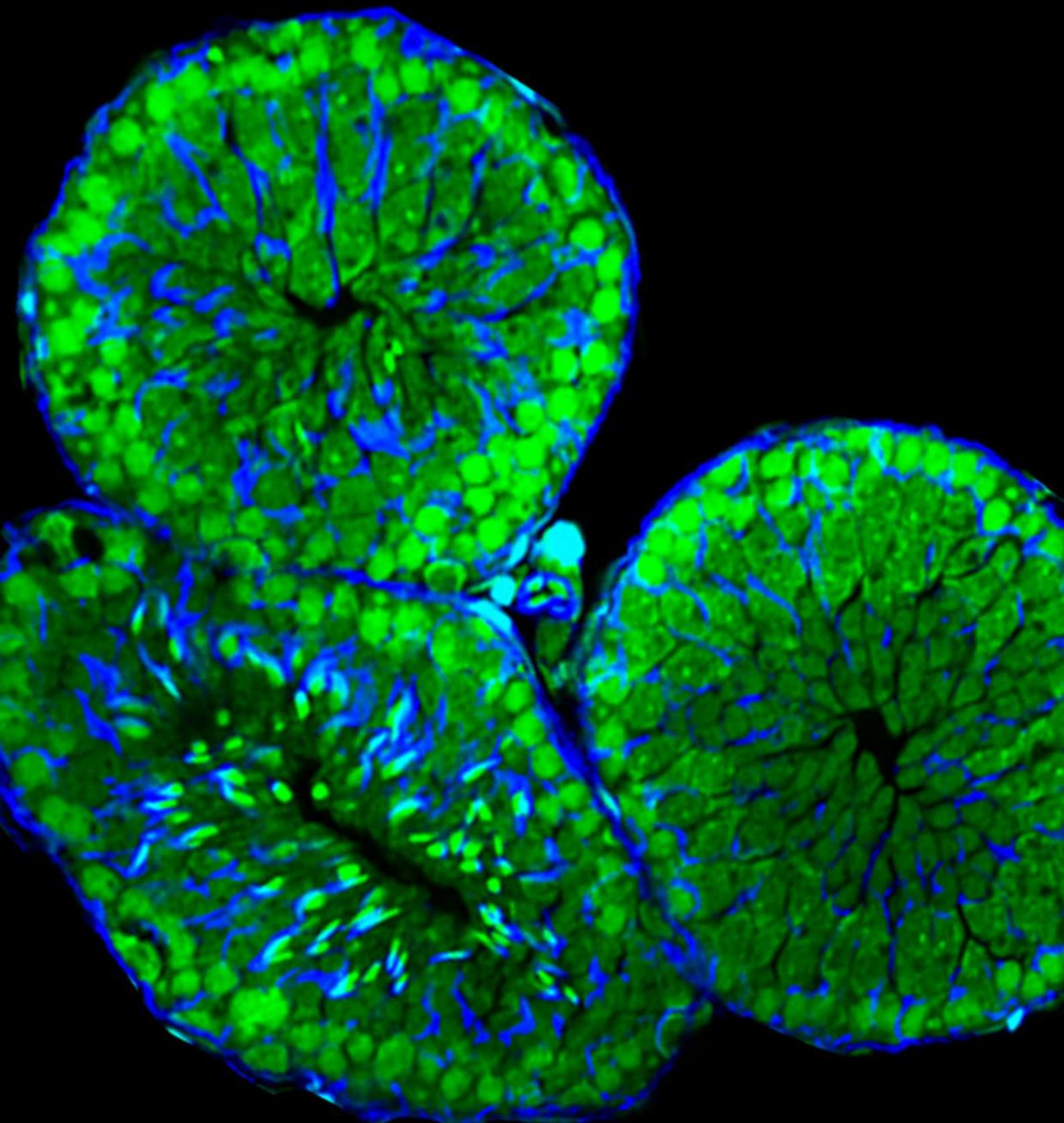
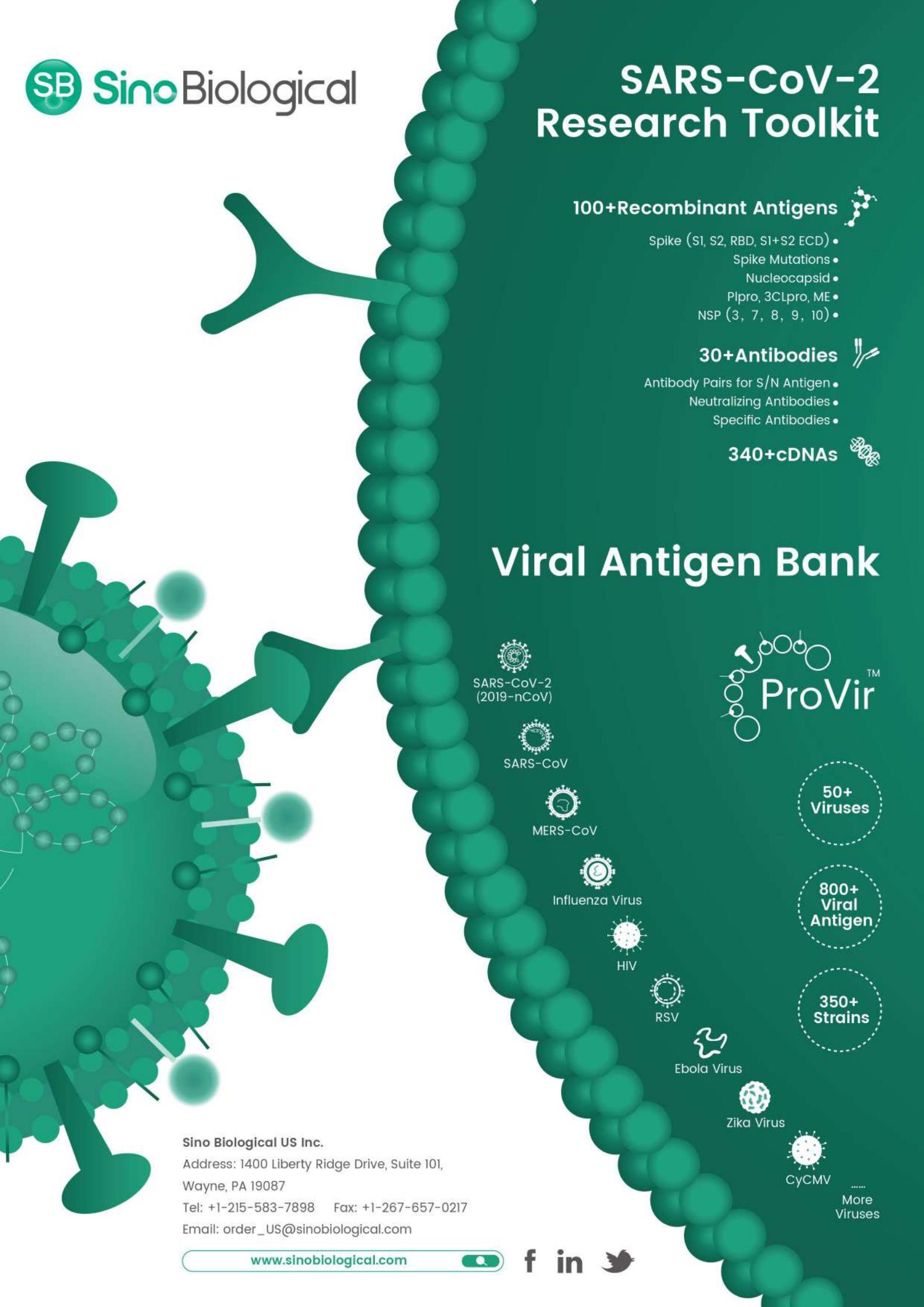


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Foreword

We are pleased to launch Bio-protocol first series of reprint collections, which consist of most widely used protocols published in 2018 and 2019, and reprint them as “Protocol Selections” highlighting a given research area or application. In this series, it is Protocol Selections focusing on immunology.

Established in 2011 by a group of Stanford scientists, Bio-protocol’s mission is to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. One primary method for Bio-protocol to publish this content is to invite contributions from authors that have published methods in brief that are used in results-oriented literature (called “Original research article” in the Protocol Selections) but not described in sufficient detail for others to replicate. Our survey carried out in 2018 showed that over 91% of users (2166 users) who tried their downloaded bio-protocols were able to successfully reproduce the experiment. This users’ feedback indicates that indeed most of bio-protocols (if not all) are highly reproducible.

In this reprint collection, we have selected 16 of the most-used immunology protocols based on published dates (published in last 2 years) as well as some metrics such as view number, download number and citations. We have to admit that the measurement of the “most-used” protocols is not absolutely accurate, in particular, given the fact that it would take time to cite a relatively new published protocol. That said, we believe this Protocol Selections well represents a collection of high-quality protocols contributed by outstanding researchers in the community.

Hopefully, you will find this collection intriguing and visit www.bio-protocol.org to check out the entire collection of protocols. Please feel free to email us (eb@bio-protocol.org) your feedback. Also, look forward to your contribution of protocols to Bio-protocol in the future.

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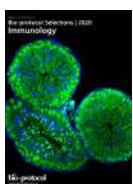
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Murine Pancreatic Islets Transplantation under the Kidney Capsule

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[Abstract] Type 1 diabetes (T1D) is an autoimmune disease caused by the lack of insulin-producing pancreatic beta cells leading to systemic hyperglycemia. Pancreatic islet transplantation is a valid therapeutic approach to restore insulin loss and to promote adequate glycemic control. Pancreatic islet transplantation in mice is an optimal preclinical model to identify new therapeutic strategies aiming at preventing rejection and optimizing post-transplant immuno-suppressive/-tolerogenic therapies.

Islet transplantation in preclinical animal models can be performed in different sites such the kidney capsule, spleen, bone marrow and pancreas. This protocol describes murine islet transplantation under the kidney capsule. This is a widely accepted procedure for research purposes. Stress caused in the animals is minimal and it leads to reliable and reproducible results.

Keywords: Type 1 diabetes, Pancreatic islets, Islet transplantation, Murine model

[Background] Many alternative sites for islet implantation have been reported so far in small animal models and the ideal site must be selected according to the technical advantages of the procedure to be used and for the purpose of the experiments. Bearing in mind that the kidney capsule is an extravascular site and it is not immunoprotected, pancreatic islet transplantation under the kidney capsule remains a surgical procedure with low mortality rates leading to hyperglycemia reversion within a few days. In addition, transplantation under the kidney capsule allows histological studies and formal demonstration of islet function (Cantarelli and Piemonti, 2011; Elisa Cantarelli *et al.*, 2013).

Materials and Reagents

1. Cotton applicators, sterile (CARLO ERBA Reagents, catalog number: 9.413 161)
2. 30 G x ½ in. needle (BD, catalog number: 305106)
3. 2 ml slip tip syringe (BD, catalog number: 302204)
4. BD INTRAMEDIC™ PE 50 (BD, catalog number: B427411)
5. Petri dish, Falcon® 50 x 9 mm Sterile (Corning, Falcon®, catalog number: 351006)
6. P200 pipette tip (SARSTEDT, catalog number: 70.760.002)
7. Eppendorf tubes volume 1.5 ml (Sigma-Aldrich, catalog number: T9661-1000EA)

Manufacturer: Eppendorf, catalog number: 022363204.

8. Silicone tubing adapter (2Biological Instruments, catalog number: SFM3-1550)
9. 1 ml syringe with 25 G needle (Ettore Pasquali, catalog number: 11.3500.05)
10. Suture Dermalon 5/0 19 MM (Covidien, catalog number: 1756-21)
11. Collagenase P (Roche Diagnostics, catalog number: 11213865001)
12. Betadine (MEDA PHARMA SpA, Farmacie Coli, catalog number: 023907076)
13. Avertin (Sigma-Aldrich, catalog number: T48402)
14. Sodium chloride ((NaCl) (CARLO ERBA Reagents, catalog number: FC72101100000)
15. Ketoprofen (PFIZER ITALIA Srl DIV.VET)
16. RPMI 1640 medium (Lonza, catalog number: 12-167F)
17. L-glutamine (Lonza, catalog number: 17-605E)
18. HEPES buffer (Lonza, catalog number: 17-737E)
19. Pen-Strep (Lonza, catalog number: 17-602E)
20. Fetal bovine serum (Euroclone, catalog number: ECS0180L)
21. Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific, GibcoTM, catalog number: 14175079)
22. Calcium chloride dihydrate (CaCl₂·2H₂O) (Sigma-Aldrich, catalog number: C5080)
23. Histopaque®-1077 (Sigma-Aldrich, catalog number: H8889)
24. RPMI/glutamine/HEPES/Pen-Strep/FBS (see Recipes)
25. HBSS/Ca/HEPES (see Recipes)

Equipment

1. Forceps (Graefe forceps, 100 mm, curved (ProSciTech, catalog number: T131C), Tweezers, style 3 (ProSciTech, catalog number: T03-212)
2. Scissor (ProSciTech, catalog number: TS103-200SB)
3. Surgical Shaver (2Biological Instruments, catalog number: 2BTOSRC)
4. Cautery (Global medical solutions, catalog number: BAA00)
5. Thermostatic bath (Thermo Fisher Scientific, Thermo ScientificTM, model: TSGP02)
6. Inverted microscope (Compact, Modular Stereo, Leica, model: Leica M60)
7. Incubator at 37 °C with 5% CO₂, relative humidity ambient to 80% [e.g., Series II 3110 Water-Jacketed CO₂ incubators (Thermo Fisher Scientific, Thermo ScientificTM, model: FormaTM II 3110 Series)]
8. Pipetman P20/P200/P1000 (Gilson)
9. Microsyringe 25 µl Hamilton syringe (Hamilton, catalog number: 80401)
10. Heating pad 25 x 40 cm, Two Temperature Range (2Biological Instruments, LCPH)
11. Herasafe KS, Class II biological safety cabinet with UV surface disinfection irradiator (Thermo Fisher Scientific, Thermo ScientificTM, model: HerasafeTM KS II, catalog number: 51022481)

Software

1. Prism software (GraphPad, USA)

Procedure

A. Preparation of islets for transplantation

1. Isolation of pancreatic islet using the appropriate protocol (Graham *et al.*, 2016)
 - a. Briefly, sacrifice animal by cervical dislocation, open the mouse abdominal cavity and cut through the peritoneum.
 - b. Place 3 ml of collagenase P into a 2 ml slip tip syringe with a 30 G needle. Perform the bile duct injection procedure and dissect the inflated pancreas, being careful not to cut the stomach, the intestines or other abdominal organs.
 - c. Pour the inflated pancreases in a collection tube on ice. When all pancreata have been resected, incubate for 15 min at 37 °C in water-bath. Add ice-cold HBSS/Ca/HEPES as quick as possible to stop the digestion process. Disrupt the pancreases by vigorously hand shaking the tubes.
 - d. Upon obtaining the digested tissue, the islets are separated by density gradient, recovered from the gradient interface, washed with RPMI 10% FBS and collected in a 50 ml Falcon tube containing 30 ml of RPMI 10% FBS.

Note: RPMI with serum is demonstrated to maintain (or even augment) glucose-stimulated insulin secretion in murine islets (Andersson, 1978).

2. Afterwards, 5 ml of islets is transferred into a Petri dish that is gently swirled to collect the islets at the center.
3. Under an inverted microscope, healthy islets are picked with a P200 sterile pipette tip. Healthy islets have smooth borders in the absence of dark centers (Figure 1). Visual examination of the islets can provide basic information regarding health. Hand-picked healthy islets are then transferred into a new Petri dish containing 3 ml of fresh RPMI 10% FBS.

Anticipate using one Petri dish for the totality of islets to be injected to one mouse.

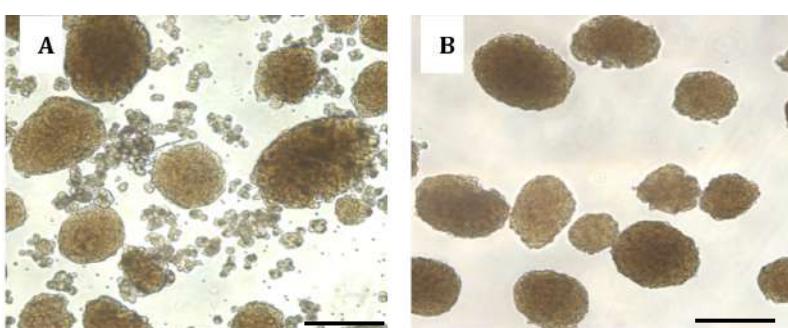


Figure 1. Images of pancreatic islets. Islets isolated before hand-picking (A). Purified islets after hand-picking (B). Scale bars represent 150 µm.

- Isolated islets are highly stressed due to physical and chemical processing. To improve overall islet viability, islets cultured in Petri dishes are rested overnight in an incubator at 37 °C with 5% CO₂. This allows a better discrimination between dead and alive islets as well as an optimum islets recovery. In case transplantation needs to be performed the same day of islet collection, due to experimental requirements, healthy islets are placed directly into a 1.5 ml Eppendorf tube with 1 ml of sterile RPMI 10% FBS and kept on ice until transplantation. The following day islets are checked under the microscope to confirm viability. If needed, healthy islets are re-collected, leaving behind dead cells. Healthy islets are then transferred from each Petri dish into a sterile 1.5 ml Eppendorf tube containing 1 ml of fresh sterile RPMI 10% FBS (Figure 2).

