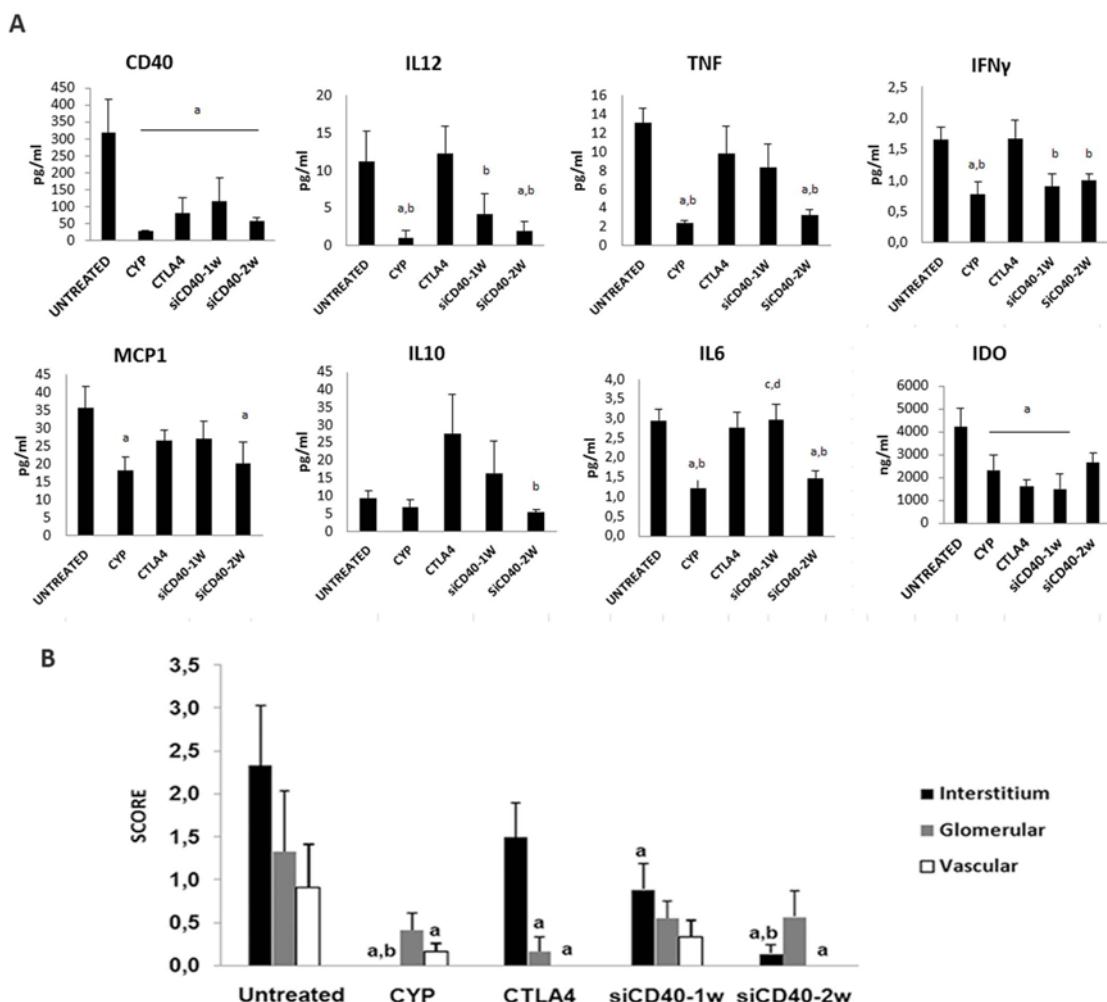


Figure 4. Systemic circulating inflammatory cytokines and local CD40 immunostaining.

A. Lupus nephritis promoted the overexpression of CD40 in the serum; this immune modulatory protein was reduced in all therapies. Lupus nephritis also induced an increase in other inflammatory cytokines; treatment with anti-CD40 siRNA reduced some of them. B. Immune localization and quantitation of CD40 protein in different kidney compartments; Chol-siRNA reduced CD40 expression, especially in interstitial cells and vessels. Data are expressed as the mean \pm SEM. a P < 0.05 vs. untreated; b vs. CTLA4; c vs. CYP; and d vs. siCD40-2w. doi:10.1371/journal.pone.0065068.g007.

Recipes

1. Annealing buffer 10×

1 M Tris-HCl (pH 7.5) 5 mL
5 M NaCl 2 mL
DEPC-treated water 43 Ml

2. DEPC-treated water

Diethyl pirocarbonate (DEPC) 0.1% 1 ml
Distilled water 1,000 ml
Mix well and leave at room temperature for 1 h

Autoclave

Let cool to room temperature prior to use

3. 1 M Tris-HCl, pH 7.5

Tris 121.14 g

Distilled water 800 mL

Adjust the pH to 7.5 with the appropriate volume of concentrated HCl

Bring the final volume to 1 L with deionized water

4. 12% acrylamide gel

For 15 mL, enough for a 13 cm × 15 cm × 0.75 mm thick gel:

10× TBE 1.5 mL

40% acrylamide (acrylamide: bis acrylamide = 29:1) 4.5 mL

Distilled and deionized water 9 mL

Stir to mix, then add:

10% ammonium persulfate 150 µL

TEMED 15 µL

Mix briefly after adding the last 2 ingredients and pour the gel immediately

5. Non-denaturing gel loading buffer

Sucrose 50%

Bromophenol Blue 0.25%

Xylene cyanole 0.25%

6. 4% PFA solution in PBS

Caution: Formaldehyde is toxic

Requirements: Gloves, safety glasses, fume hood

- a. Place PBS 1× 800 ml in a glass beaker on a stir plate in a ventilated hood
- b. Heat to 60°C while stirring (take care that the solution does not boil)
- c. Add 40 g paraformaldehyde powder
- d. Slowly raise the pH by adding 1 N NaOH dropwise from a pipette until the solution clears
- e. Cool the solution and filter
- f. Adjust the volume of the solution to 1 L with 1× PBS
- g. Recheck the pH and adjust with small amounts of diluted HCl to 6.9
- h. Solution can be aliquoted and frozen or stored at 2.8°C for up to one month

7. 0.1 M citrate stock buffer, pH 6

Distilled water 800 mL

Citric acid (MW = 192.1 g/mol) 11.341 g

Sodium citrate (MW = 294.1 g/mol) 12.044 g

Adjust the solution to the final desired pH using HCl or NaOH

Add distilled water to 1,000 mL

Store at room temperature (shelf life up to 3 months)

8. 0.01 M citrate buffer

0.1 M citrate buffer 100 mL

Distilled water to 900 mL

9. Complete culture medium

RPMI 1640 medium 500 mL

100 U/ml penicillin

100 µg/mL streptomycin
2 M L-glutamine
10% heat-inactivated and filtered FBS
20 ng/mL GM-CSF

10. 0.3% Oil Red O stock solution

Oil Red O 0.3 g
Isopropanol 99% 100 mL
Dissolve the solution at 56°C for 1 h
Filter the solution through Whatman No. 1 filter paper and keep at 4°C
This solution is stable for 1 year

11. Oil Red O working solution

Prepare in a fume hood
Stock solution 60 mL
Distilled water 40 mL
Filter the solution through Whatman No. 1 filter paper
The solution is stable for no longer than 2 h and must be prepared 15 min before use

12. Phosphate-buffered saline (PBS), pH 7.5

0.1 M phosphate
0.15 M NaCl

13. PBS-Triton (PBST)

Triton-X 2 mL
PBS 1,000 mL
PBST/Jelly
Jelly from porcine skin 0.1 g
PBST 50 mL

14. PS Buffer

Formalin 10% 200 mL
Water, quality MilliQ or similar 200 mL
Heat 60-70°C
Add 1 pellet NaOH
Cool in water to 24°C
Add 37.5 g D+ sucrose
Add 2 mL 0.5 M EDTA and 2.5 mL BHT (butylated hidroxytoluene)
Adjust to pH 7.4 with 1 M NaOH
Add MilliQ water to 500 ml and filter using filter paper with a medium filtration rate particle retention of 10-20 µm

15. Secondary antibodies (for a 1:200 dilution)

Antibody 0.5 µL
Serum normal goat (or horse) 20% 1 µL
PBST/Jelly 98.5 mL

16. Serum normal goat or horse 20%

Serum from normal goat or horse 20 µL
PBST/Jelly 80 µL

Acknowledgments

This study was partially funded by Instituto de Salud Carlos III (Co-funded by the European Regional Development Fund. ERDF, a way to build Europe) through the projects PI11/00556, PI14/00762, and PI18/01108 and by REDinREN (12/0021). We thank REDinREN and the CERCA program/Generalitat de Catalunya for institutional support.

Competing interests

There are no conflicts of interest.

Ethics

The experiments were carried out in accordance with EU legislation on animal experimentation and were approved by CEEA: Animal Experimentation Ethics Committee, the Institutional Ethics UB Committee for Animal Research.

References

- Arranz, A., Reinsch, C., Papadakis, K. A., Dieckmann, A., Rauchhaus, U., Androulidaki, A., Zacharioudaki, V., Margioris, A. N., Tsatsanis, C. and Panzner, S. (2013). [Treatment of experimental murine colitis with CD40 antisense oligonucleotides delivered in amphoteric liposomes](#). *J Control Release* 165(3): 163-172.
- Bohula, E. A., Salisbury, A. J., Sohail, M., Playford, M. P., Riedemann, J., Southern, E. M. and Macaulay, V. M. (2003). [The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor \(IGF1R\) is influenced by secondary structure in the IGF1R transcript](#). *J Biol Chem* 278(18): 15991-15997.
- Bosmans, L. A., Bosch, L., Kusters, P. J. H., Lutgens, E. and Seijkens, T. T. P. (2020). [The CD40-CD40L Dyad as Immunotherapeutic Target in Cardiovascular Disease](#). *J Cardiovasc Transl Res* 3..
- Centa, M., Ketelhuth, D. F. J., Malin, S. and Gistera, A. (2019). [Quantification of Atherosclerosis in Mice](#). *J Vis Exp*(148).
- de Ramon, L., Ripoll, E., Merino, A., Lucia, M., Aran, J. M., Perez-Rentero, S., Lloberas, N., Cruzado, J. M., Grinyo, J. M. and Torras, J. (2015). [CD154-CD40 T-cell co-stimulation pathway is a key mechanism in kidney ischemia-reperfusion injury](#). *Kidney Int* 88(3): 538-549.
- Elgueta, R., Benson, M. J., de Vries, V. C., Wasiuk, A., Guo, Y. and Noelle, R. J. (2009). [Molecular mechanism and function of CD40/CD40L engagement in the immune system](#). *Immunol Rev* 229(1): 152-172.
- Hueso, M., de Ramon, L., Navarro, E., Ripoll, E., Cruzado, J. M., Grinyo, J. M. and Torras, J. (2016) [Silencing of CD40 in vivo reduces progression of experimental atherogenesis through an NF-κB/miR-125b axis and reveals new potential mediators in the pathogenesis of atherosclerosis](#). *Atherosclerosis* 255:80-89.
- Karnell, J. L., Rieder, S. A., Ettinger, R. and Kolbeck, R. (2019). [Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond](#). *Adv Drug Deliv Rev* 141: 92-103.
- Kretschmer-Kazemi Far, R. and Sczakiel, G. (2003). [The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides](#). *Nucleic Acids Res* 31(15): 4417-4424.
- Lutgens, E., Lievens, D., Beckers, L., Wijnands, E., Soehnlein, O., Zernecke, A., Seijkens, T., Engel, D., Cleutjens, J., Keller, A. M., Naik, S. H., Boon, L., Oufella, H. A., Mallat, Z., Ahonen, C. L., Noelle, R. J., de Winther, M. P., Daemen, M. J., Biessen, E. A. and Weber, C. (2010). [Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response toward an antiinflammatory profile](#). *J Exp Med* 207(2): 391-404.
- Parasuraman, S., Kumar, E., Kumar, A. and Emerson, S. (2010). [Free radical scavenging property and diuretic effect](#)

- [of triglize, a polyherbal formulation in experimental models.](#) *J Pharmacol Pharmacother* 1(1): 38-41.
- Pluvinet, R., Petriz, J., Torras, J., Herrero-Fresneda, I., Cruzado, J. M., Grinyo, J. M. and Aran, J. M. (2005) [RNAi-mediated silencing of CD40 prevents leukocyte adhesion on CD154-activated endothelial cells.](#) *Blood* 104(12): 3642-3646.
- Remer, M., White, A., Glennie, M., Al-Shamkhani, A. and Johnson, P. (2017). [The Use of Anti-CD40 mAb in Cancer.](#) *Curr Top Microbiol Immunol* 405: 165-207.
- Ripoll, E., Merino, A., Herrero-Fresneda, I., Aran, J. M., Goma, M., Bolanos, N., de Ramon, L., Bestard, O., Cruzado, J. M., Grinyo, J. M. and Torras, J. (2013). [CD40 gene silencing reduces the progression of experimental lupus nephritis modulating local milieu and systemic mechanisms.](#) *PLoS One* 8(6): e65068.
- Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R. and Saigo, K. (2004). [Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference.](#) *Nucleic Acids Res* 32(3): 936-948.
- Hueso, M., Casas, A., Mallén, A., de Ramón, L., Bolaños, N., Varela, C., Cruzado, JM., Torras, J., Navarro, E. (2019). [The double edge of anti-CD40 siRNA therapy: It increases renal microcapillary density but favours the generation of an inflammatory milieu in the kidneys of ApoE^{-/-} mice.](#) *J Inflamm (Lond)* 16: 25-34.

Isolation of Microglia and Analysis of Protein Expression by Flow Cytometry: Avoiding the Pitfall of Microglia Background Autofluorescence

Jeremy C. Burns^{1,2}, Richard M. Ransohoff³, Michaël Mingueneau^{1,*}

¹Multiple Sclerosis and Neurorepair Research Unit, Biogen, Cambridge, USA;

²Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, USA;

³Third Rock Ventures, Boston, USA

*For correspondence: michael.mingueneau@biogen.com

Abstract

Microglia are a unique type of tissue-resident innate immune cell found within the brain, spinal cord, and retina. In the healthy nervous system, their main functions are to defend the tissue against infectious microbes, support neuronal networks through synapse remodeling, and clear extracellular debris and dying cells through phagocytosis. Many existing microglia isolation protocols require the use of enzymatic tissue digestion or magnetic bead-based isolation steps, which increase both the time and cost of these procedures and introduce variability to the experiment. Here, we report a protocol to generate single-cell suspensions from freshly harvested murine brains or spinal cords, which efficiently dissociates tissue and removes myelin debris through simple mechanical dissociation and density centrifugation and can be applied to rat and non-human primate tissues. We further describe the importance of including empty channels in downstream flow cytometry analyses of microglia single-cell suspensions to accurately assess the expression of protein targets in this highly autofluorescent cell type. This methodology ensures that observed fluorescence signals are not incorrectly attributed to the protein target of interest by appropriately taking into account the unique autofluorescence of this cell type, a phenomenon already present in young animals and that increases with aging to levels that are comparable to those observed with antibodies against highly abundant antigens.

Keywords: Microglia isolation, Flow cytometry, Neuroimmunology, Autofluorescence, Neuroscience

This protocol was validated in: eLife (2020), DOI: 10.7554/eLife.57495

Background

Microglia are a type of tissue-resident macrophages residing in the central nervous system (CNS) and account for 10% to 15% of all cells within the CNS. While displaying some canonical macrophage activities, such as the phagocytosis of debris and apoptotic bodies, microglia are also endowed with functions specific to the CNS microenvironment, such as synaptic remodeling, neuronal support, and oligodendrogenesis (Ransohoff and Khoury, 2016; Clayton *et al.*, 2017; Li and Barres, 2018). This wide array of functions implies the existence of a diverse set of microglia phenotypes, states, and subsets well suited to characterization by single-cell analytical approaches. Fluorescence-based flow cytometry, a technique routinely used in immunological studies, allows for high-throughput, multiparametric analysis of single cells in suspension, isolated from blood or from dissociated tissues. However, the dissociation of CNS tissues generates a large amount of debris, principally myelin fragments, which must be removed before flow cytometry analysis. Commonly cited isolation techniques dissociate CNS tissue through enzymatic digestion and utilize magnetic bead-based strategies to remove myelin or isolate microglia. However, commonly used proteases in enzymatic tissue digestion, such as collagenase and trypsin, can lead to the unintended cleavage of surface antigens on microglia (Autengruber *et al.*, 2012) and promote cellular transcriptional changes during the 37°C incubations required for enzymatic activity (O'Flanagan *et al.*, 2019, Mattei *et al.*, 2020). In addition, magnetic bead isolation is costly, limits study throughput, and, in our hands, does not improve the yield or viability compared to this protocol, which in young (~3 month) mice yields approximately 2.5×10^5 microglia for analysis by flow cytometry. In this study, we describe the preparation of CNS single-cell suspensions for flow cytometry by utilizing a simple mechanical tissue dissociation procedure followed by myelin removal via density centrifugation. Cost-efficient and easy to perform, all steps of this protocol are carried out on ice or at 4°C, limiting cellular changes that would otherwise occur during isolation at higher temperatures.

By utilizing two surface markers (CD45 and CD11b), this protocol yields a population of microglia with uniformly high expression of core the homeostatic markers CX3CR1, P2RY12, and TMEM119 (Burns *et al.*, 2020, Figure 1D) and has also been successfully employed to isolate microglia in multiple states, both homeostatic and activated (Burns *et al.*, 2020, Figure 6—figure supplement 1A), from mice as old as 24 months of age. Although this workflow is optimized for cell analysis by flow cytometry, we highly recommend utilizing an alternative 2-phase Percoll protocol (described in Burns *et al.*, 2020) for fluorescence-activated cell sorting (FACS), as sorting instruments are more sensitive to the amount of debris remaining post-isolation, which can negatively impact FACS purity and yield.

While flow cytometry has become a routine method used in the analysis of immune cells, the analysis of microglia (minimally defined as CD45^{dim}, CD11b⁺) presents a particular challenge, as they exhibit a uniquely intense level of autofluorescence compared to other cell types, including CNS-resident macrophages (CD45^{bright}, CD11b⁺), which do not emit any detectable autofluorescence. In addition, the distribution of the autofluorescence signal in microglia is bimodal and biologically dynamic, with about two-thirds of the microglia showing a high autofluorescence signal (autofluorescence-positive) and the remaining third showing no or very low levels of autofluorescence (autofluorescence-negative) (Burns *et al.*, 2020). Interestingly, both autofluorescence subsets are differentially impacted by aging and genetic perturbations, which adds further complexity to their analysis (Burns *et al.*, 2020). In this protocol, we provide key considerations and analytical strategies to avoid the issues associated with microglia autofluorescence.

Materials and Reagents

1. 25G × ¾" butterfly needle (EXELINT, catalog number: 26768)
2. 20 mL luer-lock syringe (BD, catalog number: 302830)
3. 7 mL dounce homogenizer (Wheaton, catalog number: 57542)
4. 15 mL polypropylene conical tubes (ThermoFisher, catalog number: 339651)
5. 96 well v-bottom assay plates (Corning, catalog number: 3897)
6. 96 well 40 µm mesh filter plates (MilliporeSigma, catalog number: MANMN4010)
7. Cluster tubes (Corning, catalog number: 4411)

8. 10 cm plastic Petri dishes (Fisher Scientific, catalog number: FB0875713)
9. Ultracomp eBeads Plus Compensation Beads (ThermoFisher, catalog number: 01-333-42)
10. Fetal bovine serum (FBS) (ThermoFisher, catalog number: 26140079)
11. 10% FBS/HBSS
12. Flow staining buffer (ThermoFisher, catalog number: 00-4222-26)
13. DAPI (ThermoFisher, catalog number: 62248)
14. Percoll (GE Healthcare, catalog number: 17-0891-01)
15. 10× Hanks Balanced Salt Solution (ThermoFisher, catalog number: 14185052)
16. 1 M HEPES (ThermoFisher, catalog number: 15630080)
17. 1× HBSS (ThermoFisher, catalog number: 14175095)
18. 0.5 M EDTA (ThermoFisher, catalog number: 15575020)
19. TruStain FcX (Biolegend, catalog number: 101320)
20. Anti-CD45 BV785 (Biolegend, catalog number: 103149)
21. Anti-CD11b BV510 (Biolegend, catalog number: 101263)
22. 33% Isotonic Percoll (see Recipes)
23. 1× Hanks Balanced Salt Solution (HBSS) with 25mM HEPES (see Recipes)
24. Phosphate buffered solution (PBS) with 3mM EDTA (see Recipes)
25. Fc receptor blocking solution (see Recipes)
26. 2× Microglia Antibody Panel (see Recipes)

Equipment

1. Disposable scalpels (Fisher Scientific, catalog: 3120032) or common single-edge razor blades
2. 13 mm extra fine Bonn scissors (Fine Science Tools, catalog number: 14084-08)
3. Iris forceps (Fine Science Tools, catalog number: 11370-31)
4. 7 ml glass homogenizer (Wheaton, catalog number: 57542)
5. Refrigerated tabletop centrifuge (ThermoFisher Sorvall Legend XTR, Rotor TX-1000)
6. 5-laser LSR Fortessa X-20 (Becton Dickinson)
7. Vacuum line for aspirating

Procedure

A. Isolation of microglia

1. Keep all solutions ice-cold through the procedure.
2. Immediately following CO₂ euthanasia, open the chest cavity to expose the heart. Insert the 25G butterfly needle into the left ventricle, make a small incision in the right atrium, and slowly perfuse the mouse with 20 mL of PBS with 3 mM EDTA.
3. Cut and peel back the skin to expose the skull. Using scissors, cut the spine at the base of the skull. Starting from the brain stem, cut rostrally along the sagittal suture. Peel the two halves of the skull away to the side. Using tweezers, scoop out the brain and transfer into a 15 mL conical tube containing 5 ml of cold HBSS with 25 mM HEPES and keep on ice.
4. Transfer the brain to a fresh Petri dish on ice and mince the tissue with a scalpel or razor blade into pieces approximately 1 mm in size.
5. Transfer the minced tissue into the 7 mL dounce homogenizer and add 5 mL of HBSS with 25 mM HEPES.
6. The 7 mL dounce homogenizer is supplied with two pestles of slightly different sizes and labeled by the manufacturer. Using the pestle marked “loose,” gently disrupt the tissue, on ice, for approximately 10 strokes. Repeat with the pestle marked “tight” for another 10 strokes.

7. Pour the single-cell suspension into a fresh 15 mL conical tube. Rinse the homogenizer with 5 ml of HBSS with 25 mM HEPES and transfer to the same 15 mL conical tube.
8. Centrifuge the single-cell suspension at $600 \times g$ for 5 min at 4°C.
9. Aspirate the supernatant and gently resuspend the cell pellet in 1 mL of 100% FBS.
10. Add 9 mL of 33% isotonic Percoll solution and mix.
11. Gently add 1 mL of 10% FBS/HBSS over the cell suspension.
12. Centrifuge the cell suspension at $800 \times g$ for 15 min at 4°C with full acceleration and no brake.
13. Carefully aspirate the resulting myelin layer located at the interface and down to the cell pellet.

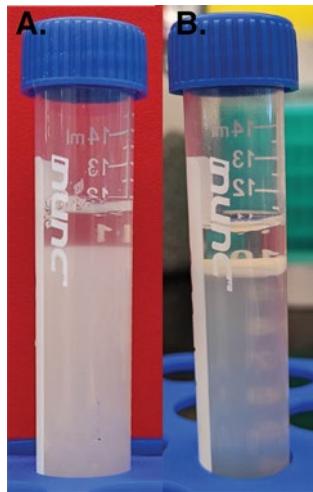


Figure 1. Percoll isolation of microglia.

A 30% Percoll cell-suspension overlaid with 1 ml 10% FBS/HBSS solution, A. before centrifugation and B. after centrifugation.

14. Resuspend the cell pellet in 1 mL of HBSS with 25 mM HEPES.
15. Add 9 mL of HBSS with 25 mM HEPES and centrifuge the single-cell suspension at $600 \times g$ for 5 min at 4°C with full acceleration and brake on.
16. Aspirate the cell pellet in a final volume of 1 mL of HBSS with 25 mM HEPES. Cells are now ready for antibody staining for flow cytometry.

B. Fluorochrome-conjugated antibody staining for flow cytometry analysis of cell surface markers on microglia

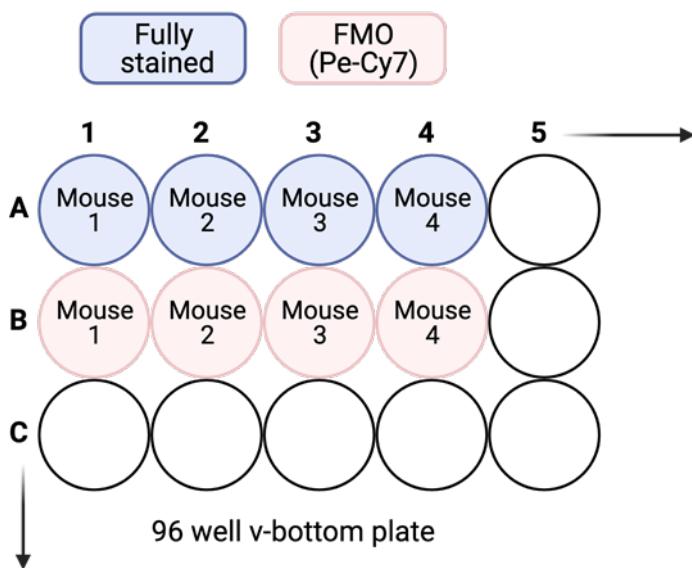


Figure 2. Example plate layout for anti-CD11c PE-Cy7 stained microglia and accompanying FMO controls

1. Transfer 150 μ L (approximately 1/7) of the single-cell suspension (approximately 300,000 live cells) from Step A16 to a well of a 96 well v-bottom plate. Repeat for any additional samples. Make sure to include wells for fluorescence-minus-one (FMO) controls for antigens of interest. For example, if the staining panel includes anti-CD11c PE-Cy7, include a well where cells are stained with all other antibodies EXCEPT for anti-CD11c PE-Cy7. This will account for background fluorescence in that specific channel for each sample.
2. Centrifuge the plate at 300 $\times g$ for 3 min at 4°C.
3. In one swift motion, decant the plate by flicking into a sink. Do not dab the plate dry. Do not flick the plate twice.
4. Resuspend the cells in the plate with 25 μ L of diluted FcBlock solution. Incubate at 4°C for 10 min.
5. Add 25 μ L of the 2 \times Microglia Antibody Panel to each well and mix well by pipetting. Incubate at 4°C for 30 min.
6. Add 150 μ L of Flow Staining Buffer to each well. Centrifuge the plate at 300 $\times g$ for 3 min at 4°C.
7. In one swift motion, decant the plate by flicking into a sink. Do not dab the plate dry. Do not flick the plate twice.
8. Resuspend the cells in 200 μ L of Flow Staining Buffer. Centrifuge the plate at 300 $\times g$ for 3 min at 4°C.
9. In one swift motion, decant the plate by flicking into a sink. Do not dab the plate dry. Do not flick the plate twice.
10. Repeat Steps B8 and B9.
11. Resuspend the cells in 200 μ L of Flow Staining Buffer containing 0.1 μ g/mL DAPI.
12. Transfer the samples to a 40 μ m mesh filter plate and centrifuge for 1 min at 100 $\times g$ to bring the samples to the lower chamber.
13. Transfer the samples to cluster tubes and keep on ice, protected from light. Promptly proceed with Section C: single-color compensation controls.

C. Preparation of single-color compensation controls

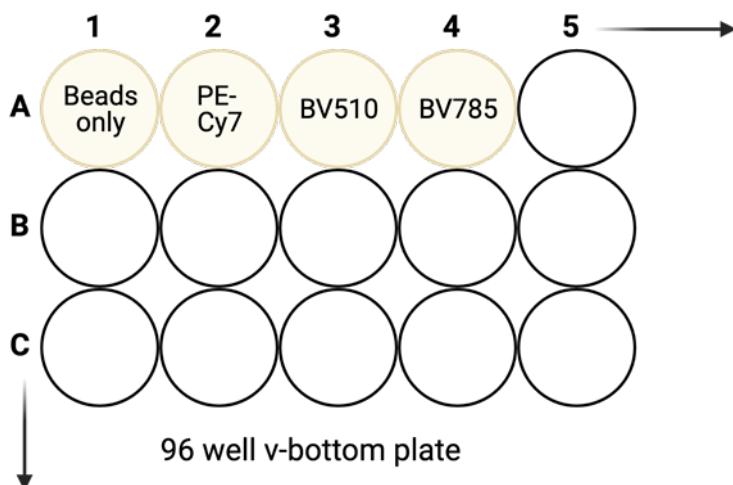


Figure 3. Sample plate layout for compensation control beads

1. For the number of fluorochromes used, add one drop of compensation beads to the same number of wells on a separate 96 well v-bottom plate. Include one extra well of beads for an unstained compensation control. Refer to Figure 3 for a sample layout.
2. Add 150 μ L of Flow Staining Buffer to each well.
3. Add 1 μ L of a single fluorochrome-conjugated antibody to a single well. Repeat for the remaining fluorochromes and wells. Do not add any antibody to the well containing the unstained compensation control. Incubate 15 min at room temperature, protected from light.
4. Centrifuge the plate at 300 $\times g$ for 3 min at 4°C.
5. In one swift motion, decant the plate by flicking into a sink. Do not dab the plate dry. Do not flick the plate twice.
6. Resuspend the beads in 200 μ L of flow staining buffer and transfer them to cluster tubes. Acquire promptly on the flow cytometer along with stained samples from Section B.

D. Sample acquisition on a 5-laser LSR Fortessa X-20

1. In Diva, create your experiment and select the detection channels corresponding to the fluorochrome panel used to stain your samples in Section B. For microglia, it is highly recommended to include an additional unoccupied detection channel (*e.g.*, 488 nm laser, 710/50 nm bandpass) to record background autofluorescence.
2. Create compensation controls for your experiment in Diva and acquire each individual single-color control, including the unstained beads, from Section C.
3. Calculate the compensation matrix and then run the samples of interest from Section B.

Data analysis

1. Identification of microglia from a CNS single-cell suspension

After sample acquisition, data analysis is performed in FlowJo V10. If a compensation matrix was not generated on the flow cytometer during sample acquisition, complete the compensation wizard in FlowJo before continuing. By utilizing the hierarchical gating strategy illustrated in Figure 4, singlets are gated first, followed by live cells (DAPI); lastly, microglia are identified by their relatively low CD45 expression and high CD11b expression.

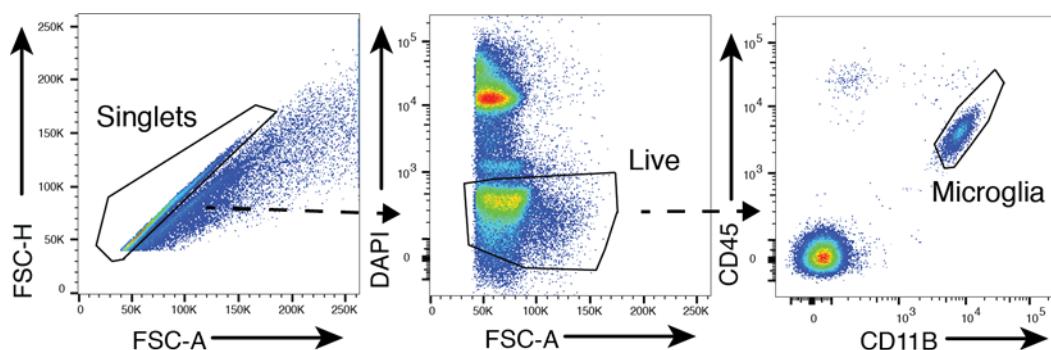


Figure 4. Hierarchical gating strategy to identify microglia from a CNS single-cell suspension. FSC, forward scatter. Reproduced from Burns *et al.*, 2020.

2. Analysis of protein target expression in microglia

Microglia subsets were recently identified based on their high or low/negligible levels of autofluorescence (Burns *et al.*, 2020). Because of the unusually high intensity, broad spectral properties, and bimodality of the autofluorescence signal in the microglia population (Figure 5), flow cytometry analysis of microglia poses specific challenges as experimental observations can be very easily confounded by background autofluorescence. Experiments should include “fluorescence-minus-one” controls (FMO) for antigen-fluorochrome combinations of interest to avoid this issue. In addition, when working with microglia, it is critical to include one or multiple unoccupied cytometer channels (i.e., with no fluorescent antibody/dyes in those channels) during sample acquisition, preferably a channel equivalent to PerCP-Cy5.5 (488 nm blue laser line, 710/50 nm bandpass filter), which is one of the most sensitive channels for microglia autofluorescence (Figure 5).

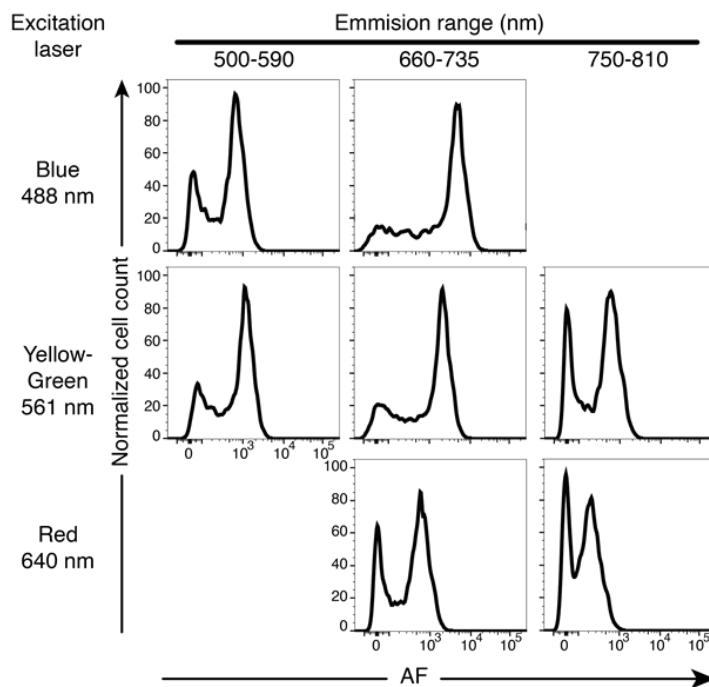


Figure 5. Representative flow cytometry histograms of autofluorescence intensity of the entire microglia population, in a single sample, from multiple combinations of excitation lasers and emission filters.

AF, autofluorescence. Reproduced from Burns *et al.*, 2020.

To illustrate these challenges, we present two examples where analysis of target protein expression in microglia can be easily confounded by autofluorescence if proper controls are not included to consider the fraction of the fluorescence signal that is attributable to autofluorescence rather than the target protein marker of interest.

In this first example, we selected the surface antigen CD11c, which is an integrin transcriptionally expressed at relatively high levels on a small subset of microglia during development, injury, disease, and aging. In Figure 6A, $CD45^{\text{dim}}CD11b^+$ microglia stained with anti-CD11c PE-Cy7 showed a clear bimodal distribution of signal in the PE-Cy7 channel, indicative at face value of positive CD11c staining in 57% of the microglia population. However, the histogram from the FMO control sample revealed a similar, bimodal pattern of fluorescence intensity with 44% of microglia gated as positive, reflective of the cellular autofluorescence in this cytometer channel (Figure 6A). When overlaid with the histogram from the CD11c PE-Cy7 stained sample, it was not possible to delineate an adequate gate to identify $CD11c^+$ microglia given the bimodality of the autofluorescence signal in microglia and the existence of two subsets of microglia, one with high levels of autofluorescence and one with no or very low levels of autofluorescence (Figure 6B). Instead, visualizing the data in an XY dot plot format using an empty cytometer channel to measure autofluorescence (Blue laser, 710/50 nm) on one axis against the cytometer channel for the target antigen of interest on the other axis (CD11c PE-Cy7 in this example) allowed us to distinguish $CD11c^+$ microglia previously confounded by the autofluorescence signal and use distinct thresholds for CD11c positivity for the autofluorescent negative and positive microglia populations (Figure 6C). Using this strategy, CD11c was found to be only expressed in 15% of the overall microglial population (Figure 6C). This result is markedly different from the one that mistakenly identified 57% of microglia as $CD11c^+$ while ignoring the autofluorescence signal (Figure 6A). In imaging studies of young, healthy CD11c-YFP reporter mice, YFP⁺ cells were present as a minor population in the adult brain (Sato-Hashimoto *et al.*, 2019), and in a recent review (Benmamar-Badel *et al.*, 2020), it was estimated that approximately 2% of microglia are $CD11c^+$ in these animals. Therefore, analyzing microglial CD11c expression by flow cytometry without taking AF into account could lead to misleading conclusions.

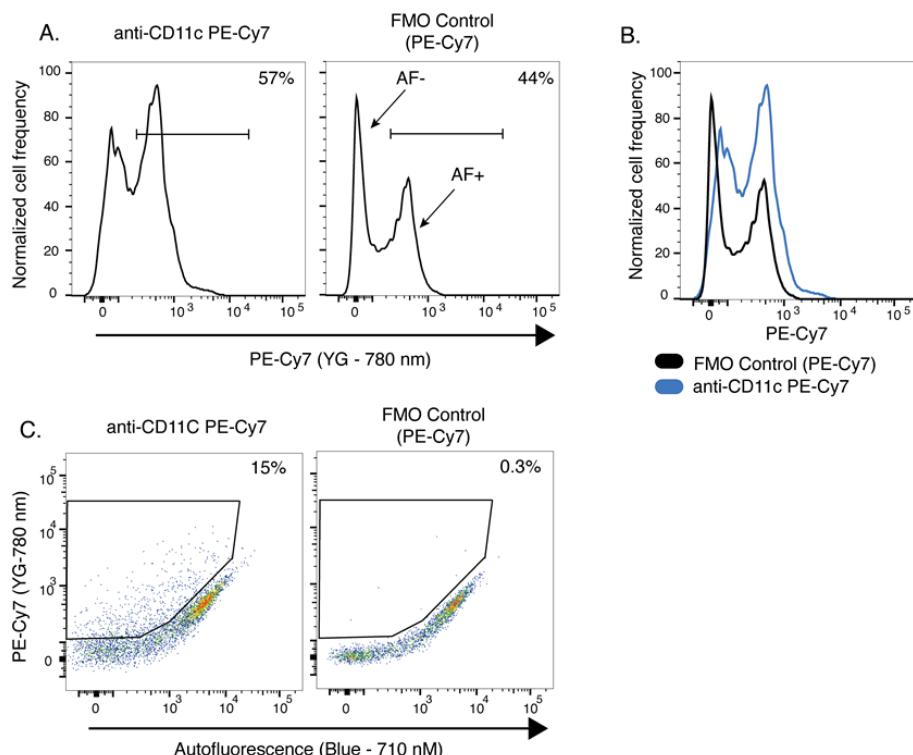


Figure 6. Analysis of microglia from 5-month-old mice by flow cytometry.

A. Histograms of fluorescence signal from FMO control and CD11c-stained microglia samples in the PE-Cy7 channel (Yellow-green laser, 780/60 nm). B. Overlay of the two previous histograms. C. Pseudocolor dot plots of signal in the PE-Cy7 fluorescence channel versus an unoccupied fluorescence channel (Blue laser, 710/50 nm). AF⁺, autofluorescence-positive; AF⁻, autofluorescence-negative; FMO, fluorescence-minus-one; Blue, 488 nm laser; YG, 561 nm yellow-green laser.

As a second example, we analyzed the impact of microglia autofluorescence on the detection of proteins expressed in the entire microglia population (as opposed to a subset) with modest to high levels of expression at the single-cell level. For this, we specifically chose the receptor family for the Fc region of IgG immunoglobulins: CD16, CD32, and CD64. Fc-receptors comprise a large family of transmembrane proteins expressed at the plasma membrane of immune cells that bind the Fc region of antibodies and activate downstream signaling cascades. Microglia are known to express the gamma family of activating Fc receptors, which includes CD16/32 and CD64 (Pellerin *et al.*, 2021). *Fcer1g*-deficient mice lack the common signaling FCER1G chain required for surface expression of all activating Fc receptors. When stained with FITC conjugated anti-CD16/32 antibody and without considering microglia autofluorescence, wild-type microglia showed a clear positive staining with a bi-modal distribution (Figure 7A). However, microglia from *Fcer1g*^{-/-} mice retained a large fraction of this signal with a wider bimodal distribution, indicating that a significant fraction of the signal detected was not attributable to CD16/32 expression but to autofluorescence (Figure 7A). Accordingly, when the analytical strategy described in the prior example was used, and anti-CD16/32 stained samples were displayed on dot plots featuring CD16/32 expression on the y-axis against an empty channel on the x-axis, a large fraction of the signal associated with CD16/32 expression in Figure 7A became clearly attributable to the microglia autofluorescence signal in the *Fcer1g*^{KO} mice (Figure 7B). Inclusion and overlay of each sample's accompanying FMO control revealed the accurate expression levels of CD16/32 and the expected lack of CD16/32 staining in microglia from *Fcer1g*^{KO} mice (Figure 7B). Gating autofluorescence-positive (AF⁺) and autofluorescence-negative (AF⁻) microglia subsets (Burns *et al.*, 2020) using the autofluorescence channel further allowed the analysis of CD16/32 expression levels on these AF microglia subsets (Figure 7C).

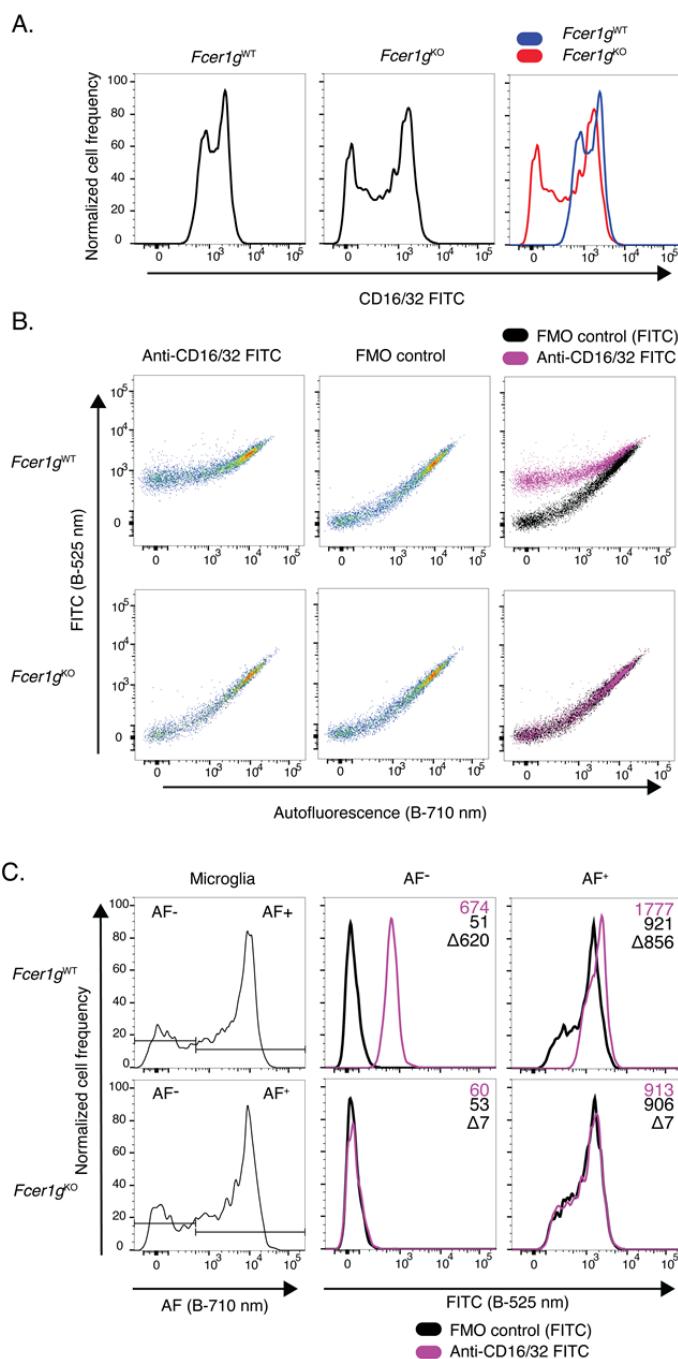


Figure 7. Analysis of Fc-receptor (CD16/32) surface expression levels in 6-month-old *Fcer1g* wild-type and knockout microglia.

A. Individual and overlaid histograms of CD16/32 surface levels on microglia isolated from wild-type and *Fcer1g*^{KO} animals. B. Individual and superimposed pseudocolor dot-plots of fluorescence signal detected in PerCP-Cy5.5 (B-710/50 nm, x-axis) and FITC (B-525/30 nm, y-axis) channels from anti-CD16/32 FITC stained and FMO control microglia isolated from wild-type or *Fcer1g*^{KO} mice. C. Gating of microglia AF subsets by AF intensity in the B-710/50 nm channel. Histograms of FITC signal intensity in autofluorescence⁺ (AF⁺) and autofluorescence⁻ (AF⁻) gated microglia subsets. Inset labels indicate geometric mean fluorescence intensity in the FITC channel for indicated population and the calculated difference between anti-CD16/32 FITC stained and FMO control. AF, autofluorescence; FMO, Fluorescence minus one; B, 488 nm blue laser.

In contrast to the modest levels of expression of CD16/32, microglia express high levels of CD64, which exceed the autofluorescence intensity level seen in microglia. As a result, the detection of this highly expressed antigen is much less subject to autofluorescence confounding issues. This is highlighted by the bright, unimodal CD64 signal observed on wild-type microglia, which clearly surpasses the signal observed in *Fcer1g^{KO}* microglia (Figure 8A). This is confirmed by displaying the data in a pseudocolor dot plot, as the signal observed in anti-CD64 stained samples exceeds that of background autofluorescence (Figure 8B). Gating AF+ and AF- microglia subsets using the autofluorescence channel further allowed the analysis of CD64 expression levels on these AF microglia subsets (Figure 8C). These results highlight that autofluorescence represents a major confounding factor for the detection of protein expression in microglia using fluorescence-based methods, such as flow cytometry, and that care should be taken to account for autofluorescence using empty cytometer channels to achieve accurate detection and quantification of protein expression in this cell type.

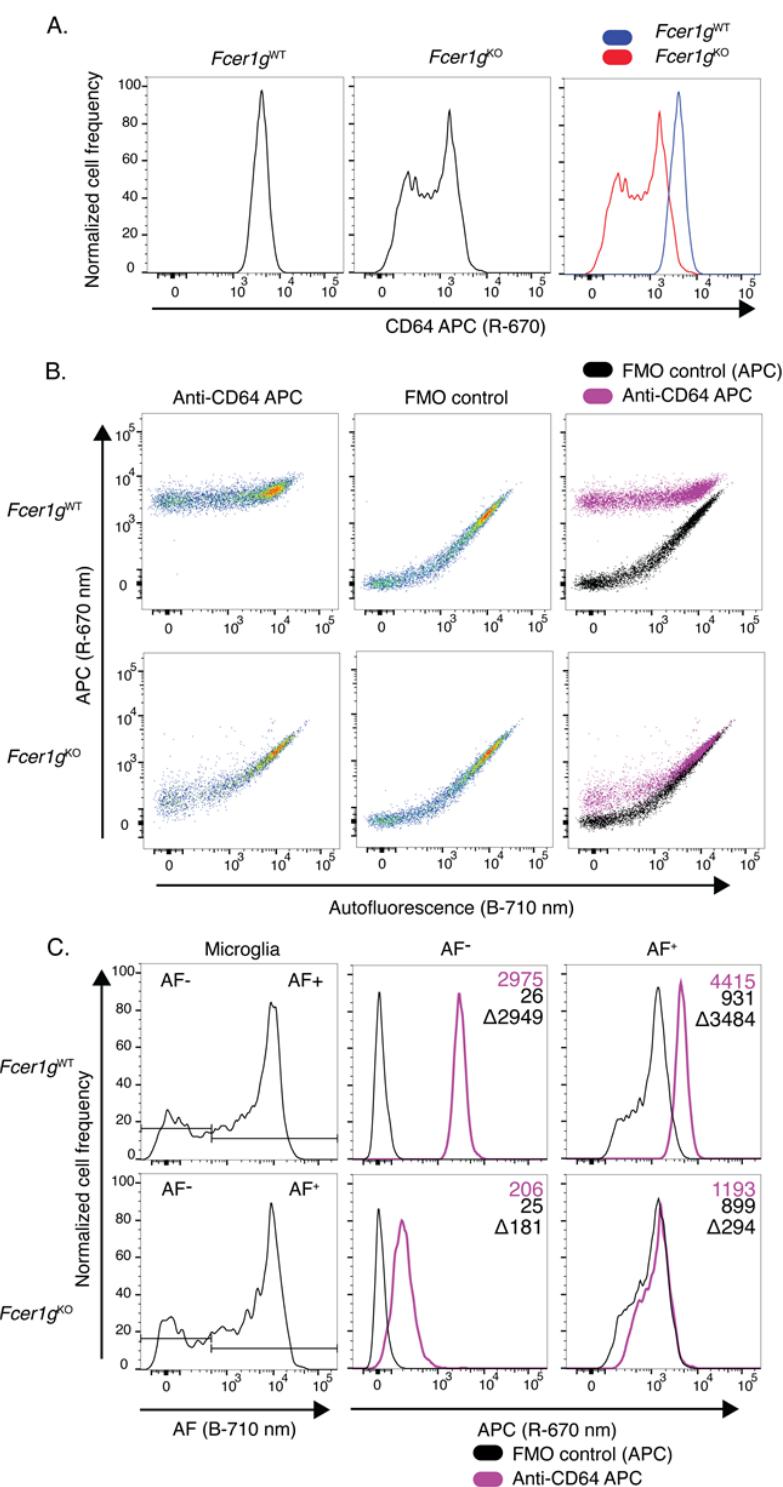


Figure 8. Analysis of Fc receptor (CD64) surface expression levels in 6-month-old *Fcerg1* wild-type and knockout microglia.

A. Individual and overlaid histograms of CD64 surface levels on microglia isolated from wild-type and *Fcer1g*^{KO} animals. B. Individual and superimposed pseudocolor dot-plots of fluorescence signal detected in PerCP-Cy5.5 (B-710/50 nm, x-axis) and APC (R-670/30 nm, y-axis) channels from anti-CD64 APC stained and FMO control microglia isolated from wild-type or *Fcer1g*^{KO} mice. C. Gating of microglia AF subsets by AF intensity in the B-710/50 nm channel. Histograms of APC signal intensity in AF⁺ and

AF⁻ gated microglia subsets. Inset labels indicate geometric fluorescence intensity in the APC channel for indicated population and the calculated difference between anti-CD64 APC stained and FMO control. AF, autofluorescence; FMO, Fluorescence minus one; B, 488 nm blue laser; R, 640 nm red laser.

Notes

1. During the wash steps of the flow cytometry staining procedure, flicking the plate twice or dabbing it dry after flicking may dislodge the cell pellet and increase cell loss.
2. Although microglia AF is detectable in all flow cytometer excitation/emission combinations we have tested, we have found that fluorescence detection channels for red-laser (640 nm) excited fluorochromes exhibit relatively lower levels of AF signal, which may minimize the confounding effects of cell autofluorescence on protein marker detection. In contrast, we found that blue-laser (488 nm) fluorescence detection channels were the more sensitive to detect microglia autofluorescence, and PerCP-Cy5.5 (710/50 nm bandpass) provided the highest resolution between AF microglia subsets.
3. If the microglial expression of an antigen of interest is expected to be low, it is advisable to test with brighter fluorochromes (*e.g.*, AlexaFluor 647); however, relative fluorochrome intensity will vary depending on the cytometer instrument ultimately used.
4. When generating single-color compensation controls, microglia should not be used, as the brightness level and bimodal nature of autofluorescence will result in an aberrant compensation matrix. Ideally, compensation beads should be used, but if unavailable, a surrogate non-autofluorescent cell population, such as splenocytes, may be used instead.
5. On the cytometer, PMT voltage levels should not be decreased to artificially minimize microglial autofluorescence, as this will also decrease the signal from antigens of interest.
6. Although we do not explicitly cover the methods to probe intracellular antigens, such as LAMP1 and Ki-67, we have successfully used the eBioscience FoxP3 Transcription Factor (ThermoFisher, catalog number: 00-5523-00) staining kit, per manufacturer protocol. However, because this strong fixation/permeabilization protocol alters the detection of several key markers to identify microglia, we recommend using the nuclear antigen PU.1 to accurately identify microglia within these fixed and permeabilized single-cell suspensions.
7. For murine samples, additional antibodies against microglia-specific cell surface markers have been successfully used (Burns *et al.*, 2020, Figure 1), including anti-P2RY12 (Biolegend, catalog number: 848004), anti-TMEM119 (AbCam, catalog number: 225494), and anti-CX3CR1 (Biolegend, catalog number: 149023).
8. Although this protocol has been applied primarily to mouse brain tissue, we have successfully used it for mouse spinal cord and brain tissue from rats and cynomolgus monkeys. When isolating CNS microglia from species other than mice, the tissue weight being processed should be kept under < 450 mg.
9. Aging critically impacts levels of autofluorescence observed in microglia. Although autofluorescence can be detected with a bimodal distribution in microglia as early as postnatal day 30, autofluorescence levels dramatically increase with aging and become more and more confounding when mice get into mature adulthood and older (Burns *et al.*, 2020).

Recipes

1. 33% isotonic Percoll

9 mL of Percoll
10× Hanks Balanced Salt Solution
20 mL of 1× HBSS with 25 mM HEPES

2. HBSS with 25 mM HEPES

12.5 mL 1 M HEPES

487.5 mL 1× HBSS

3. PBS with 3 mM EDTA

6 mL 0.5 M EDTA

494 mL 1× HBSS

4. Fc receptor blocking solution (for 10 samples)

245 µL Flow Staining buffer

5 µL TruStain FcX

5. 2× microglia antibody panel (for 10 samples)

2.5 µL of 0.2 mg/mL anti-CD45 BV785

2.5 µL of 0.2 mg/mL anti-CD11b BV510

245 µL Flow Staining buffer

6. DAPI working solution

1 µL of 1 mg/mL DAPI solution

10 mL of Flow Staining buffer

Acknowledgments

We thank the authors of Burns *et al.* (2020), from which this protocol was originally derived from.

Competing interests

JCB and MM are full-time employees of Biogen and Biogen shareholders. RMR is a full-time employee of Third Rock Ventures. No authors were provided compensation or free products by any vendors utilized in this protocol.

Ethics

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Research animals at Biogen were housed in an AAALAC accredited facility and handled according to an approved institutional animal care and use committee (IACUC) protocol (#756).

References

- Autengruber, A., Gereke, M., Hansen, G., Hennig, C. and Bruder, D. (2012). [Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function](#). *Eur J Microbiol Immunol (Bp)* 2(2): 112-120.
- Benmamar-Badel, A., Owens, T. and Wlodarczyk, A. (2020). [Protective Microglial Subset in Development, Aging, and Disease: Lessons From Transcriptomic Studies](#). *Front Immunol* 11: 430.
- Burns, J. C., Cotleur, B., Walther, D. M., Bajrami, B., Rubino, S. J., Wei, R., Franchimont, N., Cotman, S. L., Ransohoff, R. M. and Mingueneau, M. (2020). [Differential accumulation of storage bodies with aging defines discrete subsets of microglia in the healthy brain](#). *Elife* 9: e57495.
- Clayton, K. A., Van Enoo, A. A. and Ikezu, T. (2017). [Alzheimer's Disease: The Role of Microglia in Brain](#)

- [Homeostasis and Proteopathy.](#) *Front Neurosci* 11: 680.
- Li, Q. and Barres, B. A. (2018). [Microglia and macrophages in brain homeostasis and disease.](#) *Nat Rev Immunol* 18(4): 225-242.
- Mattei, D., Ivanov, A., van Oostrum, M., Pantelyushin, S., Richetto, J., Mueller, F., Beffinger, M., Schellhammer, L., Vom Berg, J., Wollscheid, B., Beule, D., Paolicelli, R. C. and Meyer, U. (2020). [Enzymatic Dissociation Induces Transcriptional and Proteotype Bias in Brain Cell Populations.](#) *Int J Mol Sci* 21(21).
- O'Flanagan, C. H., Campbell, K. R., Zhang, A. W., Kabeer, F., Lim, J. L. P., Biele, J., Eirew, P., Lai, D., McPherson, A., Kong, E., Bates, C., Borkowski, K., Wiens, M., Hewitson, B., Hopkins, J., Pham, J., Ceglia, N., Moore, R., Mungall, A. J., McAlpine, J. N., Team, C. I. G. C., Shah, S. P. and Aparicio, S. (2019). [Dissociation of solid tumor tissues with cold active protease for single-cell RNA-seq minimizes conserved collagenase-associated stress responses.](#) *Genome Biol* 20(1): 210.
- Pellerin, K., Rubino, S. J., Burns, J. C., Smith, B. A., McCarl, C. A., Zhu, J., Jandreski, L., Cullen, P., Carlile, T. M., Li, A., Rebollar, J. V., Sybulski, J., Reynolds, T. L., Zhang, B., Basile, R., Tang, H., Harp, C. P., Pellerin, A., Silbereis, J., Franchimont, N., Cahir-McFarland, E., Ransohoff, R. M., Cameron, T. O. and Mingueau, M. (2021). [MOG autoantibodies trigger a tightly-controlled FcR and BTK-driven microglia proliferative response.](#) *Brain*. doi: 10.1093/brain/awab231.
- Ransohoff, R. M. and El Khoury, J. J. (2016). [Microglia in health and disease.](#) *Cold Spring Harb. Perspect. Biol* 8(1): a020560.
- Sato-Hashimoto, M., Nozu, T., Toriba, R., Horikoshi, A., Akaike, M., Kawamoto, K., Hirose, A., Hayashi, Y., Nagai, H., Shimizu, W., Saiki, A., Ishikawa, T., Elhanbly, R., Kotani, T., Murata, Y., Saito, Y., Naruse, M., Shibasaki, K., Oldenborg, P. A., Jung, S., Matozaki, T., Fukazawa, Y. and Ohnishi, H. (2019). [Microglial SIRPalpha regulates the emergence of CD11c\(+\) microglia and demyelination damage in white matter.](#) *Elife* 8: e42025.

One-step White Blood Cell Extracellular Staining Method for Flow Cytometry

Ines Ait Belkacem^{1,2, #}, Pénélope Bourgoin^{2, #}, Jean Marc Busnel², Franck Galland¹ and Fabrice Malergue^{2,*}

¹Aix Marseille University, Centre d'Immunologie de Marseille-Luminy, Marseille, France; ²Department of Research and Development, Beckman Coulter Life Sciences-Immunotech, Marseille, France

*For correspondence: fmalergue@beckman.com

#Contributed equally to this work

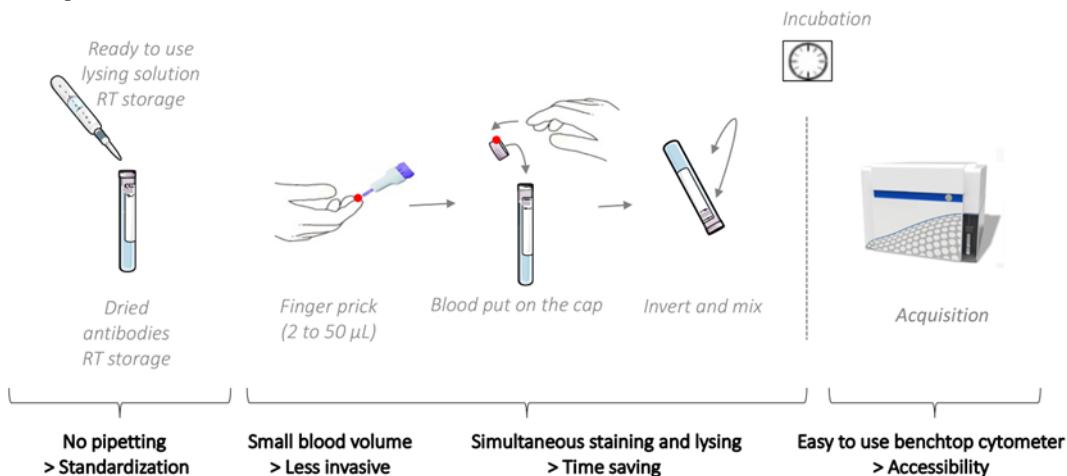
Abstract

Flow cytometry is a powerful analytical technique that is increasingly used in scientific investigations and healthcare; however, it requires time-consuming, multi-step sample procedures, which limits its use to specialized laboratories. In this study, we propose a new universal one-step method in which white blood cell staining and red blood cell lysis are carried out in a single step, using a gentle lysis solution mixed with fluorescent antibody conjugates or probes in a dry or liquid format. The blood sample may be obtained from a routine venipuncture or directly from a fingerprick, allowing for near-patient analysis. This procedure enables the analysis of common white blood cell markers as well as markers related to infections or sepsis. This simpler and faster protocol may help to democratize the use of flow cytometry in the research and medical fields.

Keywords: Blood, Leukocytes, Flow cytometry, One-step method, Point-of-care

This protocol was validated in: Cytometry B Clin Cytom (2019). DOI: 10.1002/cyto.b.21839.

Graphical Abstract:



One-step White Blood Cell Extracellular Staining Method for Flow Cytometry

Background

Flow cytometry is increasingly used in scientific investigations and healthcare; however, it requires a time-consuming, multi-step sample preparation procedure, which limits its use to specialized laboratories. Most whole blood sample preparation protocols for extracellular staining consist of at least three steps: using a venous blood sample collected in an anticoagulated sampling tube, 50-100 μ L is pipetted into a reaction tube; as a second step, fluorescent antibodies are mixed and incubated with the blood to allow white blood cell staining; as a third step, the lysis solution is added to the reaction tube to allow for red blood cell removal; finally, an optional washing step is performed to reduce non-specific fluorescence.

Due to these multiple timed steps that need to be carried out in specialized laboratories, the use of flow cytometry is limited. It is, for example, not yet routinely used in the clinical field, even though the assessment of patient immune cells has proven helpful in the indication of potential disease activity (Brown and Wittwer, 2000). Some authors have tried to establish protocols with only one step, proposing to remove the lysis step; however, this method necessitates a dedicated flow cytometer, restricting its use (Petriz *et al.*, 2018).

In this study, we propose a new universal method in which white blood cell staining and red blood cell lysis are carried out in a single step. The lysis solution is stable at room temperature (RT) and does not interfere with the staining owing to its composition, which comprises a substrate that is specifically cleaved by an enzyme uniquely present in RBC membranes (van Agthoven, 2007). White cells are maintained at neutral pH under isotonic conditions and are therefore not affected. The lysis solution can include low-dose formaldehyde (0.05%) to stabilize markers of interest (Bourgoin *et al.*, 2020c).

The fine titration of the conjugates and their direct dilution in the lysis solution enable the final washing step to be eliminated while maintaining low background levels. Using the new Dried Unitized Reagent Assays (DURA) Innovations technology, the conjugates can be dried, which enables RT storage and removes the need for pipetting, thereby supporting better standardization. The blood sample may be of venous origin or from a fingerprick, allowing for less invasive sampling suitable for a point-of-care setting. This approach is possible thanks to an excess volume of lysis solution, which permits efficient RBC lysis in up to 50 μ L of blood. The conjugate quantities have also been adjusted to stain white blood cells contained in 2-50 μ L blood, with a small quantity of blood no longer being a limitation because 2 μ L blood contains an average of 10,000 leukocytes. As a 100-cell subpopulation is usually considered statistically representative, using 2 μ L of blood allows for analysis of subpopulations as low as 1%, which is sufficient for most routine applications.

Since management of patients with infections in the Emergency Department (ED) is challenging for practitioners, we developed a panel that enables rapid patient triage in the ED using flow cytometry. It has been shown that

monitoring the expression of CD169 on monocytes (mCD169), CD64 on neutrophils (nCD64), and HLA-DR on monocytes (mHLA-DR) by flow cytometry can be indicative of viral or bacterial infection, or sepsis, respectively. We therefore established a panel consisting of antibodies targeting these three markers and evaluated the one-step method in subjects with infection and septic conditions by measuring the expression of the three infection-related markers (Bourgoin *et al.*, 2019a, 2019b, 2020a, 2020b, 2020c, and 2021; Bedin *et al.*, 2020; Michel *et al.*, 2020).

Many other applications can be envisioned in fields where flow cytometry is routinely performed, such as measuring T, B, and NK cell proportions, detecting leukemias and lymphomas, and enumerating CD34⁺ stem cells for transplantation.

Materials and Reagents

1. Regular flow cytometry 5-mL test tubes, polypropylene or polystyrene (12 × 75 mm), or 1.4-mL microtubes (e.g., from Micronic), or deep-well plates (any vendor).
2. Lysis solution (Beckman Coulter, VersaLyse™, catalog number: IM3648, store at 18–24°C, 2-year shelf-life)
3. Fixative solution (IOTest®3 10×; Beckman Coulter, catalog number: A07800, store at 2–8°C, 1-year shelf-life)
4. Antibody cocktail (IOTest Myeloid Activation CD169-PE (Phycoerythrin)/HLA-DR-APC (Allophycocyanin)/CD64-PB (Pacific-Blue) Antibody Cocktail from Beckman Coulter, catalog number: C63854, store at 2–8°C, 1-year shelf-life)
5. Easy batch preparation of the lysis buffer (see Recipes)
6. Batch preparation of the staining and lysis buffer for n tests (see Recipes)

Equipment

1. Pipettes (Gilson, PIPETMAN®, catalog numbers: FA10003M [2–20 µL] and FA10006M [100–1,000 µL])
2. 3-laser, 10-color cytometer (Beckman Coulter, Navios, catalog number: B47904) or 3-laser 13-color cytometer (Beckman Coulter, CytoFLEX, catalog number: B53000). Any other 3-laser cytometer can be used (e.g., Becton Dickinson FACSCanto, Cytek Aurora)

Software

1. Kaluza Software version 2.1 (Beckman Coulter, <https://www.beckman.fr/flow-cytometry/software/kaluza>)
Note: Any other flow cytometry software can be used (e.g., Becton Dickinson Flowjo and Cytek SpectroFlo).