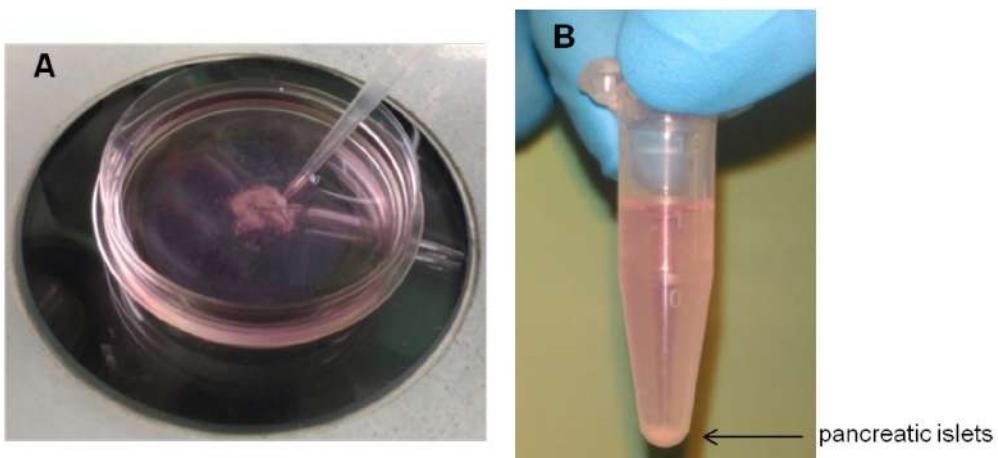


Figure 2. Collection and preparation of pancreatic islets. Healthy pancreatic islets transferred into a Petri dish are collected with a P200 (A) and transferred into a sterile 1.5 ml Eppendorf tube (B).

- The number of hand-picked islets to be transplanted under the kidney capsule varies from 300 to 600 depending on the experimental conditions required or the mouse strain used (e.g., chemically-induced or spontaneous diabetes, C57BL/6 or NOD, transgenic or knockout) (Battaglia *et al.*, 2006; Gagliani *et al.*, 2011 and 2015; Fousteri *et al.*, 2015a and 2015b) (see Table 1).

Table 1. Number of pancreatic islets required to reach normoglycemia in different mouse models

DONOR	RECIPIENT	DIABETES MOUSE MODEL	NUMBER OF ISLETS
Balb/c	C57BL/6	Chemically-induced	300
C57BL/6	Balb/c	Chemically-induced	300
Balb/c	NOD	Spontaneous	600
Nod scid	NOD	Spontaneous	600

B. Transplantation under the kidney capsule

1. All instruments and reagents used must be sterile. Ideal transplant recipients should be between 6 to 10 weeks old.

2. The recipient mouse is anesthetized by an intraperitoneal injection of a weight-adjusted dose of avertin (240 mg/kg).

Note: Avertin is a quick-acting, non-pharmaceutical grade anesthetic that is used for short duration surgical procedures in mice. It provides rapid and deep anesthesia, followed by fast and full postoperative recovery. This agent is listed on an approved Animal Protocol of the Italian Ministry of Health and procedures are performed by appropriately trained personnel. All guidelines for preparation and storage of avertin are followed.

3. The level of anesthesia can be tested by pinching the animal's toes. The mouse's eyes are kept continuously humid with a physiologic saline solution, 0.9 % NaCl until the mouse awakens to prevent corneal drying.

4. When anesthesia takes effect, islets are prepared for transplantation. All the islets sedimented (by gravity effect) in the 1.5 ml Eppendorf tube are transferred into a 25 µl Hamilton syringe connected to a P200 tip using the screw mechanism that allows for a slow less strenuous aspiration. Place a silicone tube adapter over the syringe tip. Insert PE50 tubing into the silicone adapter. Turn the Hamilton syringe the opposite site to let the islets into the PE 50 tube. Be careful to not lose the islets from the edge of the tip (Video 1 and Figure 3).

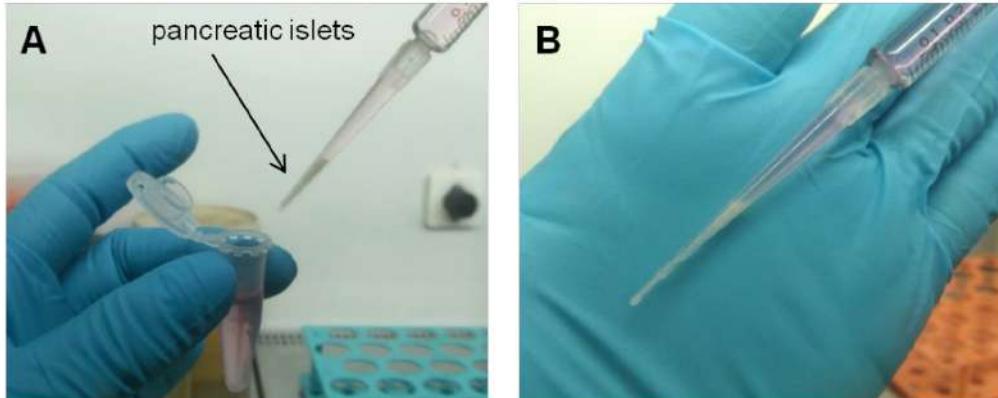


Figure 3. Collection of pancreatic islets with Hamilton syringe. Pancreatic islets are collected with the Hamilton syringe into a P200 tip (A) and then moved into the PE 50 tube (B).



Video 1. Preparation of islets for transplantation. Pancreatic islets are collected into a P200 tip and transferred into the PE50 tubing by slowly turned the Hamilton syringe.

5. While the mouse is under anesthesia, the transplant area is shaved with the electric shears and disinfected with betadine (Video 2).



Video 2. Mouse preparation for islet transplantation. The transplant area is disinfected and the mouse's eyes are kept humid.

6. Localize the kidney using your fingers and make a 1-1.5 cm incision in the skin on the left back side to visualize the peritoneum. Make the 0.5-1 cm incision in the peritoneum to expose the kidney. Slight pressure is applied to both sides of the incision, to allow the kidney to slide out of the abdominal cavity. Keep the surface of the exposed kidney wet with sterile saline using soaked cotton-tipped applicator. Repeat wetting as many times as necessary to prevent the kidney capsule from drying out (Video 3).



Video 3. Incision and kidney exposure. A small incision is made in the skin and in the peritoneum exposing the kidney.

7. Make a small scratch on the right flank of the kidney capsule using a syringe 25 gauge needle, to allow the PE50 filled with islets and attached to a Hamilton syringe to be inserted. Reach the posterior end of the capsule and carefully create some space by moving the PE50 tube and slowly inject the islets. Once all islets are injected, carefully remove the PE50 tube, dry the area and cauterize.

Note: It is highly recommended to use new PE50 tubes for each set of islets to be transplanted to avoid blood clotting. (Video 4 and Figure 4).

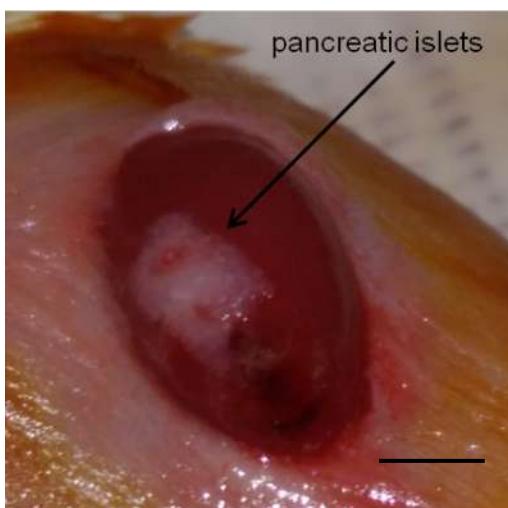


Figure 4. Islets under the kidney capsule. Islets (white) are localized under the kidney capsule. Scale bar represent 3 mm.



Video 4. Islet transplantation. Pancreatic islets are transplanted under the kidney capsule.

8. The kidney is then placed back into the cavity and residual blood is cleaned with physiological solution. Both incisions (peritoneum and skin) are sutured with 3 or 4 stitches based on the size of the incision (Video 5 and Figure 5).



Figure 5. Surgical seams



Video 5. Closing and mouse revival. The peritoneum and the skin are sutured. Subcutaneous injection of ketoprofen is made and the mice are placed on a heating pad until active.

9. The mice are treated with ketoprofen (5 mg/kg) subcutaneously and placed on a heating pad. Animals should be kept warm until they recover from anesthesia and must be monitored until maintaining upright posture and walking normally. Blood glucose levels usually normalize 24 h after the procedure.

Data analysis

1. In islet transplantation, blood glucose levels are used to define the outcome of islet engraftment, survival, rejection and tolerance according to the transplant model. Islet engraftment is usually defined as the achievement of normoglycemia, while graft rejection is defined by the subsequent development of hyperglycemia. Both in the syngeneic and allogeneic model, islet transplantation failure should be defined either as the inability to reach non-fasting blood glucose levels < 250 mg/dl or death within the first 7 d after islet transplantation (*i.e.*, surgical death). (Cantarelli *et al.*, 2013)
2. Several drug-based treatments (Gagliani *et al.*, 2011), as well as regulatory cell-based therapies have proved their efficacy in the establishment of long-term tolerance allogeneic murine islet transplantation. To test the development of an active state of tolerance and discard the possibility that treated mice are immune suppressed, mice that do not reject the graft 100 days after transplantation (graft rejection is considered after two consecutive glucose measurements with value > 250 mg/dl) are commonly boosted *in vivo* with donor-origin splenocytes. A total of 30×10^6 splenocytes isolated from the original islet donor are injected intraperitoneally (i.p.), and blood glucose levels are monitored daily thereafter. Mice still normoglycemic 30-50 days from boosting have developed a long-term tolerance to the allogenic islets (Gagliani *et al.*, 2011).
3. Statistical analysis
Islet allograft survival is commonly determined by Kaplan-Meier survival curves and compared using the logrank test. Prism software (GraphPad, USA) is used for all analyses (Fousteri *et al.*, 2015b). When multiple comparisons are made, post hoc comparisons use ANOVA with Bonferroni correction. For two-group comparisons, unpaired two-tailed *t*-tests with unequal variance are used. In all cases, a two-tailed *P* value of < 0.05 is considered significant (Battaglia *et al.*, 2006).

Recipes

1. RPMI/glutamine/HEPES/Pen-Strep/FBS
500 ml RPMI 1640 (RPMI)
5 ml L-glutamine
5 ml HEPES buffer
5 ml Pen-Strep

- 50 ml fetal bovine serum
Combine all reagents in a sterile biosafety cabinet
Store at 4 °C
2. HBSS/Ca/HEPES
1 L of Hank's balanced salt solution (HBSS)
2 mM CaCl₂
20 mM HEPES
Combine all reagents in a sterile biosafety cabinet
Store at 4 °C

Acknowledgments

This protocol has been used by members of our laboratory since it was first published (Battaglia *et al.*, 2006) and it was adapted by Gregori and colleagues (Gregori *et al.*, 2015).

The experiments using the mice were performed with approval of and strictly following the guidelines of the Animal Care and Use Committee of the Ospedale San Raffaele and communicated to the Ministry of Health. We would like to thank the members of the group for the support. This protocol is commonly used in our studies of optimizing post-transplant therapeutic strategies in wild-type or transgenic mouse models of type 1 diabetes (Battaglia *et al.*, 2006; Gagliani *et al.*, 2011 and 2015; Fousteri *et al.*, 2015a and 2015b). No potential conflicts of interest were disclosed.

References

1. Andersson, A. (1978). [Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets](#). *Diabetologia* 14(6): 397-404.
2. Battaglia, M., Stabilini, A., Draghici, E., Gregori, S., Mocchetti, C., Bonifacio, E. and Roncarolo, M. G. (2006). [Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance](#). *Diabetes* 55(1): 40-49.
3. Cantarelli, E., Citro, A., Marzorati, S., Melzi, R., Scavini, M. and Piemonti, L. (2013). [Murine animal models for preclinical islet transplantation: No model fits all \(research purposes\)](#). *Islets* 5(2): 79-86.
4. Cantarelli, E. and Piemonti, L. (2011). [Alternative transplantation sites for pancreatic islet grafts](#). *Curr Diab Rep* 11(5): 364-374.
5. Fousteri, G., Jofra, T., Di Fonte, R. and Battaglia, M. (2015a). [Combination of an antigen-specific therapy and an immunomodulatory treatment to simultaneous block recurrent autoimmunity and alloreactivity in non-obese diabetic mice](#). *PLoS One* 10(6): e0127631.

6. Fousteri, G., Jofra, T., Di Fonte, R., Gagliani, N., Morsiani, C., Stabilini, A. and Battaglia, M. (2015b). [Lack of the protein tyrosine phosphatase PTPN22 strengthens transplant tolerance to pancreatic islets in mice.](#) *Diabetologia* 58(6): 1319-1328.
7. Gagliani, N., Gregori, S., Jofra, T., Valle, A., Stabilini, A., Rothstein, D. M., Atkinson, M., Roncarolo, M.G., and Battaglia, M. (2011). [Rapamycin combined with anti-CD45RB mAb and IL-10 or with G-CSF induces tolerance in a stringent mouse model of islet transplantation.](#) *PLoS One* 6(12): e28434.
8. Gagliani, N., Jofra, T., Posgai, A. L., Atkinson, M. A. and Battaglia, M. (2015). [Immune depletion in combination with allogeneic islets permanently restores tolerance to self-antigens in diabetic NOD mice.](#) *PLoS One* 10(11): e0142318.
9. Graham, K. L., Fynch, S., Papas, E. G., Tan, C., Kay, T. W. and Thomas, H. E. (2016). [Isolation and culture of the islets of langerhans from mouse pancreas.](#) *Bio-protocol* 6(12): e1840.
10. Gregori S, Mangia P, Bacchetta R, Tresoldi E, Kolbinger F, Traversari C, Carballido JM, de Vries JE, Korthäuer U, Roncarolo MG. (2005). [An anti-CD45RO/RB monoclonal antibody modulates T cell responses via induction of apoptosis and generation of regulatory T cells.](#) *J Exp Med* 201(8): 1293-305.

Mono Sodium Urate Crystal-induced Peritonitis for *in vivo* Assessment of Inflammasome Activation

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[Abstract] Due to its particulate material, mono-sodium urate (MSU) crystals are potent activators of the NOD-like receptor NLRP3. Upon activation, NLRP3 induces the formation of inflammasome complexes, which lead to the production and release of mature IL-1 β . Bioactive IL-1 β is a potent activator of innate immune responses and promotes recruitment of inflammatory cells, including neutrophils from the blood into damaged/inflamed tissues. This protocol describes a method to study *in vivo* inflammasome activation via intraperitoneal injection of MSU crystals. MSU-injection results in a drastic increase of intraperitoneal IL-1 β levels, promoting neutrophil infiltration. Early-stage neutrophil numbers correlate with the amount of released IL-1 β and can be used as a read-out for the extent of *in vivo* inflammasome activation. In addition, this protocol might also be used as a sterile peritonitis model, to investigate mechanisms of neutrophil recruitment to the peritoneum, or as a means to obtain large numbers of *in vivo* activated neutrophils.

Keywords: (sterile) Peritonitis, Inflammasome, IL-1, NLRP3, NOD-like receptors, Innate immunity, Neutrophil recruitment

[Background] Innate immune cells recognize pathogens through a set of pattern recognition receptors (PRR), which bind to evolutionarily conserved structures on the pathogen surfaces or through ligation of other danger-associated molecular patterns. One family of these receptors are the NOD-like receptors (NLR), which react to the intracellular presence of invading pathogens and/or intracellular danger signals (Meylan *et al.*, 2006). Several PRR, including some NLRs are capable of inducing the formation of so-called inflammasome complexes, which mediate the proteolytic activation of pro-IL-1 β , pro-IL-18, and other IL-1 family cytokines (Martinon *et al.*, 2002). Due to the potent pro-inflammatory nature of IL-1 β and IL-18, inflammasome activation is a highly regulated, two-step process, involving limited transcription of pro-IL-1 β /pro-IL-18, and highly regulated activation of inflammasome receptors (Martinon *et al.*, 2009). NLRP3, one of the most studied inflammasome receptors, responds to a great variety of intracellular danger-associated molecular patterns, including bacterial cell wall components (Martinon *et al.*, 2004), damaged mitochondria (Zhou *et al.*, 2011), and particulate materials (Martinon *et al.*, 2006). Due to their particulate structure, mono sodium urate (MSU) crystals are very potent NLRP3 activators (Martinon *et al.*, 2006), which are widely used for *in vitro* studies of NLRP3 activation.

In addition to its use for *in vitro* experiments, MSU can also be used to study the *in vivo* relevance of inflammasome activation. Here, we described an MSU-induced peritonitis model to easily and quickly

study the *in vivo* relevance and extent NLRP3-inflammasome activation, e.g., upon genetic deletion of proteins that are involved in NLRP3 activation (Chen *et al.*, 2006; Spalinger *et al.*, 2016). In the MSU-induced peritonitis, the first wave of infiltrating immune cells consists mainly of neutrophils, and in the early phase of peritonitis, the number of infiltrating neutrophils correlates with the extent of inflammasome activation and with the production of mature IL-1 β (Chen *et al.*, 2006; Spalinger *et al.*, 2016).

Materials and Reagents

1. Pipette tips
2. Insulin syringes (BD, catalog number: 324826)
3. 5 ml syringes (BD, catalog number: 302187)
4. 25 G needles (Terumo, catalog number: GS-351)
5. 50 ml tubes (Corning, Falcon®, catalog number: 352070)
6. FACS tubes with lid (Corning, Falcon®, catalog number: 352058)
7. Mice: C57BL/6 adult females (THE JACKSON LABORATORY, catalog number: 000664)
Note: This protocol has been developed for C57BL/6 mice. For other mouse strains, MSU concentration and optimal time until peritoneal lavage should be titrated.
8. Mono-sodium urate (MSU) crystals (InvivoGen, catalog number: t1rl-msu)
9. Fluorescent antibody against Ly6G (for example, AlexaFluor647 anti-Ly6G [clone 1A8], BioLegend, catalog number: 127609)
10. Fluorescent antibody against Ly6B.2 (also known as 7/4 antigen; for example Fitc anti-Ly6B.2 [clone REA115], Miltenyi Biotec, catalog number: 130-103-318)
11. Fluorescent antibody against CD3ε (for example, PE-CF594 anti-CD3ε [clone 145-2C11], BD, BD Biosciences, catalog number: 562286)
12. Fluorescent antibody against CD45 (for example, Pacific Blue anti-CD45 [clone 30F11], BioLegend, catalog number: 103126)
13. Live-dead discriminator (for example Zombie NIR Fixable Viability Kit, BioLegend, catalog number: 423105)
14. Mouse IL-1 beta/IL-1F2 DuoSet ELISA kit (R&D Systems, catalog number: DY401)
15. Substrate Reagent Pack (R&D Systems, catalog number: DY999) for ELISA
16. Dulbecco's modified PBS (Sigma-Aldrich, catalog number: D8537-500ML)
17. Fetal calf serum (for example, PAN-Biotech, catalog number: P40-47100)
18. FACS buffer (see Recipes)

Equipment

1. Pipettes
2. Dissection tools (sharp scissors and forceps)
3. Neubauer cell counting chamber or automated cell counter
4. Refrigerated benchtop centrifuge
5. Flow cytometer
6. ELISA plate reader

Procedure

The whole procedure is summarized in Figure 1. All animal experiments were performed in accordance to Swiss animal welfare legislation.

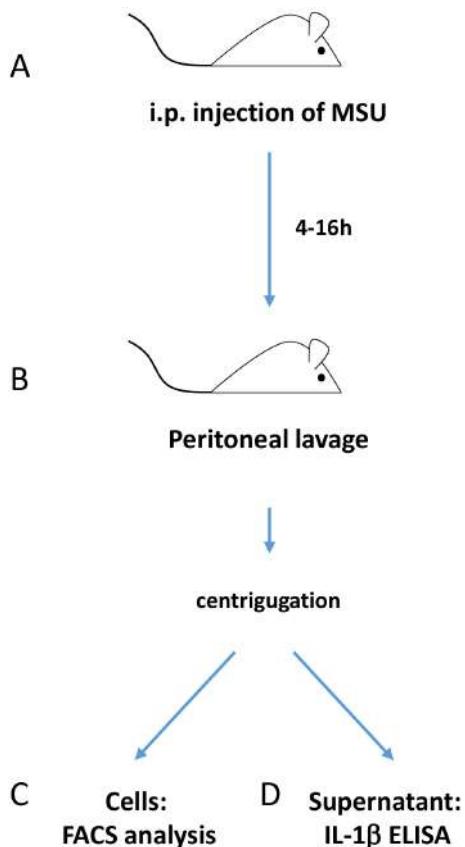


Figure 1. Overview of the procedure. The scheme summarizes the principal steps of this protocol for assessing *in vivo* inflammasome activation via peritoneal injection of MSU.

- A. MSU injection into the peritoneal cavity (see Video 1, which shows how to perform intraperitoneal injections)



Video 1. Procedure to perform intra-peritoneal injections

1. Prepare MSU suspension: add 0.5 ml of sterile PBS to one vial of MSU crystals (5 mg) and vortex thoroughly (> 5 min for initial resuspension, later vortex for 1 min is sufficient) to obtain a suspension of 10 mg/ml MSU.
Note: MSU crystals do NOT dissolve in PBS and are injected as a suspension. Do not centrifuge; vortex for 1 min prior to use.
2. Mark each mouse by ear punch or toe clipping as per local animal welfare legislation and animal experimental license.
Note: Since the experiment lasts max 16 h, mice can also be marked transiently using a waterproof bench marker.
3. Inject 180 µl of MSU suspension or 180 µl sterile PBS (control mice) into the peritoneal cavity using an insulin syringe:
 - a. Vortex the MSU suspension before drawing into the syringe.
 - b. Hold the mouse slightly inclined towards its head.
 - c. Insert the needle at a 30°-45° angle. Make sure that you are in the peritoneal cavity and slowly inject the suspension.

B. Peritoneal lavage and collection of cells

Perform peritoneal lavage as shown in Video 2 at the desired time-point of analysis (typically 4 h, 8 h, and 16 h after MSU injection):

**Video 2. Procedure to perform a peritoneal lavage**

1. Euthanize the mouse by cervical dislocation or CO₂-asphyxiation.

Note: Process one mouse after each other, the mice should not become stiff before the cell harvest is complete. Take care that no blood vessels bleed into the peritoneal cavity when euthanizing by cervical dislocation.

2. Open the skin of the belly carefully without damaging the peritoneum.
3. Inject 5 ml PBS into the peritoneal cavity using a 25 G needle.
4. Shake the mouse for 2-3 min.
5. Aspirate the PBS from the peritoneal cavity using the same syringe, transfer into a 50 ml conical tube, measure the amount of recovered PBS.
Note: An experienced experimenter recovers approx. 4 ml of the injected PBS.
6. Determine the cell concentration per ml using a Neubauer counting chamber or an automated cell counter.
Note: Red cell lysis is not required, but make sure not to count red blood cells, debris, or dead cells.

C. Flow cytometry to characterize cell infiltrate

1. Take 1 x 10⁶ cells from each lavage, spin down, transfer to FACS tube.
2. Resuspend the cells in 50 µl PBS containing:
 - a. AlexaFluor anti-Ly6G antibody, 1 µg/ml;
 - b. Fitc anti-Ly6B.2 antibody, 1 µg/ml;
 - c. PE-CF594 anti-CD3ε antibody, 0.5 µg/ml;
 - d. Pacific Blue anti-CD45 antibody, 0.5 µg/ml;
 - e. Zombie-NIR Live-dead discriminator (dilute 1:800).
3. Incubate for 20 min on ice in the dark.
4. Add 100 µl FACS buffer to each tube.
5. Spin down at 350 x g for 5 min.
6. Remove supernatant and wash once more with 100 µl FACS buffer.

7. Resuspend in 100 μ l FACS buffer and proceed to analysis at Flow cytometer. Figure 2 shows typical results and gating strategy used to identify live, single, CD45 $^{+}$ cells.

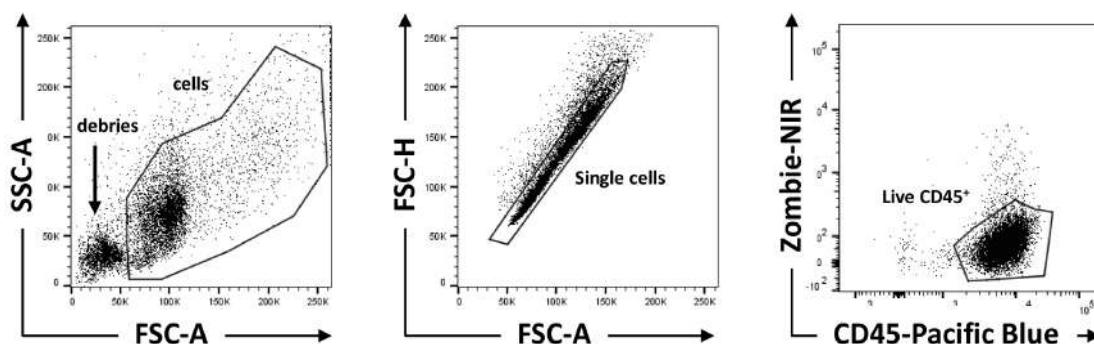


Figure 2. Gating strategy. Gating strategy to exclude debris, doublets, and dead cells from the analysis.

8. CD45 $^{+}$, Ly6G $^{+}$, Ly6B.2 $^{+}$ cells are neutrophils. Figure 3A shows typical flow cytometry dot plots when gated on single, live CD45 $^{+}$ cells.

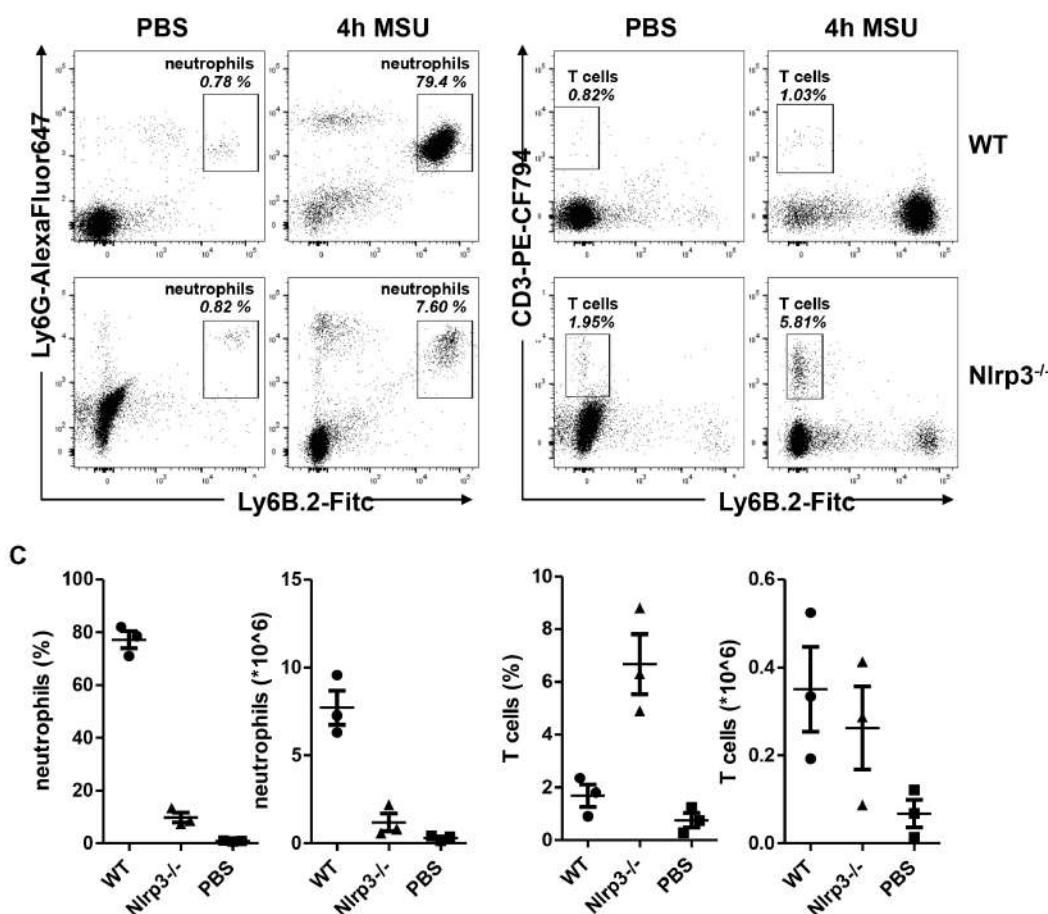


Figure 3. Representative data from flow cytometry. A. Representative flow cytometry plots of peritoneal cells collected 4 h after intraperitoneal injection of MSU. B. Representative results

of relative and absolute numbers of infiltrating neutrophils (left) and T cells (right). Each point represents one mouse.

D. Quantification of IL-1 β in peritoneal lavage

1. Take 0.5 ml of peritoneal lavage from Step B6.
2. Spin the cells down, use supernatant for the analysis.

Note: IL-1 β levels can be rather low; do not dilute the supernatant for ELISA analysis.

3. Perform IL-1 β ELISA according to the manufacturer's instructions.

Figure 4 shows typical results of IL-1 β ELISA on peritoneal lavages.

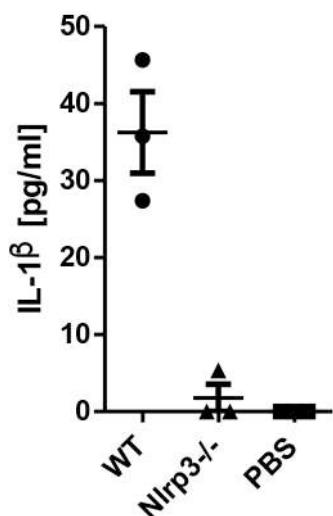


Figure 4. Representative data from ELISA measurement. IL-1 β ELISA from peritoneal lavage collected from WT and NLRP3^{-/-} mice 8 h after intra-peritoneal MSU injection. For the lavage, 3 ml of PBS was injected into the peritoneal cavity. Each dot represents one mouse.

Data analysis

The number of infiltrating neutrophils is calculated as follows:

1. Calculate the absolute number of neutrophils: cell concentration (cell count per ml) obtained in Step B6 x 5 = total number of cells.
2. Calculate the number of infiltrating neutrophils: the total number of cells x frequency of Ly6G⁺, Ly6C⁺ cells of single, live, CD45⁺ cells = absolute number of infiltrating neutrophils.

Accordingly, the calculation would be:

$$\text{cell conc.} \times 5\text{ml} \times \text{frequency}$$

With cell conc. = concentration of cells obtained in Step B6; and frequency = proportion of cells in the Lys6G⁺/Ly6B.2⁺ gate (see Figure 3A, left panels).

If desired, the same approach is used to determine absolute numbers of T cells in the peritoneum, using the frequency of CD3⁺ cells within live, single, CD45⁺ cells. Figure 3B shows typical results obtained with this method.

Notes

1. All animal experiments should be carried out according to local animal welfare legislation.
2. The used concentration of MSU was tested for C57BL/6 mice and may require titration if other mouse strains are used.
3. As a measure for inflammasome activation, 4 h and/or 8 h are typical time-points of analysis. However, the experiment can also be performed for up to 24 h. However, at later time-points, secondary factors influence the results and number of infiltrating cells might no longer correlate directly with inflammasome activity.
4. IL-1 β levels in the peritoneal lavage can be rather low. If IL-1 β measurement is the primary read-out, lavage can be performed with as little as 2.5 to 3 ml PBS. However, this results in reduced numbers of recovered cells, since only approx. 2.0 to 2.5 ml of the injected PBS can be recovered.
5. We typically use Nlrp3^{-/-} mice as a negative control for MSU-induced peritonitis, since these mice are defective for the inflammasome receptor primarily involved in recognizing MSU.

Recipes

1. FACS buffer
Supplement Dulbecco's PBS with 2% fetal calf serum
Keep sterile and store at 4 °C for up to 2 months

Acknowledgments

This protocol was first described by Chen *et al.* (2006) and was further developed for a study by our group (Spalinger *et al.*, 2016), which was supported by the Swiss National Science Foundation (314730-146204; CRSII3_154488/1; 310030-120312), the Zürcher Universitäts-Verein, and the Swiss Philanthropy Foundation. The authors declare no conflicts of interest or competing financial interests.

References

1. Chen, C. J., Shi, Y., Hearn, A., Fitzgerald, K., Golenbock, D., Reed, G., Akira, S. and Rock, K. L. (2006). [MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals](#). *J Clin Invest* 116(8): 2262-2271.

2. Martinon, F., Agostini, L., Meylan, E. and Tschopp, J. (2004). [Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome.](#) *Curr Biol* 14(21): 1929-1934.
3. Martinon, F., Burns, K. and Tschopp, J. (2002). [The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- \$\beta\$.](#) *Mol Cell* 10(2): 417-426.
4. Martinon, F., Mayor, A. and Tschopp, J. (2009). [The inflammasomes: guardians of the body.](#) *Annu Rev Immunol* 27: 229-265.
5. Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. and Tschopp, J. (2006). [Gout-associated uric acid crystals activate the NALP3 inflammasome.](#) *Nature* 440(7081): 237-241.
6. Meylan, E., Tschopp, J. and Karin, M. (2006). [Intracellular pattern recognition receptors in the host response.](#) *Nature* 442(7098): 39-44.
7. Spalinger, M. R., Kasper, S., Gottier, C., Lang, S., Atrott, K., Vavricka, S. R., Scharl, S., Gutte, P. M., Grutter, M. G., Beer, H. D., Contassot, E., Chan, A. C., Dai, X., Rawlings, D. J., Mair, F., Becher, B., Falk, W., Fried, M., Rogler, G. and Scharl, M. (2016). [NLRP3 tyrosine phosphorylation is controlled by protein tyrosine phosphatase PTPN22.](#) *J Clin Invest* 126(5): 1783-1800.
8. Zhou, R., Yazdi, A. S., Menu, P. and Tschopp, J. (2011). [A role for mitochondria in NLRP3 inflammasome activation.](#) *Nature* 469(7329): 221-225.

Intravenous Labeling and Analysis of the Content of Thymic Perivascular Spaces

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[Abstract] Following development in the thymus, T cells are thought to exit into the periphery predominantly through perivascular spaces (PVS). This exit route is used by conventional T cells, and likely also applies to unconventional T cell subsets, such as precursors of CD8 $\alpha\alpha$ and TCR δ intraepithelial lymphocytes, regulatory T cells and natural killer T cells. Additional cell types might also be found in the PVS and initiate interactions with exiting T cells. The exact content of the PVS, and the processes within, are not well studied. To distinguish vascular from resident cells within various tissues by flow cytometry, intravenous (i.v.) labeling is becoming a commonly employed method. We recently used anti-CD45.2 antibodies and magnetic enrichment to further evaluate this technique, and compared labeled and unlabeled cells in the thymus and blood. This assay can be used to specifically investigate hematopoietic cell subsets within the PVS of the thymus.

Keywords: Perivascular spaces, Thymus, Thymic emigration, Recent thymic emigrants, Intravenous labeling

[Background] Immature thymocytes undergo a series of maturation steps, including positive and negative selection, which eliminate the majority of developing T cells. The resulting mature T cell pool is thereby shaped towards a higher proportion of beneficial clones and a reduced proportion of dangerous self-reactive clones. The thymus also produces less abundant T cell subsets that generally act to maintain immune system, tissue, and metabolic homeostasis, including: TCR δ cells, regulatory T (Treg) cells, natural killer T (NKT) cells, intraepithelial lymphocyte (IEL) precursors, and mucosal associated invariant T (MAIT) cells. Mature thymocytes poised to emigrate into the periphery upregulate expression of the receptor (S1PR1) that recognizes sphingosine-1 phosphate (S1P), a lipid molecule present at high concentrations in the blood. S1PR1 $^+$ T cells migrate along an S1P gradient and wind up in vascular circulation.