Procedure

1. Prepare the lysis buffer (wear common Personal Protective Equipment). Dilute 1:200 the fixative solution in the lysis solution under chemical hood protection and mix.
2. Perform the lysis–staining step.
3. In a tube containing 500 µL lysis buffer and 10 µL conjugate panel, add 2–50 µL blood sample (venous or capillary), mix, and incubate for 15 min in the dark at RT (18–25°C).
4. Acquire the data on a 3-laser cytometer (then dispose of the waste in containers dedicated to biohazard waste).

Data analysis

A. Acquiring data on the flow cytometer

Unstained cells are used to set the parameters of the flow cytometer.

1. Turn on the flow cytometer.
2. Open the acquisition software and create four dot plots:
 - a. Side Scatter (SSC) on the x-axis and Forward Scatter (FSC) on the y-axis.
 - b. Channel dedicated to PE (FL2 on Navios) detection on the x-axis and SSC on the y-axis.
 - c. Channel dedicated to APC (FL6 on Navios) detection on the x-axis and SSC on the y-axis.
 - d. Channel dedicated to PB (FL9 on Navios) detection on the x-axis and SSC on the y-axis.
3. Set the voltages/gains for the SSC-FSC plot such that most of the cell population is in the middle of the graph (Figure 1a). The discriminant/threshold should be set up in such a way that minimum debris is acquired.
4. Set all the compensations to 0.
5. Set the voltages for FL9, FL2, and FL6 such that the lymphocyte mean or median fluorescence intensity is around 0.3 (Figure 1b).

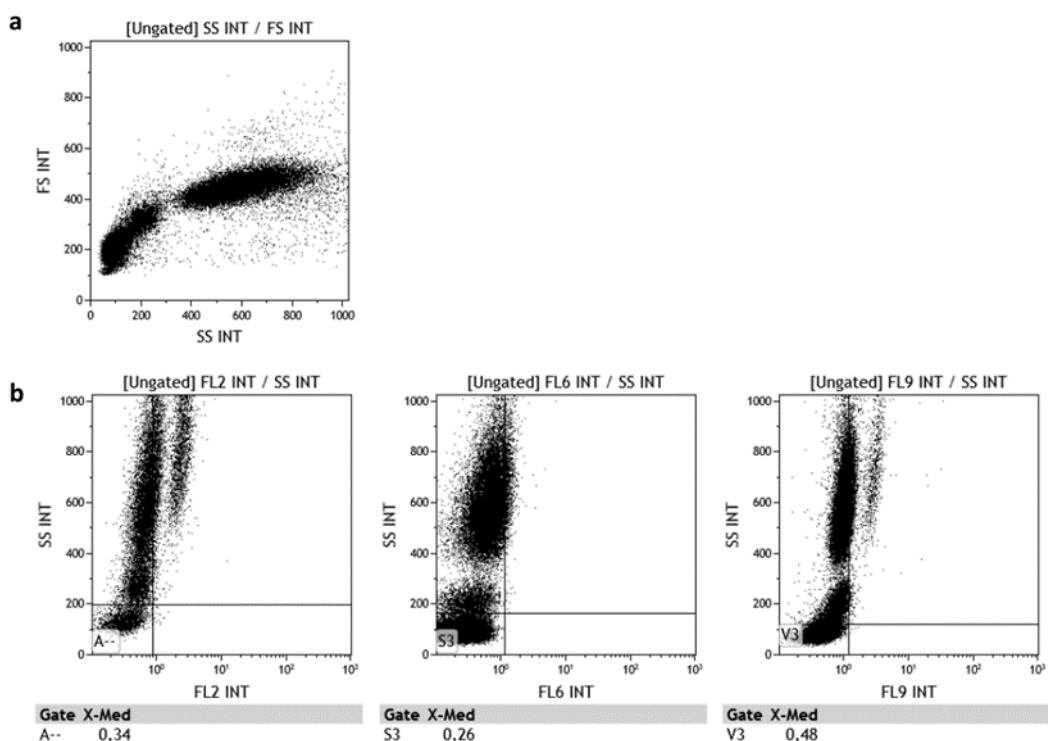
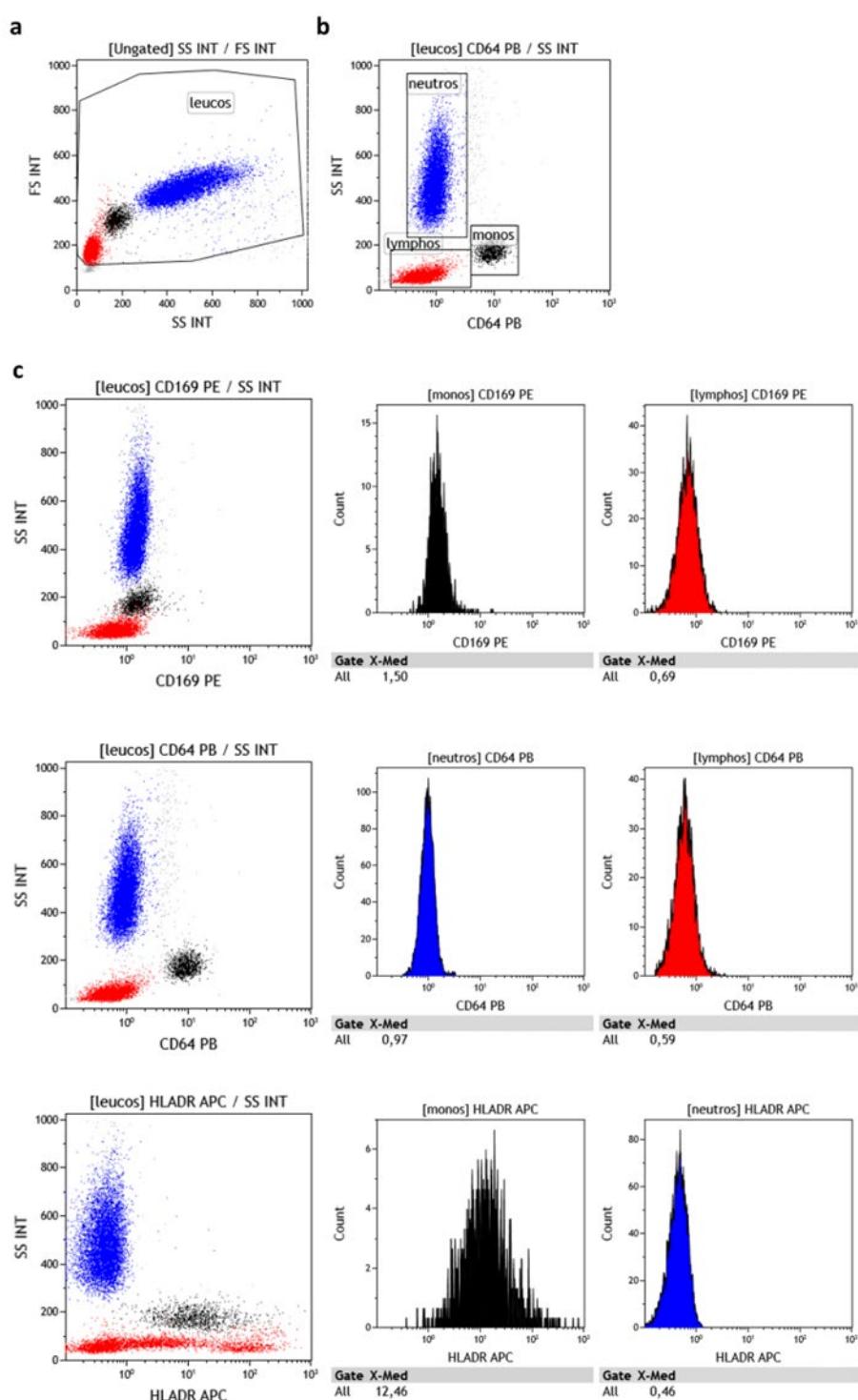


Figure 1. Flow cytometry parameters on (a) SSC-FSC plot and (b) FL2-, FL6-, and FL9-SSC plots

6. Save the protocol.
7. Acquire the first stained sample using a high flow rate (~1 $\mu\text{L}/\text{s}$) without any compensation. Make sure to acquire at least 500 monocytes for a robust analysis (usually 30 to 90 s).
8. On the SSC-FSC plot, draw a leukocyte gate, avoiding debris (Figure 2a).
9. On the SSC-FL9 (CD64-PB) plot, draw the lymphocyte, monocyte, and neutrophil gates (Figure 2b).
10. CD169 and HLA-DR expression levels should be assessed on monocytes. CD64 expression levels should be assessed on neutrophils (Figure 2b and 2c).

**Figure 2. Data analysis procedure.**

(a and b) Gating strategy of lymphocytes, monocytes, and neutrophils. (c) CD169, CD64, and HLA-DR expression level assessment strategy.

- Marker levels should be expressed as the Mean of Fluorescence Intensity, Median of Fluorescence Intensity, or Signal-to-noise. Signal-to-noise is calculated as follows: CD169 expression levels on monocytes should be divided by CD169 expression levels on lymphocytes; CD64 expression levels on

- neutrophils should be divided by CD64 expression levels on lymphocytes; and HLA-DR expression levels on monocytes should be divided by HLA-DR expression levels on neutrophils (Figure 3).
12. Results are considered as follows: High mCD169 expression is indicative of viral stimuli, high nCD64 expression is indicative of bacterial stimuli, high mHLA-DR expression is indicative of infectious stimuli, and low mHLA-DR expression is indicative of immune exhaustion (Figure 3).

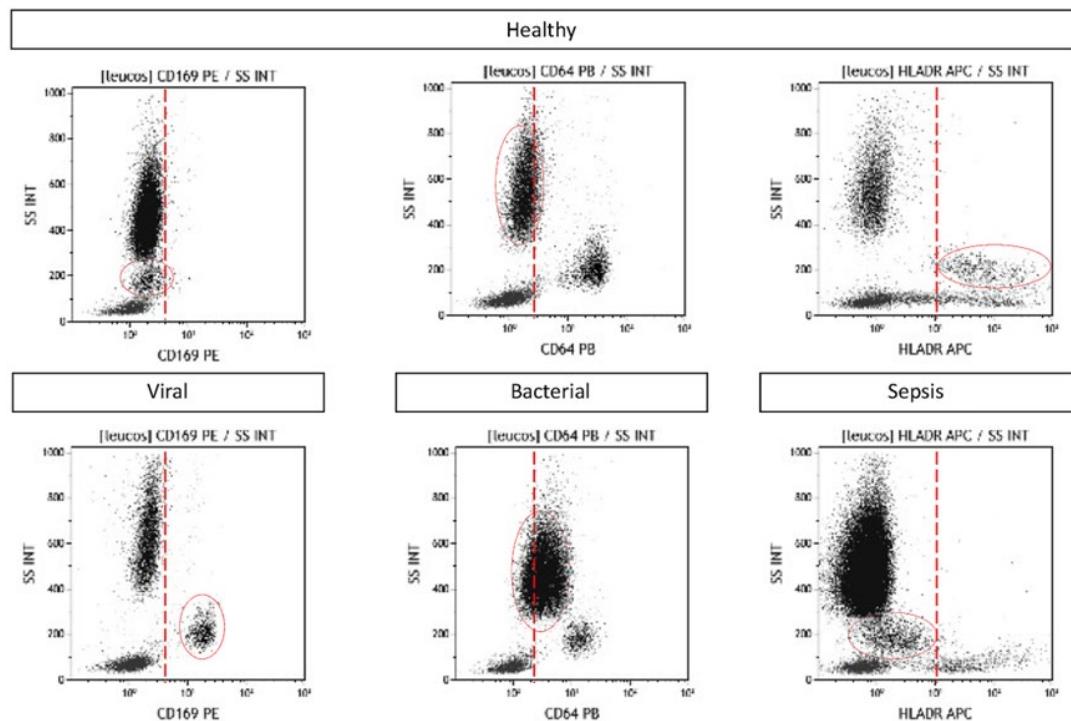


Figure 3. mCD169, nCD64, and mHLA-DR expression assessment in whole blood using the one-step method.

Examples are given for one healthy volunteer, one virus-infected patient, one bacteria-infected patient, and one patient with sepsis.

13. More research will be necessary to precisely set the thresholds in each laboratory setting, but as an indication, previous evaluations have shown that thresholds should be close to 3 for the CD169 and CD64 indexes.
14. Further analysis can be performed using software such as Kaluza (re-analysis, other gating and auto-gating, other histograms or dot plots, coloring, compensation adjustments, refined percentages and fluorescent values, overlay and merge data sets, batch analysis for higher throughput, etc.).

Notes

Depending on the target marker, application, and required stability, the method should be adapted in each laboratory setting. For example, and only for indication and not as an established performance, it has been observed in some applications that:

1. Blood sample can be venous or capillary.
2. Blood can be anticoagulated with EDTA or heparin.
3. Thanks to the gentle lysis reagent and added fixative, blood can be processed up to 3-4 days after sampling when stored at RT or 2-8°C. Moreover, samples can be analyzed on the cytometer up to 3 days after processing when stored at 2-8°C.
4. The lysis solution can include 0.05% fixative solution.

5. The lysis buffer (lysis solution containing fixative solution) can be stored for up to one month at RT.
6. The targeted marker can be any membrane marker.
7. Antibodies can be liquid or dried depending on product availability.

WARNING:

- a. If a liquid cocktail is used, an antibody volume higher than 100 µL could impact lysis efficiency.
- b. If a commercial DURAclone format is used, the lysis volume should be increased to 1 mL (for 2-50 µL blood) or 2 mL (for 50-100 µL blood as per IFU).

8. Antibodies can be premixed with the lysis buffer up to a few days prior.

Recipes

Note: To be adapted depending on each application; performances are not established.

1. Easy batch preparation of lysis buffer

One bottle Versalyse solution (100 mL)
500 µL fixative solution (10×)
Mix and store at RT

2. Batch preparation of the staining and lysis buffer for n tests

Pipet (n+1) × 500 µL lysis buffer
Add (n+1) × 10 µL myeloid activation cocktail (or any other panel at its optimal dose)
Mix well and distribute 510 µL per reaction tube, store in the dark
Tube is now ready to receive the blood sample

Acknowledgments

IAB is the recipient of a CIFRE Ph.D. grant (N°2018/1212) from the ANRT (National Agency for Research and Technology). This work was supported by Beckman Coulter through donation of the research reagents used in the flow cytometry experiments and participation of the four employees: IAB, PB, JMB, and FM. The method described was modified from ‘VersaLyse and the mechanism of ammonium chloride lysis’ (van Agthoven, 2007) and resulted in recent publications (Bourgoin *et al.*, 2019a, 2019b, 2020a, 2020b, 2020c, and 2021; Bedin *et al.*, 2020; Michel *et al.*, 2020).

Competing interests

There are no conflicts of interest or competing interests.

Ethics

All enrolled patients provided informed consent, and the procedures followed were in accordance with the Helsinki Declaration. Routine care of the subjects was not modified; analyses were performed on anonymized left-over blood, and all data collected in the study were part of routine clinical practice and retrieved from subject records. Results of this study had no influence on subject management.

References

- Bedin, A.S., Makinson, A., Picot, M.C., Mennechet, F., Malergue, F., Pisoni, A., Nyiramigisha, E., Montagnier, L., Bollore, K. and Debiesse, S. J. (2020). [Monocyte CD169 expression as a biomarker in the early diagnosis of COVID-19](#). *J Infect Dis* 223(4): 562-567.
- Bourgoin, P., Biechele, G., Ait Belkacem, I., Morange, P. E. and Malergue, F. (2020a). [Role of the interferons in CD64 and CD169 expressions in whole blood: Relevance in the balance between viral- or bacterial-oriented immune responses](#). *Immun Inflamm Dis* 8(1): 106-123.
- Bourgoin, P., Hayman, J., Rimmelle, T., Venet, F., Malergue, F. and Monneret, G. (2019a). [A novel one-step extracellular staining for flow cytometry: Proof-of-concept on sepsis-related biomarkers](#). *J Immunol Methods* 470: 59-63.
- Bourgoin, P., Lediagon, G., Arnoux, I., Bernot, D., Morange, P. E., Michelet, P., Malergue, F. and Markarian, T. (2020b). [Flow cytometry evaluation of infection-related biomarkers in febrile subjects in the emergency department](#). *Future Microbiol* 15: 189-201.
- Bourgoin, P., Soliveres, T., Ahriz, D., Arnoux, I., Meisel, C., Unterwalder, N., Morange, P. E., Michelet, P., Malergue, F. and Markarian, T. (2019b). [Clinical research assessment by flow cytometry of biomarkers for infectious stratification in an Emergency Department](#). *Biomark Med* 13(16): 1373-1386.
- Bourgoin, P., Soliveres, T., Barbaresi, A., Loundou, A., Belkacem, I. A., Arnoux, I., Bernot, D., Loosveld, M., Morange, P. E., Michelet, P., Malergue, F. and Markarian, T. (2021). [CD169 and CD64 could help differentiate bacterial from CoVID-19 or other viral infections in the Emergency Department](#). *Cytometry A* 99(5): 435-445.
- Bourgoin, P., Taspinar, R., Gossez, M., Venet, F., Delwarde, B., Rimmelle, T., Morange, P. E., Malergue, F. and Monneret, G. (2020c). [Toward Monocyte HLA-DR Bedside Monitoring: A Proof of Concept Study](#). *Shock* 55(6): 782-789.
- Brown, M. and Wittwer, C. (2000). [Flow cytometry: principles and clinical applications in hematology](#). *Clin Chem* 46(8 Pt 2): 1221-1229.
- Michel, M., Malergue, F., Belkacem, I. A., Bourgoin, P., Morange, P.-E., Arnoux, I., Miloud, T., Million, M., Tissot-Dupont, H., Mege, J. L., Busnel, J. M. and Vitte, J. (2020). [An ultra-sensitive, ultra-fast whole blood monocyte CD169 assay for COVID-19 screening](#). *medRxiv*.
- van Agthoven, A. (2007). [VersaLyse and the mechanism of ammonium chloride lysis](#). *Int J Lab Hematol* 29: 65-66.

Identification and Quantitation of Neutrophil Extracellular Traps in Human Tissue Sections

Coraline Radermecker^{1,2}, Alexandre Hego³, Philippe Delvenne^{4,5} and Thomas Marichal^{1,2,*}

¹Laboratory of Immunophysiology, Grappe Interdisciplinaire de Genoproteomique Appliquee (GIGA) Institute, Liege University, Liege, Belgium

²Faculty of Veterinary Medicine, Liege University, Liege, Belgium

³Cell Imaging platform, Grappe Interdisciplinaire de Genoproteomique Appliquee (GIGA), Liege, Belgium

⁴Department of Pathology, Clinique Hospitalo-Universitaire (CHU) University Hospital, Liege University, Liege, Belgium

⁵Laboratory of Experimental Pathology, Grappe Interdisciplinaire de Genoproteomique Appliquee (GIGA) Institute, Liege University, Liege, Belgium

*For correspondence: t.marichal@uliege.be

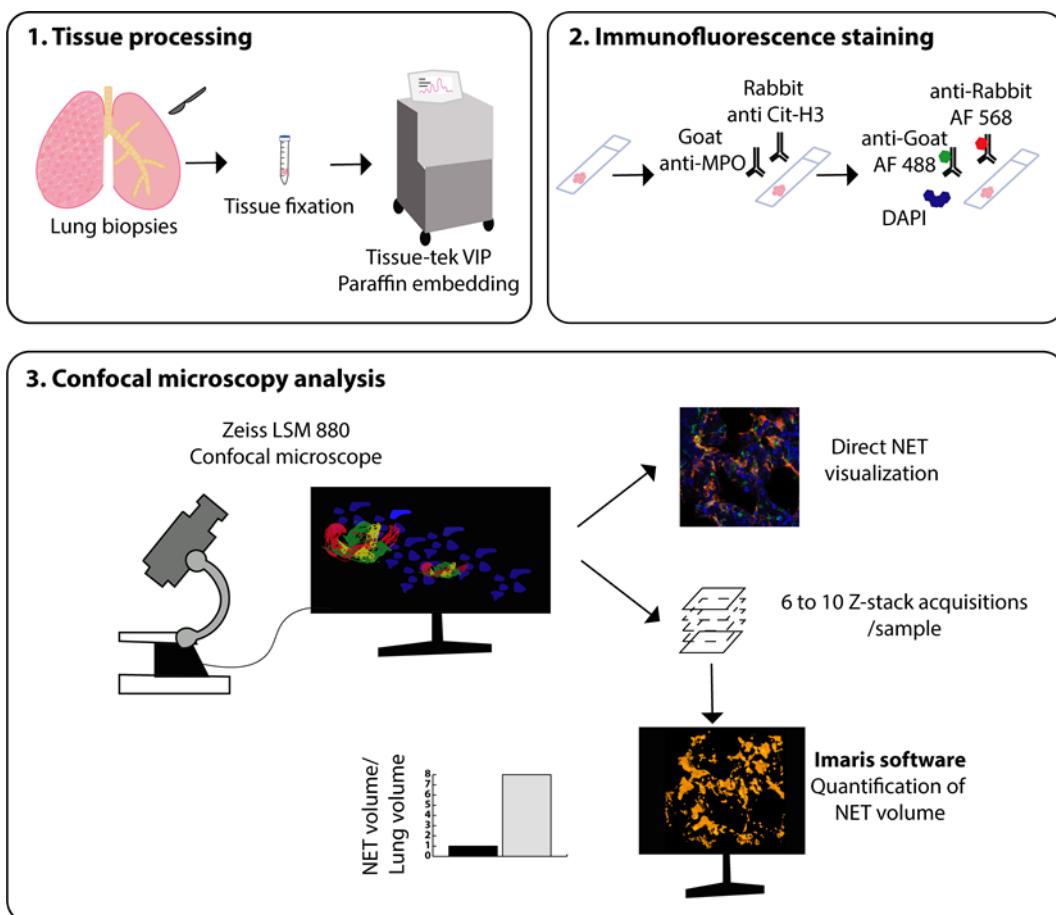
Abstract

Neutrophils are one of the first innate immune cells recruited to tissues during inflammation. An important function of neutrophils relies on their ability to release extracellular structures, known as Neutrophil Extracellular Traps or NETs, into their environment. Detecting such NETs in humans has often proven challenging for both biological fluids and tissues; however, this can be achieved by quantitating NET components (e.g., DNA or granule/histone proteins) or by directly visualizing them by microscopy, respectively. Direct visualization by confocal microscopy is preferably performed on formalin-fixed paraffin-embedded (FFPE) tissue sections stained with a fluorescent DNA dye and antibodies directed against myeloperoxidase (MPO) and citrullinated histone 3 (Cit-H3), two components of NETs, following paraffin removal, antigen retrieval, and permeabilization. NETs are defined as extracellular structures that stain double-positive for MPO and Cit-H3. Here, we propose a novel software-based objective method for NET volume quantitation in tissue sections based on the measurement of the volume of structures exhibiting co-localization of Cit-H3 and MPO outside the cell. Such a technique not only allows the unambiguous identification of NETs in tissue sections but also their quantitation and relationship with surrounding tissues.

Keywords: Neutrophil, Neutrophil extracellular traps, Lung, Tissue sections, Confocal microscopy, NET quantitation

This protocol was validated in: J Exp Med (2020), DOI: 10.1084/jem.20201012

Graphical Abstract:



Graphical representation of the methodology used to stain and quantitate NETs in human lung tissue.

Background

Neutrophils are the major innate immune cells in the blood (50-70% of white blood cells in humans) (Mestas and Hughes, 2004). These cells have a diameter of 5-7 μm and are characterized by a segmented nucleus, secretory vesicles in their cytoplasm, and a short lifespan (Borregaard, 2010). Neutrophils are produced in the bone marrow in response to G-CSF secretion under the control of key transcription factors such as PU.1, C/EBP α , GFI-1, and C/EBP ϵ (Hidalgo *et al.*, 2019). Neutrophils participate in pathogen destruction by releasing the cytotoxic contents of their secretory vesicles or by phagocytosis (Borregaard, 2010). Recently, several discoveries have shed light on neutrophil biology and function. First, a study using radiolabeling of neutrophils demonstrated an unexpected longer half-life of these cells, which can reach 5.4 days in humans and 18 hours in mice (Pillay *et al.*, 2010). Second, in 2004, Brinkmann and colleagues discovered a novel bactericidal action of neutrophils. Indeed, it was observed that the release of particular structures could trap bacteria and limit their dissemination throughout the organism (Brinkmann *et al.*, 2004). These new structures were named Neutrophil Extracellular Traps or NETs. NETs are web-like structures composed of extracellular free DNA associated with a particular form of citrullinated histone 3 (Cit-H3), in which arginine residues have been replaced by citrulline, and various antimicrobial peptides from secretory vesicles, such as myeloperoxidase (MPO), neutrophil elastase (NE), or cathelicidins (Papayannopoulos, 2018). The release of these structures can be triggered by various stimuli derived from bacteria, viruses, parasites, and fungi, but also by immune complexes, crystals, pro-inflammatory cytokines, and chemokines (Papayannopoulos, 2018).

Within neutrophils, the activation of pyruvate kinase C, formation of reactive oxygen species (ROS), and the activation of peptidyl arginase 4 and neutrophil elastase allow chromatin decondensation, nuclear membrane disruption, and NET release (Papayannopoulos, 2018). NETs can immobilize pathogens and prevent their dissemination but are also able to kill them directly (Brinkmann *et al.*, 2004; Papayannopoulos, 2018). Unfortunately, excessive NET release or inappropriate NET accumulation can trigger important tissue damage and lesions (Narasaraju *et al.*, 2011) or inadequate activation of immune cells, thereby promoting various immune-mediated disorders (Hakkim *et al.*, 2010; Radermecker *et al.*, 2019) or coagulation issues (Middleton *et al.*, 2020). Thus, NETs are increasingly implicated in various pathological processes, as evidenced by experimental work in mouse models (Radermecker *et al.*, 2019; Villanueva *et al.*, 2011). The investigation into their potential contributions to human disease is therefore of great interest. NET detection in humans is quite challenging and can be performed either indirectly in physiological fluids or directly in tissue sections. To date, NETs have been mainly measured in physiological fluids such as serum and bronchoalveolar lavage fluid (BALF) (Toussaint *et al.*, 2017; Middleton *et al.*, 2020). NET measurements in fluids rely on the detection of one or two of their components (extracellular DNA, citrullinated histone 3, or complexes of DNA/MPO or DNA/NE). Extracellular free DNA can be detected using DNA quantitation assays on serum or BALF supernatant (Toussaint *et al.*, 2017). Methods for the detection of Cit-H3 have been developed, including western blotting (Liu *et al.*, 2016; Thålin *et al.*, 2017). A more specific technique relies on the detection of complexes formed by DNA and one of the antimicrobial peptides released from secretory vesicles, like MPO or NE, by ELISA (Caudrillier *et al.*, 2012; Caldarone *et al.*, 2019). These techniques are rapid but lack specificity and do not provide any information about the location of NETs in the tissue. Here, we describe a protocol allowing the direct visualization and quantitation of NETs *in situ* by confocal microscopy. NETs are identified as extracellular structures exhibiting a co-localization of Cit-H3 and MPO, two important components of NETs. This technique is currently considered the gold standard of NET detection. Furthermore, we propose a novel software-based objective method to quantitate NETs in tissues based on the measurement of the volume of structures exhibiting a co-localization of Cit-H3 and MPO outside the cell (Radermecker *et al.*, 2019). This technique is specific, quantitative, and provides information about the location of NETs in tissues (Radermecker *et al.*, 2020). Identification of specific NET-rich areas in human tissues may promote a better understanding of their pathological roles in disease.

Materials and Reagents

1. Coverslips, 22 × 22 mm (Fisher Scientific, ThermoFisher Scientific, catalog number: 15727582)
2. Microscope slides, Superfrost (VWR, catalog number: 631-0113)
3. Pipette tips
4. Bovine serum albumin (Sigma-Aldrich, catalog number: 9048-46-8), stored at 4–8°C
5. Donkey serum (Sigma-Aldrich, catalog number: D9663), stored at -20°C
6. Goat anti-human/mouse myeloperoxidase antibody (Novus Biologicals, catalog number: AF3667), stored at -20°C
7. Rabbit anti-histone H3 (citrulline R2+R8+R17) antibody (Abcam, catalog number: ab5103), stored at -20°C
8. Donkey anti-goat IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488-conjugated (ThermoFisher Scientific, catalog number: A32814), stored at 4–8°C
9. Donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568-conjugated (ThermoFisher Scientific, catalog number: A10042), stored at 4–8°C
10. Neo Clear (Sigma-Aldrich, catalog number: 64741-65-7)
11. Absolute ethanol (Fisher Chemical, catalog number: E/0600DF/15)
12. Xylene (VWR, catalog number: 28973.363)
13. Ultrapure DNase/RNase-free distilled water (ThermoFisher Scientific, Invitrogen, catalog number: 10977049)
14. DAPI (Sigma-Aldrich, catalog number: 28718-90-3), stored at -20°C
15. ProLong Gold antifade mountant (ThermoFisher Scientific, catalog number: P10144), stored at -20°C
16. Goat IgG isotype control (ThermoFisher Scientific, catalog number: 31245), stored at 4–8°C
17. Rabbit IgG isotype control (ThermoFisher Scientific, catalog number: 31235), stored at 4–8°C
18. Formaldehyde (Sigma-Aldrich, catalog number: 47608)

19. HIER Tris-EDTA Buffer, pH 9.0 (10×) (Zytomed Systems, catalog number: ZUC029-500), stored at 4-8°C
20. Tween-20 (Fisher Scientific, Acros Organics, catalog number: 10491081), stored at room temperature (RT)
21. Triton X-100 (Sigma-Aldrich, catalog number: 9002-93-1), stored at room temperature (RT)
22. Antigen Retrieval Buffer (see Recipes)
23. Permeabilization Buffer (see Recipes)
24. Blocking Buffer (see Recipes)

Equipment

1. Tissue-Tek-VIP 5 (Sakura)
2. Rotary microtome (Leica)
3. Zeiss LSM 880 Airyscan Elyra s.1. confocal microscope (Zeiss)
4. StainTray slide staining system (Sigma-Aldrich, catalog number: Z670146)
5. Kartell PMPHellendhal staining jar (ThermoFisher Scientific, Fisher Scientific, catalog number: 10375681)
6. Portable steam sterilizer (Prestige Medical, 2100 classic)
7. Pipettes
8. Refrigerator (4°C)
9. Freezer (-20°C)
10. Chemical fume hood

Software

1. ImageJ (version 15.1n, NIH, <https://imagej.nih.gov/ij/download.html>)
2. Imaris (version 7.5.2, oxford instruments)
3. Zen black (Zeiss)

Procedure

A. Sample collection, fixation, and paraffin embedding

1. Collect lung post-mortem biopsies and fix them for three hours at RT in 4% formaldehyde.
2. After fixation, embed the biopsies in paraffin, following these three steps:
 - a. Dehydration: The fixation agent is eliminated by successive baths of increasing ethanol concentration: three times for 30 min in 70% ethanol, followed by three times for 30 min in 85% ethanol, and three times for 30 min in 100% ethanol.
 - b. Clearing: Immerse the tissues in a xylene bath three times for 20 min.
 - c. Embed in liquid paraffin at 59°C.

Note: These steps can be performed by an automated instrument like the “Tissue-Tek-VIP.”

3. Cut 4-μm-thick tissue sections using a rotary microtome and place on Superfrost slides

B. Removal of paraffin

1. Rinse the slides in Neo Clear twice for 10 min.
2. Place the slides in a 100% ethanol bath for 5 min.
3. Place the slides in a 96% ethanol bath for 5 min.

4. Place the slides in an 80% ethanol bath for 5 min.
5. Place the slides in a 70% ethanol bath for 5 min.
6. Place the slides in a 50% ethanol bath for 5 min.
7. Rinse in ultrapure distilled water three times for one minute.
8. Rinse in 1× PBS.

C. Antigen retrieval

1. Place the slides in a vertical, plastic staining rack and add antigen retrieval buffer to totally immerse the paraffin-embedded samples (around 50 mL buffer for an 8-slide rack).
2. Fill the sterilizer with an adequate amount of distilled water.
3. Place the rack in the sterilizer and close it.
4. Allow the sterilizer to heat to 120–135°C, and incubate for 10–15 min.
5. After 10–15 min, open the lid and allow the slides to cool to RT on the bench for approximately 20 min.
6. Rinse twice with 1× PBS.

D. Permeabilization

1. Immerse the slides in permeabilization buffer for 2 min.
2. Rinse twice with 1× PBS.

E. Blocking

1. Place the slides in the staining rack and incubate with blocking buffer for one hour at RT.
2. Keep the excess blocking buffer at 4°C.
3. During the blocking step, prepare the StainTray slide staining system. Place moistened paper in the tray.
4. Rinse the slides once with 1× PBS.

F. Primary staining

1. Prepare the primary staining mix. Use 80 µl staining mix for every 1 cm² of sample. Adapt the quantity of staining mix according to the surface area of the sample. Add goat anti-MPO at a 1/40 dilution and rabbit anti-histone 3 at a 1/100 dilution in blocking buffer.
2. Place the slides in the staining system tray.
3. Place the staining mix on the tissue sections to completely immerse them.
4. Close the staining system and incubate at RT for one hour.
5. Transfer the slides to a plastic rack.
6. Rinse three times with 1× PBS.

G. Secondary staining

1. Prepare the secondary staining mix. Add anti-goat Alexa Fluor 488 at a 1/200 dilution, anti-rabbit Alexa Fluor 568 at a 1/200 dilution, and DAPI at a 1/1,000 dilution in blocking buffer.
2. Place the slides in the staining system tray.
3. Place the staining mix on the tissue sections to completely immerse them.
4. Close the staining system and incubate for two hours at RT.
5. Transfer the slides to a plastic rack.
6. Rinse three times with 1× PBS.
7. Remove the moistened paper from the staining system tray.
8. Dry the slides.

Important: Never touch the tissue sections.

9. From this point onward, work in a chemical fume hood to allow for optimal drying. Place a drop of ProLong Gold onto the tissue sections and add a coverslip. Allow the slides to dry overnight, protected from the light, but without totally closing the staining system to facilitate air circulation. In some experiments using other mounting media, we observed a rapid loss of the Cit-H3 staining. Since ProLong Gold is described to protect fluorescent dyes from fading and photobleaching, we chose this mounting medium.
10. Analyze the slides and acquire images using a confocal microscope within two days of staining.

H. Controls

You can perform two types of negative control. You can perform primary staining with isotype control antibodies, *i.e.*, goat IgG and rabbit IgG isotype control antibodies instead of anti-MPO and anti-citrullinated Histone 3, at similar concentrations. Alternatively, you can replace the primary staining with an incubation with sera from the host species in which the primary antibodies were produced (*i.e.*, rabbit and goat serum). In both cases, the secondary staining remains the same.

Data analysis

A. Image acquisition

Images are acquired using a confocal microscope (here, a Zeiss LSM 880). Select the appropriate lasers; in this case, 488 nm (MPO), 561 nm (Cit-H3), and 405 nm (DAPI). Adjust the laser voltages to identify stained structures. To ensure the specificity of your staining, compare positive samples with control samples. Once the lasers have been set up, be sure to always use the same settings for all the slides that you want to analyze and compare. NETs are identified as extracellular structures that stain positive for MPO and Cit-H3. DAPI staining is not always observed in NETs, as NETs are composed of decondensed chromatin (Figure 1). To further quantitate the NETs, we performed Z-stack acquisition of 6-10 random fields on each section at 20 \times magnification.

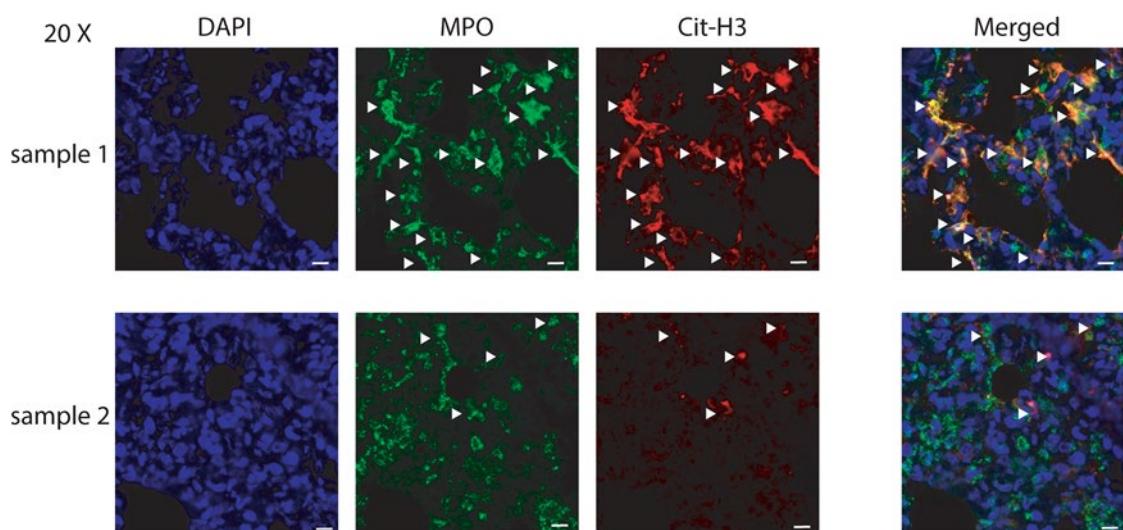


Figure 1. Representative images of lung NETs.

NETs are identified as extracellular structures where MPO (AF-488, green) and Cit-H3 (AF568, red) co-

localize (shown by white arrows). Co-localization with DAPI could not always be observed. Images were acquired at 20 \times magnification. Scale bars, 10 μm .

B. Quantitation of NET volume

To quantitate NETs in lung tissue sections (Figure 2), we analyzed 6-10 (20 \times magnification) per section, and one section per patient (Radermecker *et al.*, 2020). We acquired Z-stack images using the Imaris software. We performed a three-dimensional reconstruction of structures that stained double-positive for Cit-H3 (red) and MPO (green) (Figure 2), and Imaris provided quantitation of the volume of these structures. The co-localization method uses three Imaris script/macro tools (Table 1). The first script processes all the .ims files in a folder, then thresholds the red Cit-H3 and green MPO staining. During this step, we used a threshold to separate voxels from background, and voxels from staining, in each separate channel. We defined true staining as all the staining above 10 voxels. Then, the thresholding method replaced each pixel in an image with a black pixel, if the image intensity was less than the threshold previously fixed (10 voxels), or a white pixel, if the image intensity was greater than that threshold. Subsequently, we created a new channel, *i.e.*, the intersection between the red and green thresholds, where all the voxels have a co-localization equal to 1. The second script then measured the volume of the intersection between the red and green thresholds. Finally, the third script measured the total volume of the image and created an Excel .xlsx file containing all the measurements. Finally, we divided the volume obtained (volume of the intersection of red and green channels) by the total volume of lung tissue analyzed in each sample to yield the cubic micrometers of NETs per cubic micrometer of lung tissue. All the procedures for quantitation are represented in Figure 3.

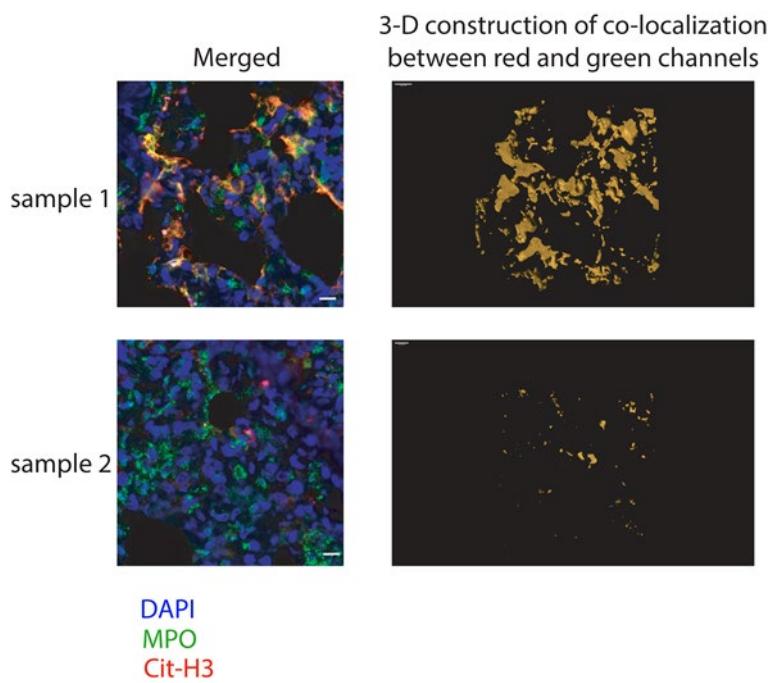


Figure 2. Example of a 3D construction of volumes, where co-localization of MPO and Cit-H3 is present. Confocal microscopy staining (left) and three-dimensional modeling (right) of Cit-H3+MPO+ NETs. Scale bars, 10 μm .

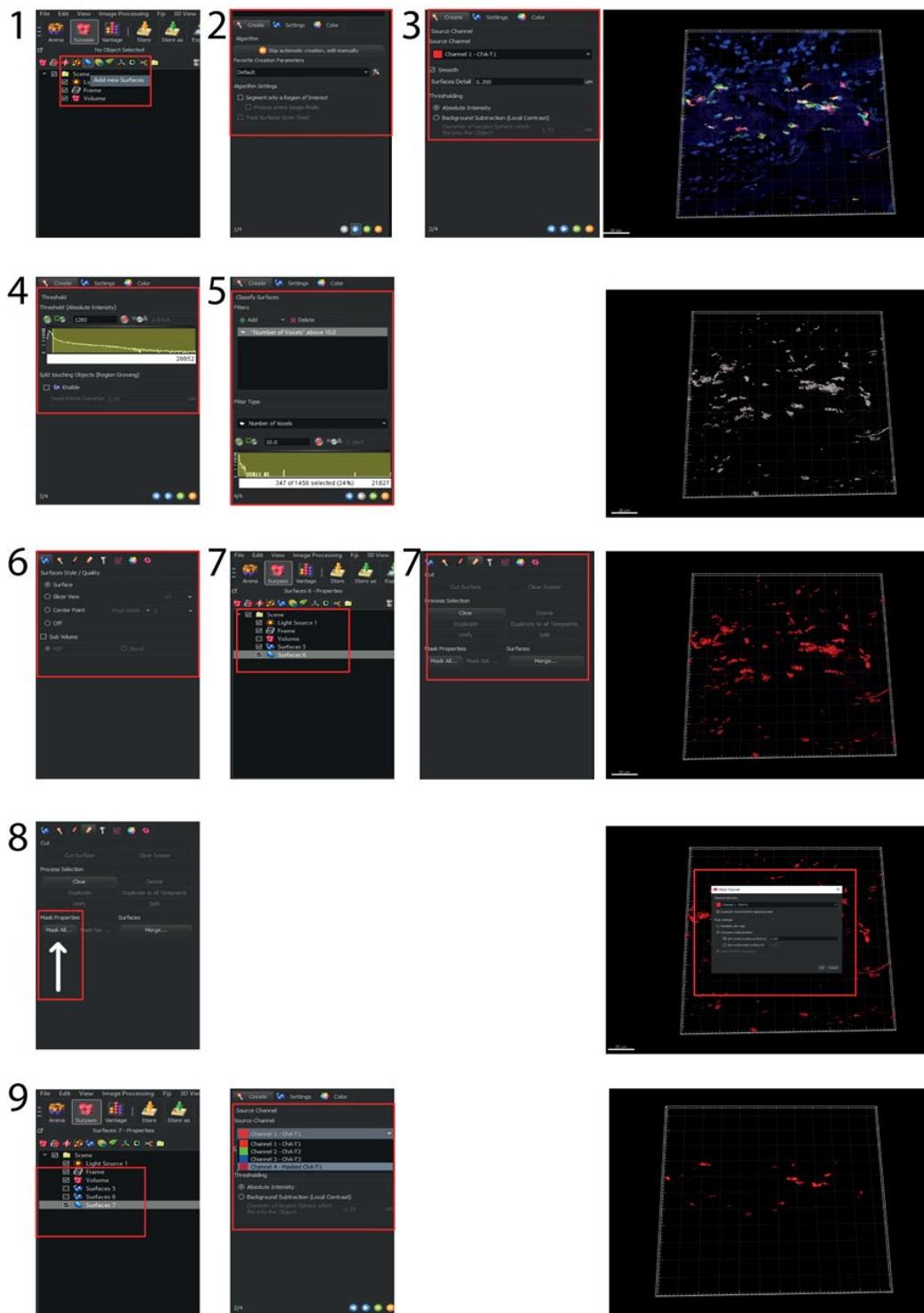


Figure 3. Procedure for the 3D reconstruction of volumes, where co-localization of MPO and Cit-H3 is present on Imaris.

- 1) Add a new red surface; 2) Initiate; 3) Choose the red channel, smooth 0.2, absolute intensity; 4) Set the threshold to 1280; 5) Classify the surface above 10; 6) Validate the surface; 7) Repeat steps 2-6 with the green channel; 8) Select channel green > cut > Mask all > Channel 1 and duplicate the channel before applying mask > constant outside to 0; 9) Repeat steps 2-6 with Mask of red channel.

Table 1. Scripts used for the quantitation of NET volume

Green algorithm	Red algorithm
Enable Region Of Interest = false	Enable Region Of Interest = false
Enable Region Growing = false	Enable Region Growing = false
Enable Tracking = false	Enable Tracking = false
[Source Channel]	[Source Channel]
Source Channel Index = 4	Source Channel Index = 1
Enable Smooth = true	Enable Smooth = true
Surface Grain Size = 0.200 μm	Surface Grain Size = 0.200 μm
Enable Eliminate Background = false	Enable Eliminate Background = false
Diameter Of Largest Sphere = 1.00 μm	Diameter Of Largest Sphere = 1.00 μm
[Threshold]	[Threshold]
Enable Automatic Threshold = false	Enable Automatic Threshold = false
Manual Threshold Value = 1200	Manual Threshold Value = 1030
Active Threshold = true	Active Threshold = true
Enable Automatic Threshold B = true	Enable Automatic Threshold B = true
Manual Threshold Value B = 10964.9	Manual Threshold Value B = 6835.53
Active Threshold B = false	Active Threshold B = false
[Classify Surfaces]	[Classify Surfaces]
"Number of Voxels" above 10.0	"Number of Voxels" above 10.0

C. Statistical analysis

We represented the data as the mean for each section and analyzed it for statistical significance using a non-parametric Mann-Whitney U test on the mean values. P values < 0.05 were considered statistically significant.

Notes

- While the current protocol has been mainly employed in human lung formalin-fixed paraffin-embedded (FFPE) sections, it has also been applied to human liver, pancreas, kidney, and heart FFPE sections (Radermecker *et al.*, 2020). While we did not detect NET structures in those organs of severe Covid-19 patients, neither did we detect non-specific staining, suggesting that the protocol could also be applied to these organs.
- Of note, MPO can also be expressed by other phagocytes, such as human macrophages or eosinophils, which may also form extracellular traps and stain for Cit-H3; therefore, the present protocol would be more specific for neutrophils and NETs if an additional stain directed against a neutrophil marker, such as Ly-6G, was used. From our experience, anti-Ly-6G antibodies do not work properly on FFPE sections, and some clones also detect Ly-6C, which does not help to solve the specificity issue. In laboratory mice, selective depletion of neutrophils, such as an anti-Ly-6G-mediated depletion, can be used to confirm the specificity of the method, as performed previously (Radermecker *et al.*, 2019). Indeed, NETs were shown to be present in lungs from neutrophil-sufficient mice exposed intranasally to a low dose of lipopolysaccharide, but NETs were absent in neutrophil-depleted mice (Radermecker *et al.*, 2019).

Recipes

1. Antigen Retrieval Buffer

Make a 1/10 dilution of HIER Tris-EDTA Buffer pH 9.0 (10 \times) in distilled water. Add Tween-20 at a final concentration of 0.05%.

2. Permeabilization Buffer

Add Triton X-100 to PBS at a final concentration of 0.5%.

3. Blocking Buffer

Add BSA at a final concentration of 2% and donkey serum at a final concentration of 2% in PBS.

Acknowledgments

The immunofluorescence protocol was adapted from an article by Toussaint and colleagues (Toussaint *et al.*, 2017). The quantitation method is described in Radermecker *et al.* (2019 and 2020).

Competing interests

The authors declare no conflict of interest.

Ethics

The use of human specimens was approved in 2020 by the Ethics Review Board of the University Hospital of Liege (ref 2020/119).

References

- Borregaard, N. (2010). [Neutrophils, from marrow to microbes](#). *Immunity* 33(5): 657-670.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. and Zychlinsky, A. (2004). [Neutrophil extracellular traps kill bacteria](#). *Science* 303(5663): 1532-1535.
- Caldarone, L., Mariscal, A., Sage, A., Khan, M., Juvet, S., Martinu, T., Zamel, R., Cypel, M., Liu, M., Palaniyar, N. and Keshavjee, S. (2019). [Neutrophil extracellular traps in ex vivo lung perfusion perfusate predict the clinical outcome of lung transplant recipients](#). *Eur Respir J* 53(4).
- Caudrillier, A., Kessenbrock, K., Gilliss, B. M., Nguyen, J. X., Marques, M. B., Monestier, M., Toy, P., Werb, Z. and Looney, M. R. (2012). [Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury](#). *J Clin Invest* 122(7): 2661-2671.
- Hakkim, A., Furnrohr, B. G., Amann, K., Laube, B., Abed, U. A., Brinkmann, V., Herrmann, M., Voll, R. E. and Zychlinsky, A. (2010). [Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis](#). *Proc Natl Acad Sci U S A* 107(21): 9813-9818.
- Hidalgo, A., Chilvers, E. R., Summers, C. and Koenderman, L. (2019). [The Neutrophil Life Cycle](#). *Trends Immunol* 40(7): 584-597.
- Liu, S., Su, X., Pan, P., Zhang, L., Hu, Y., Tan, H., Wu, D., Liu, B., Li, H., Li, H., Li, Y., Dai, M., Li, Y., Hu, C. and Tsung, A. (2016). [Neutrophil extracellular traps are indirectly triggered by lipopolysaccharide and contribute to acute lung injury](#). *Sci Rep* 6: 37252.
- Mestas, J. and Hughes, C. C. W. (2004). [Of Mice and Not Men: Differences between Mouse and Human Immunology](#). *J Immunol* 172(5): 2731-2738.
- Middleton, E. A., He, X. Y., Denorme, F., Campbell, R. A., Ng, D., Salvatore, S. P., Mostyka, M., Baxter-Stoltzfus, A., Borczuk, A. C., Loda, M., Cody, M. J., Manne, B. K., Portier, I., Harris, E. S., Petrey, A. C., Beswick, E. J., Caulin, A. F., Iovino, A., Abegglen, L. M., Weyrich, A. S., Rondina, M. T., Egeblad, M., Schiffman, J. D. and Yost, C. C. (2020). [Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome](#). *Blood* 136(10): 1169-1179.

- Narasaraju, T., Yang, E., Samy, R. P., Ng, H. H., Poh, W. P., Liew, A. A., Phoon, M. C., van Rooijen, N. and Chow, V. T. (2011). [Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis](#). *Am J Pathol* 179(1): 199-210.
- Papayannopoulos, V. (2018). [Neutrophil extracellular traps in immunity and disease](#). *Nat Rev Immunol* 18(2): 134-147.
- Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Tesselaar, K. and Koenderman, L. (2010). [In vivo labeling with \$^{2}\text{H}_2\text{O}\$ reveals a human neutrophil lifespan of 5.4 days](#). *Blood* 116(4): 625-627.
- Radermecker, C., Detrembleur, N., Guiot, J., Cavalier, E., Henket, M., d'Emal, C., Vanwinge, C., Cataldo, D., Oury, C., Delvenne, P. and Marichal, T. (2020). [Neutrophil extracellular traps infiltrate the lung airway, interstitial, and vascular compartments in severe COVID-19](#). *J Exp Med* 217(12).
- Radermecker, C., Sabatel, C., Vanwinge, C., Ruscitti, C., Maréchal, P., Perin, F., Schyns, J., Rocks, N., Toussaint, M., Cataldo, D., Johnston, S. L., Bureau, F. and Marichal, T. (2019). [Locally instructed CXCR4hi neutrophils trigger environment-driven allergic asthma through the release of neutrophil extracellular traps](#). *Nat Immunol* 20(11):1444-1455.
- Thålin, C., Daleskog, M., Goransson, S. P., Schatzberg, D., Lasselin, J., Laska, A. C., Kallner, A., Helleday, T., Wallen, H. and Demers, M. (2017). [Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma](#). *Immunol Res* 65(3): 706-712.
- Toussaint, M., Jackson, D. J., Swieboda, D., Guedan, A., Tsourouktsoglou, T. D., Ching, Y. M., Radermecker, C., Makrinioti, H., Aniscenko, J., Bartlett, N. W., Edwards, M. R., Solari, R., Farnir, F., Papayannopoulos, V., Bureau, F., Marichal, T. and Johnston, S. L. (2017). [Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation](#). *Nat Med* 23(6): 681-691.
- Villanueva, E., Yalavarthi, S., Berthier, C. C., Hodgin, J. B., Khandpur, R., Lin, A. M., Rubin, C. J., Zhao, W., Olsen, S. H., Klinker, M., Shealy, D., Denny, M. F., Plumas, J., Chaperot, L., Kretzler, M., Bruce, A. T. and Kaplan, M. J. (2011). [Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus](#). *J Immunol* 187(1): 538-552.

Intact *in situ* Preparation of the *Drosophila melanogaster* Lymph Gland for a Comprehensive Analysis of Larval Hematopoiesis

Diana Rodrigues^{1, 2, 3}, K. VijayRaghavan^{2, 3}, Lucas Waltzer⁴ and Maneesha S. Inamdar^{1, *}

¹Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

²National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

³Shanmuga Arts, Science, Technology and Research Academy, Tamil Nadu, India

⁴University of Clermont Auvergne, CNRS, Inserm, GReD, Clermont-Ferrand, France

*For correspondence: inamdar@jncasr.ac.in

Abstract

Blood cells have a limited lifespan and are replenished by a small number of hematopoietic stem and progenitor cells (HSPCs). Adult vertebrate hematopoiesis occurs in the bone marrow, liver, and spleen, rendering a comprehensive analysis of the entire HSPC pool nearly impossible. The *Drosophila* blood system is well studied and has developmental, molecular, and functional parallels with that of vertebrates. Unlike vertebrates, post-embryonic hematopoiesis in *Drosophila* is essentially restricted to the larval lymph gland (LG), a multi-lobed organ that flanks the dorsal vessel. Because the anterior-most or primary lobes of the LG are easy to dissect out, their cellular and molecular characteristics have been studied in considerable detail. The 2-3 pairs of posterior lobes are more delicate and fragile and have largely been ignored. However, posterior lobes harbor a significant blood progenitor pool, and several hematopoietic mutants show differences in phenotype between the anterior and posterior lobes. Hence, a comprehensive analysis of the LG is important for a thorough understanding of *Drosophila* hematopoiesis. Most studies focus on isolating the primary lobes by methods that generally dislodge and damage other lobes. To obtain preparations of the whole LG, including intact posterior lobes, here we provide a detailed protocol for larval fillet dissection. This allows accessing and analyzing complete LG lobes, along with dorsal vessel and pericardial cells. We demonstrate that tissue architecture and integrity is maintained and provide methods for quantitative analysis. This protocol can be used to quickly and effectively isolate complete LGs from first instar larval to pupal stages and can be implemented with ease.

Keywords: *Drosophila* hematopoiesis, Complete larval lymph gland, Blood progenitor, Dissection, Posterior lobes, Secondary lobes, Tertiary lobes

This protocol was validated in: eLife (2021), DOI: 10.7554/eLife.61409

Cite as: Rodrigues, D. et al. (2021). Intact *in situ* Preparation of the *Drosophila melanogaster* Lymph Gland for a Comprehensive Analysis of Larval Hematopoiesis. Bio-protocol 11(21): e4204. DOI: 10.21769/BioProtoc.4204.

161

Background

Vertebrate hematopoietic stem and progenitor cells (HSPCs) give rise to various kinds of mature blood cell types. HSPCs can be identified by surface marker expression, staining properties of vital dyes, proliferative ability, and in vivo differentiation potential (Granick *et al.*, 2012; Ema *et al.*, 2014; Crisan and Dzierzak, 2016). Murine and zebrafish in vivo models have proved extremely useful in understanding various aspects of vertebrate HSPC biology. Adult mouse HSPCs primarily reside in the bone marrow, although recent studies show that HSPCs can circulate in the peripheral blood (Wright *et al.*, 2001; Granick *et al.*, 2012). Bone marrow is primarily located in the flat bones of the pelvis, vertebrae, ribs, and cranium, and in the long bones of the tibia, femur, and humerus. However, for post-embryonic analysis, HSPCs are obtained mainly from the long bones of the tibia and femur that represent a subset of the entire progenitor population. Distribution of HSPCs across various anatomical sites makes it difficult to study the entire progenitor pool, especially in post-embryonic stages and in larger animals such as mice and humans. The *Drosophila* hematopoietic system has proved helpful in addressing various aspects of hematopoiesis, owing to conserved signaling mechanisms and transcriptional factors that regulate hematopoiesis (Banerjee *et al.*, 2019).

Drosophila hematopoiesis occurs in two successive waves. First, blood cell progenitors emerge from the procephalic/head mesoderm in the early embryo and give rise to larval circulating and sessile hemocytes, which persist until adulthood (Tepass *et al.*, 1994; Holz *et al.*, 2003; Honti *et al.*, 2010; Ghosh *et al.*, 2015; Sanchez Bosch *et al.*, 2019). The second wave of hematopoiesis takes place in a specialized larval hematopoietic organ called the lymph gland (LG), located dorsally, flanking the anterior cardiac tube/dorsal vessel (Rugendorff *et al.*, 1994; Lanot *et al.*, 2001; Mandal *et al.*, 2004; Grigorian *et al.*, 2013). Blood cell progenitors that form the LG are derived from the embryonic dorsal mesoderm. Clonal analysis suggests the presence of hemangioblast precursor cells that can give rise to LG blood cells and cells of the dorsal vessel (Mandal *et al.*, 2004). By stage 11, Odd-skipped (Odd) is expressed in the thoracic and the abdominal segments, T1-A6 (Ward and Skeath, 2000); the thoracic clusters form the LG, and the abdominal clusters give rise to the pericardial cells (Mandal *et al.*, 2004). At stages 11-12, expression of the homeotic gene Antennapedia is restricted to segment T3 (Mandal *et al.*, 2007). By stages 13-16, Odd+ cells in the thoracic segment (T1-T3) coalesce to form the LG, whereas Antennapedia is expressed in 5-6 cells at the posterior boundary of the LG primordium (Mandal *et al.*, 2007). Two Collier expressing clusters appear in the thoracic segments T2 and T3, which coalesce following germ-band retraction (Crozatier *et al.*, 2004). Collier expression is maintained at high levels at the posterior tip of the developing LG in 3-5 cells, whereas the remaining LG cells express Collier at low levels (Crozatier *et al.*, 2004). In the late embryo, the LG consists of a single pair of lobes, the primary/anterior lobes, each lobe containing approximately 20 cells that express Serpent and Odd (Jung *et al.*, 2005). At the first instar larval stage, primary lobe cells proximal to the dorsal vessel, termed pre-progenitors, express Serpent, Notch, Dorothy, and STAT92e, and lack expression of domeless (dome) (Jung *et al.*, 2005; Dey *et al.*, 2016; Banerjee *et al.*, 2019). By the second instar, primary lobes have increased in size, consisting of approximately 200 cells in each lobe. Additionally, 2-3 pairs of smaller lobes are formed posterior to the primary lobes and are referred to as the secondary, tertiary, and quaternary lobes (Jung *et al.*, 2005; Banerjee *et al.*, 2019; Rodrigues *et al.*, 2021).

Based on morphology and molecular marker analysis, third instar larval LG primary lobes are compartmentalized into three zones. The posterior signaling center (PSC) acts as the signaling niche. The medullary zone (MZ) towards the cardiac tube consists of multipotent progenitors. A peripheral cortical zone (CZ) mainly harbors phagocytic plasmacytocytes and a few crystal cells. Intermediate zone (IZ) progenitors reside in the region between the MZ and the CZ, are identified by the expression of progenitor and early differentiation markers, and lack the expression of late markers (Jung *et al.*, 2005; Banerjee *et al.*, 2019). The multiple posterior lobes harbor progenitors that resist differentiation upon immune challenge (Rodrigues *et al.*, 2021). Under steady state conditions, blood cells produced in the LG are released in circulation only at the pupal stage, contributing to the pupal and adult blood cell populations (Holz *et al.*, 2003; Grigorian *et al.*, 2011; Ghosh *et al.*, 2015; Sanchez Bosch *et al.*, 2019).

While primary lobes are well characterized, the identity of the posterior lobes was ill-characterized until recently (Rodrigues *et al.*, 2021). Based on the expression of a limited set of markers and mutant analysis, a few studies proposed that the secondary lobes are essentially composed of blood cell progenitors that differentiate at the larval/pupal transition (Jung *et al.*, 2005; Grigorian *et al.*, 2011; Kulkarni *et al.*, 2011; Benmimoun *et al.*, 2015).

Studies on secondary/posterior lobes used preparation of LG samples detached from their brain/ring gland anterior attachment site with thin tungsten needles and placed on glass slides (Lanot *et al.*, 2001). This method of sample preparation causes damage to the delicate organ and might be the reason for the partial analysis of LG lobes. To obtain the entire intact LG, we use the larval fillet method of dissection described in this protocol, which helps maintain primary and posterior lobes intact. This protocol has been invaluable for a comprehensive analysis in our previous studies (Kulkarni *et al.*, 2011; Sinha *et al.*, 2013; Khadilkar *et al.*, 2014; Sinha *et al.*, 2019; Rodrigues *et al.*, 2021). For instance, we could show that depletion of *asrij*, *arfl*, or *garz* and overexpression of *arflGAPI* leads to severe phenotypes of hyperproliferation and premature differentiation in the posterior lobes, as compared to the primary lobes (Kulkarni *et al.*, 2011; Khadilkar *et al.*, 2014). We also employed this method of dissection for whole LG proteomic analysis, which provided a resource to identify novel regulators of hematopoiesis (Sinha *et al.*, 2019). Furthermore, differential RNA sequencing analysis for the primary and the posterior lobes helped to identify novel progenitor markers and regulators of hematopoiesis, and unveiled the molecular heterogeneity, as well as functional compartmentalization of the LG progenitor pool present in the different lobes (Rodrigues *et al.*, 2021). Thus far, our studies suggest that analysis of the whole LG is crucial for exploring the complete application of *Drosophila* LG hematopoiesis. Here, we describe detailed protocols for whole LG sample preparation that can be used for GFP expression screens, immunostaining, RNA *in situ*, and high-throughput analyses.

Materials and Reagents

A. Fly stocks

1. *Canton-S* was used as the wild-type reference strain
2. *dome-Gal4, UAS2xEGFP* (provided by Utpal Banerjee, University of California Los Angeles)
3. *srpHemo-Gal4-UAS-GFP* (National Centre for Biological Sciences (NCBS), Fly Facility)

B. Materials

1. Fine paint brush (No. 2)
2. Glass cavity dish 40 × 40 mm (Atom Scientific, catalog number: SDCE4040-1)
3. Sylgard (Sigma-Aldrich, catalog number: 761036) or equivalent
4. Micro test plate, 96-well (Tarsons, catalog number: 941196) or equivalent
5. 35 mm Petri dish (Tarsons, catalog number: 460035) or equivalent
6. 1.5 ml microcentrifuge tube (Axygen, catalog number: MCT-150-C)

C. Reagents

Tissue dissection and fixation

1. NaCl (Fisher Scientific, catalog number: S25542)
2. Na₂HPO₄ (Merck, catalog number: S9763)
3. NaH₂PO₄·2H₂O (Merck, catalog number: 71505)
4. Paraformaldehyde (Fisher Scientific, catalog number: 23995)
5. 10× Phosphate Buffered Saline (PBS, pH 7) stock (see Recipes)
6. 4% Paraformaldehyde (PFA) (see Recipes)

Immunostaining and mounting

1. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
2. Normal Goat serum (GeNei, catalog number: NS1)
3. Primary antibodies: Mouse anti-P1/NimC1 and mouse anti-Hemese (provided by Istvan Ando, Biological Research Center of the Hungarian Academy of Sciences), rabbit anti-Asrij (Kulkarni *et al.*, 2011)

4. Secondary antibodies: Alexa fluor 568 goat anti-mouse (Invitrogen, catalog number: A11004)
Alexa fluor 488 goat anti-rabbit (Invitrogen, catalog number: A11008)
5. Phalloidin conjugated to Alexa fluor 633 (Invitrogen, catalog number: A22284)
6. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, ThermoFisher Scientific, catalog number: D1306)
7. Glycerol (Merck, catalog number: BP229-1)
8. Neutral red (Merck, catalog number: N4638)
9. 0.1% Triton X-100 in PBS (PTX) (see Recipes)

RNA *in situ* hybridization

1. *In situ* hybridization probe: tep4 (Rodrigues *et al.*, 2021)
2. Methanol (Merck, catalog number: 34860)
3. Tween-20 (Sigma-Aldrich, catalog number: P9416)
4. Sodium citrate (Na3C6H5O7) (HiMedia, catalog number: TC249)
5. Molecular grade water-UltraPure DNase/RNase-free distilled water (Invitrogen, catalog number: 10977015)
6. Formamide (Sigma-Aldrich, catalog number: 11814320001)
7. tRNA (Sigma-Aldrich, catalog number: 10109517001)
8. Heparin (Sigma-Aldrich, catalog number: H3149)
9. Roche blocking agent (Sigma-Aldrich, catalog number: 11096176001)
10. CHAPS (Sigma-Aldrich, catalog number: C9426)
11. EDTA (Fisher Scientific, catalog number: S311-100)
12. NaCl (Fisher Scientific, catalog number: S25542)
13. MgCl₂ (Fisher Scientific, catalog number: BP214-500)
14. Anti-DIG conjugated to alkaline phosphatase (Sigma-Aldrich, catalog number: 11093274910)
15. NBT/BCIP (Promega, catalog number: S3771)
16. FastRed substrate kit (Abcam, catalog number: ab64254)
17. 0.1% Tween-20 in PBS (PBS-T) (see Recipes)
18. 20× Saline-Sodium Citrate buffer (20× SSC, pH 7) (see Recipes)
19. Hybridization buffer (see Recipes)
20. Staining buffer (see Recipes)

Equipment

1. Stereomicroscope (Olympus SZ51, magnification range 0.8-4×)
2. Fine forceps (Fine Science Tools, Dumont #5, catalog number: 11252-20)
3. Spring scissors 2.5 mm cutting edge (Fine Science Tools, catalog number: 15000-08)
4. Insect pins (Fine Science Tools, Minutien, 0.1 mm, stainless steel, catalog number: 26002-10)
5. Confocal microscope (Zeiss, model: LSM 880)

Software

1. ImageJ
2. Adobe Photoshop CS5 (Adobe Systems)

Procedure

A. Larval fillet preparation for obtaining intact lymph gland (LG)

Whole LG preparations can be obtained for the first, second, and third instar larvae, as well as for pupae, using this method of dissection. Fly breeding and crosses were performed using standard protocols. Larvae were reared to the appropriate stage on standard cornmeal agar medium, under non saturating density. Figure 1A shows the relative size of the first, second, and third instar larvae.