The thymic perivascular spaces (PVS) are basement membrane-separated compartments between the parenchyma and the vasculature. They are thought to facilitate trafficking of cells, especially mature T cells emigrating from the thymus (Mori *et al.*, 2007; Weinreich and Hogquist, 2008; Zachariah and Cyster, 2010). The exact content of the PVS is not well characterized yet, and could include antigen presenting cells such as dendritic cells and macrophages, that carry antigens not normally expressed in the thymus, into this tissue to contribute to thymocyte selection processes.

Intravenous (i.v.) labeling is a technique commonly used to distinguish vasculature-associated circulating cells from those residing within tissues at the time of analysis (Anderson *et al.*, 2014). Cyster and colleagues have used this approach to identify CD4 $^+$ emigrating T cells within the PVS, and

showed that upon tail-vein injection of CD4-labeling antibody, CD4 T cells in the PVS are positively labeled within 3 min (Zachariah and Cyster, 2010). We sought to establish whether thymic precursors of CD8 $\alpha\alpha$ IEL, an agonist selected T cell subset that downregulates both CD4 and CD8 expression during thymic maturation, can also be found in the PVS. In order to do so, we adapted the i.v. labeling approach, using phycoerythrin (PE)-conjugated anti-CD45.2 (for C57BL/6 mice). As CD45 is not T cell specific, but is expressed by hematopoietic cells in general, various cells can be identified within the i.v.-labeled (IV $^+$) fraction. Furthermore, we combined this with magnetic enrichment for the PE-conjugated antibodies. This allowed us to more closely evaluate the perivascular contents.

Materials and Reagents

1. 1.5 ml microcentrifuge tubes (DOT Scientific, catalog number: 509-FTG)
2. Aluminum foil (Spring Grove)
3. 6-well plates (Corning, Costar $^{\circledR}$, catalog number: 3506)
4. 70 μ m cell strainers (Corning, Falcon $^{\circledR}$, catalog number: 352350)
5. 1 ml insulin syringes (BD, catalog number: 329420)
6. 3 ml syringes (Covidien, catalog number: 8881513918)
7. 5 ml polystyrene round-bottom tubes (flow cytometry tubes; Corning, Falcon $^{\circledR}$, catalog number: 352008)
8. 15 ml conical centrifuge tubes (Corning, Falcon $^{\circledR}$, catalog number: 352097)
9. 96 round-bottom well plates (SARSTEDT, catalog number: 82.1582.001)
10. MACS LS columns (Miltenyi Biotec, catalog number: 130-042-401)
11. Mice, C57BL/6J, 5-6 weeks old (THE JACKSON LABORATORY, catalog number: 000664)
12. Anti-CD45.2 PE clone 104 (Tonbo Biosciences, catalog number: 50-0454-U100; 0.2 mg/ml)
13. Heparin sodium injection (Sagent Pharmaceuticals, NDC 25021-400-10; 10,000 USP units per 10 ml)
14. Phosphate buffered saline (PBS) (Corning, Mediatech, catalog number: 21-040-CV)
15. Isoflurane (Piramal Healthcare, 001725CS)
16. Anti-PE MicroBeads (Miltenyi Biotec, catalog number: 130-048-801)
17. Live/Dead Fixable Aqua kit (Thermo Fisher Scientific, Invitrogen $^{\text{TM}}$, catalog number: L34957)
18. CD1d-tetramer (PBS57-loaded CD1d-monomers and tetramers available from NIH tetramer core facility; <http://tetramer.yerkes.emory.edu>)
19. Anti-CD25 clone PC61 (e.g., BioLegend, catalog number: 102024)
20. Anti-TCR β clone H57-597 (e.g., BD, BD Biosciences, catalog number: 562841)
21. Anti-CD4 clone RM4-5 (e.g., BioLegend, catalog number: 100548)
22. Anti-CD8 α clone 53-6.7 (e.g., BD, BD Biosciences, catalog number: 563332)
23. Anti-CD5 clone 53-7.3 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 47-0051-82)

24. Anti-CD122 clone TM-b1 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 46-1222-82)
25. Anti-H-2K^b clone AF6-88.5 (e.g., BD, BD Biosciences, catalog number: 562942)
26. Anti-PD-1 clone J43 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 17-9985-82)
27. Anti-NK1.1 clone PK136 (e.g., BioLegend, catalog number: 108705)
28. Anti-CD11c clone N418 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 25-0114-82)
29. Anti-I-A^b clone AF6-120.1 (e.g., BioLegend, catalog number: 116421)
30. Anti-CD19 clone 1D3 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 56-0193-82)
31. Anti-CD11b clone M1/70 (e.g., Thermo Fisher Scientific, eBioscience, catalog number: 11-0112-41)
32. Anti-GR1 clone Gr-1 (e.g., BioLegend, catalog number: 108411)
33. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150), heat inactivated at 65 °C
34. Ethylenediamine tetraacetate acid (EDTA) (Fisher Scientific, catalog number: BP120-500)
35. Sodium azide (Fisher Scientific, catalog number: BP922I-500)
36. Ammonium chloride (NH₄Cl) (Sigma-Aldrich, catalog number: A4514-500G)
37. Potassium bicarbonate (KHCO₃) (Fisher Scientific, catalog number: P235-500)
38. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A7906)
39. FACS buffer (see Recipes)
40. ACK lysis buffer (see Recipes)
41. MACS buffer (see Recipes)

Equipment

1. Class-II biosafety cabinet/laminar flow hood
2. MACS Multistand (Miltenyi Biotec, catalog number: 130-042-303)
3. QuadroMACS Separator (Miltenyi Biotec, catalog number: 130-090-976)
4. Heat lamp
5. Timer (Fisher Scientific, catalog number: 14-649-17)
6. Benchtop centrifuge (Beckman Coulter, model: Allegra X-12-R)
7. 2,000 ml drop glass jar
8. Refrigerator (4 °C) (Fisher Scientific, model: Isotemp™ General-Purpose Series Lab Refrigerator)
9. Hemacytometer (Sigma-Aldrich, catalog number: Z359629-1EA)
10. FACS flow cytometer (BD, model: LSR-II, H10.10)

Software

1. FlowJo version 10.4.0

Procedure

Note: All animal procedures must be approved by your institution's ethics committee.

A. Intravenous labeling and sample preparation

Note: Perform Steps A6-A8 in a class-II biosafety cabinet/laminar flow hood with exhaust. Figure 1 shows an outline of the procedure up to the tissue collection.

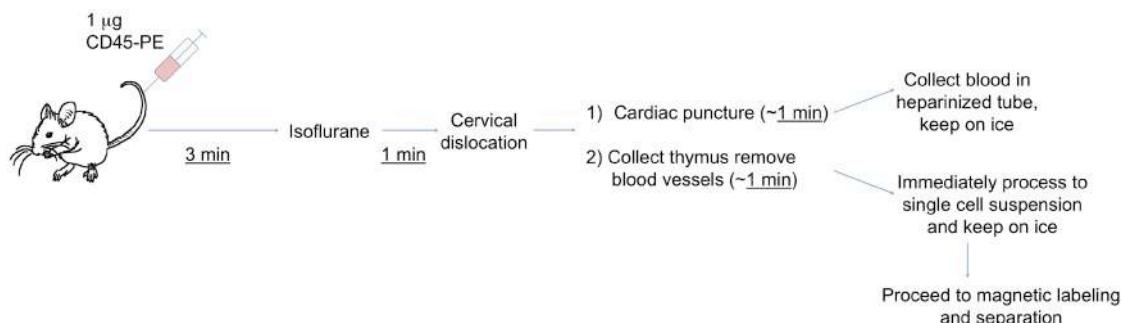


Figure 1. Outline of IV labeling and tissue collection. Experimental procedures prior to magnetic cell sorting are depicted as explained in the text. It is important to work quickly and do one animal at a time, such that the time from injection to single cell suspension does not vary greatly between samples.

1. Prepare anti-CD45-PE/PBS solution (1 µg/200 µl): Dilute 5 µl anti-CD45.2-PE in 195 µl PBS in a 1.5 ml microcentrifuge tube, vortex, place on ice, covered with aluminum foil.

Note: It is advisable to prepare 5-10% more solution than needed to account for solution loss within the syringe dead space.

2. Prepare a 6-well plate with 5 ml FACS buffer (see Recipes) and a 70 µm cell strainer per well for collection of thymi, place on ice.

3. Prepare 1.5 ml microcentrifuge tubes with 20 µl heparin for blood collection, place on ice.

4. Working one animal at a time, warm up mice with a heat lamp until tail vein is dilated (usually ~5 min), constantly monitoring mice to avoid overheating or burning.

5. Set timer 3 min. When tail vein is dilated, inject 200 µl of the anti-CD45-PE/PBS solution into the tail-vein, using a 1 ml insulin syringe. Immediately after injection, start the timer.

Note: Include mice injected with 200 µl PBS as negative controls.

6. While the timer is running, prepare a glass jar inside a class-II biosafety cabinet with exhaust for the isoflurane drop method: Place a folded tissue soaked with 2 ml isoflurane into the bottom part of a 2,000 ml drop glass jar, and place insert on top, ensuring physical separation of isoflurane-soaked tissue and the mouse.

7. When the timer goes off 3 min after the injection, place the mouse inside the prepared glass jar and close the lid.
8. After 1 min (mouse should be anesthetized), euthanize mouse by cervical dislocation.
9. Open the chest of the animal and collect blood by cardiac puncture, using a 1 ml insulin syringe: Insert the needle about 5 mm into the heart, and gently pull the plunger (Figures 2A and 2B). Empty the syringe content (usually around 0.2-0.5 ml) into a heparinized 1.5 ml microcentrifuge tube, and place the collection tube with blood on ice. Cover samples with aluminum foil to protect from light until further processing.
10. Collect the thymus and remove blood vessels attached to the outside of the thymus (Figures 2C-2E). Suspend in 5 ml cold FACS buffer (6-well plate with 70 μ m cell strainer) on ice, and immediately dissociate the tissue by mashing it with the plunger of a 3 ml syringe and pressing it through the cell strainer into the well. Discard the strainer with the remaining connective tissue. Repeat for each thymus. Cover samples with aluminum foil to protect from light until further processing.
11. Blood samples: Transfer blood into 5 ml flow cytometry tubes, add 2 ml ACK lysis buffer (see Recipes), keep at room temperature (RT) for 2 min, spin for 3 min at 524 $\times g$ (1,500 RPM), 4 °C, discard supernatant. Repeat 2 times (resuspending pellets in ACK lysis buffer) and finally resuspend cells in 1 ml cold FACS buffer and place on ice.

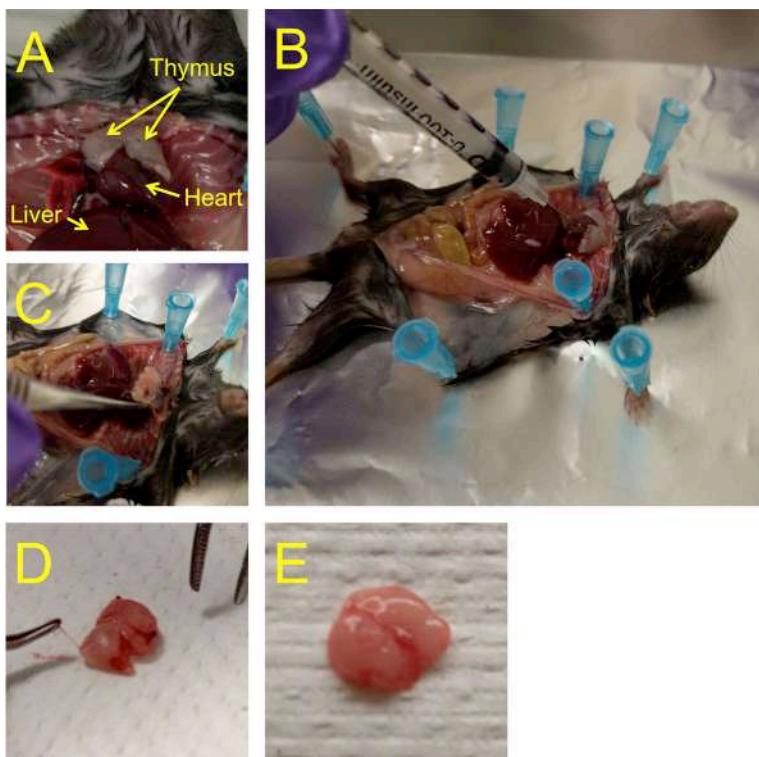


Figure 2. Outline of cardiac blood and thymus collection. A. The thymus is localized right above the heart. B. Blood is drawn from the heart with an insulin syringe. C. To remove the thymus, tweak it at the base with a curved forceps and gently pull. D. The thymus is covered

with blood vessels, which should be removed with a curved forceps as best as possible. E. Thymus after removal of lining blood vessels.

B. MACS enrichment of i.v.-labeled (IV⁺) cells from thymic samples

1. Determine cell count of each sample by hemacytometer (usually 150-250 × 10⁶).
2. Resuspend cells to 1 × 10⁷/80 µl MACS buffer (see Recipes).
3. Set 40 µl (5 × 10⁶) of each sample aside as an untouched control, and fill up to 1 ml with FACS buffer, keep on ice. Cover with aluminum foil.
4. In accordance with the Miltenyi protocol (included with the anti-PE MicroBeads), add 20 µl anti-PE beads per 80 µl to the remaining suspensions.

Note: In our experience, this protocol also works with lower amounts of beads. E.g., resuspend cells to 1 × 10⁷/40 µl MACS buffer and add 10 µl beads. Further reduction of beads might be possible. If looking at abundant cells, a fraction of the thymic sample could be sufficient for enrichment.

5. Mix, and incubate in the refrigerator (4 °C) for 15 min, protected from light.
6. Wash samples 2 x with 1 ml MACS buffer (spin for 5 min at 524 × g (1,500 RPM), 4 °C, discard supernatant, resuspend in MACS buffer).
7. After the second wash step, resuspend the samples at up to 1 × 10⁸ in 500 µl MACS buffer.
8. Perform magnetic separation using LS columns and according to the Miltenyi protocol.
9. Collect the magnetically-labeled (IV⁺) fractions in 15 ml centrifuge tubes.

Note: We use untouched fractions of samples as controls. The flowthrough from the magnetic separation step can also be collected as a negative control. We generally observed no differences between the untouched and flowthrough IV⁺ fractions when analyzed.

C. Antibody- and tetramer-labeling for flow cytometry

1. Determine cell count of each IV⁺ eluted sample (and IV⁻ flowthrough sample, if collected)
2. Spin samples (including untouched fractions) for 5 min at 524 × g RPM (1,500), 4 °C, discard supernatant, resuspend in 200 µl FACS buffer and transfer each sample into a well of a 96 round-bottom well plate, place on ice.

Note: We stain samples at up to 5 × 10⁶ cells per 50 µl master-mix (see below). The IV⁺ fraction per thymus, and the untouched fractions are generally within this range. If collecting flowthrough fractions, we recommend to determine the cell number and stain 5 × 10⁶ cells.

3. Determine cell numbers of the blood samples and transfer 5 × 10⁶ cells per sample into individual wells.
4. Live/Dead discriminator: In our lab we frequently use Live/Dead Fixable Aqua kit. We use this kit prior to labeling with markers of interest, and stain cells for 20 min on ice in PBS (without added protein) with 1:1,000 Live/Dead discriminator. This amine-binding discriminator stains cells that have lost their membrane integrity, and positively stained (dead) cells should be excluded when analyzing the FACS data.

5. Wash samples 3 x with 200 μ l FACS buffer (spin for 5 min at 524 $\times g$ (1,500 RPM), 4 °C, discard supernatant, resuspend in FACS buffer).
6. Prepare master mix for labeling for FACS. We use most antibodies at 1:200 μ l and CD1d-tetramer at 1:400 in FACS buffer, 50 μ l per 5×10^6 cells.
We typically include the following set of antibodies to analyze CD4 $^+$ CD8 α^- (CD4 single-positive, SP), CD4 $^+$ CD8 α^+ (CD8 SP), CD4 $^+$ CD8 α^+ (double-positive, DP) and IEL-precursor (CD1d-tetramer \cdot CD25 \cdot CD4 \cdot CD8 $\alpha\cdot$ CD5 \cdot TCR $\alpha\cdot$ CD122 \cdot H-2K b^+ PD-1 $^+$ or PD-1 $^+$ NK1.1 $^+$) T cells (panel 1), or B cells (CD19 $^+$ I-Ab $^+$), dendritic cells (CD11c $^+$ I-Ab $^+$) and CD11b $^+$ GR-1 $^+$ myeloid cells (panel 2), and these panels can be extended/substituted according to the cells of interest:
Panel 1: CD1d-tetramer, CD25 TCR β , CD4, CD8 α , CD5, CD122, H-2K b , PD-1, NK1.1; Panel 2: CD11c, I-A b , CD19, CD11b, GR1.
Note: If B cells or myeloid cells are the specific focus of investigation, it is advisable to include an Fc receptor blocker (anti-CD16/32).
7. Spin 96-well plate with samples for 5 min at 524 $\times g$ (1,500 RPM), 4 °C, discard supernatant, resuspend in 50 μ l master mix per 5×10^6 cells. Keep on ice, covered with aluminum foil, for 20 min.
8. Wash samples 3 x with 200 μ l FACS buffer (spin for 5 min at 524 $\times g$ (1,500 RPM), 4 °C, discard supernatant, resuspend in FACS buffer).
9. After the final wash step, resuspend each sample in 200 μ l FACS buffer and transfer into polystyrene FACS tubes. Keep on ice and cover with aluminum foil until acquisition.

D. Flow cytometry

1. Set up compensation on a flow cytometer.
2. Acquire samples.

Data analysis

1. Samples are analyzed with FlowJo. Within singlet Live/Dead-negative thymocytes, gates are set on the IV $^-$ and IV $^+$ fractions (see Figure 3A).
2. The IV $^-$ fraction of the untouched or flowthrough samples should be compared to the IV $^+$ fraction of the enriched and blood samples (Figure 3).
3. Identify and analyze cells of interest.

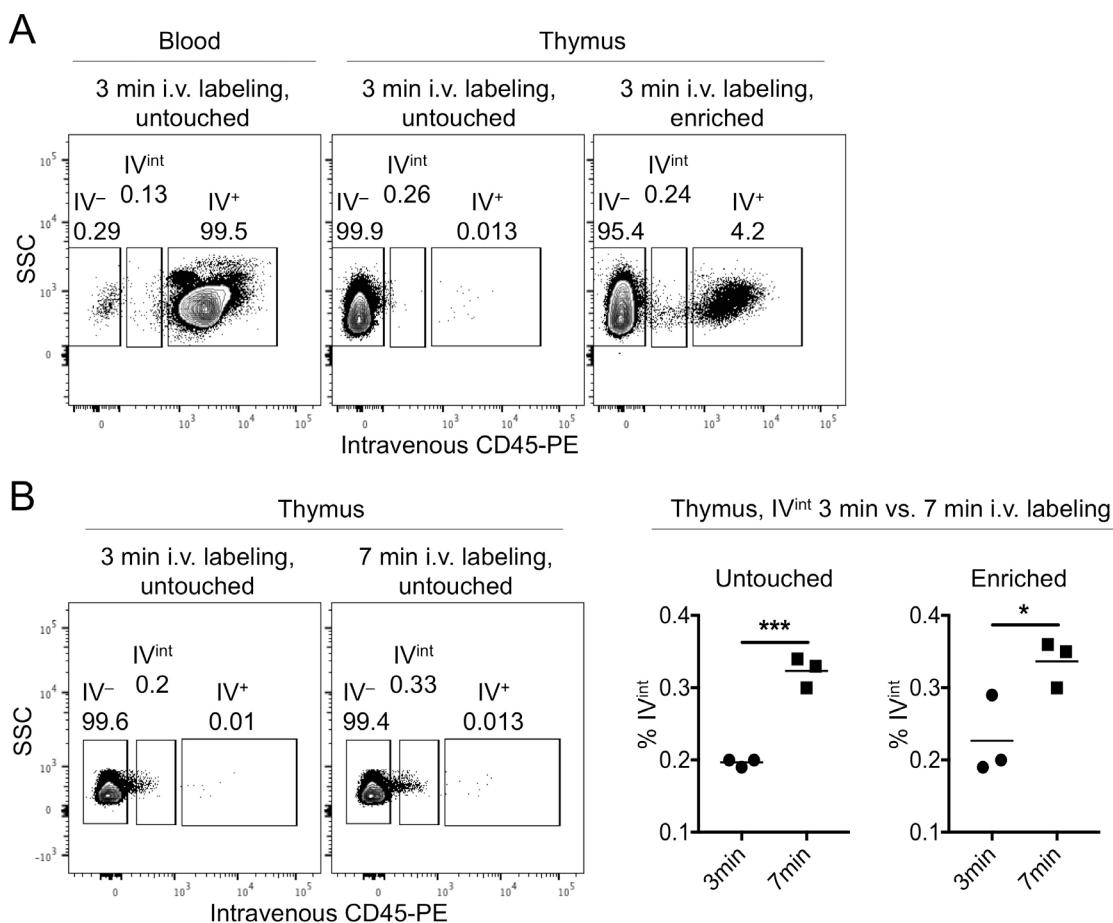


Figure 3. Gating strategy for IV⁺ and IV⁻ cells. A. The IV-labeled (IV⁺) or–unlabeled (IV⁻) cells were identified within the thymic untouched (left) or MACS-enriched samples (middle), or in the blood (right). Further analysis of cells of interest can be undertaken with an appropriate staining panel. B. 3 min versus 7 min i.v. labeling times were compared in one experiment (dot plots). Graphs (right) show the percent i.v.-intermediate (IV^{int}) cells in the untouched (left graph) and enriched (right graph) fractions. *P < 0.05, *** P < 0.001, Student's t-test. Each symbol represents an individual mouse.

Notes

- For reference: From 6-9 week old mice (predominantly females), we normally obtain between 200-300 x 10⁶ total cells and recover between 1.5-4.5 x 10³ total enriched IV⁺ cells. Within the enriched IV⁺ cells, we found about 48.15 (\pm 2.52)% TCR β ⁺, 2.97 (\pm 0.31)% CD19⁺I-Ab⁺ B cells and 2.21 (\pm 0.54)% CD11b⁺GR1⁺ cells. Also see reference Ruscher *et al.* (2017), Figure 5 and Suppl. Figure 4.
- For first time users of this protocol, we recommend using up to three mice.
- We tested 3 min versus 7 min IV labeling time in one experiment. In our hands there was an increased ‘leakage’ of labeling antibody into the thymic parenchyma, as observed by a PE⁺

shoulder of the IV⁻ fraction (see IV^{int} in Figure 3B). We recommend adhering to the suggested 3 min labeling time.

4. We also tested the effects of perfusion with 50 ml PBS immediately after the 3 min i.v. labeling and 1 min isoflurane steps, and did not observe differences in the proportions of cell types within the PVS with or without perfusion. Furthermore, some results suggest that perfusion can damage tissue architecture in a way that affects i.v. labeling (Anderson *et al.*, 2014).
5. Zachariah *et al.* confirmed accuracy of i.v. labeling of CD4⁺ T cells in the thymic PVS with FTY720 (an analog of S1P that works as an antagonist) treatment, and in RAG-GFP models (Zachariah and Cyster, 2010). We compared the percentages of T cell subsets, B cells and CD11b⁺GR-1⁺ cells within the blood IV⁺, untouched IV⁻ (parenchyma) and enriched IV⁺ fractions (Ruscher *et al.*, 2017). We further have unpublished data using CD24 and additional markers, and investigated the %GFP⁺ and GFP median fluorescent intensity after i.v. labeling of *Rag2*^{GFP} [*Rag2*^{GFP} indicates the age of T cells after T cell receptor recombination events, with GFP fluorescence being high at the DP stage and after positive selection decreasing over time (Boursalian *et al.*, 2004; McCaughtry *et al.*, 2007)]. The differences indicated that the enriched IV⁺ fractions were distinct from the blood IV⁺ fractions, suggesting that this protocol predominantly identifies cells that are in the thymic PVS.
6. In addition to the anti-CD45 antibodies used in our PVS labeling studies, we and others (Zachariah and Cyster, 2010; Anderson *et al.*, 2014) have employed anti-CD4 (clone RM4-4, eBioscience; clone RPA-T4, BD Biosciences) and anti-CD8a (clone 53-6.7, eBioscience) for intravenous labeling of vascular content in various peripheral tissues.
7. Similarly, while not tested specifically for PVS content labeling, we have used fluorochromes such as allophycocyanin (APC) or Brilliant Violet 421 (BV421) for i.v. labeling of peripheral tissue vasculature-associated T cells. These fluorochromes might also work for the purpose of identifying the thymic PVS content by i.v. labeling.

Recipes

1. FACS buffer

Supplement 1 L PBS with:

1% FBS

2 mM EDTA

0.02% sodium azide

2. ACK lysis buffer

Supplement 1 L ddH₂O with:

150 mM NH₄Cl

10 mM KHCO₃

0.1 mM EDTA

Adjust pH to 7.2-7.4

3. MACS buffer

Supplement 1 L PBS with:

0.5% BSA

2 mM EDTA

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References

1. Anderson, K. G., Mayer-Barber, K., Sung, H., Beura, L., James, B. R., Taylor, J. J., Qunaj, L., Griffith, T. S., Vezys, V., Barber, D. L. and Masopust, D. (2014). [Intravascular staining for discrimination of vascular and tissue leukocytes](#). *Nat Protoc* 9(1): 209-222.
2. Boursalian, T. E., Golob, J., Soper, D. M., Cooper, C. J. and Fink, P. J. (2004). [Continued maturation of thymic emigrants in the periphery](#). *Nat Immunol* 5(4): 418-425.
3. McCaughtry, T. M., Wilken, M. S. and Hogquist, K. A. (2007). [Thymic emigration revisited](#). *J Exp Med* 204(11): 2513-2520.
4. Mori, K., Itoi, M., Tsukamoto, N., Kubo, H. and Amagai, T. (2007). [The perivascular space as a path of hematopoietic progenitor cells and mature T cells between the blood circulation and the thymic parenchyma](#). *Int Immunol* 19(6): 745-753.
5. Ruscher, R., Kummer, R. L., Lee, Y. J., Jameson, S. C. and Hogquist, K. A. (2017). [CD8aa intraepithelial lymphocytes arise from two main thymic precursors](#). *Nat Immunol* 18(7): 771-779.
6. Weinreich, M. A. and Hogquist, K. A. (2008). [Thymic emigration: when and how T cells leave home](#). *J Immunol* 181(4): 2265-2270.
7. Zachariah, M. A. and Cyster, J. G. (2010). [Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction](#). *Science* 328(5982): 1129-1135.

Nab Escaping AAV Mutants Isolated from Mouse Muscles

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[Abstract] Neutralizing antibodies (Nabs) are a major challenge in clinical trials of adeno-associated virus (AAV) vector gene therapy, because Nabs are able to inhibit AAV transduction in patients. We have successfully isolated several novel Nab-escaped AAV chimeric capsids in mice by administrating a mixture of AAV shuffled library and patient serum. These AAV chimeric capsid mutants enhanced Nab evasion from patient serum with a high muscle transduction efficacy. In this protocol, we describe the procedures for selection of the Nab-escaped AAV chimeric capsid, including isolation and characterization of Nab-escaping AAV mutants in mice muscle.

Keywords: AAV, Nab-escaping AAV, Chimeric capsid, Mouse muscle

[Background] Adeno-associated virus (AAV) vectors have been used in many preclinical studies and clinical trials. Many diseases have received eventual treatment using AAV gene therapy. However, the presence of neutralizing antibodies in circulation poses a major challenge for AAV vector application in future clinical trials. Many approaches have been explored to evade activities of Nab. Herein, we described the approach with directed evolution for selection of Nab-escaping mutants from an AAV shuffling library.

DNA shuffling is a powerful strategy for generating diverse mutants. Through successive rounds of phenotypic selection, DNA shuffling libraries were characterized by higher quality and more targeted diversification. High-throughput selection of capsid mutants from AAV shuffling libraries has been used as a promising strategy to explore AAV mutants with the abilities to target specific tissues and evade Nabs. However, most of these selecting methods were only tested *in vitro*; some studies even used rabbit anti-AAV2 sera or human intravenous immunoglobulin. The approach of *in vivo* selection of capsid mutants could provide a platform to generate more effective AAV mutants that not only escape Nab from patient serum but also enhance transduction in specific tissues.

Materials and Reagents

1. GeneMate individual 0.2 ml PCR tubes (BioExpress, catalog number: T-3035-1)
2. BD Veo insulin syringes with BD Ultra-Fine 6 mm x 31G needle (BD, catalog number: 324910)
3. Corning™ 96-Well Clear Bottom Black Polystyrene Microplates (Corning, catalog number: 3904)

4. VWR® Tissue Culture 48-well Plate (VWR, catalog number: 10861-560)
5. BALB/c mice, typically of 6 weeks old female mice
6. ElectroMAX™ DH10B™ Cells (Thermo Fisher Scientific, catalog number: 18290015)
7. Adherent HEK 293 cells and Huh7 cells
8. MAX Efficiency™ DH10B™ Cells (Thermo Fisher Scientific, Invitrogen™, catalog number: 18297010)
9. JBS DNA-Shuffling Kit (Jena Bioscience, catalog number: PP-103)
10. DNase I, RNase-free (1 U/ μ l) (Thermo Fisher Scientific, catalog number: EN0521)
11. EDTA
12. QIAquick PCR Purification Kit (250) (QIAGEN, catalog number: 28106)
13. Purified single-stranded AAV (any serotype) (Xiao *et al.*, 1998)
14. PfuUltra High-Fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA)
15. Wild type AAV2 plasmid psub201, plasmid pXR2 (RepCap plasmid) and pXX6-80 (a helper plasmid contains the genes E4, E2a and VA from adenovirus)
16. 1x PBS (Thermo Fisher Scientific, Gibco™, catalog number: 14190144)
17. Patient serum from clinical study for Duchenne Muscular Dystrophy (Bowles *et al.*, 2012)
18. Adenovirus dl309
19. DNeasy Blood and Tissue Kit (QIAGEN, catalog number: 69504)
20. Swal (New England Biolabs, catalog number: R0604S)
21. XbaI (New England Biolabs, catalog number: R0145S)
22. Passive lysis buffer (Promega, catalog number: E1941)
23. Luciferase Assay Substrate (Promega, catalog number: E151A)
24. XenoLight D-Luciferin - Bioluminescent Substrate (PerkinElmer, catalog number: 122799)
25. 25 mg/ml D-luciferin substrate (see Recipes)

Equipment

1. Pipettes
2. Bio-Rad Thermal Cyclers (Bio-Rad Laboratories, catalog number: 1709703)
3. Tabletop centrifuge (Eppendorf, model: 5424, catalog number: 022620401)
4. VICTOR Multilabel Plate Reader (PerkinElmer)
5. Small animal anesthesia system (Xenogen, XGI-8 Gas Anesthesia System)
6. IVIS Lumina In Vivo imaging system (PerkinElmer)

Software

1. Living Image software (PerkinElmer)

Procedure

A. The generation of a new AAV shuffling library

Note: There are multiple methods that can be used to generate DNA shuffling library, and a handful of kits available from manufacturers. DNA shuffling library can be generated using JBS DNA shuffling kit. The process of generation of AAV shuffling library is shown in Figure 1.

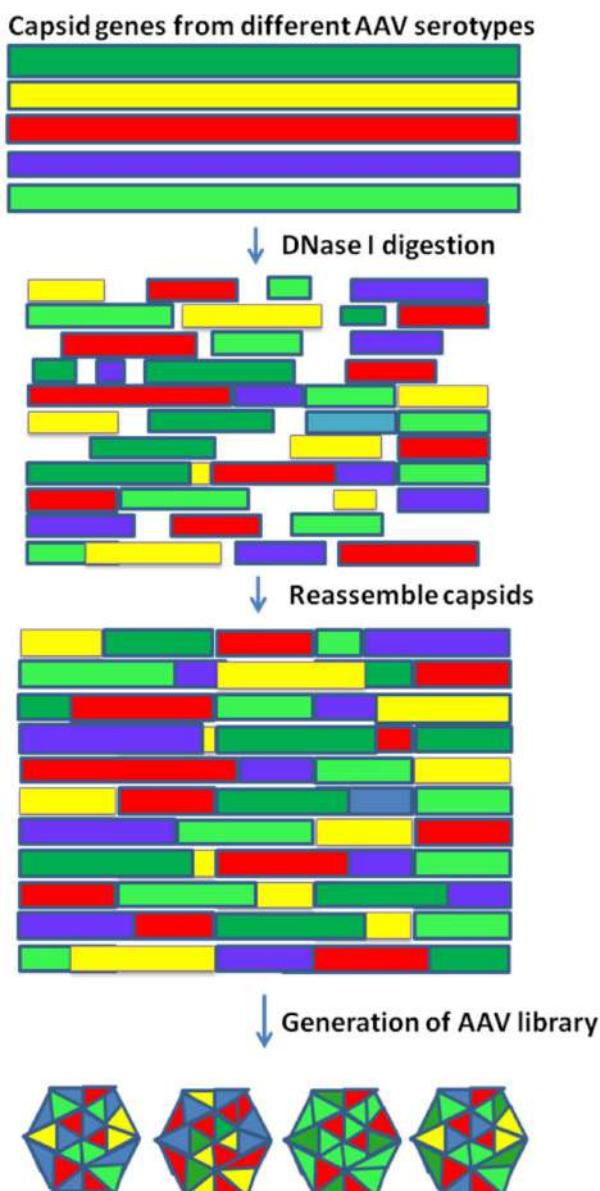


Figure 1. Flow chart for generation of AAV shuffling library

1. Amplify the capsid genes by PCR assay using AAV serotypes 1, 2, 3, 6, 8 and 9 mixed in equal ratios as templates. The primers sequences were shown in Table 2 of Li *et al.* (2008).
2. Treat a total of 4 µg of the DNA templates by 0.4 U of DNase I at 37 °C for 5 min. Stop DNase activation by adding 1 µl 25 mM EDTA, and then heat inactivation at 75 °C for 10 min.

3. Purify DNA fragments in size of 100-300 bp using DNA purification Kit following standard procedures.
4. Denature, re-anneal and repair the purified DNA fragments by Pfu Ultra High-Fidelity DNA polymerase to reassemble random capsid genes in 0.2 ml PCR tube. Run the PCR assay using the program in Table 1 (Li *et al.*, 2008; Yang *et al.*, 2009).