1. Using a fine paint brush, transfer the larvae to a cavity dish/Petri plate containing water, then rinse the larvae to get rid of any food particles.
2. Transfer the larvae to a clean cavity dish and place on ice for 20-30 min to immobilize the larvae. Immobilization (optional) helps to pin the larvae (see Step A4).
3. Place cooled larva with the dorsal side up on the Sylgard dish and view it through a stereomicroscope at magnification 4 \times (zoom range 0.8-4 \times), focusing on the dorsal cuticle. All subsequent steps are to be performed while viewing larval tissue under the microscope.
4. Restrain larva by inserting insect pins firmly through it, near the anterior and the posterior spiracles, and through the Sylgard dish (Figure 1B). Add a drop of PBS (approximately 200 μ l) to prevent the larva from desiccating.
5. Using fine dissection scissors, make a small incision in the cuticle on the right side near the posterior end. Insert the scissors into the incision end and slit the cuticle laterally (Figure 1C and 1D).
6. Lift the loose end of the cuticle with the help of fine forceps, extend it to the left side, and cut along the left lateral edge of the cuticle (Figure 1E).
7. Carefully remove the viscera. Locate the LG that is attached to the brain lobes in the anterior region flanking the dorsal vessel, followed by rows of pericardial cells at the posterior end (Figure 1F). Side illumination with dual goose-neck light source can help distinguish the refringent LG.

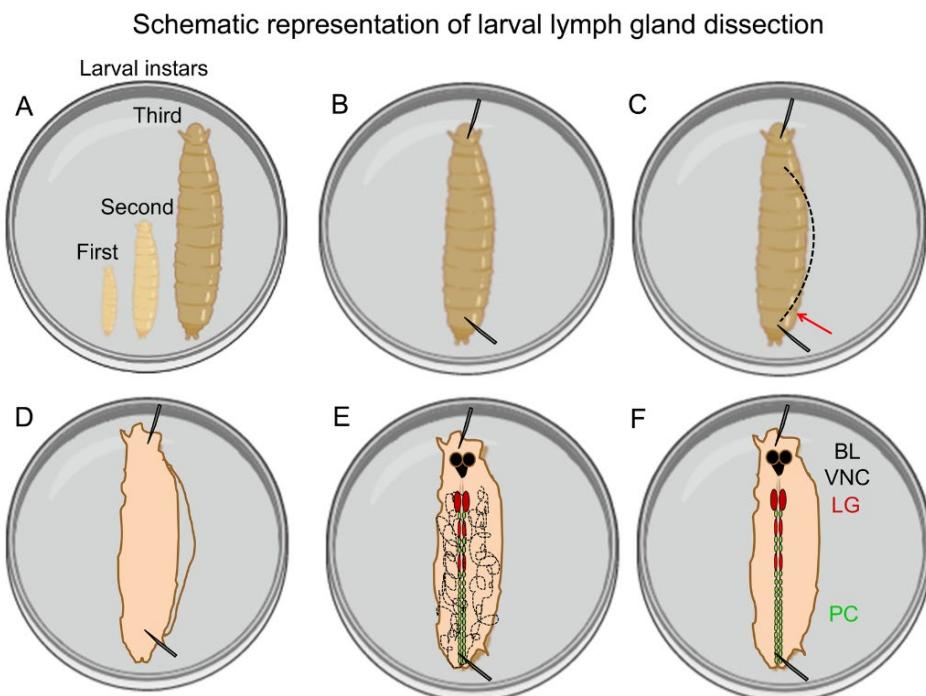


Figure 1. Schematic representation of larval LG dissection.

(A) Representative image of first, second, and third instar larvae. (B-F) Stepwise schematic representation of LG dissection from wandering third instar larvae. BL: brain lobes, VNC: ventral nerve cord, LG: lymph gland,

and PC: pericardial cells. The number of pericardial cells may vary between LG lobes. Larvae and LG images are not depicted to scale but enlarged for clarity.

8. Add a fresh drop of PBS and gently remove it to get rid of any tissue debris. Repeat if required.
9. Replace PBS with 200 μ l of fixative (4% paraformaldehyde, see Recipes) and incubate for 20 min at room temperature. Remove the fixative and wash three times with PBS. Carefully remove the pins, slowly lift the LG preparation (with the underlying ventral cuticle) from the posterior end, and transfer to a 96-well plate or a 1.5 ml microcentrifuge tube for immunostaining or *in situ* procedures.

B. Pupal fillet preparation for obtaining intact lymph gland (LG)

Whole LG can be prepared from 0 to 20 h after pupa formation (APF). In our experience, most lobes histolyze by 15 h APF (Rodrigues *et al.*, 2021).

1. Place pupa on the Sylgard dish with the dorsal side facing up. All subsequent steps are to be performed while viewing the pupal tissue under the stereomicroscope.
2. Insert a fine insect pin firmly near the anterior spiracle through the Sylgard dish (Figure 2A). Add a drop of PBS (approximately 200 μ l) to prevent the pupa from desiccating.
3. Using fine dissection scissors, cut horizontally along the posterior spiracles (Figure 2A).
4. Insert the scissor into the incised end and make a slit laterally along the right side of the cuticle, followed by an incision along the anterior part as shown by the dotted lines (Figure 2B).
5. Lift the loose end of the cuticle and carefully extend it to the left side, and cut along the left lateral edge of the cuticle along the dotted lines (Figure 2B).
6. Carefully remove the visceral organs without damaging the LG (Figure 2C). The cardiac tube and pericardial cells are a good landmark to locate the remaining LG lobes (Figure 2D). Add a fresh drop of PBS to remove any tissue debris and repeat if required.

Schematic representation of pupal lymph gland dissection

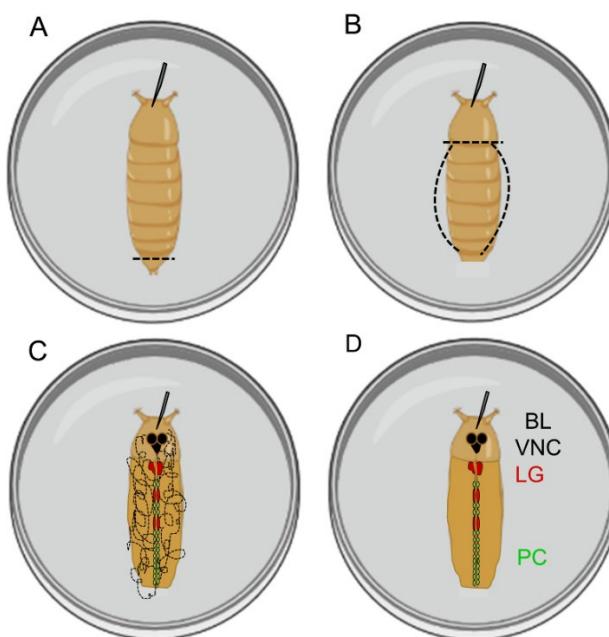


Figure 2. Schematic representation of pupal LG dissection.

(A-D) Stepwise schematic representation of pupal LG dissection. BL: brain lobes, VNC: ventral nerve cord, LG: lymph gland, and PC: pericardial cells. The number of pericardial cells may vary between LG lobes. Pupa and LG images are not depicted to scale but enlarged for clarity.

7. Follow Step A9 onwards of larval LG dissection and proceed for immunostaining or *in situ* procedures.

C. Neutral red staining of lymph gland (LG) for easier visualization

1. Dissect and fix larval or pupal LG as described above. Wash thrice in PBS for 8-10 min each.
2. Add 100 μ L of 0.2% neutral red solution (0.2% neutral red in PBS) and incubate at room temperature for 5-7 min or till LG is visibly stained.
3. Wash off neutral red with PBS. Brain lobes and LG lobes should be easily visible. First, second, and third instar, and pupal LG can be easily identified by neutral red staining (Figure 3A-3D).

Neutral red staining for visualization of lymph gland

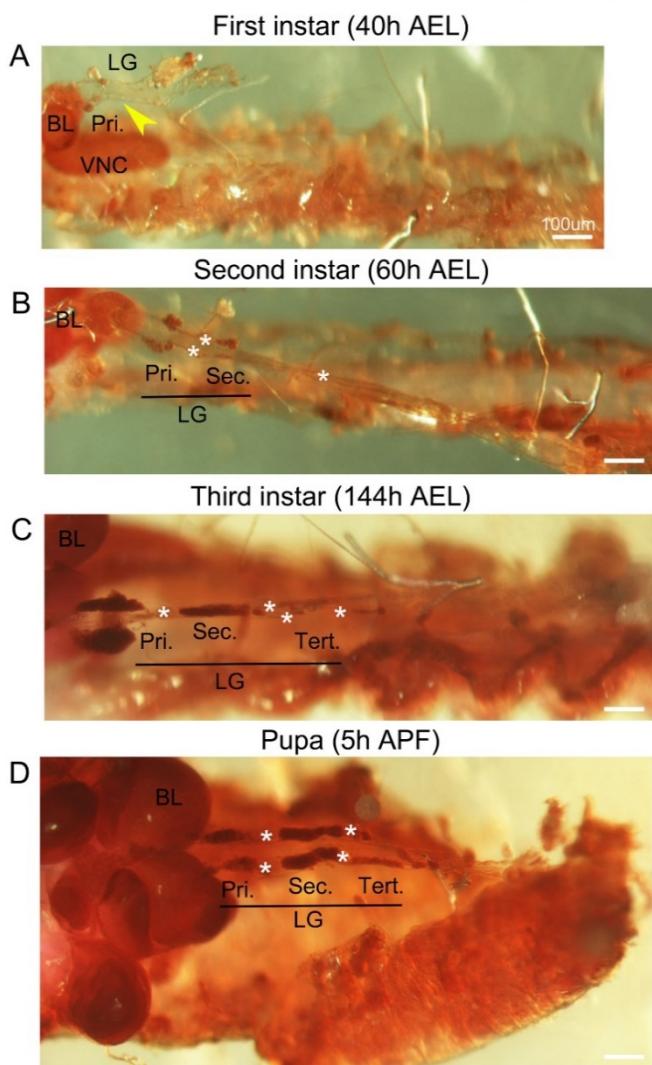


Figure 3. LG preparations stained with neutral red.

(A-D) Hemi-dissected fillet preparation with intact primary and posterior lobes stained with neutral red. LG lobes are intensely stained (red) as shown for first instar (A), second instar (B), third instar (C), larvae and pupa (D). Yellow arrowhead indicates lymph gland. BL: brain lobes, VNC: ventral nerve cord, LG: lymph gland, Pri: primary lobes, Sec: secondary lobes, Tert: tertiary lobes. Asterisks indicate pericardial cells. Scale bar: 100 μ m.

D. Immunostaining of lymph gland (LG)

1. All steps are performed by placing the 96-well dish on a flat-bed rocker.
2. Wash 10-12 fixed LG preparations in a 96-well dish in 200 µL of PBS, three times for 10-15 min each.
3. Add 200 µL of 0.1% Triton X-100 in PBS (0.1% PTX, see Recipes) as a permeabilizing agent to the LG preparations incubate for 15-20 min.
4. Discard PTX and add 100 µL of 20% normal goat serum in PBS as a blocking agent for 30 min.
5. Remove the blocking agent and add 40 µL of primary antibody at the desired concentration diluted in PBS (or blocking agent). Incubate at 4°C overnight.
6. Remove the primary antibody and wash the LG preparations with PBS three times for 10-15 min each.
7. Discard PBS, add 100 µL of 20% normal goat serum, and incubate for 30 min.
8. Replace with 40 µL of secondary antibody (usually 1:400) in PBS and incubate for 2 h at room temperature.
9. Remove secondary antibody and wash in 200 µL of PBS, three times for 10-15 min each. Proceed to the mounting step (see below, procedure section F).

E. *In situ* hybridization of lymph gland (LG)

1. Wash 15-20 fixed LG preparations in a microcentrifuge tube in 500 µL of PBS, three times for 10-15 min each.
2. For long term storage, wash LG preparations in methanol four times and store in methanol at -20°C. To resume the experiment, rinse the samples once with a mixture of methanol and PBS (1:1) and three times in PBS.
3. Wash LG preparations in 500 µL of 0.1% Tween-20 in PBS (PBS-T), three times for 10-15 min each (see Recipes for 0.1% PBS-T).
4. Equilibrate LG preparations in 500 µL of equal volumes of 0.1% PBS-T and hybridization buffer (HB) (see Recipes for hybridization buffer).
5. Discard the PBS-T-HB solution and pre-incubate in 500 µL HB for 1 h at 65°C.
6. Remove HB and replace with 200 µL HB with 1 µL of Digoxigenin (DIG)-labeled RNA probe (Dilution of the probe needs to be adjusted empirically depending on the probe concentration and level of expression of the gene of interest). Hybridize overnight at 65°C.
7. Remove HB carefully and wash the LG preparations in 500-800 µL of HB for 1 h at 65°C.
8. Wash LG preparations in 500-800 µL equal volumes of 0.1% PBS-T and HB for 30 min at 65°C, followed by three quick washes in 0.1% PBS-T at room temperature.
9. Follow up with three washes in 0.1% PBS-T for 10 min each at room temp.
10. Block non-specific binding with 1% bovine serum albumin (BSA) or normal goat serum (NGS) in 0.1% PBS-T for 30 min.
11. Incubate with anti-DIG antibody coupled to Alkaline Phosphatase diluted (1:1,000) in the blocking solution for 2 h at room temp.
12. Remove the anti-DIG blocking solution mixture, followed by three quick rinses in 0.1% PBS-T. Then, wash in 0.1% PBS-T, three times for 10 min.
13. Equilibrate for 10 min in 500 µL freshly prepared staining buffer (SB) (see Recipes).
14. Visualize with NBT/BCIP in 1 ml SB (6.5 µL NBT + 3.5 µL BCIP) at room temperature or 37°C until staining is visible (Figure 4).
15. Wash three times in PBS and mount the LG preparations as indicated (see below, procedure section F).

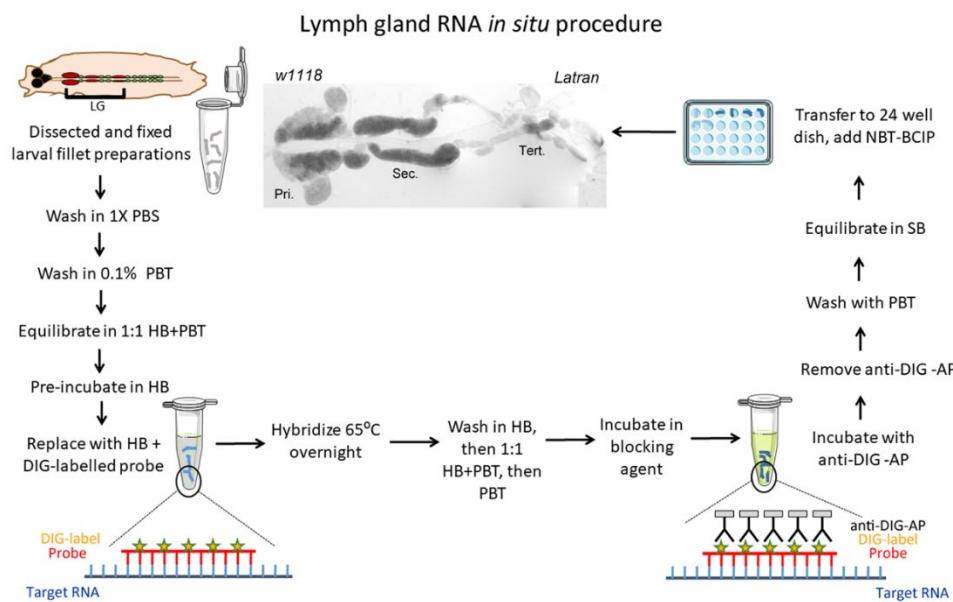


Figure 4. Schematic representation of RNA *in situ* hybridization procedure.

Stepwise representation of lymph gland RNA *in situ* hybridization protocol. Pri: primary lobes, Sec: secondary lobes, Tert: tertiary lobes. Mounted LG preparation shows expression of *Latran* as revealed by NBT/BCIP.

F. Mounting intact lymph gland (LG)

1. Place the fixed and stained larval fillet preparation in a coverslip bottom 35 mm Petri dish. These can also be made by punching a hole of 1 cm diameter in a 35 mm Petri dish and gluing a coverslip to the bottom of the dish (Figure 5). Add a small drop (10-20 μ L) of 70% glycerol with DAPI (1:500) or mounting medium to prevent the samples from desiccating.
2. Using a pair of forceps, hold the filleted cuticle at the anterior end. With another fine forceps, carefully detach the brain lobes from the rest of the cuticle. The LG is attached via the ring gland to the brain lobes and flanks the dorsal vessel (Figure 1F). This procedure will partially dislodge it from the cuticle.
3. Next, using a pair of forceps, hold the cuticle at the posterior end and, with another forceps, carefully detach and slide the posterior part of the dorsal vessel along with the pericardial cells onto the coverslip. The tissue is very fragile, and the whole procedure must be done gently while viewing through a stereomicroscope.
4. Once the LG is loosened from the anterior and the posterior ends, carefully move the LG away from the ventral cuticle. Do not directly lift or hold the LG. When moving the LG, grasp the brain lobes at the anterior end and the pericardial cells at the posterior end.
5. Multiple lymph glands can be mounted in this way in a single dish (Figure 5) and can be subjected to high resolution imaging analysis.

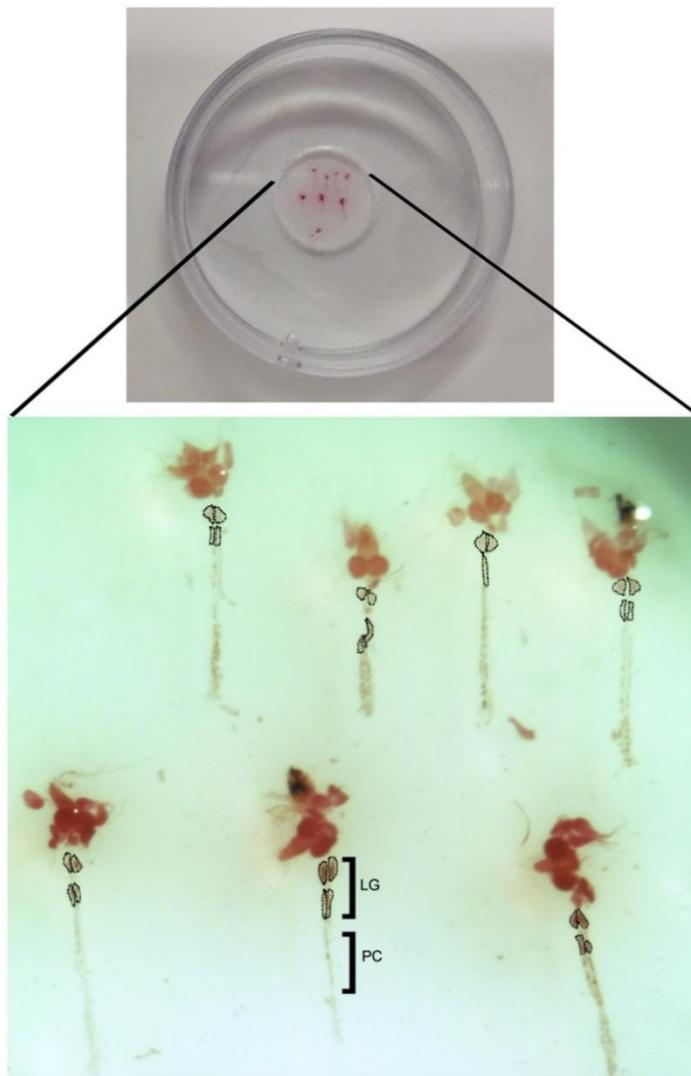


Figure 5. LG preparations mounted in a 35 mm coverslip bottom dish.

Seven third instar larval LG preparations stained with neutral red at a 35 mm coverslip bottom dish. Black dotted lines indicate LG lobes. LG: lymph gland, and PC: pericardial cells.

Data analysis

A. Representative results

The *Drosophila* third instar larval LG is a multi-lobed organ that flanks the dorsal vessel. The first pair of lobes, called primary lobes, are followed by 2-3 pairs of posterior lobes- the secondary, tertiary, and (rarely) quaternary lobes- separated by pericardial cells that function as nephrocytes. For simplicity, we refer to the secondary, tertiary, and quaternary lobes as the posterior lobes. Rows of pericardial cells line the dorsal vessel at the posterior end.

To demonstrate that this method of dissection maintains tissue integrity, we analyzed markers for the whole LG, cardiac tube, and the pericardial cells. Phalloidin marks actin and is useful for visualizing the integrity of LG lobes and the dorsal vessel (Figure 6A). Hemese, a generic blood cell marker (Kurucz *et al.*, 2003), is

expressed in all cells of the primary and most cells of the posterior lobes (Figure 6B). Asrij (Kulkarni *et al.*, 2011), another pan-hemocyte marker, marks LG hemocytes of the primary and the posterior lobes (Figure 6C). srpHemo-Gal4, containing a regulatory region of the serpent gene, is active in the embryonic hemocytes (Bruckner *et al.*, 2004) and strongly expressed in the pericardial cells (Figure 6D). However, very few cells in the primary lobes express srpHemo-Gal4 (Figure 6D). With help from the above-mentioned markers, we confirmed that this method of dissection is useful for isolating intact LG primary and posterior lobes.

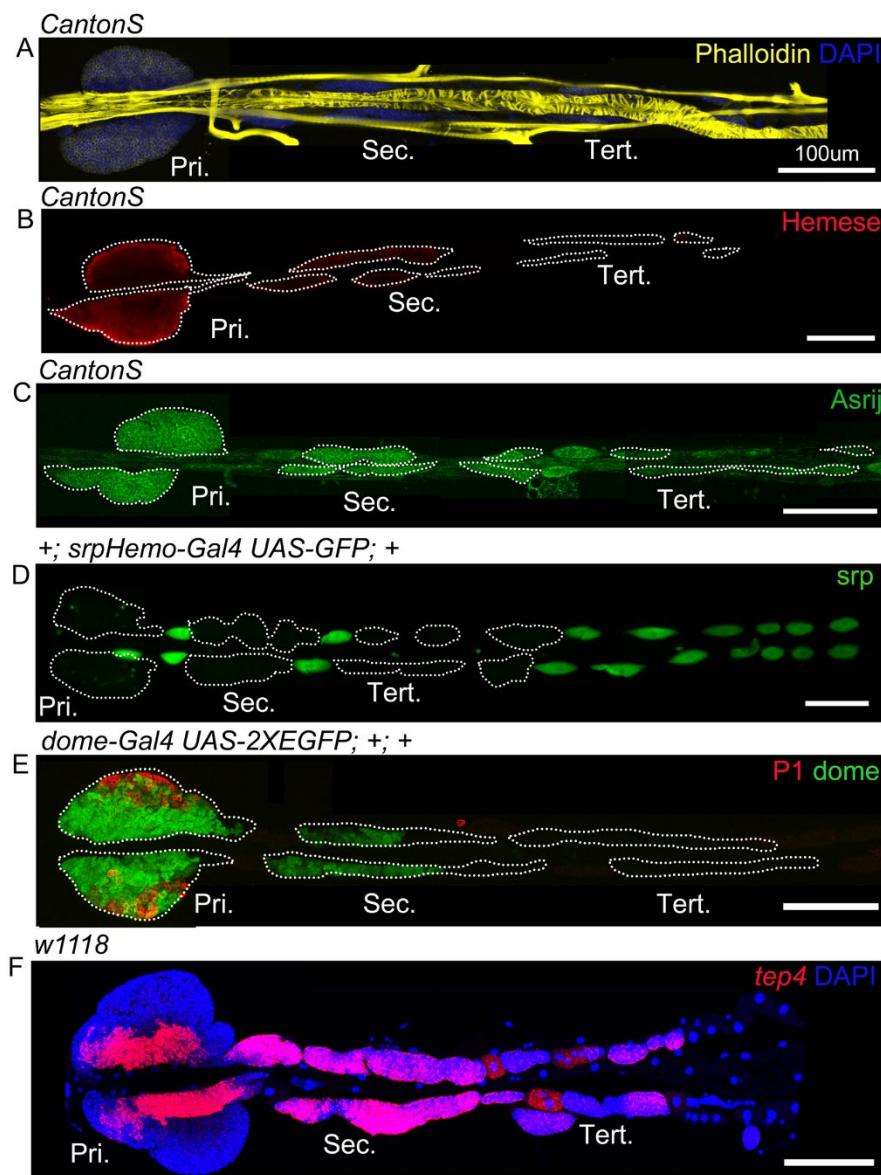


Figure 6. Validation of intact whole LG preparations using pan hemocyte, progenitor, and differentiation markers.

Whole LG preparations from wandering third larval instar include primary and posterior lobes. (A) Phalloidin (yellow) marks actin and is used for identifying LG blood cells and the cardiac tube. Pan hemocyte markers (B), Hemese (red), and (C) Asrij (green) are expressed in the LG lobes and help to identify intact lobes. (D) srpHemo-Gal4 (green) is strongly expressed in the pericardial cells. (E) dome (green) marks progenitors in the primary and the posterior lobes, P1/Nimrod C1 (red) marks plasmacytoid cells. (F) *In situ* hybridization for *tep4* (red) shows high expression in the MZ, secondary lobes, and some cells of the tertiary lobes. Pri: primary lobes,

Sec: secondary lobes, Tert: tertiary lobes. (A-F) Scale bar: 100 μ m.

For in-depth analysis of tissue integrity, we further examined known progenitor (MZ) and differentiation (CZ) markers, as defined in the anterior lobes. We performed reporter, immunostaining, or RNA *in situ* analysis to analyze marker expression. As previously described, the progenitor marker domeless (Jung *et al.*, 2005) is expressed in the MZ, secondary lobes, and in some cells of the tertiary lobes (Figure 6E). Differentiation marker P1/Nimrod C1 (Kurucz *et al.*, 2007) expression is restricted to the CZ of the primary lobes (Figure 6E). RNA *in situ* for another progenitor marker, *tep4* (Rodrigues *et al.*, 2021), reveals high expression in the MZ, the secondary lobes, and in some cells of the tertiary lobes (Figure 6F). Using the fillet method of LG dissection, we obtained intact primary and posterior lobes, as observed with MZ and CZ marker staining. Intact LG lobes are also useful in the analysis of the number of niche cells, progenitors, and differentiated blood cells in each lobe. Therefore, 3D images can be reconstructed using the IMARIS software. Using the 2D Slice module, the diameter is defined for cell nuclei marked by DAPI. The Spots module is employed to estimate the number of DAPI⁺ nuclei. Differentiated cells marked by P1 or progenitors marked by dome are analyzed using the surface module. The surface module is also used for 3D rendering, with the distance between the 3D surface (P1 or dome) and spot (DAPI⁺) defined by the 2D slice module. Next, using the module “Find spots closer to surface” or “Find spots away from surface,” the number of spots (DAPI⁺) positive or negative for a particular marker can be determined.

Using this method of dissection, we have successfully separated primary and posterior lobes and performed RNA sequencing analysis (Figure 7). This has led to the identification of new markers of LG blood cells (Rodrigues *et al.*, 2021). Maintenance of tissue integrity and quality of the sample depends on critical steps in the dissection process. Though the dissections are demanding, identifying the LG and performing quick dissections (2-3 min) has been achieved routinely with 2-3 days of practice, even by inexperienced summer interns. This detailed protocol aimed to establish a standard method for LG dissection and comprehensive analysis that can also be adapted for isolating LG from early larval instars and pupal stages.

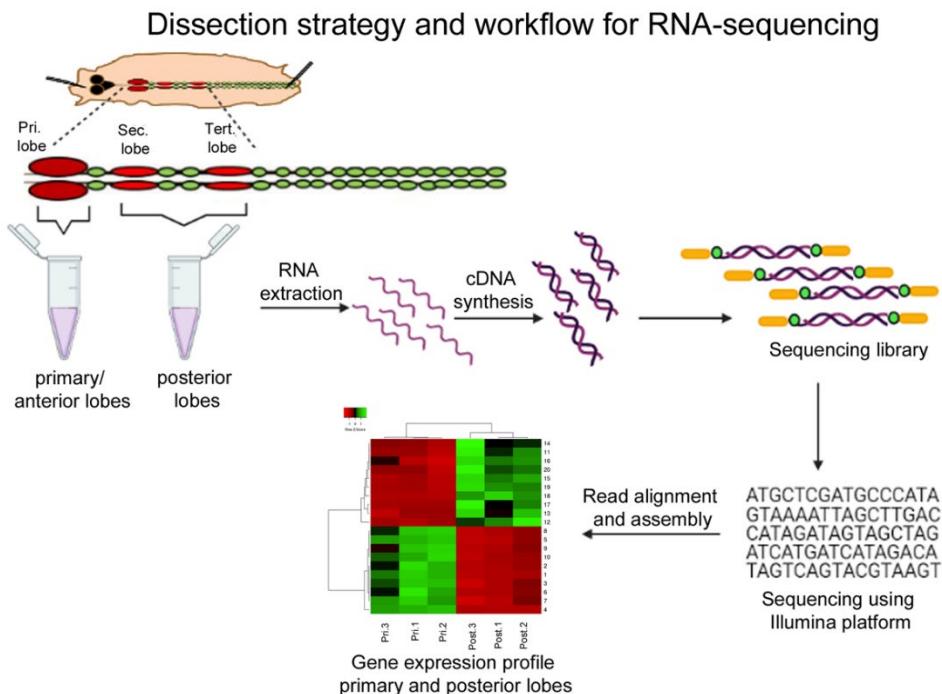


Figure 7. Schematic representation of dissection strategy and workflow for RNA-seq analysis.
Stepwise representation of dissection strategy and RNA-seq for obtaining transcriptomic profiles for the primary and the posterior lobes separately. Pri: primary lobes, Sec: secondary lobes, Tert: tertiary lobes, and Post: posterior lobes.

B. Discussion

Unlike vertebrate HSPCs, most *Drosophila* blood progenitors reside in a single hematopoietic organ, the lymph gland, which also harbors their differentiated progeny. The small size of the LG (~1.5-2 mm in length) makes a complete and comprehensive *in situ* analysis of its resident progenitor and differentiated pools possible. Yet, most of our understanding of larval LG hematopoiesis is derived from in-depth analysis restricted to the anterior-most lobes, called the primary lobes. Until recently, cellular and molecular mechanisms that operate in the primary lobe were thought to be applicable to the posterior lobes. However, anterior and posterior lobes have significant differences in gene expression and mutant phenotypes (Kulkarni *et al.*, 2011; Khadilkar *et al.*, 2014; Rodrigues *et al.*, 2021). Additionally, the posterior lobes are physically separated from the anterior lobe PSC, and differentiated hemocytes are involved in regulating progenitor fate in the anterior lobes (Figure 1F). Furthermore, a larger pool of blood progenitors resides in the posterior lobes (Rodrigues *et al.*, 2021). Hence, incomplete analysis of the LG overlooks important developmental and functional information regarding hematopoiesis, which could be relevant to vertebrate hematopoiesis. To aid dissection and analysis of the complete intact LG, we describe a detailed protocol that will also be useful in analyzing the entire progenitor population. LG primary lobes are specified at the embryonic stage, whereas the posterior lobes form during the second instar larval stage and continue to develop until the wandering larval stage. Hence, this method of dissection allows simultaneous analysis of developmental hematopoiesis in a single animal (Rodrigues *et al.*, 2021).