Table 1. PCR cycling parameter

Number of cycles	Temperature	Duration
1 cycle	96 °C	90 sec
	94 °C	30 sec
	65 °C	90 sec
	62 °C	90 sec
	59 °C	90 sec
	56 °C	90 sec
35 cycles	53 °C	90 sec
	50 °C	90 sec
	47 °C	90 sec
	44 °C	90 sec
	41 °C	90 sec
	72 °C	4 min
1 cycle	72 °C	7 min

5. Clone the capsids from DNA shuffling library into wild-type AAV2 plasmid psub201 to generate AAV capsid DNA library (Li *et al.*, 2008; Li *et al.*, 2016).
 6. Produce AAV shuffling library in HEK293 cells by co-transfection of two plasmids: pXX6-80 and AAV capsid DNA library.
 7. Purify single-stranded AAV with CsCl gradient ultracentrifugation and measure virus titer of shuffled AAV by quantitative PCR (Xiao *et al.*, 1998).
- B. Isolation of Nab escaping AAV mutants from mouse muscles
1. Mix 1×10^{10} particles of the AAV shuffling library virus in 50 μ l PBS with 50 μ l undiluted patient serum at 4 °C for 2 h.
 2. Inject the AAV/serum mixture using insulin syringes *via* intramuscular (i.m.) route into the hind leg muscle of 6-week-old female BALB/c mice (injection volume 100 μ l).
 3. Three days post-treatment, inject 10^7 virus particles (vg) of adenovirus (Adv) dl309 *via* i.m. into the same muscle to amplify AAV genomes *in vivo*.
- Note: Any replicable Adv can be used in this step.*
4. Collect muscles from injected mice at 2 days post Adv administration, and extract total DNA using DNeasy Blood and Tissue Kit.

Note: There are several methods that can be used to isolate DNA from tissues, and a handful of kits available from many manufacturers to do it. Here, we performed a genomic DNA isolation from mouse muscle using DNeasy Blood and Tissue Kit. In this protocol, the isolation of genomic DNA from muscle relies upon the columns and spin steps. Please follow the protocol of the manufacturer.

5. Amplify AAV mutant capsids by PCR assay using the genomic DNA from the muscle as templates. The sequences of the primers are F1 5'-CAACTCCATCACTAGGGTTC and R1 5'-CATGGGAAAGGTGCCAGA, which are localized at the AAV2 rep and AAV2 ITR, respectively. Run PCR assay using PfuUltra High-Fidelity DNA polymerase, following the program in Table 2.

Table 2. PCR cycling parameters

Number of cycles	Temperature	Duration
1 cycle	95 °C	2 min
	95 °C	30 sec
35 cycles	53 °C	30 sec
	72 °C	3 min
1 cycle	72 °C	10 min

6. Purify PCR products by the QIAquick PCR Purification Kit.
7. Digest with Swal and XbaI and ligate into pXR2 digested with the same endonucleases. Transform the constructed plasmids into a culture of DH10b competent cells.
Note: The plasmid pXR2 can be any expression plasmid with Rep gene of AAV for further AAV package.
8. Sequence clones and generate AAV/luc mutant vectors (Xiao *et al.*, 1998).

C. Nab escaping ability of AAV mutants *in vitro*

Note: Cell lines that can be transduced by AAV can be used for neutralizing assay. Add the serum/AAV mixture when the cells are suspended.

1. Seed Huh7 cells into a 48-well plate at a density of 10^5 cells for each well.
2. Incubate two-fold dilutions of the serum (from 1:4 to 1:1,024) with AAV-Luc (1×10^8 vg) for 1 h at 37 °C.
3. Add the mixture (diluted serum and AAV) into Huh7 cells and incubate for 48 h at 37 °C.
4. Discard the culture media.
5. Lyse cells with 100 µl 1x of passive lysis buffer for 30 min.
6. Transfer the 20 µl lysed medium into 96-well black plate.
7. Add luciferase substrate (100 µl) into 96-well black plate and briefly mix with lysed medium.
8. Measure luciferase activity using luminometer reader (VICTOR Multilabel Plate Reader).
9. Define Nab titers as the highest dilution for which luciferase activity is 50% lower than

serum-free controls.

D. Nab escaping ability of AAV mutants in mice

1. Incubate 1×10^{10} vg of AAV/luc mutants isolated from muscles with the 5-fold diluted patient serum or PBS for 2 h at 4 °C.
2. Inject the AAV/serum mixture (total volume 100 μ l) in the legs of six-week-old female BALB/c mice *via* i.m.
3. Image luciferase expression in mice at 3 weeks post-injection. Anesthetize mice using an isoflurane vaporizer and inject with 120 mg/kg of D-Luciferin substrate intraperitoneally 5 min before imaging. Place the mice inside the camera box of the IVIS system. Start imaging with an exposure capturing in mice.

Data analysis

1. After the mixture of AAV shuffling library and patient serum was injected into the muscles of the mice, imaging was carried out by the IVIS Lumina In Vivo imaging system, and the photon signal was measured by Living Image software. The results were shown in Figure 1b of the original paper (Li *et al.*, 2016).
2. The AAV mutant capsids were recovered and sequenced. The results were shown in Figure 1a of the original paper (Li *et al.*, 2016).
3. The recovered AAV mutant capsids were used to package the AAV mutants. The mixtures of the AAV mutants and patient serum were administrated into muscles of mice. The imaging was carried out by the IVIS Lumina In Vivo imaging system and the photon signal was measured by Living Image software. The results were shown in Figure 2 of the original paper (Li *et al.*, 2016).

Recipes

1. 25 mg/ml D-luciferin substrate
Prepare 25 mg/ml D-luciferin solution in DPBS

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References

1. Bowles, D. E., McPhee, S. W., Li, C., Gray, S. J., Samulski, J. J., Camp, A. S., Li, J., Wang, B., Monahan, P. E., Rabinowitz, J. E., Griege, J. C., Govindasamy, L., Agbandje-Mckenna, M., Xiao, X., and Samulski, R. J. (2012) [Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector.](#) *Mol Ther* 20(2), 443-455.
2. Li, W., Asokan, A., Wu, Z., Van Dyke, T., DiPrimio, N., Johnson, J. S., Govindaswamy, L., Agbandje-McKenna, M., Leichtle, S., Eugene Redmond, D., Jr., McCown, T. J., Petermann, K. B., Sharpless, N. E. and Samulski, R. J. (2008). [Engineering and selection of shuffled AAV genomes: A new strategy for producing targeted biological nanoparticles.](#) *Mol Ther* 16(7): 1252-1260.
3. Li, C, Wu S, Albright B, Hirsch M, Li W, Tseng YS, Agbandje-Mackenna M, McPhee S, Asokan A, Samulski, R.J., (2016).[Development of patient-specific AAV vectors after neutralizing antibody selection for enhanced muscle gene transfer.](#) *Mol Ther* 24(1): 53-65.
4. Xiao, X., Li, J. and Samulski, R. J. (1998). [Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus.](#) *J Virol* 72(3): 2224-2232.
5. Yang, L., Jiang, J., Drouin, L. M., Agbandje-McKenna, M., Chen, C., Qiao, C., Pu, D., Hu, X., Wang, D. Z., Li, J. and Xiao, X. (2009). [A myocardium tropic adeno-associated virus \(AAV\) evolved by DNA shuffling and in vivo selection.](#) *Proc Natl Acad Sci U S A* 106(10): 3946-3951.

Immunohistochemical Identification of Human Skeletal Muscle Macrophages

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[Abstract] Macrophages have well-characterized roles in skeletal muscle repair and regeneration. Relatively little is known regarding the role of resident macrophages in skeletal muscle homeostasis, extracellular matrix remodeling, growth, metabolism and adaptation to various stimuli including exercise and training. Despite speculation into macrophage contributions during these processes, studies characterizing macrophages in non-injured muscle are limited and methods used to identify macrophages vary. A standardized method for the identification of human resident skeletal muscle macrophages will aide in the characterization of these immune cells and allow for the comparison of results across studies. Here, we present an immunohistochemistry (IHC) protocol, validated by flow cytometry, to distinctly identify resident human skeletal muscle macrophage populations. We show that CD11b and CD206 double IHC effectively identifies macrophages in human skeletal muscle. Furthermore, the majority of macrophages in non-injured human skeletal muscle show a ‘mixed’ M1/M2 phenotype, expressing CD11b, CD14, CD68, CD86 and CD206. A relatively small population of CD11b+/CD206- macrophages are present in resting skeletal muscle. Changes in the relative abundance of this population may reflect important changes in the skeletal muscle environment. CD11b and CD206 IHC in muscle also reveals distinct morphological features of macrophages that may be related to the functional status of these cells.

Keywords: Skeletal muscle, Macrophages, Immune cells, Immunohistochemistry, CD68, CD11b, CD206, Flow cytometry

[Background] Macrophages are pleotropic immune cells capable of adapting to changes in the local microenvironment. Over the last several years, research has shown that macrophage phenotype is dynamic, existing on a continuum (Mosser and Edwards, 2008, Italiani and Boraschi, 2014, Martinez and Gordon, 2014). However, to date macrophage populations continue to be described using the

restrictive M1 and M2 classifications. It is commonly accepted that these designations are an oversimplification of macrophage phenotype and represent opposite extremes of a continuum (Mosser and Edwards, 2008; Gordon *et al.*, 2014; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Murray *et al.*, 2014). M1 macrophages are classically activated, have pro-inflammatory functions and are involved in host responses to pathogens and tissue injury. M2 macrophages are alternatively activated, exhibit anti-inflammatory functions and are involved in wound healing and tissue repair. In addition to the functional definition of M1 and M2, cell surface markers have been identified to distinguish between these populations. Surface markers associated with M1 macrophages include CD40, CD64 and the co-stimulatory molecules CD80/CD86 (Lolmede *et al.*, 2009; Ambarus *et al.*, 2012), whereas M2 macrophages have been shown to express high levels of CD163, CD206 and galactose receptors (Lolmede *et al.*, 2009; Ambarus *et al.*, 2012; Roszer, 2015).

From tissue to tissue, macrophage populations are heterogeneous adopting different functional roles depending on the local environment (Gordon *et al.*, 2014; Italiani and Boraschi, 2014). This, coupled with the macrophage continuum, has led to inconsistencies with regard to identification and nomenclature across fields and across species (Murray *et al.*, 2014). Skeletal muscle macrophages have primarily been studied in rodent models of injury, where the M1 versus M2 macrophage classification has proven useful (Smith *et al.*, 2008; Chazaud *et al.*, 2009; Tidball and Villalta, 2010; Kharraz *et al.*, 2013; Novak and Koh, 2013; Saclier *et al.*, 2013b; Rigamonti *et al.*, 2014; Tidball *et al.*, 2014; Wang *et al.*, 2014; Sciorati *et al.*, 2016; Varga *et al.*, 2016; Mackey and Kjaer, 2017). The skeletal muscle response to injury is characterized by highly orchestrated temporal processes. Initially, M1 macrophages phagocytize damaged skeletal muscle fibers and debris, followed by M2 macrophage-facilitated repair and regeneration (Chazaud *et al.*, 2009; Tidball and Villalta, 2010; Kharraz *et al.*, 2013; Saclier *et al.*, 2013a; Tidball *et al.*, 2014; Sciorati *et al.*, 2016). Recent work nicely details fiber repair in human skeletal muscle *in vivo*, showing the presence of macrophages, using a pan-macrophage intracellular marker (CD68+), in regenerating zones along injured fibers (Mackey and Kjaer, 2017). Direct interaction between macrophages and satellite cells (Dumont and Frenette, 2013; Ceafalan *et al.*, 2017; Du *et al.*, 2017; Wehling-Henricks *et al.*, 2018), and defects in skeletal muscle regeneration in the absence of macrophage participation (Arnold *et al.*, 2007; Melton *et al.*, 2016), highlight the necessity of these cells for skeletal muscle repair. *In vitro*, M1 macrophages promote skeletal muscle cell proliferation and M2 macrophages promote differentiation, suggesting that macrophages may play a role in skeletal muscle growth adaptations, as well as repair (Arnold *et al.*, 2007; Saclier *et al.*, 2013b).

Skeletal muscle is a highly adaptable tissue, able to respond to a wide range of external stimuli, such as exercise, inactivity, hormones and nutritional signals. In contrast to the clearly defined, strongly polarizing responses elicited by acute skeletal muscle injury, the role of tissue resident macrophages during less polarizing processes, such as responses to the aforementioned stimuli, is relatively unknown. Under non-damaging exercise conditions, animal studies report an increase in macrophage populations following aerobic and resistance exercise, linked to both metabolic and growth adaptations (DiPasquale *et al.*, 2007; Ikeda *et al.*, 2013). However, the mechanisms by which macrophages in

skeletal muscle influence training adaptations remain to be explored. It has also been reported that resident human skeletal muscle macrophage abundance is affected by aging, obesity and diabetes (Przybyla *et al.*, 2006; Hong *et al.*, 2009; Varma *et al.*, 2009; Tam *et al.*, 2012; Fink *et al.*, 2014; Reidy *et al.*, 2017); however, the inconsistent use of macrophage markers across studies has made the interpretation of these findings difficult. Further, the applicability of the distinctive M1/M2 markers of polarized macrophages to tissue resident macrophages is unclear, as surface markers may not be mutually exclusive on resident macrophages under non-polarizing conditions (Italiani and Boraschi, 2014). Thus, there is a need in the field for a standardized, validated method for identifying and quantifying macrophages in human skeletal muscle. Establishing a simple and reproducible protocol for studying muscle macrophages will aide in the characterization of their role in muscle adaptations to various stimuli, independent of injury.

Although results from studies of skeletal muscle macrophages in animal models are informative, these studies often use macrophage markers that are not directly translatable for use in humans. Even when human homologs do exist, the same surface markers in mouse and rat skeletal muscles often identify different populations in human skeletal muscles, complicating the extrapolation of findings from rodent models to human studies (Murray *et al.*, 2014). For example, CD68 is used as a pan-macrophage marker in humans and an M1 marker in mice. There is a need in the field for a standardized, validated method for identifying and quantifying macrophages in human skeletal muscle. Taking into account the limited mass of frozen muscle tissue available for analyses from human skeletal muscle biopsies (normally in the range of 100 mg), an immunohistochemical method is the most feasible approach to identifying and quantifying human skeletal muscle macrophage populations.

A variety of markers have been used to characterize human macrophages by flow cytometry. The most detailed studies have been performed utilizing peripheral blood mononuclear cells (PBMCs), artificially polarized to an M1 or M2 phenotype (Martinez *et al.*, 2006; Ambarus *et al.*, 2012; Iqbal, 2015). These *in vitro* studies characterize the expression of various marker combinations on M1 and M2 macrophages and provide a good starting point for choosing markers to identify macrophage populations in frozen human skeletal muscle tissue. CD14 has been identified as a monocyte marker, expressed mainly by macrophages but also neutrophils and dendritic cells (Table 1). In blood, CD14 co-staining with CD16 is used to stratify monocytes into three subsets: classical (CD14++/CD16-), intermediate (CD14++/CD16+) and non-classical (CD14+/CD16++) (Sprangers *et al.*, 2016; Boyette *et al.*, 2017). It is thought that classical monocytes give rise to tissue macrophages under homeostatic conditions; however, during an inflammatory insult all monocyte populations differentiate into macrophages (Italiani and Boraschi, 2014; Sprangers *et al.*, 2016). In tissue, CD16 is predominantly used to identify NK cells, but is also expressed on neutrophils, granulocytes, dendritic cells and some macrophage populations (Table 1). CD11b is a commonly used marker and is expressed on subsets of lymphocytes and monocytes, these include natural killer (NK) cells, granulocytes and macrophages (Table 1). CD68 is expressed by cells in the monocyte lineage, including macrophages, and is the most commonly used macrophage marker in human skeletal muscle tissue (Table 1) (Stupka *et al.*, 2001; Beaton *et al.*, 2002; Peterson *et al.*, 2003; Crameri *et al.*, 2004; Przybyla *et al.*, 2006; Crameri *et al.*,

2007; Mahoney *et al.*, 2008; Mikkelsen *et al.*, 2009; Varma *et al.*, 2009; Paulsen *et al.*, 2010a; Paulsen *et al.*, 2010b; MacNeil *et al.*, 2011; Tam *et al.*, 2012; Chistiakov *et al.*, 2017; Mackey and Kjaer, 2017; Reidy *et al.*, 2017). CD68 is a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family of proteins, which are mainly associated with the endosomal/lysosomal compartment. Though largely intracellular, CD68 can traffic to the cell surface. Of note, other cell types have been reported to express CD68, including hematopoietic cells, fibroblasts and endothelial cells (Table 1) (Kunisch *et al.*, 2004; Gottfried *et al.*, 2008; Paulsen *et al.*, 2013; Chistiakov *et al.*, 2017). CD206, the mannose receptor, is a well-accepted macrophage marker in skeletal muscle and is widely used to identify M2 macrophage subsets (Lolmede *et al.*, 2009; Ambarus *et al.*, 2012; Italiani and Boraschi, 2014; Roszer, 2015), although CD206 expression by other cell types (including satellite cells) has been reported (Table 1) (Jansen and Pavlath, 2006). M2 macrophages also express CD163 (Table 1) (Lolmede *et al.*, 2009; Ambarus *et al.*, 2012; Roszer, 2015). CD80 and CD86 are co-stimulatory molecules expressed by antigen presenting cells upon activation and have been used to identify M1 macrophage populations (Table 1) (Mosser and Edwards, 2008; Lolmede *et al.*, 2009; Ambarus *et al.*, 2012). Using three grams of discarded human hamstring muscle from patients undergoing anterior cruciate ligament (ACL) reconstruction surgery, we isolated and labeled mononuclear cells with antibodies against some of the markers described above (CD11b, CD14, CD16, CD86 and CD206) and performed multichannel flow cytometry. Due to the intracellular expression of CD68, we were not able to include CD68 in flow cytometry analyses. Mononuclear cells from skeletal muscle did not express CD16, but co-expressed the other 4 markers tested (Figures 1A-1E). Thus, human skeletal muscle macrophages have a ‘mixed’ phenotype, co-expressing both M1 (CD86) and M2 (CD206) cell surface markers (Figure 1D).

Table 1. Overview of monocyte and macrophage markers

Marker	Synonyms	Tissue cell populations	Monocyte/macrophage subset	Notes
CD11b	CR3, C3biR, Integrin alphaM, ITGAM, Mac-1, Mo1	B cells, dendritic cells, granulocytes, macrophages, monocytes, NK cells and T cells	Pan-macrophage	
*CD14	Monocyte differentiation antigen CD14, LPS receptor	B cells, dendritic cells, granulocytes, macrophages and monocytes	Pan-monocyte	*Co-expression with CD16 identifies monocyte subsets
*CD16	FcyRIIIA	B cells, macrophages, monocytes, neutrophils, NK cells T cells	CD14++/CD16-- classical CD14++/CD16+ - intermediate CD14+/CD16++ - non-classical	
CD68	gp110, LAMP4, Macrosialin, SCARD1	B cells, dendritic cells, endothelial cells, fibroblasts, granulocytes, hematopoietic stem cells (LT), macrophages, monocytes, NK cells and T cells	Pan-macrophage	Intracellular expression
CD80	B7, B7-1, B7.1 BB1, CD28LG, CD28LG1, LAB7	B cells, dendritic cells, macrophages, monocytes and T cells	M1	Co-stimulatory with CD86
CD86	B7-2, B7.2, B70, CD28LG2, LAB72	B cells, dendritic cells, macrophages, monocytes and T cells	M1	
CD163	D11, D1 1C163A, GHI/61, M130, MM130, RM3/1, SCARI	B cells, macrophages and monocytes	M2c	Identifies a subset of CD206+ macrophages in skeletal muscle
CD206	CLEC13D, mannose receptor, MMR, MRC1	Dendritic cells, certain skin cells, endothelial cells, macrophages and monocytes	M2	In rare instances, Pax7+ skeletal muscle satellite cells co-express CD206
Markers in non-injured human skeletal muscle				
CD11b+/CD206-	M1-like macrophages			FC - Also expresses CD14
CD11b+/CD206+	M2-like macrophages			FC - Also express CD14 and CD86
CD163+/CD206+	M2c macrophages			
FC = multichannel flow cytometry analysis				

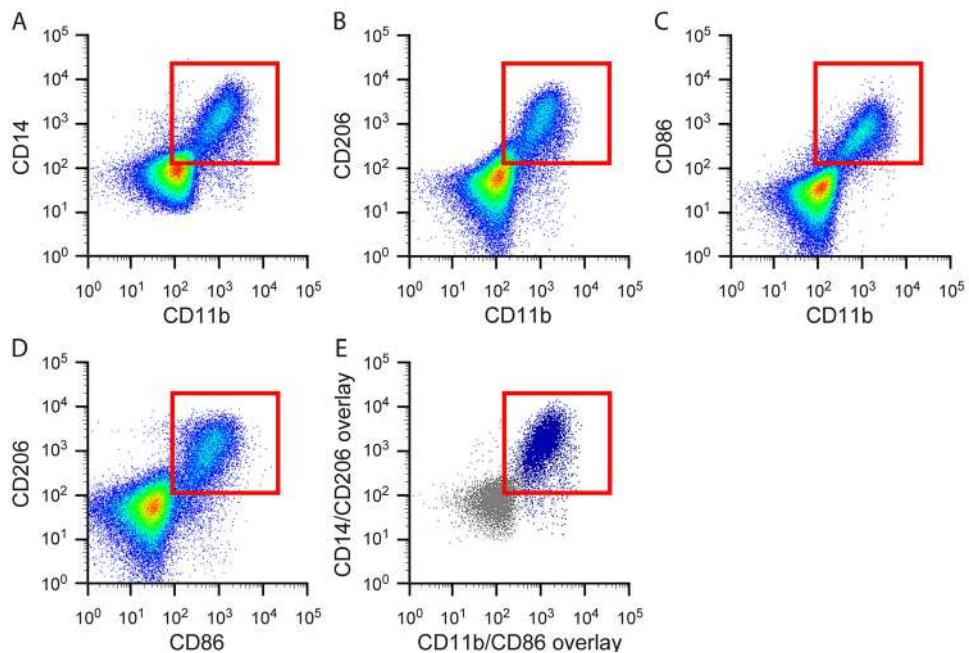


Figure 1. Flow cytometry from discarded human hamstring muscle showing co-expression of both M1 and M2 macrophage markers. Mononuclear cells isolated from human skeletal muscle express A. Both pan-monocyte markers CD11b and CD14; B. Both the M2 macrophage marker, CD206, and the pan marker, CD11b; C. Both the M1 marker, CD86, and the pan marker CD11b; D. Both the M2 marker, CD206, and the M1 marker, CD86; E. Overlay of CD206+/CD86+ populations from panel D onto CD14/CD11b flow plot shown in panel A. This overlay shows that CD206+/CD86+ macrophages (denoted in dark blue) also express the pan-monocyte markers CD11b and CD14. Red boxes indicate cells that are double positive for the markers shown.

Using these 4 cell surface antibodies against CD11b, CD14, CD86 and CD206 that label skeletal muscle resident macrophages, we sought to develop a simple and reproducible immunohistochemical method for identification of macrophages in fresh frozen human skeletal muscle sections. CD68 is a commonly used pan-macrophage marker in human skeletal muscle. However, CD68 expression is predominantly intracellular, requiring permeabilization steps to perform immunohistochemistry (IHC). These permeabilization steps lead to inconsistent results and compromise staining with additional antibodies for cell surface markers. The cell surface localization of CD11b results in better morphological definition of macrophages and more consistent staining across samples than intracellular CD68 staining. Moreover, CD11b can readily be combined with CD206 and other cell surface markers for IHC. For these reasons, we compared CD11b and CD68 staining, and found CD11b to be comparable to CD68 as a pan-macrophage marker in human skeletal muscle (Figures 2A-2E; CD11b and CD68 antibodies cannot be used to label sections simultaneously for technical reasons, see General Note 12). Furthermore, distinct morphological features of muscle macrophages that may be related to functional status was revealed through CD11b and CD206 double IHC (Figures 8A-8C) (Durafourt *et al.*, 2012; McWhorter *et al.*, 2013). We describe here a detailed method for

combined IHC using CD11b and CD206 antibodies on frozen human skeletal muscle sections. We also describe in detail our approach to quantifying macrophage subsets in non-injured skeletal muscle. We find the majority of human muscle macrophages are CD11b+/CD206+, whereas a small subset are CD11b+/CD206- (Figures 4A-4C). We were unable to obtain IHC results with antibodies against M1 cell surface markers (CD80 or CD86). Of note, anti-CD163 works well on frozen human skeletal muscle and can be used in place of CD11b and in combination with CD206 with this protocol (see General Note 13). Moreover, combining Pax7 (a satellite cell marker) IHC with CD206 shows very little co-expression, supporting the conclusion that CD206 co-localizes with CD11b in human skeletal muscle and is a valid macrophage marker. This protocol allows reproducible quantification of the relative abundance of CD11b+/CD206+ and CD11b+/CD206- macrophage populations in human skeletal muscle, which can be extended to human skeletal muscle adaptations, aging and disease, enabling comparison of results across studies, across labs and across diverse human populations.

Materials and Reagents

1. Pipette tips:
 - 101-1,000 µl (USA Scientific, TipOne, catalog number: 1122-1832)
 - 0.1-10 µl (USA Scientific, TipOne, catalog number: 1120-3812)
2. Gloves (VWR, catalog number: 82026-424)
3. Nalgene Dewar (for liquid nitrogen) (Sigma-Aldrich, catalog number: F9401)
4. Tri-Pour Polypropylene beaker (for cooling isopentane in liquid nitrogen) (VWR, catalog number: 89011-786)
Manufacturer: MEDEGEN MEDICAL PRODUCTS, catalog number: PB5935-400.
5. MX35 Ultra Low-Profile blades for cryotomy (Thermo Fisher Scientific, catalog number: 3053835)
6. Superfrost Plus slides (Fisher Scientific, catalog number: 12-550-15)
7. Shandon Single Cytoslides (Thermo Fisher Scientific, catalog number: 5991056)
8. Shandon Single Cytofunnel (Thermo Fisher Scientific, catalog number: 5991040)
9. ImmEdge PAP pen (Vector Laboratories, catalog number: H-4000)
10. 1.5 ml microcentrifuge tubes (USA Scientific, catalog number: 1615-5599)
11. 15 ml conical tube (VWR, catalog number: 89039-666)
12. 24 x 50 mm, No. 1 coverglass (VWR, catalog number: 48393-081)
13. Kimwipes (KCWW, Kimberly-Clark, catalog number: 34120)
14. Cork stoppers for making muscle mounts (Fisher Scientific, catalog number: 07-782J)
15. PTFE (Teflon) coated stainless steel spatula (Fisher Scientific, catalog number: 21-401-50A)
Manufacturer: Saint-Gobain Performance Plastics, catalog number: D1069292.
16. #10 curved blade disposable scalpel (Sklar Surgical Instruments, catalog number: 06-3310)
17. Dumont #7 curved forceps (Fine Science Tools, catalog number: 11270-20)
18. Cryo Tongs (Thermo Fisher Scientific, catalog number: 4000388)

19. Tragacanth gum, powder (Sigma-Aldrich, catalog number: G1128-500G)
20. Fisher O.C.T compound (Fisher Scientific, catalog number: 23-730-571)
21. Isopentane (2-methylbutane) (Merck, catalog number: MX0760-1)
22. Liquid nitrogen (Scott Gross, catalog number: SG #347)
23. Dry ice (Scott Gross, no catalog number available)
24. Ice cold acetone, stored at -20 °C (Fisher Scientific, catalog number: A18-4)
25. Streptavidin/Biotin blocking kit (Vector Laboratories, catalog number: SP-2002)
26. 2.5% normal horse serum (NHS) (Vector Laboratories, catalog number: S-2012)
27. 50% 1x PBS (see 21 and Recipes)/50% glycerol mounting medium (glycerol - VWR, catalog number: BDH1172-1LP) or Vectashield (Vector Laboratories, catalog number: H-1000)
28. Antibodies for IHC, dilutions made with 2.5% normal horse serum or PBS (Table 2)
29. ImmPRESS-AP Anti-Mouse IgG (alkaline phosphatase) polymer detection kit (Vector Laboratories, catalog number: MP-5402)
30. ImmPACT Vector Red Alkaline Phosphatase (AP) Substrate (Vector Laboratories, catalog number: SK-5105)
31. Antibodies for multichannel flow cytometry (Table 3)

Table 2. Detailed IHC Antibody Information

Primary Antibody	Company	Catalog Number	Reactivity	Host /Isotype	Dilution/Diluent
CD68	Dako	M0814	Human/Mouse/Rat/Monkey	Mouse/IgG1	(1:100)/2.5% NHS
CD11b	Cell Sciences	MON1019	Human	Mouse/IgG1	(1:100)/2.5% NHS
Purified IgG1, κ	BD	555746	N/A	Mouse/IgG1	(1:500)/2.5% NHS
C206	R&D Systems	AF2534	Human	Goat/IgG (polyclonal)	(1:200)/2.5% NHS
CD163	Hycult Biotech	HM2157	Human/Monkey	Mouse/IgG1	(1:50)/2.5% NHS
Pax7	Developmental Studies Hybridoma Bank	Pax7	Human/Mouse/Rat	Mouse/IgG1	(1:100)/2.5% NHS
Biotinylated goat anti-mouse IgG1	Jackson ImmunoResearch	115-065-205	Mouse IgG1	Goat/N/A	(1:1,000)/2.5% NHS
ImmPRESS -AP	Vector Laboratories	MP-5402	Mouse IgG	N/A	Neat/no dilution
Biotinylated rabbit anti-goat IgG	Vector Laboratories	BA-5000	Goat	Rabbit/N/A	(1:500)/2.5% NHS
Streptavidin HRP (SA-HRP)	Thermo Fisher Scientific	S911	Biotin	N/A	(1:500)/PBS
Streptavidin Alexa Fluor 594 (SA-594)	Thermo Fisher Scientific	S32356	Biotin	N/A	(1:200)/PBS
Superboost TSA Alexa Fluor 488 (TSA 488)	Thermo Fisher Scientific	B40953	HRP	N/A	(1:500)/PBS
ImmPACT Vector Red kit	Vector Laboratories	SK-5105	Alkaline Phosphatase	N/A	According to manufacturer's instructions

Table 3. Detailed antibody information for multichannel flow cytometry

Antigen	Fluorophore	Laser	Filter/Bandpass	Ex (nm)	Em (nm)	Concentration ($\mu\text{g}/\mu\text{l}$)	$\mu\text{l}/10^6 \text{ cells (500 }\mu\text{l total volume)}$	Company	Catalog Number
CD11b	Alex Fluor 488	Blue (488)	530/30	490	525	0.4*	25	BioLegend	301317
CD14	Pacific Blue	Violet (407)	450/50	410	455	0.5	20	BioLegend	325615
CD16	VioGreen	Violet (407)	525/50	405	520	NA	50	Miltenyi Biotec	130-113-959
CD86	Phycoerythrin (PE)	Blue (488)	575/26	496	578	0.1*	25	BioLegend	305405
CD206	PerCP/Cy5.5	Blue (488)	695/40	482	695	0.1*	25	BioLegend	321121
LIVE/DEAD	Fixable Blue	UV (325)	450/50	350	450	NA	0.5	Thermo Fisher Scientific	L34961
Mouse IgG1, κ	Alexa Fluor 488	Blue (488)	530/30	490	525	0.2*	50	BioLegend	400132
Mouse IgG1, κ	Pacific blue	Violet (407)	450/50	410	455	0.5	20	BioLegend	400131
REA Control (S)	VioGreen	Violet (407)	525/50	405	520	NA	50	Miltenyi Biotec	130-104-608
Mouse IgG2b, κ	Phycoerythrin (PE)	Blue (488)	575/26	496	578	0.2	12.5	BioLegend	400311
Mouse IgG1, κ	PerCP/Cy5.5	Blue (488)	695/40	482	695	0.2*	12.5	BioLegend	400149

*Concentration is not specific and varies by batch

32. LIVE/DEAD Fixable Blue Dead Cell Stain Kit, for UV excitation (Thermo Fisher Scientific, catalog number: L34961) (Table 3)
33. UltraComp eBeads Compensation Beads (Thermo Fisher Scientific, catalog number: 01-2222-41)
34. Polyurethane ice bucket (Fisher Scientific, catalog number: 02-591-45)
35. 1x phosphate-buffered saline (PBS) (see Recipes)
 - a. Deionized (DI) water
 - b. Sodium Chloride (NaCl) (VWR, catalog number: 97061-266)
 - c. Disodium hydrogen phosphate heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) (VWR, catalog number: 200007-704)
Manufacturer: Acros Organic, catalog number: 206515000.
 - d. Potassium phosphate monobasic (KH_2PO_4) (Aldon, catalog number: PP0730-500GR)
 - e. Sodium hydroxide 10 N (NaOH) (VWR, catalog number: BDH7247-1)
 - f. Hydrochloric acid, 6 N (HCl) (VWR, catalog number: 97064-758)
36. 30% Hydrogen peroxide, ACS, Stabilized (VWR, catalog number: BDH7690-1) (see Recipes)
37. DAPI for staining cell nuclei (4',6-diamidino-2-phenylindole) (Thermo Fisher Scientific, Molecular Probes™, catalog number: D1306) (see Recipes)
38. Rabbit polyclonal anti-Laminin (Sigma-Aldrich, catalog number: L9393). Use at a dilution of 1:100 in 2.5% NHS

Equipment

1. Epifluorescent microscope with automated stage (ZEISS, model: Axio Imager M1)
2. Cryostat (Thermo Fisher Scientific, model: HM525 NX)
3. P1000 pipetman (Gilson, catalog number: F123602)
4. P10 pipetman (Gilson, catalog number: F144802)
5. P2 pipetman (Gilson, catalog number: F144801)
6. Glass Coplin jars (VWR, catalog number: 470175-194)
Manufacturer: VARIETY GLASS, catalog number: 674.
7. Humidifying slide chamber (10 slide staining tray with black lid) (Electron Microscopy Sciences, catalog number: 71396-B)
8. Variable speed 2D rocker, 14 x 12 work surface (USA Scientific, catalog number: 2527-2000)
9. 4 °C refrigerator (Fisher Scientific, model: Isotemp Value Lab, catalog number: 17LREEFSA)
10. -80 °C freezer (Thermo Fisher Scientific, model: Revco® Elite Plus)
11. Class II Type A/B3 Biological safety cabinet (NuAire)
12. LSR II flow cytometer equipped with a 355 nm, 405 nm and 488 nm lasers (BD Biosciences)
13. Shandon Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific, catalog number: A78300003)

Software

1. Image capture software (Zeiss, Zen blue)
2. Image processing software with an event count tool (Zeiss, Zen blue or Zen lite)
3. Prism7 or equivalent graphing software (GraphPad)
4. FlowJo v10 (FlowJo)

Procedure

- A. Multichannel flow cytometry validation of immunohistochemistry (IHC) macrophage markers
1. Approximately 3 g of human skeletal muscle tissue is cleaned from a patient's hamstring tendon that is being utilized for ACL reconstruction surgery (performed in the operating room by trained medical personnel).
 2. Place skeletal muscle tissue in an ice bucket, on ice for transport back to the laboratory, then place inside a UV sterilized biosafety cabinet and perform all possible remaining steps inside the biosafety cabinet.
 3. Split the skeletal muscle specimen into multiple chunks as needed (1-2 g each) to allow for optimal tissue digestion.
 4. Isolate skeletal muscle mononuclear cells according to the established protocol referenced here (Liu *et al.*, 2015).
 5. After obtaining a single cell suspension, centrifuge at 500 x g (rcf) (relative centrifugal force or g-force) for 5 min at 4 °C with gentle braking to pellet cells.
 6. Wash the cell pellet by resuspending with 1 ml sterile (autoclaved) 1x PBS, at this step, cell suspensions from multiple muscle chunks can be recombined back into one sample if desired.
 7. Repeat Step A5 to pellet cells and resuspend in 500 µl of fluorophore-conjugated antibody cocktail (Table 3).
 8. Mix well by pipetting up and down 6-8 times and incubate at 4 °C in the dark for 1 h.
 9. Centrifuge at 500 x g (rcf) for 5 min at 4 °C with gentle braking to pellet cells, then resuspend in sterile 1x PBS to wash.
 10. Repeat Step A9 for a second wash then resuspend cell pellet in 1 ml sterile 1x PBS for FACS.
 11. Store in an ice bucket, on ice until fluorescence-activated cell sorting (FACS).
 - a. Incubate 1 drop of UltraComp eBeads Compensation Beads with each individual antibody and follow the manufacturer's protocol to set single channel compensation parameters for each fluorophore-conjugated antibody.
 - b. An unstained sample of cell suspension should always be run to determine the location of negative cell populations (Liu *et al.*, 2015).
 - c. Determine the dead cells by LIVE/DEAD Fixable blue staining and exclude them from analyses by gating on live cells only.