We demonstrated the utility of our method by analyzing pan-hemocyte, progenitor, and differentiation markers at the RNA and protein levels. Obtaining whole intact LG lobes is essential for understanding progenitor heterogeneity in physiological and immune conditions (Rodrigues *et al.*, 2021). This protocol can be used for studying the ontogeny, development, and immune aspects of LG blood cells. It can also be used for isolating distinct lobes for follow up biochemical and omics assays (Sinha *et al.*, 2019; Rodrigues *et al.*, 2021). Therefore, this protocol will be particularly valuable to gain new and more complete insights into the regulation of blood cell progenitor fate.

Notes

1. We use neutral red stained preparations only for the ease of visualization during training and practicing LG dissection and not for immunostaining or *in situ* hybridization experiments.
2. Avoid placing the coverslip on the LG preparation as this can damage the tissue. We prefer to use coverslip-bottom dishes for mounting. With experience, 8-10 LG preparations can be mounted in a single coverslip-bottom dish (Figure 5).
3. The fillet method of LG dissection retains the ventral larval cuticle containing sessile hemocyte clusters that can be retrieved at the sample mounting step (see Step F4), allowing simultaneous analysis of blood cells emerging from distinct anlage (Khadilkar *et al.*, 2014).
4. This protocol helps to isolate three organ systems: dorsal vessel, pericardial cells, and LG from the same animal in a single sample preparation.
5. As anterior/primary lobe progenitors are specified by the first instar, while secondary and tertiary lobes only develop at subsequent stages, this method allows analysis of developmental stages in a single animal.

Recipes

1. 10× PBS

NaCl 18.9 g
Na₂HPO₄ 2.48 g
NaH₂PO₄·2H₂O 1.17 g
Dissolve in autoclaved distilled water.

Adjust volume to 250 mL.

Adjust pH to 7.0.

Store at room temperature.

2. 4% Paraformaldehyde (PFA)

Dissolve 4 g PFA powder in 80 mL of 1× PBS.

Adjust the volume to 100 mL with 1× PBS solution.

Place the mixture at 60°C in a water bath to dissolve the PFA.

Store at 4°C.

3. 0.1% Triton X-100 in PBS (PTX)

Add 0.1 mL Triton X-100 in 100 mL PBS.

Store at room temperature.

4. 0.1% Tween-20 in PBS (PBS-T)

Add 0.1 mL Tween-20 in 100 mL PBS.

Store at room temperature.

5. 20× Saline-sodium citrate buffer (SSC)

NaCl 43.82 g

Na₃C₆H₅O₇ 22.05 g

Dissolve in molecular grade (DNase/RNase free) water.

Adjust volume to 200 mL with molecular grade (DNase/RNase free) water.

Adjust pH to 7.0.

Autoclave and store at -20°C.

6. Hybridization buffer

Formamide 50 mL

20× SSC 10 mL

Yeast RNA (50 mg/mL) 2 mL

Heparin (0.05 g/mL) 100 μL

Roche blocking reagent (10%) 20 mL (prepared according to the manufacturer's instructions).

CHAPS (10%) 1 mL

EDTA (0.5M) 1 mL

Tween-20 (10%) 1 mL

Adjust the volume to 100 mL using molecular grade water (DNase/RNase free).

Store at -20°C.

7. Staining buffer

5 M NaCl 200 μL

1 M MgCl₂ 500 μL

Tris-HCl pH 9.5 1 mL

Tween-20 (10%) 1 μL

Adjust volume to 10 mL with autoclaved distilled water.

Acknowledgments

We thank the *Drosophila* community for fly stocks and antibodies; the National Centre for Biological Sciences, Fly Facility for stocks, the JNCASR Imaging facility, and our laboratory members for valuable input and suggestions. Schematics were created with BioRender.com. This work was funded by the Indo-French Centre for the Promotion

Cite as: Rodrigues, D. et al. (2021). Intact *in situ* Preparation of the *Drosophila melanogaster* Lymph Gland for a Comprehensive Analysis of Larval Hematopoiesis. Bio-protocol 11(21): e4204. DOI: 10.21769/BioProtoc.4204.

of Advanced Research (IFCPAR/CEFIPRA) grant to MSI and LW. MSI's work was also supported by a SERB grant, J C Bose award project, and Jawaharlal Nehru Centre for Advanced Scientific Research.

Author contributions: DR, LW, and MSI performed experiments; DR, KVR, LW, and MSI wrote the manuscript; MSI and LW obtained funding and facilities for the work. Original research paper: Differential activation of JAK-STAT signaling reveals functional compartmentalization in *Drosophila* blood progenitors. DOI: 10.7554/eLife.61409.

Competing interests

The authors declare no conflicts of interest.

References

- Banerjee, U., Girard, J. R., Goins, L. M. and Spratford, C. M. (2019). [Drosophila as a Genetic Model for Hematopoiesis](#). *Genetics* 211(2): 367-417.
- Benmimoun, B., Polesello, C., Haenlin, M. and Waltzer, L. (2015). [The EBF transcription factor Collier directly promotes Drosophila blood cell progenitor maintenance independently of the niche](#). *Proc Natl Acad Sci U S A* 112(29): 9052-9057.
- Bruckner, K., Kockel, L., Duchek, P., Luque, C. M., Rorth, P. and Perrimon, N. (2004). [The PDGF/VEGF receptor controls blood cell survival in Drosophila](#). *Dev Cell* 7(1): 73-84.
- Crisan, M. and Dzierzak, E. (2016). [The many faces of hematopoietic stem cell heterogeneity](#). *Development* 143(24): 4571-4581.
- Crozatier, M., Ubeda, J. M., Vincent, A. and Meister, M. (2004). [Cellular immune response to parasitization in Drosophila requires the EBF orthologue collier](#). *PLoS Biol* 2(8): E196.
- Dey, N. S., Ramesh, P., Chugh, M., Mandal, S., Mandal, L. (2016). [Dpp dependent Hematopoietic stem cells give rise to Hh dependent blood progenitors in larval lymph gland of Drosophila](#). *eLife* 5: e12295.
- Ema, H., Morita, Y. and Suda, T. (2014). [Heterogeneity and hierarchy of hematopoietic stem cells](#). *Exp Hematol* 42(2): 74-82 e72.
- Ghosh, S., Singh, A., Mandal, S. and Mandal, L. (2015). [Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response](#). *Dev Cell* 33(4): 478-488.
- Granick, J. L., Simon, S. I. and Borjesson, D. L. (2012). [Hematopoietic stem and progenitor cells as effectors in innate immunity](#). *Bone Marrow Res* 2012: 165107.
- Grigorian, M., Liu, T., Banerjee, U. and Hartenstein, V. (2013). [The proteoglycan Trol controls the architecture of the extracellular matrix and balances proliferation and differentiation of blood progenitors in the Drosophila lymph gland](#). *Dev Biol* 384(2): 301-312.
- Grigorian, M., Mandal, L. and Hartenstein, V. (2011). [Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymph gland](#). *Dev Genes Evol* 221(3): 121-131.
- Holz, A., Bossinger, B., Strasser, T., Janning, W. and Klapper, R. (2003). [The two origins of hemocytes in Drosophila](#). *Development* 130(20): 4955-4962.
- Honti, V., Csordas, G., Markus, R., Kurucz, E., Jankovics, F. and Ando, I. (2010). [Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster](#). *Mol Immunol* 47(11-12): 1997-2004.
- Jung, S. H., Evans, C. J., Uemura, C. and Banerjee, U. (2005). [The Drosophila lymph gland as a developmental model of hematopoiesis](#). *Development* 132(11): 2521-2533.
- Khadilkar, R. J., Rodrigues, D., Mote, R. D., Sinha, A. R., Kulkarni, V., Magadi, S. S. and Inamdar, M. S. (2014). [ARF1-GTP regulates Asrij to provide endocytic control of Drosophila blood cell homeostasis](#). *Proc Natl Acad Sci U S A* 111(13): 4898-4903.
- Kulkarni, V., Khadilkar, R. J., Magadi, S. S. and Inamdar, M. S. (2011). [Asrij maintains the stem cell niche and controls differentiation during Drosophila lymph gland hematopoiesis](#). *PLoS One* 6(11): e27667.

- Kurucz, E., Markus, R., Zsamboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacovich, T., Gateff, E., et al. (2007). [Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes.](#) *Curr Biol* 17(7): 649-654.
- Kurucz, E., Zettervall, C. J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedus, Z., Ando, I. and Hultmark, D. (2003). [Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila.](#) *Proc Natl Acad Sci U S A* 100(5): 2622-2627.
- Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001). [Postembryonic hematopoiesis in Drosophila.](#) *Dev Biol* 230(2): 243-257.
- Mandal, L., Banerjee, U. and Hartenstein, V. (2004). [Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm.](#) *Nat Genet* 36(9): 1019-1023.
- Mandal, L., Martinez-Agosto, J. A., Evans, C. J., Hartenstein, V. and Banerjee, U. (2007). [A Hedgehog- and Antennapedia-dependent niche maintains Drosophila haematopoietic precursors.](#) *Nature* 446(7133): 320-324.
- Rodrigues, D., Renaud, Y., VijayRaghavan, K., Waltzer, L. and Inamdar, M. S. (2021). [Differential activation of JAK-STAT signaling reveals functional compartmentalization in Drosophila blood progenitors.](#) *Elife* 10: e61409.
- Rugendorff, A., Younossi-Hartenstein, A. and Hartenstein, V. (1994). [Embryonic origin and differentiation of the Drosophila heart.](#) *Roux's Arch Dev Biol* 203(5): 266-280.
- Sanchez Bosch, P., Makhijani, K., Herboso, L., Gold, K. S., Baginsky, R., Woodcock, K. J., Alexander, B., Kukar, K., Corcoran, S., Jacobs, T., et al. (2019). [Adult Drosophila Lack Hematopoiesis but Rely on a Blood Cell Reservoir at the Respiratory Epithelia to Relay Infection Signals to Surrounding Tissues.](#) *Dev Cell* 51(6): 787-803 e785.
- Sinha, A., Khadilkar, R. J., S, V. K., Roychowdhury Sinha, A. and Inamdar, M. S. (2013). [Conserved regulation of the Jak/STAT pathway by the endosomal protein asrij maintains stem cell potency.](#) *Cell Rep* 4(4): 649-658.
- Sinha, S., Ray, A., Abhilash, L., Kumar, M., Sreenivasamurthy, S. K., Keshava Prasad, T. S. and Inamdar, M. S. (2019). [Proteomics of Asrij Perturbation in Drosophila Lymph Glands for Identification of New Regulators of Hematopoiesis.](#) *Mol Cell Proteomics* 18(6): 1171-1182.
- Tepass, U., Fessler, L. I., Aziz, A. and Hartenstein, V. (1994). [Embryonic origin of hemocytes and their relationship to cell death in Drosophila.](#) *Development* 120(7): 1829-1837.
- Ward, E. J. and Skeath, J. B. (2000). [Characterization of a novel subset of cardiac cells and their progenitors in the Drosophila embryo.](#) *Development* 127(22): 4959-4969.
- Wright, D. E., Wagers, A. J., Gulati, A. P., Johnson, F. L. and Weissman, I. L. (2001). [Physiological migration of hematopoietic stem and progenitor cells.](#) *Science* 294(5548): 1933-1936.

Suppression of Human Dendritic Cells by Regulatory T Cells

Qing Huang^{1, 2, #}, Avery J. Lam^{1, 2, #}, Dominic A. Boardman^{1, 2, #}, Nicholas A. J. Dawson^{1, 2} and Megan K. Levings^{1, 2, 3, *}

¹Department of Surgery, University of British Columbia, Vancouver, Canada

²BC Children's Hospital Research Institute, Vancouver, Canada; ³School of Biomedical Engineering, University of British Columbia, Vancouver, Canada

*For correspondence: mlevings@bcchr.ca

#Contributed equally to this work

Abstract

Regulatory T cells (Tregs) suppress immune responses via a variety of mechanisms and can be used as a cellular therapy to induce tolerance. The function of Tregs is commonly assessed *in vitro* using assays that measure suppression of effector T cell proliferation and/or cytokine production. However, Tregs can also suppress the function of antigen presenting cells, creating a need for methodology to routinely measure this aspect of their function. This protocol describes a method to measure human Treg-mediated suppression of CD80 and CD86 expression on mature, monocyte-derived dendritic cells. Representative data show suppression mediated by polyclonal Tregs as well as antigen-specific Tregs generated using chimeric antigen receptor (CAR) technology. This method can be used in parallel to T cell suppression assays to measure the functional activity of human Tregs.

Keywords: Regulatory T cells, Dendritic cells, CD80, CD86, Chimeric antigen receptor

This protocol was validated in: Sci Transl Med (2020), DOI: 10.1126/scitranslmed.aaz3866

Background

Regulatory T cells (Tregs) are immunosuppressive cells that play a fundamental role in maintaining peripheral tolerance. Tregs inhibit the action of many immune cells, including effector T cells and antigen presenting cells (APC), via cell contact-dependent and contact-independent mechanisms. The suppressive function of Tregs is typically assessed *in vitro* by measuring their ability to inhibit the proliferation of polyclonally stimulated T cells. However, methods to measure how Tregs suppress APCs are limited.

One strategy Tregs use to inhibit the function of APCs is the removal of co-stimulatory molecules from the APC, thereby reducing their ability to stimulate effector T cells. Tregs achieve this by expressing CTLA-4, which binds CD80 and CD86 with a high affinity and allows the Treg to physically remove these molecules from the APC cell surface (Walker and Sansom, 2011). We have also previously reported the ability of human Tregs to suppress the expression of co-stimulatory molecules on both immature and mature monocyte-derived DCs (moDCs) (Wang *et al.*, 2011).

This protocol describes a method to test Treg-mediated suppression of CD80 and CD86 and is modified from a previously published mouse-based protocol (Onishi *et al.*, 2008). Our protocol focuses on the ability of human Tregs to suppress the expression of CD80 and CD86 by moDCs. In this assay, polyclonal Tregs transduced with a truncated nerve growth factor receptor (Δ NGFR) reporter can reduce CD80 and CD86 expression in moDCs. Furthermore, when using HLA-A2 $^+$ target moDCs, antigen-specific Tregs expressing an HLA-A2-specific chimeric antigen receptor (CAR) are more potent than polyclonal Tregs (Dawson *et al.*, 2020; Fung *et al.*, 2021).

Materials and Reagents

Materials

1. 5 mL polystyrene round-bottom tubes (Corning, catalog number: 352052)
2. TC-coated 6-well plates (Corning, catalog number: 353502)
3. TC-coated 12-well plates (Corning, catalog number: 353503)
4. 96-well U- and V-bottom plates (Corning, catalog numbers: 353077, 3894)
5. 1.5 mL microcentrifuge tubes (Fisher Scientific, catalog number: 229442)
6. 15 mL and 50 mL conical tubes (Corning, catalog numbers: 352096, 352070)
7. Sterile 1 mL or 3 mL syringe (BD, catalog numbers: 309628, 309657)

Media and Buffers

1. Lymphoprep™ (STEMCELL Technologies, catalog number: 07801)
2. Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, catalog number: 14190), 1 \times
3. X-VIVO 15 (Lonza, catalog number: BEBP02-054Q)
4. Human serum (Wisent Bio Products, catalog number: 022210)
5. Penicillin/streptomycin (Gibco, catalog number: 15140-122)
6. GlutaMAX (Gibco, catalog number: 35050-061)
7. Sodium pyruvate (Gibco, catalog number: 11360-070)
8. Fetal bovine serum (Gibco, catalog number: 12483020)
9. Ethylenediaminetetraacetic acid solution (EDTA) (Sigma-Aldrich, catalog number: 03690)
10. EasySep Buffer (see Recipes)
11. Dendritic Cell Medium (see Recipes)

Reagents

1. Stericup-GP Sterile Vacuum Filtration System (Millipore, catalog number: SCGPU05RE), 500 mL
2. EasySep Human CD14 Positive Selection Kit II (STEMCELL Technologies, catalog number: 17858)
3. Acridine Orange/Propidium Iodide (AO/PI; Nexcelom, catalog number: NEX-CS201065ML)

Cite as: Huang, Q. et al. (2021). Suppression of Human Dendritic Cells by Regulatory T Cells. Bio-protocol 11(21): e4217.
DOI: 10.21769/BioProtoc.4217.

Cytokines

1. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (STEMCELL Technologies, catalog number: 78015), 20 µg/mL
2. Recombinant human interleukin (IL)-4 (STEMCELL Technologies, catalog number: 78045), 20 µg/mL
3. Recombinant human tumour necrosis factor (TNF)- α (eBioscience, catalog number: 14-8329-63), 20 µg/mL
4. Prostaglandin E2 (PGE2) (Tocris, catalog number: 2296), 100 mM
5. Recombinant human IL-1 β (STEMCELL Technologies, catalog number: 78041), 20 µg/mL
6. Recombinant human IL-6 (STEMCELL Technologies, catalog number: 78148), 20 µg/mL
7. Recombinant human interferon (IFN)- γ (eBioscience, catalog number: 14-8319-80), 20 µg/mL
8. Recombinant human IL-2 (Proleukin) (Novartis, DIN# 02130181)

Antibodies

1. Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience, catalog number: 14-9161-73)
2. Fixable Viability Dye (FVD) (eBioscience, catalog number: 65-0865-18)
3. Anti-human CD3 (UCHT1) APC (BD Biosciences, catalog number: 564465)
4. Anti-human CD3 (UCHT1) BB515 (BD Biosciences, catalog number: 564465)
5. Anti-human CD4 (OKT4) APC (eBioscience, catalog number: 17-0048-42)
6. Anti-human CD4 (RPA-T4) BV711 (BioLegend, catalog number: 300558)
7. Anti-human CD8a (RPA-T8) BV711 (BioLegend, catalog number: 301044)
8. Anti-human CD11c (B-ly6) PE (BD Biosciences, catalog number: 555392)
9. Anti-human CD14 (M5E2) BV421 (BioLegend, catalog number: 301830)
10. Anti-human CD14 (M5E2) BV786 (BD Biosciences, catalog number: 563698)
11. Anti-human CD40 (5C3) PE-Cy7 (BD Biosciences, catalog number: 561215)
12. Anti-human CD56 (CMSSB) PE (eBioscience, catalog number: 12-0567-42)
13. Anti-human CD69 (FN50) BV785 (BioLegend, catalog number: 310932)
14. Anti-human CD70 (113-16) PerCP-Cy5.5 (BioLegend, catalog number: 355107)
15. Anti-human CD80 (L307.4) FITC (BD Biosciences, catalog number: 557226)
16. Anti-human CD83 (HB15e) BV421 (BioLegend, catalog number: 305324)
17. Anti-human CD86 (2331 (FUN-1)) APC (BD Biosciences, catalog number: 555660)
18. Anti-human CD86 (HA5.2B7) PerCP-Cy5.5 (Beckman Coulter, catalog number: B30647)

Equipment

1. Type II Biosafety cabinet (NuAire, model: LabGard ES NU-540)
2. Centrifuge, microcentrifuge (Eppendorf, models: 5810R and 5452)
3. STEMCELL EasySep™ magnet (STEMCELL Technologies, catalog number: 18000)
4. Cell counter (Nexcelom, model: Cellometer Auto 2000)
5. 37°C incubator with 5% (v/v) CO₂ (Sanyo, model: MCO-18AIC)
6. Flow cytometer (BD LSRIFortessa X-20; alternative instruments can be used)

Software

1. FlowJo software (BD Biosciences, v10.7)

Procedure

Overview

- Day 0: Prepare peripheral blood mononuclear cells (PBMCs).
- Day 0: Isolate CD14⁺ monocytes from PBMCs by positive selection.
- Day 0: Differentiate monocytes into dendritic cells by culturing in the presence of GM-CSF and IL-4.
- Day 3: Replenish GM-CSF and IL-4.
- Day 5: Mature dendritic cells by adding TNF-α, PGE2, IL-1β, and IL-6 to the culture.
- Day 6: Mature dendritic cells by adding IFN-γ to the culture.
- Day 7: Confirm maturation of the DCs by flow cytometry and set up the suppression assay.
- Day 11: Collect cells, stain, perform flow cytometry, and analyse.

Detailed Procedure

A. Day 0: Prepare PBMCs

1. Prepare PBMCs: Either freshly isolated from blood or thawed PBMCs. PBMCs can be isolated from human peripheral blood by density gradient centrifugation using Lymphoprep™, according to the manufacturer's protocol.

Note: Using batch-frozen PBMCs from one donor to differentiate moDCs will reduce donor-to-donor variability. Expect 5-10% fresh/frozen PBMCs or 50-100% CD14⁺ cells to become moDCs. See [Notes](#) for further details.

2. Optional: Set aside $\sim 5 \times 10^3$ - 10×10^3 PBMCs for purity check (see Table 1, Figure 1, and [Notes](#) for further information).

Table 1. Day 0 Purity Check Panel.

Stain PBMCs in parallel, if desired. See [Figure 1](#) for representative data.

Marker	Dilution	Clone	Fluorophore
Fc Receptor Binding Inhibitor (preincubate for 10 min and do not wash)	1:5	Polyclonal	N/A
FVD	1:1,000	N/A	eF780
CD3	1:100	UCHT1	BB515
CD4	1:100	OKT4	APC
CD8a	1:100	RPA-T8	BV711
CD14	1:200	M5E2	BV421
CD56	1:25	CMSSB	PE

B. Day 0: Isolate CD14⁺ monocytes using the STEMCELL EasySep Human CD14 Positive Selection Kit II, as per the manufacturer's protocol and described below, unless stated otherwise:

1. Resuspend PBMCs at 100×10^6 /mL in EasySep Buffer (0.1-2 mL). The total starting number of PBMCs should be between 10×10^6 and 200×10^6 cells. For x number of CD14⁺ monocytes, start with $10\times$ (fresh) or $\sim 20\times$ (frozen) PBMCs.
2. Transfer PBMCs to a 5 mL polystyrene round-bottom tube.
3. Add 100 µL of Selection Cocktail per millilitre of cells and incubate 10 min at room temperature (RT).

4. Vortex RapidSpheres for 30 s, add 100 μ L of RapidSpheres per ml cells, mix tube by gentle rotation, and incubate for 3 min at RT.
5. Top up tube with EasySep Buffer to 2.5 mL, mix by pipetting, place in an EasySep™ magnet, and incubate for 3 min at RT.
6. Gently pour tube while in magnet to discard supernatant. The round-bottom tube contains CD14 $^{+}$ cells (keep).
7. Wash remaining RapidSpheres by removing the round-bottom tube from the magnet and topping up tube with 2.5 mL of EasySep Buffer. Mix by pipetting to wash layer off tube wall, replace the tube back in the magnet, and incubate for 3 min at RT. Pour to discard supernatant and pipette last drop from tube while inverted.
8. Repeat Step B7 so that the tube of cells is incubated in the magnet a total of three times.
9. Optionally repeat Step B7 two more times (total of five times on magnet) for potentially higher enrichment.

Note: This step is not part of the original STEMCELL Technologies protocol.

10. Transfer the cells from the round-bottom tube into a 15 mL conical tube by washing the walls of the round-bottom tube with ~3 mL of DPBS. Centrifuge at 450 \times g for 5 min and discard supernatant.
11. Resuspend the enriched CD14 $^{+}$ cells in Dendritic Cell Medium (see Recipes).
12. Count cells (see protocols from Nexcelom Cellometer Auto 2000 for details; alternatively, a hemocytometer and trypan blue can be used).
13. Optional: set aside $\sim 5 \times 10^3$ - 10×10^3 monocytes for purity check (see Table 1, Figure 1, and Notes for further information).

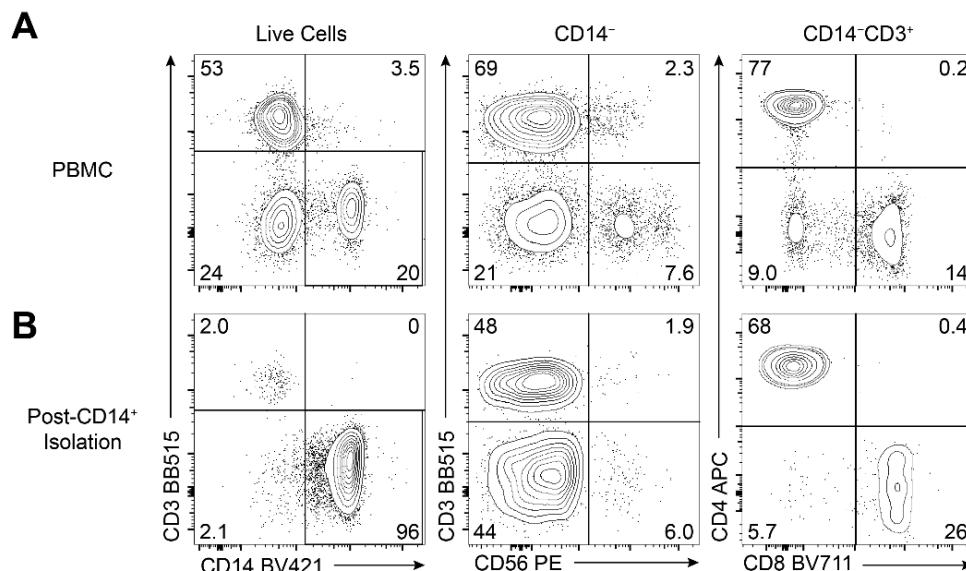


Figure 1. Day 0 CD14 $^{+}$ Purity Check.

(A) Approximately 5×10^3 - 10×10^3 total PBMCs and (B) CD14-enriched cells were stained and analysed by flow cytometry to evaluate the purity of the CD14-enriched cells and the extent of CD3 $^{+}$ cell contamination. Contaminating T cells can affect downstream results if OKT3 (anti-CD3) is included during assays (see Notes). Cells were stained as described in Table 1 and in accordance with “Guidelines for the use of flow cytometry and cell sorting in immunological studies” (Cossarizza *et al.*, 2019); they were acquired with a BD LSRII Fortessa X-20 and data analysed using FlowJo. The proportion of total live cells that were CD14 $^{+}$ CD3 $^{-}$ (CD14 $^{+}$ cell purity) is shown on the left. From the live CD14 $^{-}$ cells, CD56 expression was analysed (middle) to identify NK/NKT cells (CD56 $^{\text{med/hi}}$) and a subset of monocytes (CD56 $^{\text{lo}}$). The contaminating live CD14 $^{-}$ CD3 $^{+}$ T cells were further analysed for their expression of CD4 and CD8 (right). See Notes for more information.

C. Day 0: Culture and differentiate enriched CD14⁺ cells into dendritic cells

1. Adjust cells to 2×10^6 cells per millilitre with Dendritic Cell Medium.
2. Plate 2 mL cells per well in a 6-well plate (4×10^6 cells per well).

Note: Plate multiple wells of cells as needed.

3. Add GM-CSF (final concentration: 50 ng/mL) and IL-4 (final concentration: 100 ng/mL).
4. Incubate cells at 37°C (5% v/v CO₂) for 3 days.

Note: Cytokines can be stored at 4°C for ~1 week.

D. Day 3: Change Dendritic Cell Medium and replenish cytokines

1. Change Dendritic Cell Medium: without disturbing the cells at the bottom of the well, collect 1.5 mL of medium into sterile 1.5 mL microcentrifuge tube, centrifuge at $450 \times g$ for 5 min, discard the supernatant, resuspend the pelleted cells in 1.5 mL of fresh Dendritic Cell Medium, and re-plate into original well.
2. Fully replenish the cytokines by adding fresh GM-CSF (final concentration: 50 ng/mL) and IL-4 (final concentration: 100 ng/mL), assuming that no cytokines from day 0 remain in the culture.
3. Incubate at 37°C (5% v/v CO₂) for 2 days.

Note: Cytokines can be stored at 4°C for ~1 week.

Alternative medium change method: Centrifuge plate at $450 \times g$ for 5 min, gently replace 1.5 mL of Dendritic Cell Medium, and fully replenish the GM-CSF and IL-4 as detailed above.

E. Day 5: Mature dendritic cells

1. Collect cells: pipette up and down to detach cells and collect either into a 15 mL or 50 mL conical tube. Detach remaining cells using a 1 mL or 3 mL syringe rubber in gentle, one-way movements. Rinse well twice with DPBS to collect as many cells as possible.
2. Wash cells by filling the conical tube with DPBS, centrifuge cells at $450 \times g$ for 5 min, and discard the supernatant.
3. Resuspend cells in fresh Dendritic Cell Medium, count cells, and adjust volume so that cells are at a final concentration of 1×10^6 cells/mL in Dendritic Cell Medium.
4. Add GM-CSF (final concentration: 50 ng/mL) and IL-4 (final concentration: 100 ng/mL).
5. Plate cells in a new well by adding 2 mL of cells per well in a new 6-well plate (2×10^6 cells per well) or 1 mL of cells per well in a new 12-well plate (1 $\times 10^6$ cells per well).
6. Mature moDCs by adding TNF- α (final concentration: 50 ng/mL), PGE2 (final concentration: 1 μ g/mL, ~2.837 μ M), IL-1 β (final concentration: 10 ng/mL), and IL-6 (final concentration: 100 ng/mL).

Note: If immature moDCs are desired, skip this step. Keeping a minimum of 1×10^6 immature moDCs (GM-CSF and IL-4 with no additional cytokines) is useful for evaluating the moDC maturation at the end (day 7, see Procedure G).

7. Incubate at 37°C (5% v/v CO₂) for 1 day.

F. Day 6: Continue to mature dendritic cells

1. Add IFN- γ (final concentration: 50 ng/mL).

Note: Skip this step for immature moDCs.

2. Incubate at 37°C (5% v/v CO₂) for 1 day.

G. Day 7: Confirm maturation of the DCs before setting up the suppression assay

1. Collect cells by pipetting to resuspend and transfer to a 15 mL conical tube. Detach remaining cells using a 1 mL or 3 mL syringe rubber in gentle, one-way movements. Wash wells twice with DPBS and add this to the conical tube. Centrifuge cells at 450 × g for 5 min, resuspend in fresh Dendritic Cell Medium, count cells, and adjust cell concentration to 5 × 10⁵ cells/mL in Dendritic Cell Medium.
2. Confirm moDC maturation by collecting ~5 × 10⁶-10 × 10⁶ mature moDCs and immature moDCs for staining for flow cytometry (see Table 2, Figure 2).

Table 2. Day 7 moDC Maturation Check Panel.

Immature moDC must be stained in parallel. See Figure 2 for representative data.

Marker	Dilution	Clone	Fluorophore
Fc Receptor Binding Inhibitor (preincubate for 10 min and do not wash)	1:5	Polyclonal	N/A
FVD	1:1,000	N/A	eF780
CD3	1:50	UCHT1	APC
CD11c	1:50	B-ly6	PE
CD14	1:100	M5E2	BV785
CD80	1:50	L307.4	FITC
CD83	1:50	HB15e	BV421
CD86	1:50	HA5.2B7	PerCp-Cy5.5
CD40	1:100	5c3	PECy-7
HLA-DR	1:50	L243	BV510

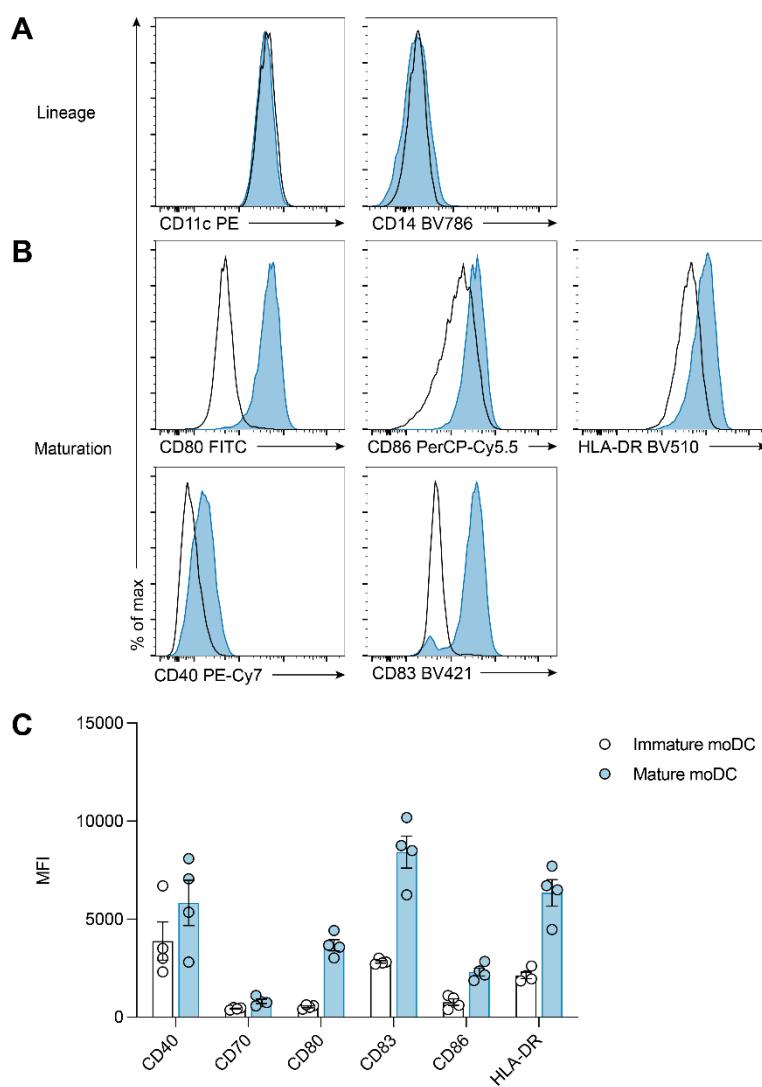


Figure 2. Day 7 phenotype of immature and mature moDCs.

(A-C) Immature (black line) and mature (blue) moDCs were analysed by flow cytometry for expression of CD11c, CD14, CD80, CD86, CD40, CD83, and HLA-DR. Cells were stained as described in Table 2 and in accordance with “Guidelines for the use of flow cytometry and cell sorting in immunological studies” (Cossarizza *et al.*, 2019); they were acquired with a BD LSRII Fortessa X-20 and data analysed using FlowJo. moDCs (gated as live, single cells) expressed similar levels of lineage markers (A) but upregulated expression of CD80, CD86, HLA-DR, CD40, and CD83 following maturation (B-C). MFI, geometric mean fluorescence intensity.

H. Day 7: Set up DC suppression assay

1. Plate 100 μ L moDC in a 96-well U-bottom plate (50,000 cells per well).
2. Collect rested Tregs, count cells, and resuspend at 2.5×10^6 cells per ml in Dendritic Cell Medium with 100 IU/mL IL-2.

*Note: Tregs should be expanded and rested using protocols established by the user's lab. Details of how Tregs were expanded and rested for this protocol are provided in Dawson *et al.* (2020). Briefly, Tregs were isolated from peripheral blood and polyclonally expanded with artificial APCs in the presence of 1,000 IU/mL IL-2 for 7 days. Tregs were then rested by culturing in fresh medium with 100 IU/mL IL-2 overnight.*

CAR Tregs were generated by lentivirally transducing polyclonal Tregs one day post-stimulation.

3. Set up moDC and Treg co-culture in a 1-to-5 ratio (50,000 DCs to 250,000 Tregs per well): add 100 µL Treg cell suspension into the wells containing moDCs from Step H1.
4. Control wells: set up one well of moDC alone and another well of Treg alone as controls in separate wells with a final volume of 200 µL per well.
5. Co-culture cells in Dendritic Cell Medium with 50 IU/mL IL-2 at 37°C (5% v/v CO₂) for 4 days.

I. Day 11: Flow cytometry

1. Collect cells: centrifuge the cell culture plate at 970 × g for 3 min, remove from centrifuge, and discard half the volume (100 µL) supernatant by pipetting. Resuspend the cells in the remaining supernatant and transfer into a new 96-well V-bottom plate. Wash the wells in the cell culture plate with 100 µL DPBS and add this to the respective wells in the V-bottom plate.
2. Centrifuge the plate at 970 × g for 3 min, discard the supernatant, and resuspend the cells in 200 µL DPBS to wash.
3. Centrifuge the plate at 970 × g for 3 min and discard the supernatant.
4. Stain cells for analysis by flow cytometry using Table 3. See Figure 3 for example data.

Table 3. Day 11 moDC Suppression Assay Panel.

moDC-alone and Treg-alone controls must be stained in parallel. See Figure 3 for representative data.

Marker	Dilution	Clone	Fluorophore
Fc Receptor Binding Inhibitor (preincubate for 10 min and do not wash)	1:5	Polyclonal	N/A
FVD	1:1,000	N/A	eF780
CD4	1:100	RPA-T4	BV711
CD69	1:50	FN50	BV785
CD11c	1:50	B-ly6	PE
CD70	1:50	113-16	PerCP-Cy5.5
CD80	1:50	L307.4	FITC
CD83	1:50	HB15e	BV421
CD86	1:50	FUN-1	APC
CD40	1:100	5c3	PE-Cy7
HLA-DR	1:50	L243	BV510

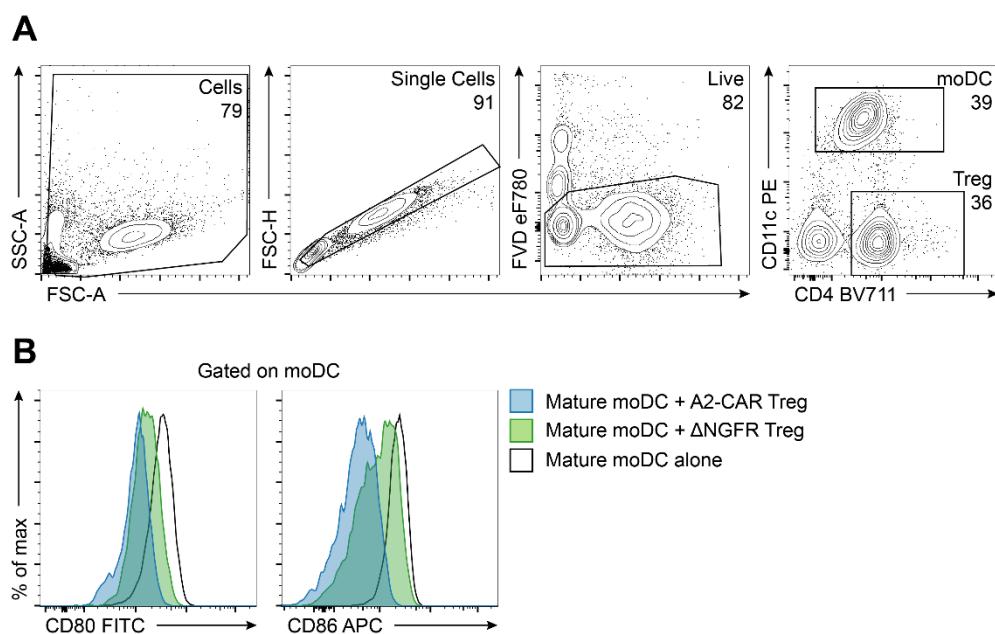


Figure 3. Day 11 analysis of CD80 and CD86 on mature moDCs following co-culture with Tregs.

(A-B) Mature moDCs and Tregs were co-cultured for 4 days, and then cells were stained as described in Table 3 and in accordance with “Guidelines for the use of flow cytometry and cell sorting in immunological studies” (Cossarizza *et al.*, 2019); they were acquired with a BD LSRFortessa X-20 and data analysed using FlowJo. (A) Gating strategy to identify mature moDCs. Live singlet cells were gated, and moDCs were identified as CD11c⁺CD4⁻ cells. (B) Expression of CD80 (left) and CD86 (right) following co-culture with Tregs. This assay was performed using HLA-A2⁺ moDCs co-cultured with either polyclonal ΔNGFR-transduced Tregs or HLA-A2-specific CAR-transduced Tregs. Compared to moDCs cultured alone (black line), polyclonal Tregs exert a moderate level of suppression (green), as determined by the decrease in CD80 and CD86 expression. This suppression is greater when co-cultures are performed with A2-CAR Tregs (blue). See Dawson *et al.* (2020) for more examples.

Notes

1. PBMC: Fresh and frozen PBMCs are similar and suitable for moDC differentiation. Frozen PBMCs yield slightly fewer CD14⁺ monocytes than fresh PBMCs. Using frozen PBMCs from a single donor can reduce donor-to-donor moDC variability. Fresh PBMCs can be prepared the day of (proceed to CD14⁺ isolation immediately) or the day before running the experiment [store overnight at 4°C in 25 mL 10% (v) fetal bovine serum-supplemented medium of choice (*e.g.*, RPMI 1640) mixed with 25 ml DPBS in a 50 ml tube placed horizontally in a fridge].
2. CD14⁺ selection performance: Post-selection (3× magnet-isolation, as per manufacturer’s protocol), 90-95% are CD14⁺ cells and 0.5-5% are CD3⁺ T cells (see Figure 1). Detection of contaminating T cells can be obscured if FSC/SSC voltages and thresholds are set too low. T cell contamination persists to day 7 and can affect results from moDC-T cell co-cultures (*e.g.*, if OKT3 is added). Thus, an anti-CD3 antibody can be added to the day 7 moDC maturation panel to evaluate purity since the cells have expanded (instead of day 0).
3. moDC lineage markers: moDCs should be CD11c⁺ and CD14⁻. moDC maturation markers: mature moDCs should be CD80^{hi}, CD86^{hi}, HLA-DR^{hi} (Ag presentation and T cell co-stimulation), CD40⁺ (promote CD40L⁺ T cell maturation and cytokine secretion), and CD83⁺ (function less clear).
4. Co-culture: Confirmation of moDC maturation is important before setting up suppression co-culture.

Recipes

1. EasySep Buffer

DPBS supplemented with 2% (v/v) fetal bovine serum, 1 mM EDTA. Contents were sterile-filtered with a Stericup-GP vacuum filtration flask.

2. Dendritic Cell Medium

X-VIVO 15 supplemented with 5% (v/v) human serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) GlutaMAX, 1% (v/v) sodium pyruvate. Contents were sterile-filtered with a Stericup-GP vacuum filtration flask.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) FDN-154304 and TxCell. AJL and NAJD were supported by a CIHR doctoral award and MKL received a salary award from the BC Children's Hospital Research Institute. This method is derived from the original publication by Dawson *et al.* (2020) (DOI: 10.1126/scitranslmed.aaz3866).

Competing interests

The authors of this manuscript have received research funding from Sangamo Therapeutics (formerly TxCell SA) to partially support this work. MKL has also received research funding from Takeda, Bristol Myers Squibb, Pfizer, and CRISPR Therapeutics for work not related to this study.

Ethics

For all studies, healthy volunteers gave written informed consent according to protocols approved by the University of British Columbia Clinical Research Ethics Board and Canadian Blood Services.

References

- Cossarizza A, Chang HD, Radbruch A, Acs A, Adam D, Adam-Klages S, Agace WW, Aghaeepour N, Akdis M, Allez M., *et al.* (2019). [Guidelines for the use of flow cytometry and cell sorting in immunological studies \(second edition\)](#). *Eur J Immunol* 49(10):1457-1973.
- Dawson, N. A. J., Rosado-Sanchez, I., Novakovsky, G. E., Fung, V. C. W., Huang, Q., McIver, E., Sun, G., Gillies, J., Speck, M., *et al.* (2020). [Functional effects of chimeric antigen receptor co-receptor signaling domains in human regulatory T cells](#). *Sci Transl Med* 12(557): eaaz3866.
- Fung, V. C. W., Rosado-Sanchez, I. and Levings, M. K. (2021). [Transduction of Human T Cell Subsets with Lentivirus](#). *Methods Mol Biol* 2285: 227-254.
- Onishi, Y., Fehervari, Z., Yamaguchi, T. and Sakaguchi, S. (2008). [Foxp3⁺ natural regulatory T cells preferentially form aggregates on dendritic cells *in vitro* and actively inhibit their maturation](#). *Proc Natl Acad Sci U S A* 105(29): 10113-10118.
- Walker, L. S. and Sansom, D. M. (2011). [The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses](#). *Nat Rev Immunol* 11(12): 852-863.

Wang, A. Y., Crome, S. Q., Jenkins, K. M., Medin, J. A., Bramson, J. L. and Leving, M. K. (2011). [Adenoviral-transduced dendritic cells are susceptible to suppression by T regulatory cells and promote interleukin 17 production](#). *Cancer Immunol Immunother* 60(3): 381-388.

Production of Recombinant Replication-defective Lentiviruses Bearing the SARS-CoV or SARS-CoV-2 Attachment Spike Glycoprotein and Their Application in Receptor Tropism and Neutralisation Assays

Nazia Thakur^{1, 2,*}, Giulia Gallo¹, Ahmed M. E. Elreafey¹ and Dalan Bailey^{1,*}

¹Viral Glycoproteins Group, The Pirbright Institute, Pirbright, Woking, UK

²The Jenner Institute, Nuffield Department of Medicine, University of Oxford, Oxford, UK

*For correspondence: Dalan.bailey@pirbright.ac.uk; nazia.thakur@pirbright.ac.uk

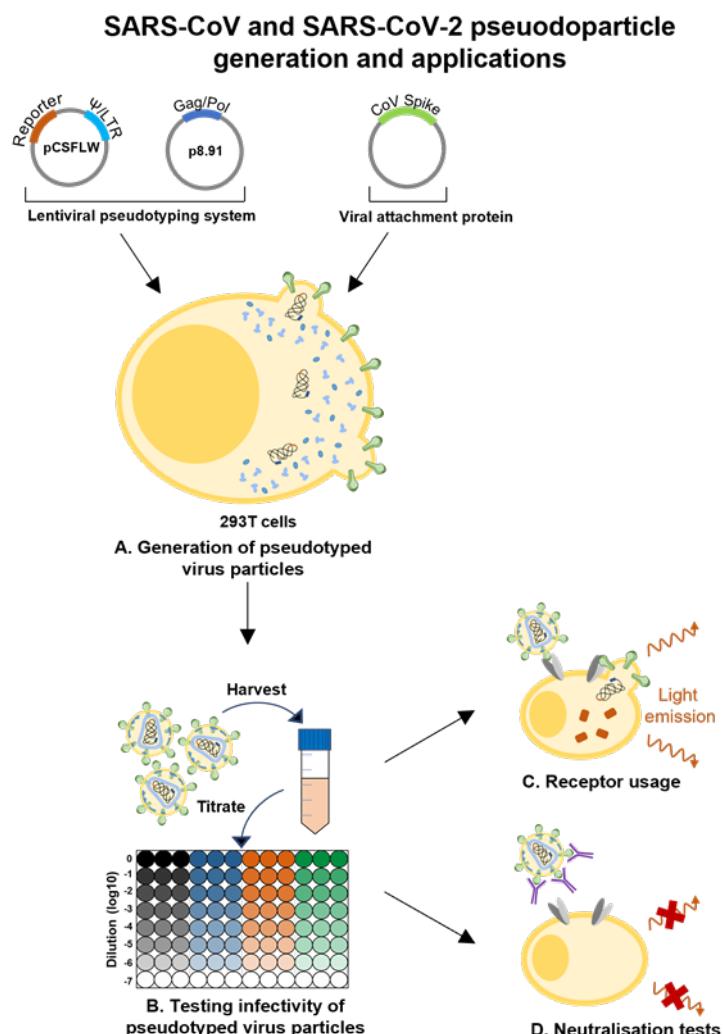
Abstract

For enveloped viruses, such as SARS-CoV-2, transmission relies on the binding of viral glycoproteins to cellular receptors. Conventionally, this process is recapitulated in the lab by infection of cells with isolated live virus. However, such studies can be restricted due to the availability of high quantities of replication-competent virus, biosafety precautions and associated trained staff. Here, we present a protocol based on pseudotyping to produce recombinant replication-defective lentiviruses bearing the SARS-CoV or SARS-CoV-2 attachment Spike glycoprotein, allowing the investigation of viral entry in a lower-containment facility. Pseudoparticles are produced by cells transiently transfected with plasmids encoding retroviral RNA packaging signals and *Gag-Pol* proteins, for the reconstitution of lentiviral particles, and a plasmid coding for the viral attachment protein of interest. This approach allows the investigation of different aspects of viral entry, such as the identification of receptor tropism, the prediction of virus host range, and zoonotic transmission potential, as well as the characterisation of antibodies (sera or monoclonal antibodies) and pharmacological inhibitors that can block entry.

Keywords: SARS-CoV-2, SARS-CoV, Pseudotyped virus, Tropism, Neutralisation

This protocol was validated in: PLoS Biol (2020), DOI: 10.1371/journal.pbio.3001016

Graphical Abstract:



SARS-CoV and SARS-CoV-2 pseudoparticle generation and applications.

Background

Pseudoparticles are replication-defective viral particles obtained through expression of viral envelope glycoproteins on the surface of a recombinant virus, which provides the core components of the particle. Vesicular stomatitis virus (VSV), a rhabdovirus, and two lentiviruses – human immunodeficiency virus-1 (HIV-1) and murine leukaemia virus (MuLV) – are commonly used as viral vectors for pseudotyping (Takada *et al.*, 1997; Wool-Lewis and Bates, 1998; Sharkey *et al.*, 2001; Negrete *et al.*, 2005; Grehan *et al.*, 2015; Thakur *et al.*, 2021). In our recent study, we successfully used a lentiviral-based system to study the interaction of severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 Spike (S) protein with its cellular receptor, angiotensin converting enzyme 2 (ACE2) (Conceicao *et al.*, 2020).

SARS-CoV-2, the etiological agent of the ongoing COVID-19 pandemic, is a highly pathogenic betacoronavirus that requires handling at BSL-3 facilities, which are not always available in research laboratories. To allow work with SARS-CoV and SARS-CoV-2 at lower containment, the generation of viral particles pseudotyped with the

Spike protein represents a safe and appealing surrogate. This technique allows (i) dissection of viral entry pathways, (ii) investigation of host cell susceptibility and tropism of the angiotensin converting enzyme 2 (ACE2) receptor, (iii) examination of inter-species transmission, (iv) assessment of the neutralising antibody responses in immunogenicity and sero-epidemiological studies, and (v) efficacy assessment of small-molecule inhibitors that block viral entry. Notably, this technique has been applied to viral glycoproteins from a wide variety of viruses such as influenza hemagglutinin (Bertram *et al.*, 2010), Nipah virus fusion and attachment proteins (Thakur *et al.*, 2021), Ebola virus glycoprotein (Simmons *et al.*, 2003), Chikungunya virus E1 (Salvador *et al.*, 2009), hepatitis C virus E2 proteins (Hsu *et al.*, 2003), and VSV glycoprotein (DePolo *et al.*, 2000).

To generate lentiviral-based pseudoparticles of HIV-1, cells are co-transfected with the following plasmids: (i) HIV-1 packaging plasmid encoding for the core genes Gag and Pol, (ii) the transfer plasmid that encodes a firefly luciferase reporter gene flanked by HIV-1 regulatory LTR regions and the packaging signal, and (iii) a third plasmid encoding for the heterologous viral glycoprotein. Pseudoparticles possessing the viral glycoprotein of interest on their surface are assembled at the cellular membrane, from which they bud (Zufferey *et al.*, 1997). Upon infection, the luciferase gene encoded by the lentivirus genome is expressed, allowing accurate quantification of viral entry.

Materials and Reagents

1. 50 mL Falcon tubes (VWR International, catalog number: 734-0448)
2. Clear bottom 6-well tissue-culture treated plate (Scientific Laboratory Supplies, FalconTM, catalog number: 353046)
3. Pipette tips (STARLAB, catalog numbers: S1110-3700 [10/20 µL XL Graduated TipOne[®]]; S1111-1206-C [200 µL Yellow Bevelled TipOne[®] Tip]; S1112-17200 [1,250 µL XL Graduated TipOne[®]])
4. Serological pipettes (Corning, catalog numbers: 4101 [10 mL StripetteTM]; 4051 [5 mL StripetteTM]; 4251 [25 mL StripetteTM])
5. Opti-MEMTM (Thermo Scientific, GibcoTM, catalog number: 11058021, storage conditions: 4°C, shelf life: 12 months)
6. Disposable weighing boats 85 × 85 × 24 mm, PS, medium, white, anti-static (VWR, catalog number: 10770-448, storage conditions: room temperature)
7. 7 mL polycarbonate polypropylene screw cap bijous (container for storage of small-volume samples) (STARLAB, catalog number: E1412-0710)
8. 96-well Delta-treated (hydrophilic surface that promotes cell attachment and growth) White flat-bottom plate (Fisher Scientific, Nunc, MicroWell, catalog number: 10182831)
9. Tissue culture flasks (Greiner Bio-One, catalog numbers: 660160 [175 cm²], 658170 [75cm²])
10. 1.5 mL Microcentrifuge sterile Eppendorf tubes (STARLAB, TubeOne[®], catalog number: S1615-5510)
11. Millex-GP syringe filter unit, 0.22 µm filter, polythersulfone, 33 mm, gamma sterilised (Merck, Millipore, catalog number: SLGP033RS, storage conditions: room temperature)
12. Human Embryonic Kidney 293T, HEK293T cells (ATCC[®], catalog number: CRL-3216TM, storage conditions: liquid nitrogen vapour phase)
13. Baby Hamster Kidney-21, BHK-21 cells (ATCC[®], catalog number: CCL-10TM, storage conditions: liquid nitrogen vapour phase)
14. Plasmid DNA: ACE2 receptors (pDISPLAY expression vector, codon-optimised, N-terminal signal peptide [the murine Ig κ-chain leader sequence], C-terminal HA-tag), SARS-CoV Spike, SARS-CoV-2 Spike (pcDNA3.1(+), codon-optimised, C-terminal FLAG-tag) (BioBasic, Canada [Conceicao *et al.*, 2020]), p8.91, CSFLW, VSV-G (pcDNA3.1(+) expression vector) (available upon request), pcDNA3.1(+) (Thermo Scientific, Invitrogen, catalog number: V79020) and pDISPLAYTM (Thermo Scientific, InvitrogenTM, catalog number: V66020)
15. Sera or antibodies for neutralisation assays, with relevant biological risk assessment and ethical approvals in place
16. Dulbecco's modified Eagle's medium, DMEM (Merck, Sigma-Aldrich, catalog numbers: D5796 [with phenol red]; D1145 [phenol red free], storage conditions: 4°C, 12 months)

17. Foetal bovine serum, FBS (Life Science Production, catalog number: S-001A-BR, -20°C)
18. Penicillin-Streptomycin, 10,000 U/mL (Thermo Scientific, Gibco™, catalog number: 15240122, storage conditions: -20°C, shelf life: 12 months)
19. Sodium pyruvate, 100 mM (Thermo Scientific, Gibco™, catalog number: 11360070, storage conditions: 4°C, shelf life: 12 months)
20. EDTA (0.5 M), pH 8.0, RNase-free (Thermo Scientific, Ambion®, catalog number: AM9269G)
21. 1× Trypsin-EDTA, 0.25%, phenol red (Thermo Scientific, Gibco™, catalog number: 2520072, storage conditions: -20°C long-term, 4°C while in use, shelf life: 24 months)
22. *TransIT-X2®* Dynamic Delivery System (Mirus, catalog number: MIR 6000, storage conditions: -20°C, shelf life: 12 months)
23. Polyethyleneimine, PEI (Merck, Sigma-Aldrich, catalog number: 408727, storage conditions: 4°C)
24. Nuclease-free, autoclaved, 0.2 µm filtered DEPC-treated water (Ambion, catalog number: AM9906, storage conditions: room temperature)
25. Hydrochloric acid 36.5-38.0%, Bioreagent, for molecular biology (Sigma-Aldrich, catalog number: H1758-100 mL, storage conditions: room temperature)
26. Bright-Glo™ Luciferase Assay System (Promega, catalog number: E2650, storage conditions: -20°C)
27. 55 mL StarTub PVC reagent reservoirs (STARLAB, sterile individually wrapped, catalog number: E2310-1010)
28. DMEM-10% (see Recipes)
29. Working solution of 1 mg/mL PEI (see Recipes)

Equipment

1. Microbiological safety cabinet, BSL-2 (CAS, Biomat 2 – class 2 complies with BS EN 12469:2000)
2. CO₂ incubator (PHC Europe B.V., PHCbi, catalog number: MCO-170AICD-PE)
3. -86°C ultra-low temperature freezer (PHCbi, Panasonic, vip plus, model: MDF-DU900V)
4. -20°C Medical freezer with 14 storage drawers (Liebherr, Profiline, model: G5216)
5. 4°C refrigerator (VDW CoolSystems, Labcold, Sparkfree, model: RLV0217)
6. Sub aqua 5 plus water bath (Fisher Scientific, Grant, model: 13251183)
7. Automated pipettor for serological pipettes (Fisher Scientific, Thermo Scientific™, S1 Pipet Fillers, catalog number: 10072332)
8. Single-channel pipettes (Gilson, Pipetman L, catalog numbers: FA1001M [P2L 0.2-2 µL], FA1003M [P20L 2-20 µL]; FA1005M [P200L 20-200 µL]; FA1006M [P1000L 100-1,000 µL])
9. Multi-channel pipettes (Thermo Scientific™, Finnpipette™ F2 multichannel pipette, catalog numbers: 4662010 [8-well 5-50 µL]; 4662070 [12-well 30-300 µL])
10. Inverted microscope for cell culture (Leica Microsystems, model: DMI1-S 40/0.45)
11. Haemocytometer (Fisher Scientific, Hirschmann™ Bright Lined Counting Chambers, catalog number: 105289616)
12. Centrifuge machine (Kendo laboratory product, Sorvall Legend RT, EASYset, model: 75004373)
13. Benchtop autoclave (Fisher Scientific, Astell scientific, catalog number: 12755375)
14. GloMax® Discover Microplate Reader (Promega, catalog number: GM3000)

Software

1. Microsoft Excel (Microsoft 365 for Windows, www.microsoft.com)
2. GraphPad Prism (Version 8.4.2, GraphPad Software for Windows, San Diego, California USA, www.graphpad.com)
3. GloMax® Discover System Software (Version 3.2.3, Promega, Southampton, United Kingdom www.promega.co.uk)

Procedure

A. Generation of SARS-CoV-2 and SARS-CoV pseudotyped virus particles

1. Maintain HEK293T cells for pseudoparticle production in 25 mL of DMEM-10% (see Recipes) in a 75 cm² tissue culture flask.
2. Seed HEK293T cells at a concentration of 7.5×10^5 cells per well in a 6-well plate in 3 mL of DMEM-10%, for the total number of wells required.
3. Agitate cells in the plate using a rapid up-down, left-right movement. This will ensure cells are evenly distributed and do not clump. Incubate at 37°C, 5% CO₂ overnight.
4. The next day, set up transfection mixes in the afternoon. The seeded HEK293T cells should be between 60-80% confluent for optimal transfection efficiency. Set up transfections for the SARS-CoV-2 S or SARS-CoV S plasmid, alongside an empty vector negative control (no glycoprotein, no GP) and a positive control. For instance, if the SARS-CoV-2 S and SARS-CoV S plasmids are in a pcDNA3.1 backbone, use an empty pcDNA3.1 plasmid as your no GP control. Generally, a VSV-G plasmid is used as a positive control, as it is trans-encapsidated into the HIV-1 particle efficiently (*i.e.*, it pseudotypes well).
5. In a sterile 1.5 mL Eppendorf tube, add 100 µL of Opti-MEM along with 0.6 µg of p8.91 plasmid (encoding for HIV-1 gag-pol), 0.6 µg of CSFLW plasmid (lentivirus backbone expressing Firefly luciferase), and 0.5 µg of glycoprotein (SARS-CoV-2 S, SARS-CoV S or VSV-G) or empty vector (pcDNA3.1) per well. Incubate for 5 min at room temperature.
6. In a separate 1.5 mL Eppendorf tube, add 100 µL of Opti-MEM plus and 10 µL of PEI (1 µg/mL) per transfection and incubate for 5 min at room temperature.
7. For each 100 µL transfection mix of DNA in Opti-MEM, add 100 µL of PEI in Opti-MEM and mix vigorously with a pipette ten times. Incubate at room temperature for 20 min.
8. Add 200 µL of the volume of the transfection mix in a dropwise manner to each well of the 6-well plate and incubate overnight at 37°C, 5% CO₂.
9. The next morning, use a serological pipette to gently remove the media from wells containing the transfection mix by tilting the dish towards you and aspirating from the edge of the well, being careful not to disturb the monolayer. Replace with 3 mL of DMEM-10%. Incubate overnight at 37°C, 5% CO₂ for 24 h.
10. Harvest cell supernatants containing pseudotyped virus particles and transfer to a 50 mL Falcon, pooling similarly transfected wells, and store at 4°C. Replace the media with 3 mL of DMEM-10% per well, and incubate at 37°C, 5% CO₂ for 24 h.
11. Harvest the cell supernatants containing pseudotyped virus particles and pool with pseudoparticles harvested the day before. Centrifuge at 2,500 × g for 10 min at 4°C to remove cellular debris.
12. Aliquot 4-5 mL of pseudoparticles into bijous and freeze at -80°C until further use.

Note: Larger volume of pseudoparticles can also be prepared in 10 cm² culture dishes. The necessary cell seeding density, DNA concentrations and volumes required for this setup can be found in Table 1, with the corresponding values for the 6-well plate format noted alongside. Steps A9-A12 remain the same regardless of the dish size used, changing only the volume of media required.

Table 1. Quick-guide to generating lentiviral-based pseudotyped viruses

		6-well plate format	10 cm² dish format
Cell seeding density	HEK293T cells	7.5×10^5 per well in 3 mL total volume DMEM-10%	2×10^6 per culture dish in 10 mL total volume DMEM-10%
DNA mix	<i>Viral glycoprotein:</i> SARS-CoV-2/SARS-CoV Spike OR pcDNA3.1 empty vector (NE, negative control) OR VSV-G (positive control)	0.9 µg	1 µg
	p8.91 (HIV-1 gag/pol)	0.6 µg	1 µg
	pCSFLW (HIV-1 LTR, Firefly luciferase gene)	0.6 µg	1.5 µg
	Opti-MEM	100 µL	200 µL
Transfection reagent mix	PEI (1 µg/mL)	10 µL	20 µL
	Opti-MEM	100 µL	200 µL
Total volume per well	Opti-DNA + Opti-PEI mix	200 µL	400 µL
Media	Volume of DMEM-10% to replace after transfection or harvest	3 mL	10 mL

B. Testing SARS-CoV-2 and SARS-CoV pseudoparticle infectivity

1. Seed HEK293T cells at a density of 7.5×10^5 per well in a 6-well plate in a total of 3 mL of DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
2. Ensure plated cells are at 60-80% confluence to ensure optimal transfection efficiency. Set up transfection mixes to test pre-generated SARS-CoV-2 pseudoparticles. In a sterile 1.5 mL Eppendorf tube, add 200 µL of Opti-MEM along with 500 ng of human ACE2 plasmid per well to be transfected. Bring the TranIT-X2 transfection reagent to room temperature before use, add 2 µL (for every 1 µg of DNA) directly to the tube, and gently flick the tube to mix. Incubate at room temperature for 20 min.
3. Add 200 µL of the transfection mix dropwise to each well of the pre-plated cells and incubate overnight at 37°C, 5% CO₂.
4. Remove the media containing the transfection mix from the wells by tilting the dish towards you and aspirating from the edge of the well using a serological pipette, being careful not to disturb the monolayer. Add 1 mL of DMEM-10% per well and harvest the transfected cells. HEK293T cells have low adherence and come off the plate easily. As such, using the force of the pipetted liquid is sufficient to harvest cells, although care should be taken to ensure a single cell suspension is achieved without clumps. Trypsin should be avoided as this will unnecessarily cleave off the receptors, hampering future experimentation. Transfer to a 50 mL Falcon and dilute cells to 2×10^5 /mL with DMEM-10%.
5. Seed 100 µL of diluted cells (2×10^4 per well) into a flat, white-bottomed 96-well plate and incubate overnight at 37°C, 5% CO₂.
6. The next day, thaw an aliquot of SARS-CoV-2 and/or SARS-CoV pseudoparticles, along with the negative (pcDNA3.1, no GP) and positive (VSV-G) controls. Titrate the pseudoparticles in a clear-bottomed 96-well plate, starting with undiluted virus in the top row, and titrating 10-fold in DMEM-10%, for a final volume of 100 µL.
7. Gently remove the media from the white-plate seeded with human ACE2-transfected cells and add 100 µL titrated pseudoparticles. Incubate for 48 h at 37°C, 5% CO₂.
8. Remove the media from the wells by tilting the dish towards you and aspirating from the edge of the well

using a multi-channel pipette and add 50 μ L Bright-GloTM diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min and then measure the luciferase signal on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.

9. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.

C. ACE2 receptor usage screen using SARS-CoV-2 and SARS-CoV pseudotyped virus particles (Conceicao *et al.*, 2020)

1. Maintain BHK-21 cells in 25 mL DMEM-10% in a 75 cm² tissue culture flask. Seed BHK-21 cells in 24-well plates at 1×10^5 /well in DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
2. Ensure plated cells are at 60-80% confluence to ensure optimal transfection efficiency. Set up transfection mixes in 100 μ L of Opti-MEM along with 500 ng of different species of ACE2-expressing constructs or an empty vector control (*e.g.*, pDISPLAY). Bring the TranIT-X2 transfection reagent to room temperature before use and add 3 μ L for every 1 μ g of DNA directly to the tube and gently flick the tube to mix. Incubate at room temperature for 20 min.
3. Add 100 μ L of the transfection mix dropwise to each well of the pre-plated BHK-21 cells and incubate overnight at 37°C, 5% CO₂.
4. Remove the media containing the transfection mix from the wells and add 0.5 mL of 2 mM EDTA in PBS per well to harvest the transfected cells. Transfer to a bijou and dilute cells to 2×10^5 /mL with DMEM-10%.
5. Seed 100 μ L of diluted cells (2×10^4 per well) into a flat, white-bottomed 96-well plate and incubate overnight at 37°C, 5% CO₂.
6. Remove media from cells and infect with SARS-CoV-2 or SARS-CoV pseudoparticles equivalent to 10^6 - 10^7 relative light units (RLU), or the no GP control at the same dilution and incubate for 48 h at 37°C, 5% CO₂.
7. Remove the media from the wells and add 50 μ L of Bright-GloTM diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min then read on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.
8. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.

D. Neutralisation assay using SARS-CoV-2 and SARS-CoV pseudotyped virus particles

1. Prior to setting up neutralization assays, seed HEK293T cells at a density of 7.5×10^5 per well in a 6-well plate in a total of 3 mL of DMEM-10%. Incubate overnight at 37°C, 5% CO₂.
2. Ensure plated cells are at 60-80% confluence to ensure optimal transfection efficiency. In a sterile 1.5 mL Eppendorf tube, add 200 μ L of Opti-MEM along with 500 ng of human ACE2 plasmid per well to be transfected. Bring the TranIT-X2 transfection reagent to room temperature before use and add 2 μ L for every 1 μ g of DNA directly to the tube and gently flick the tube to mix. Incubate at room temperature for 20 min.
3. Add 200 μ L of the transfection mix dropwise per well of pre-plated cells and incubate overnight at 37°C, 5% CO₂.
4. Set up neutralisation assays by diluting sera/monoclonal antibodies (mAbs)/inhibitors considering the dilution series to be used and the final volume after addition of pseudoparticles. For example, sera to be titrated using a 2-fold dilution series starting at a 1:10 dilution would require 10 μ L sera per well in 100 μ L serum free DMEM. The same is applicable for mAbs or inhibitors with a known concentration.
5. Add 100 μ L of diluted sera/mAbs/inhibitors in triplicate to the top row of a flat white-bottomed 96-well plate. Add 50 μ L of serum free media to all remaining wells. Remove 50 μ L from the top row and titrate 2-fold down the plate, mixing well before each titration. Do not titrate into the bottom row. This whole row will be used as the untreated control.

6. Thaw an aliquot of SARS-CoV or SARS-CoV-2 pseudoparticles and dilute in serum free DMEM, equivalent to $\sim 10^6$ RLU and add 50 μ L per well, including the untreated controls. Incubate for 1 h at 37°C, 5% CO₂.
7. Remove the media from the 6-well plates transfected with human ACE2. Add 1 mL of DMEM-10% per well and harvest the transfected cells. HEK293T cells have low adherence, so come off the plate easily; therefore, the force of the pipetted liquid should be sufficient to harvest cells (see B4 above). Transfer to a 50 mL Falcon and dilute cells to 2×10^5 /mL with DMEM-10%.
8. Seed 100 μ L of diluted cells (2×10^4 per well) onto each well containing sera/mAb/inhibitor with pseudoparticles and the untreated controls. Incubate for 48 h at 37°C, 5% CO₂.
9. Remove the media from the wells and add 50 μ L of Bright-Glo™ diluted 1:1 with serum free, phenol red free DMEM. Incubate the plate in the dark for 5 min then read on a GloMax Multi+ Detection System under the luminescence protocol with 0.5 s integration.
10. Export the CSV file generated on a USB flash drive for analysis using Microsoft Excel and plot data on GraphPad Prism.

Data analysis

A. Testing SARS-CoV-2 and SARS-CoV pseudoparticle infectivity

1. After preparing a batch of pseudoparticles, their infectivity can be tested by titrating them on target cells that have been transfected to express the host receptor (ACE2) of the pseudotyped attachment protein (Spike) for SARS-CoV and SARS-CoV-2. Undiluted pseudotyped virus (“1”) is titrated 10-fold with DMEM-10% down a 96-well plate in triplicate (“10”, “100”, “1,000” etc.) (**Figure 1A**).
2. Measuring the luciferase signal of the pseudoparticles will generate a CSV file that can be exported onto a USB flash drive and analysed on Microsoft Excel. These results can then be plotted on GraphPad Prism to show the mean \pm SD for each pseudoparticle.
3. The no GP negative control serves as an indication of background luciferase signal, and only values above this at each corresponding dilution should be considered as a true luciferase signal for the pseudoparticles being tested (**Figure 1B, black line**). Generally, a minimum of ~ 2 log dynamic range between the no GP and the pseudotyped virus and a RLU signal between $10^{5.5}$ and $10^{7.5}$ RLU (**Figure 1B, shaded area**) is sufficient for use in subsequent assays. The titration series will also help to determine the lowest usable dilution of the pseudoparticles to still obtain meaningful luciferase values above the background. Of note: the following data to be discussed was generated for illustrative purposes only.
4. For example, when considering the luciferase values obtained for SARS-CoV, although these are above the no GP control at the highest dilution, the difference between the two is only ~ 1 log, which falls outside our criteria for use (**Figure 1B, blue line**). When titrating the pseudoparticles, the luciferase values also fall quite quickly below the lower limit of the workable range at a 1:10 dilution and to the same values as the no GP control by the 1:1000 dilution, making the titre of this preparation of SARS-CoV pseudoparticles unsuitable for use in subsequent assays (Figure 1B).
5. In comparison, the luciferase signals obtained for SARS-CoV-2 are ~ 2 log above the background no GP control, and a dilution of 1:10 of the pseudoparticles would be within the workable range for use in subsequent assays, which is lost at a 1:100 dilution (**Figure 1B, orange line**).
6. The VSV-G pseudoparticles are a positive control within the assay, where the luciferase values observed should be above 107 RLU (**Figure 1B, green line**).

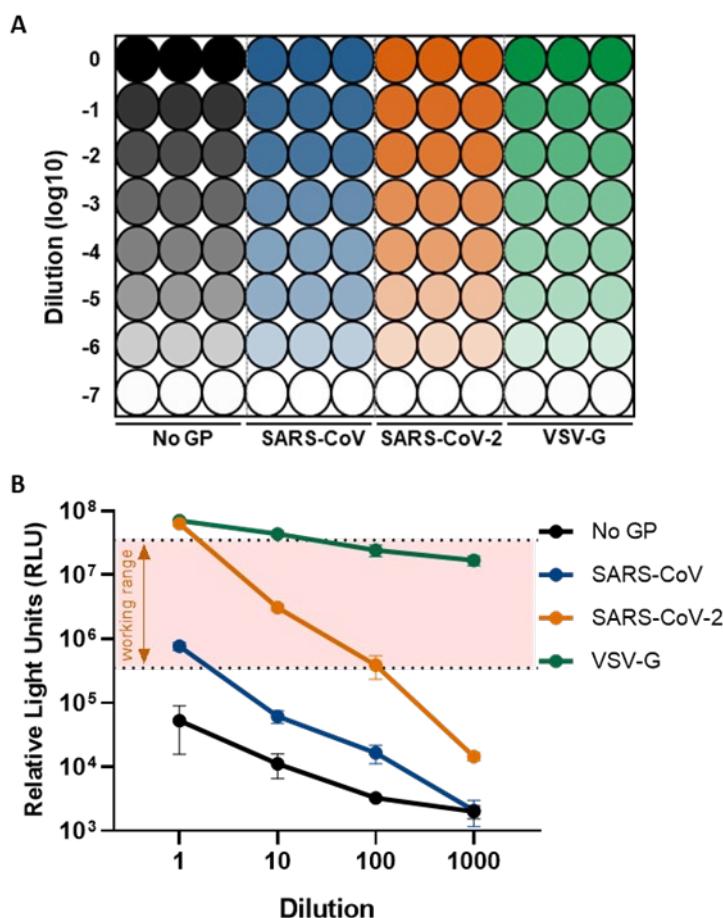


Figure 1. Testing the infectivity of SARS-CoV and SARS-CoV-2 pseudoparticles.

(A) For SARS-CoV and SARS-CoV-2 pseudoparticle titrations, 10-fold serial dilutions of the supernatant are used to infect HEK293T cells transiently expressing the human ACE2 receptor in a white flat-bottomed 96 well-plate. Negative (no GP) and positive (VSV-G) controls are also included in the experiment. Each condition is tested in triplicate. (B) Two days after infection, signal luciferase values are measured and plotted as the mean \pm SD. The no GP control is indicative of the background, and only pseudoparticle values above this should be considered as true infectivity, matched at each dilution of the virus. The use of pseudoparticles for subsequent neutralisation assays and receptor usage screens should show ~ 2 log dynamic range between the no GP and pseudotyped virus and fall between a working range of $10^{5.5}$ to $10^{7.5}$ (shaded area).

B. ACE2 receptor usage screen using SARS-CoV-2 and SARS-CoV pseudotyped virus particles

- Infection of cells expressing various species ACE2 receptors with SARS-CoV or SARS-CoV-2 pseudoparticles set up in triplicate yields luciferase signals that can be plotted alongside each other (mean \pm SD) to depict raw values. These data also give an idea of the general trend of receptor tropism across different viruses (Figure 2A).
- For example, water buffalo and goat ACE2 permit the entry of SARS-CoV and SARS-CoV-2 pseudoparticles well, which is less evident for little brown bat ACE2. Differences between viruses can be observed for civet ACE2, which permits the entry of SARS-CoV more efficiently than SARS-CoV-2 (Figure 2A). These experiments should be conducted at least three times on three separate occasions, with

- representative data shown. A subset of ACE2 receptors are shown in Figure 2A, but a more in-depth, wider analysis can be found in Supplementary Figure 3A and 3C in Conceicao *et al.* (2020).
3. Two negative controls are set up in this screen. The first is an empty vector control (pDISPLAY) to ensure any signal measured is solely from overexpression of the ACE2 receptor. The second is infection of cells with the no GP control pseudoparticle preparation to ensure luciferase signals can be attributed to the pseudotyped viruses and provides a baseline for the background (**Figure 2A**).
 4. The raw luciferase signals can then be used to determine the relative usage of non-cognate host ACE2 receptors (water buffalo, civet, goat, little brown bat) to a known or cognate host receptor, in this case, human ACE2. The mean percentage from three separate experiments performed on different days are used to obtain these values. The luciferase value for human ACE2 is set to 100%, and the luciferase values for unknown host receptors and the negative control are then expressed as a percentage relative to human ACE2.
 5. These results can also be shown as a heatmap using a colour gradient to show different trends of receptor usage. For example, human ACE2 (100%) is set as green. Expression lower than this is shaded from green to red, indicative of poorer ACE2 usage relative to human ACE2. Values above 100% are shown as a darker green, suggestive of ACE2 usage equivalent to or greater than human ACE2 (**Figure 2B**). A subset of ACE2 receptors are shown in **Figure 2B**, but a more in-depth, wider analysis can be found in Figure 2 A in Conceicao *et al.* (2020).
 6. Further analysis can be carried out to compare the receptor tropism of different species ACE2 between SARS-CoV and SARS-CoV-2 by plotting the percentage values for each virus against each other on an xy scatter graph, calculating the Pearson's correlation coefficient, and plotting a linear line of regression fitted with 95% confidence intervals (data not shown). An example of such analysis can also be found in Supplementary Figure 5 in Conceicao *et al.* (2020).

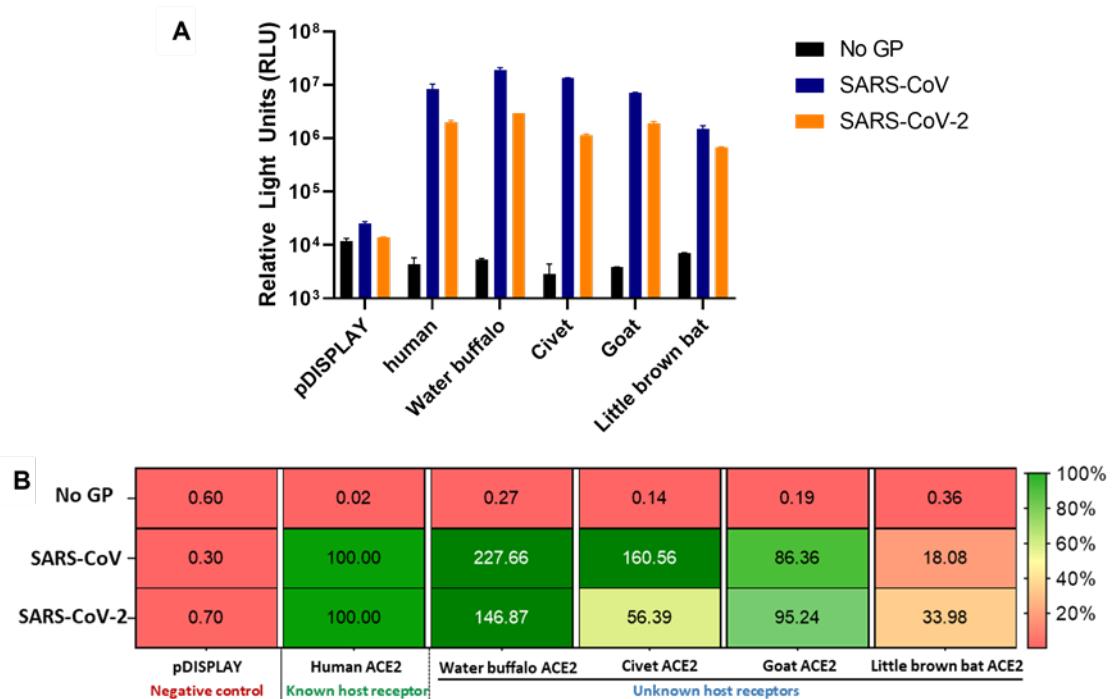


Figure 2. ACE2 receptor usage screen using SARS-CoV and SARS-CoV-2 pseudoparticles.

In our study, pseudoparticles were used as a surrogate to live viruses to assess receptor tropism of SARS-CoV and SARS-CoV-2 with different species of ACE2 receptors. Pseudoparticles were employed to assess SARS-CoV and SARS-CoV-2 Spike glycoproteins' usage of ACE2 receptors from different species and presented as (A) raw luciferase signal values or (B) a percentage relative to human ACE2.

Negative controls were included for pseudoparticles bearing an empty vector control (No GP) or mock-transfected with an empty vector in place of an ACE2 receptor (pDISPLAY). Data are presented as mean \pm SD of triplicate values, with each experiment performed three times on three separate occasions, and representative data shown.

C. Neutralisation assay using SARS-CoV-2 and SARS-CoV pseudotyped virus particles

1. Neutralisation assays using pseudotyped viruses are a low-biocontainment alternative to using live virus and can be performed in a relatively high-throughput manner. These neutralisation assays can be performed on mAbs, sera or any other drug or inhibitor that has the potential to inhibit viral entry. The data discussed herein have been generated for illustrative purposes only.
2. Inhibitors (mAbs/sera/drugs) of a known concentration can be titrated down a 96-well plate in triplicate to determine the extent of inhibition of SARS-CoV-2 entry. This is done by taking an average of the untreated controls and expressing the RLU values for each individual replicate of the mAb of interest relative to this. These can then be plotted as the mean \pm SD and should be repeated a minimum of three times, with representative data shown:
$$(\text{RLU individual replicate of mAb}/\text{RLU average of untreated}) \times 100$$
3. The inhibitory concentration of 50% (IC₅₀) should be indicated on a graph along with the untreated, no mAb control (100%). Values below this IC₅₀ line are indicative of S-mediated inhibition of entry, which can be calculated at each concentration. For example, mAb2 is able to inhibit SARS-CoV-2 S entry by ~80% (20% of untreated) at 100 $\mu\text{g}/\text{mL}$. The values obtained for mAb2 at all concentrations tested are below the IC₅₀ value, so lower concentrations would need to be tested to determine the limit of inhibition. In contrast, mAb3 inhibits SARS-CoV-2 S entry by ~90% at 100 $\mu\text{g}/\text{mL}$, but at 12.5 $\mu\text{g}/\text{mL}$, the inhibition is now above the IC₅₀ threshold (**Figure 3A**).
4. There may also be examples of mAbs that do not inhibit SARS-CoV-2 S, as with mAb1. It may be possible that when inhibition of entry is not observed, a slight increase above the 100% threshold is seen. The mechanisms causing this increase are still unknown and under investigation, but for the purposes of this assay, the conclusion that the mAb does not neutralise SARS-CoV-2 S is sufficient (**Figure 3A**). Examples of this sort of analysis can be found in Thakur *et al.* (2021).
5. When determining the inhibition of viral entry from individuals who have antibodies against SARS-CoV-2 S, whether that be following natural infection or vaccination, a neutralising antibody titre is usually calculated to enumerate the level of SARS-CoV-2 S neutralisation. The simplest method of calculating this is by calculating the average RLU of the untreated controls and determining the IC₅₀ value, i.e., 50% of the no sera control. The neutralisation titre is then calculated as the inverse of the dilution at which there is 50% inhibition of the no sera luciferase values in all triplicate wells. These titres can then be tabulated or plotted on a log scale.
6. For example, the titre of 256 for serum sample 1 and 512 for serum sample 3 indicates that IC₅₀ was calculated at a dilution of 1:256 and 1:512, respectively. The conclusion that could be drawn from this is that serum sample 3 is able to neutralise SARS-CoV-2 S-mediated entry more efficiently than serum sample 1 and therefore has higher antibody titres (**Figure 3B**).
7. For serum sample 4, this value has been plotted as 1,024, which is the upper limit of detection (ULoD) for this assay. This means that this sample was able to inhibit 50% of the luciferase signal in all wells and at the lowest dilution that was tested. This serum would have to be retitrated with a broader dilution series to determine the neutralisation titre. For serum sample 2, none of the wells in the dilution range yielded a recordable IC₅₀. The neutralisation titre is therefore plotted as an arbitrary value below the lower limit of detection (LLoD), which in this case is 40, but would be reported as <40 as the true titre is unknown (**Figure 3B**). Examples of IC₅₀ neutralisation titres using this method can be found in Figure 4F in Thakur *et al.* (2021) and in Figure 2D and 2E in Graham *et al.* (2020).

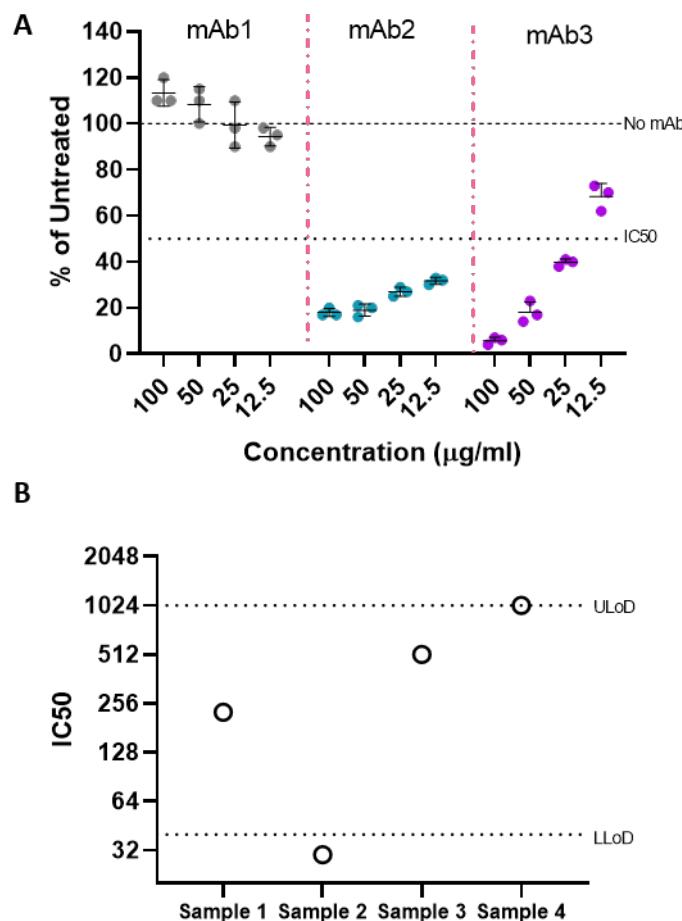


Figure 3. Neutralisation assays using SARS-CoV-2 pseudoparticles.

SARS-CoV-2 Spike neutralisation assays were performed in the presence of (A) monoclonal antibodies (mAbs), presented as a percentage relative to untreated controls or (B) sera samples, with data expressed as neutralising titres. SARS-CoV-2 pseudoparticles were incubated with mAbs or sera for 1 h prior to addition of human ACE2-expressing HEK293T cells. Inhibition of SARS-CoV-2 Spike-mediated viral entry was determined by calculating the concentration (mAbs) or dilution (sera) at which there is a 50% reduction in luciferase signal (IC₅₀). Data represent the mean \pm SD of triplicate values, with each experiment performed three times on three separate occasions, and representative data shown.

Notes

1. Procedure A, step 11 mentions centrifugation of pseudoparticle preparations prior to use in subsequent assays to remove cellular debris. Other protocols require further filtration of pseudoparticles using a 0.45 μm filter before storage. This step is not carried out in our lab, as we have observed a reduction in infectivity following filtration.
2. Manufacturers usually recommend an optimal confluence of 60-80% for transfection. Therefore, it may be necessary to change the seeding density depending on the characteristics of the cells used. For example, if the cell types used are larger (e.g., BHK-21 cells are larger than HEK293T cells) or have a high doubling rate, we recommend starting at a lower seeding density. On the other hand, if the cells are smaller, or have a slower growth rate, and are difficult to reach confluence (e.g., Calu3 cells) or indeed are suspension cells, you may want to start with a higher seeding density. In both instances, we recommend testing different seeding densities

to find the optimal for any given experiment.

3. An example of optimal confluence of HEK293T cells prior to infection (60-80%) is shown in **Figure 4**. Cells should be evenly distributed across the well (i.e., no clumping or aggregation in one area), with visible gaps in the monolayer (**Figure 4A**). The cell morphology of the HEK293T cells should appear flat and polygonal at confluence, which indicates adherence to the plastic (**Figure 4B**).

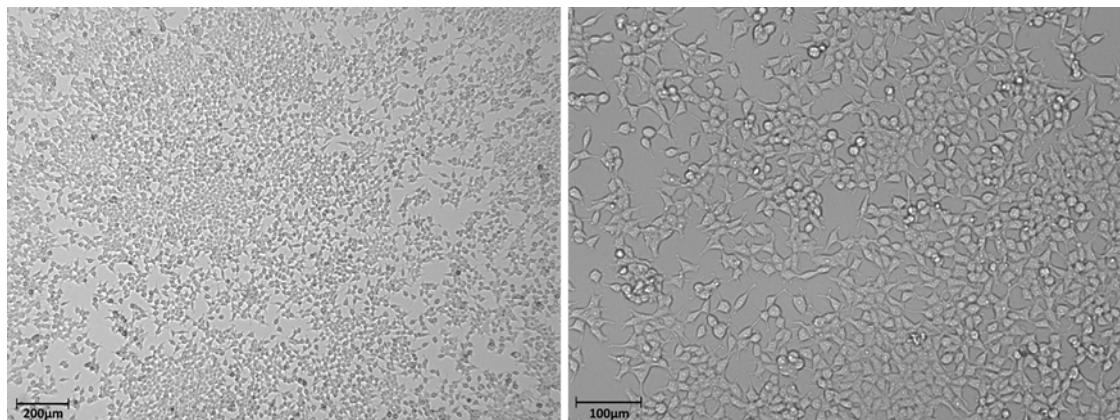


Figure 4. Brightfield image to illustrate 60-80% optimal confluence of HEK293T cells.

HEK293T cells were seeded at 7.5×10^5 in a 6-well dish in 3mL of DMEM-10%. On the following day, cells should be between 60-80% confluent for optimal transfection. (A) Example of ~80% confluent HEK293T at 4 \times magnification, with cells appearing evenly distributed across the well and visible gaps. (B) Higher power magnification (10 \times) of HEK293T cells where cells should appear bright, flat and polygonal prior to transfection.

4. The concentrations of DNA used for transfection at various steps have been optimised for use in these assays. It is important that any plasmids used are optimised to account for variability in vector platform and codon-optimisation.
5. A higher signal (in the 10^4 - 10^5 RLU range) in the no GP control can sometimes be seen, likely the result of non-specific uptake of ‘bald’ pseudoparticles or debris from producer cells. To reduce this background signal, care should be taken to ensure producer cells are not transfected at low confluence as this can cause cytopathic effects (CPE) to develop.
6. All the experiments conducted here were performed with over-expressed ACE2 only. Co-expression of the serine protease TMPRSS2, which is required for S2 protein cleavage to S2', can facilitate the fusion of viral and cellular membranes and cleavage of the Spike protein (Hoffmann *et al.*, 2020). TMPRSS2 was not included in our host range assays as we wanted to specifically examine the effects of different ACE2s – indeed, over-expression of TMPRSS2 led to ACE2 restrictions being masked (Conceicao *et al.*, 2020).
7. Other formulae can be used to determine the IC50 value, yielding different titres. This is acceptable if the same method is used throughout analyses and the method used are described in full. Other formulae for calculating neutralisation titres include (1) using a non-linear regression analysis tool on GraphPad Prism after plotting data on an XY graph to interpolate neutralisation values (Ferrara and Temperton, 2018), (2) interpolating the point at which infectivity is reduced to 50% of the value of a no serum control sample using a fixed formula (Logan *et al.*, 2016), and (3) determining the highest dilution at which complete neutralisation is seen in all replicate wells and considering other wells that also show neutralisation. Neutralisation is then calculated by inputting these values into a Spearman Karber formula (Lambe *et al.*, 2021).
8. Neutralisation titres do not always need to be recorded as IC50 values. Other cut-off points can be chosen dependent on the level of neutralisation expected in a given assay, and to provide a more stringent measure of neutralisation (e.g., 80% neutralisation, IC80).
9. The surface expression of different ACE2 receptors may differ, which may affect the level of Spike-ACE2 interaction leading to misinterpretation of results. Therefore, it is important to investigate and normalise the cell surface expression of the ACE2 receptors used. The mammalian ACE2 receptors described and used herein

were HA-tagged at the C-terminus, which allowed detection of surface expression by flow cytometry. Additionally, protein expression was assessed by Western blotting (Conceicao *et al.*, 2020).

Recipes

1. DMEM-10%

DMEM supplemented with 10% FBS, 1% penicillin/streptomycin 10,000 U/mL, and 1% 100 mM sodium pyruvate, cultured at 37°C with 5% CO₂.

2. Working solution of 1 mg/mL PEI

- a. Weigh the viscous liquid to get 50 mg/mL in water (e.g., 0.42 g PEI + 8.4 mL water) and transfer to a sterile 50 mL Falcon.
- b. Place Falcon in a water bath set to 50°C and gently pipette up and down using a 1 mL pipette until fully dissolved.
- c. Dilute to 1 mg/mL with water (e.g., take 0.5 mL of your 50 mg/mL stock and add 24.5 mL water)
- d. The solution in its current state will be very basic. Adjust pH to 7 using diluted hydrochloric acid.
- e. Filter through a 0.22 µm filter and aliquot into 1.5 mL Eppendorf tubes.
- f. Store at -20°C long-term and at 4°C for up to one month while in use.

Acknowledgments

This work was supported by the following grants to Dalan Bailey: a UK Research and Innovation (UKRI, <https://www.ukri.org/>) Medical Research Council (MRC) New Investigator Research Grant (MR/P021735/1), UKRI Biotechnology and Biological Sciences Research Council (UKRI - BBSRC, <https://www.ukri.org/>) project grants (BB/R019843/1 and BB/T008784/1, student funding), and Institute Strategic Programme Grant (ISPG) to The Pirbright Institute (BBS/E/I/00007034, BBS/E/I/00007030 and BBS/E/I/00007039). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We would like to acknowledge the research papers from which the protocols described herein have been derived from, which include: “The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins” (Conceicao *et al.*, 2020), “Micro-fusion inhibition tests: quantifying antibody neutralization of virus-mediated cell-cell fusion” (Thakur *et al.*, 2021), and “Evaluation of the immunogenicity of prime-boost vaccination with the replication-deficient viral vectored COVID-19 vaccine candidate ChAdOx1 nCoV-19” (Graham *et al.*, 2020).

We would also like to thank the following for assistance in establishing the SARS-CoV and SARS-CoV-2 pseudotype systems: Ed Wright (Viral Pseudotype Unit, University of Sussex), Nigel Temperton (Viral Pseudotype Unit, University of Kent), Brian Willett (University of Glasgow Centre for Virus Research), Emma Bentley and Giada Mattiuzzo (National Institute for Biological Standards and Control), and Michael Letko (National Institute of Allergy and Infectious Disease).

Competing interests

Authors declare no conflicts of interest.

References

Bertram, S., Glowacka, I., Blazejewska, P., Soilleux, E., Allen, P., Danisch, S., Steffen, I., Choi, S. Y., Park, Y.,

- Schneider, H., Schughart, K. and Pohlmann, S. (2010). [TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells](#). *J Virol* 84(19): 10016-10025.
- Conceicao, C., Thakur, N., Human, S., Kelly, J. T., Logan, L., Bialy, D., Bhat, S., Stevenson-Leggett, P., Zagrajek, A. K., Hollinghurst, P., et al. (2020). [The SARS-CoV-2 Spike protein has a broad tropism for mammalian ACE2 proteins](#). *PLoS Biol* 18(12): e3001016.
- DePolo, N. J., Reed, J. D., Sheridan, P. L., Townsend, K., Sauter, S. L., Jolly, D. J. and Dubensky, T. W., Jr. (2000). [VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum](#). *Mol Ther* 2(3): 218-222.
- Ferrara, F. and Temperton, N. (2018). [Pseudotype Neutralization Assays: From Laboratory Bench to Data Analysis](#). *Methods Protoc* 1(1): 8.
- Graham, S. P., McLean, R. K., Spencer, A. J., Belij-Rammerstorfer, S., Wright, D., Ulaszewska, M., Edwards, J. C., Hayes, J. W. P., Martini, V., Thakur, N., et al. (2020). [Evaluation of the immunogenicity of prime-boost vaccination with the replication-deficient viral vectored COVID-19 vaccine candidate ChAdOx1 nCoV-19](#). *NPJ Vaccines* 5(1): 69.
- Grehan, K., Ferrara, F. and Temperton, N. (2015). [An optimised method for the production of MERS-CoV spike expressing viral pseudotypes](#). *MethodsX* 2: 379-384.
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N. H., Nitsche, A., Muller, M. A., Drosten, C. and Pohlmann, S. (2020). [SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor](#). *Cell* 181(2): 271-280 e278.
- Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M. and McKeating, J. A. (2003). [Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles](#). *Proc Natl Acad Sci U S A* 100(12): 7271-7276.
- Lambe, T., Spencer, A. J., Thomas, K. M., Gooch, K. E., Thomas, S., White, A. D., Humphries, H. E., Wright, D., Belij-Rammerstorfer, S., Thakur, N., et al. (2021). [ChAdOx1 nCoV-19 protection against SARS-CoV-2 in rhesus macaque and ferret challenge models](#). *Commun Biol* 4(1): 915.
- Logan, N., McMonagle, E., Drew, A. A., Takahashi, E., McDonald, M., Baron, M. D., Gilbert, M., Cleaveland, S., Haydon, D. T., Hosie, M. J. and Willett, B. J. (2016). [Efficient generation of vesicular stomatitis virus \(VSV\)-pseudotypes bearing morbilliviral glycoproteins and their use in quantifying virus neutralising antibodies](#). *Vaccine* 34(6): 814-822.
- Negrete, O. A., Levroney, E. L., Aguilar, H. C., Bertolotti-Ciarlet, A., Nazarian, R., Tajyar, S. and Lee, B. (2005). [EphrinB2 is the entry receptor for Nipah virus, an emergent deadly paramyxovirus](#). *Nature* 436(7049): 401-405.
- Salvador, B., Zhou, Y., Michault, A., Muench, M. O. and Simmons, G. (2009). [Characterization of Chikungunya pseudotyped viruses: Identification of refractory cell lines and demonstration of cellular tropism differences mediated by mutations in E1 glycoprotein](#). *Virology* 393(1): 33-41.
- Sharkey, C. M., North, C. L., Kuhn, R. J. and Sanders, D. A. (2001). [Ross River virus glycoprotein-pseudotyped retroviruses and stable cell lines for their production](#). *J Virol* 75(6): 2653-2659.
- Simmons, G., Reeves, J. D., Grogan, C. C., Vandenberghe, L. H., Baribaud, F., Whitbeck, J. C., Burke, E., Buchmeier, M. J., Soilleux, E. J., Riley, J. L., et al. (2003). [DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells](#). *Virology* 305(1): 115-123.
- Takada, A., Robison, C., Goto, H., Sanchez, A., Murti, K. G., Whitt, M. A. and Kawaoka, Y. (1997). [A system for functional analysis of Ebola virus glycoprotein](#). *Proc Natl Acad Sci U S A* 94(26): 14764-14769.
- Thakur, N., Conceicao, C., Isaacs, A., Human, S., Modhiran, N., McLean, R. K., Pedrera, M., Tan, T. K., Rijal, P., Townsend, A., Taylor, G., Young, P. R., Watterson, D., Chappell, K. J., Graham, S. P. and Bailey, D. (2021). [Micro-fusion inhibition tests: quantifying antibody neutralization of virus-mediated cell-cell fusion](#). *J Gen Virol* 102(1).
- Wool-Lewis, R. J. and Bates, P. (1998). [Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines](#). *J Virol* 72(4): 3155-3160.
- Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. and Trono, D. (1997). [Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo](#). *Nat Biotechnol* 15(9): 871-875