- d. Set a gate excluding doublets (Liu *et al.*, 2015) using the forward scatter height (y-axis) and forward scatter area (x-axis).
 - e. Isotype control antibodies in the appropriate fluorophores are used as a control to ensure antibody staining specificity (Table 3).
12. FACS sort CD11b+/CD14+ cells for immunocytochemistry (ICC) and collect into 1x PBS.
 13. Following the sort, pellet cells by centrifugation at 500 \times g for 5 min at 4 °C and resuspend in 600 μ l of 1x PBS.
 14. Load 200 μ l of cell suspension into a Shandon Single Cytofunnel and centrifuge with a Shandon Cytospin 4 at 1,000 revolutions per minute (RPM) for 3 min to place cells onto Shandon Single Cytoslides.
 15. Use PAP pen to trace the circle printed on the slide indicating where the sample is located and allow slides to air dry on the bench at room temperature (RT) for 30 min.
 16. Follow Steps C8-C22 of the CD11b/CD206 double IHC protocol below, replacing the primary antibody against CD11b with anti-CD68 (Step C20c) at a 1:100 dilution (Table 2).
 17. Following overnight (ON) incubation with primary anti-CD68 antibody and washes, incubate cells with ImmPRESS-AP, anti-mouse IgG (alkaline phosphatase) polymer detection kit for 1 h at RT, rocking (Table 2).
 18. Discard ImmPRESS-AP reagent and wash cells by pipetting 1x PBS into PAP pen circle, then rock for 5 min at RT. Repeat wash 3 more times for a total of 4 washes.
 19. Visualize CD68+ staining by adding alkaline phosphatase substrate using the ImmPACT Vector Red kit according to the manufacturer's protocol (Table 2).
 - a. Staining intensity can be monitored using a standard light microscope to determine the optimal amount of staining time; 10-20 min was found to be sufficient.
 - b. Staining will be both chromogenic (appearing red) and fluorescent (visible using a TRITC filter cube).
 20. Repeat Step A18 (above) to wash and coverslip using 50% 1x PBS/50% glycerol.
 21. Image or store for up to a month at 4 °C in the dark (Figure 2).

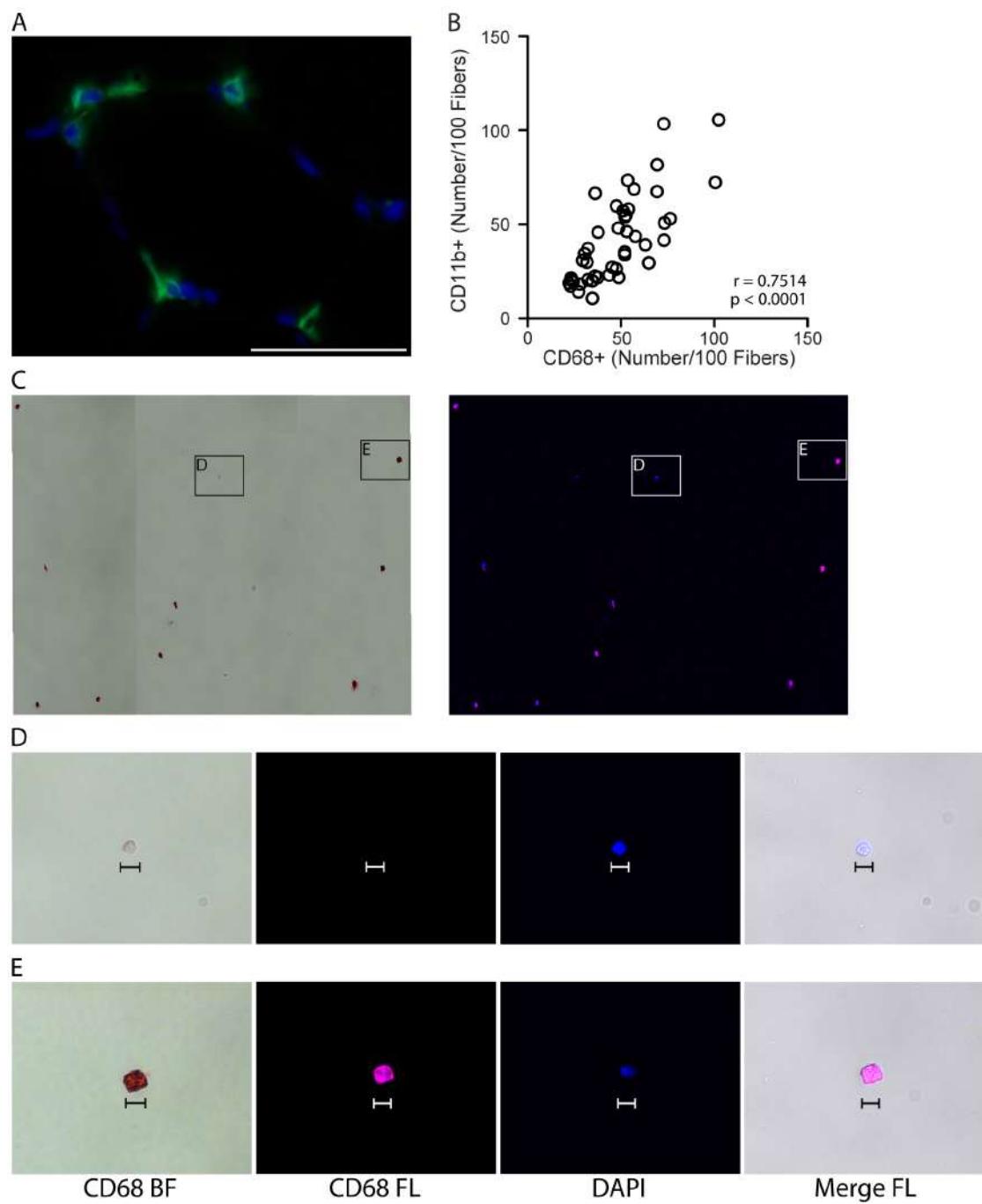


Figure 2. Validating CD11b as a pan-macrophage, cell surface marker in human skeletal muscle. A. Representative image showing IHC for the pan, intracellular macrophage marker, CD68 (green), with DAPI stained cell nuclei (blue) in human vastus lateralis muscle. Scale bar = 50 μ m. B. Correlation of CD11b+ and CD68+ macrophage numbers, identified by IHC on consecutive sections from vastus lateralis muscle, $n = 44$ muscle samples analyzed. P value determined by Pearson's correlation, $r =$ Pearson's correlation coefficient. C. CD14+/CD11b+ mononuclear cells (shown in Figure 1, panel A) were isolated by fluorescence-activated cell sorting (FACS) and stained for CD68. Positive staining is both chromogenic (red when imaged with transmitted light, bright field, BF) and fluorescent (pseudocolored pink, FL). Left image: CD68 BF; positive staining is

red. Right: CD68 FL, positive staining is pink. Cell nuclei were labeled with DAPI (blue). D-E. High magnification images showing a CD68- event (D) and CD68+ cell (E) from panel C. CD68- events were small (< 10 µm) and did not appear to be monocytes/macrophages. CD68- structures appeared to stain with DAPI, suggesting that this may be cellular debris (cell nucleus without cytoplasm). Scale bars = 10 µm.

B. Preparing IHC mounts from a Bergstrom needle human skeletal muscle biopsy (Tarnopolsky *et al.*, 2011; Shanely *et al.*, 2014)

Note: This method is optional for preparing human skeletal muscle IHC mounts.

1. Mix tragacanth gum powder and O.C.T compound with a Teflon coated spatula at a ratio of 1 part tragacanth gum powder to 1.5 parts O.C.T to make a thick paste (mounting medium).
2. Apply mounting medium to cork and mold into a donut shape, leaving a hole in the center where the muscle specimen will be placed (Figure 3A).
3. Following the skeletal muscle biopsy, remove the tissue from the Bergstrom needle and quickly identify skeletal muscle pieces free of fat and connective tissue.
4. Gently separate individual pieces, choosing the largest intact pieces for IHC (~0.5-0.75 cm in length and 50-100 mg of total tissue).
5. Trim any fat, connective tissue or accessory skeletal muscle fragments with a scalpel to clean the sample.
6. Lay each skeletal muscle piece for IHC side by side lengthwise, with the edges on one end aligned (this will become the top of the IHC mount) and trim the other end with a #10 curved blade scalpel to align (bottom of IHC mount) (Figure 3B).
7. Using fine-tipped forceps and the Teflon spatula, gently roll the skeletal muscle pieces together into a solid cylindrical shape (muscle cylinder).
8. Roll the muscle cylinder onto the tip of the spatula so that the bottom (the edge you trimmed) is aligned with the tip of the spatula.
9. Stand the muscle cylinder/spatula up so that skeletal muscle fibers are now running perpendicular to bench and the untrimmed, aligned edge (top) is facing up.
10. Gently place the muscle cylinder into the center of the mounting medium donut, against one side of the donut and use forceps to push the muscle cylinder off of the spatula. Remove the spatula. The muscle cylinder is now perpendicular to the surface of the cork with the bottom, trimmed end on the cork and the long axis of the skeletal muscle fibers sticking up (Figure 3C).
11. Using the spatula, work the mounting medium in to completely enclose the skeletal muscle specimen, closing gaps and leaving no air pockets between the muscle cylinder and the mounting medium (this protects the muscle specimen from freeze damage).
12. Sculpt the top of the mounting medium so that it is flush with the top of the skeletal muscle specimen.
13. Rapidly freeze the IHC mount by placing into liquid nitrogen cooled isopentane for 2 min.

- a. Isopentane should be a slushy consistency, with solid just beginning to form.
 - b. The cork side of the mount should be facing up and the skeletal muscle specimen facing down, completely submerged in the cooled isopentane.
14. Once frozen, use tongs to remove the IHC mount from the cooled isopentane and place on dry ice to rest for 5 min.
15. The final IHC mount is now ready to be stored at -80 °C until you are ready to section (Figures 3D-3E).

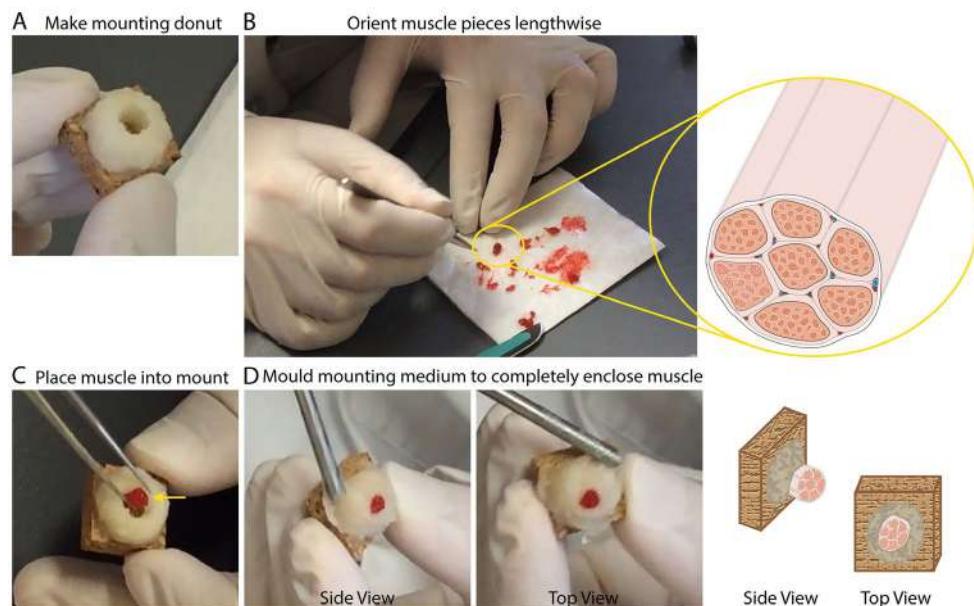


Figure 3. Preparing IHC mounts from human skeletal muscle. A. Representative image illustrating the donut, made from a mixture of Tragacanth powder and O.C.T. (mounting medium), described under Procedure B Step 2. B. Representative image and illustration depicting the lengthwise orientation of muscle pieces to be mounted for sectioning. C. Representative image showing the placement of a muscle cylinder into the donut made of mounting medium. Yellow arrow serves to illustrate that the muscle is positioned so that it is against one side/wall of the donut. D. Representative images of the finished IHC muscle mount (just prior to freezing); side view (left) and top view (right).

C. Skeletal Muscle Sectioning and CD11b/CD206 Double IHC protocol

1. Pull IHC mount from -80 °C freezer and transport to the cryostat in a Styrofoam cooler on dry ice. Set the chamber temperature in the cryostat to -25 °C and place the IHC mount into the cryostat chamber.
2. Adhere the IHC mount to a chuck by covering the top of the chuck with O.C.T Compound, setting the cork into the O.C.T using forceps and allowing the O.C.T to harden by freezing.
3. Allow the IHC mount to acclimate to the temperature of the chamber for 1 h prior to sectioning. The chamber temperature may need to be adjusted for each sample in order to achieve flat sections with the greatest number of fibers in cross-section.

4. Cross-section frozen muscle mounts with the cryostat stage angle between 8° and 10°.
 - a. The stage angle may also need adjusted for some samples; however, this is less common and stage angles outside of this range are not recommended.
 - b. Sample should be adjusted so the top of the mount is perpendicular to the blade when sectioning. In order to obtain cross-sectional fibers, small adjustments in the angle of the chuck/mount may need to be made. Once adjustments are made, quality sections should be cut containing at least 100 muscle fibers and no more than 20% longitudinal fibers.
5. Using the anti-roll plate to keep sections from curling, cut frozen sections at 7 µm and pick up onto charged Superfrost Plus slides.
6. Allow sections to dry on the benchtop at RT, then circle samples to be stained using an ImmEdge PAP pen.
7. Allow PAP pen to dry for an additional 20-30 min.
8. Fix sections at -20 °C for 3 min by submerging slides in a Coplin jar containing ice cold acetone. Acetone should be stored at -20 °C to maintain temperature.
9. Quickly dab slide edges onto a paper towel to drain excess acetone and transfer to a Coplin jar filled with 1x PBS at RT.
10. Rock the slides in 1x PBS for 5 min; repeat wash step two more times for a total of three washes.
11. Remove slides from Coplin jar, gently flick to remove excess 1x PBS, wipe the back of the slide with a paper towel and place into a humidifying slide chamber containing approximately 1.5 cm of water in the reservoir.
12. Block endogenous peroxidases by pipetting 3% hydrogen peroxide onto the sections and allow to incubate, rocking at RT for 8 min.
13. Gently dump the hydrogen peroxide onto a paper towel to discard, replace with 1x PBS and rock for 5 min at RT.
14. Repeat Step C13 two more times for a total of three washes.
15. Dump excess 1x PBS onto a paper towel, flick slide, wipe the back with a paper towel and place back in the slide chamber.
16. Perform Streptavidin/Biotin blocking using the kit per the manufacturer's instructions, carrying out all incubations in the humidifying slide chamber:
 - a. Incubate sections with Streptavidin blocking solution for 15 min at RT.
 - b. Wash briefly: two times, 2 min with 1x PBS.
 - c. Incubate sections with Biotin blocking solution for 15 min at RT.
17. Remove biotin blocking solution and replace with 1x PBS and incubate for 5 min, at RT, rocking. Repeat this wash with 1x PBS a total of three times.
18. Dump 1x PBS onto a paper towel, flick slide to remove excess 1x PBS, wipe the back of the slide with a paper towel and return to the slide chamber.
19. Add enough volume of 2.5% normal horse serum (NHS) to completely cover the sections and rock at 4 °C, overnight.

20. Remove 2.5% NHS, gently dab PAP pen with a kimwipe to re-establish hydrophobic barrier, add the primary antibody for CD11b, diluted 1:100 in 2.5% NHS, and return to the rocker at 4 °C overnight.
 - a. To ensure the specificity of the primary antibody against CD11b, an isotype specific control should also be prepared by adding purified mouse IgG1, κ to a section at the same concentration as the primary antibody against CD11b (0.1 mg/ml). The isotype control listed in Table 2 has a concentration of 0.5 mg/ml and should be diluted 1:500 in 2.5% NHS and incubated overnight at 4 °C, rocking (Figure 4A).
 - b. To determine signal produced by background staining of the tissue specimen, a no primary antibody control should be included. Prepare the no primary antibody control by covering the section in 2.5% NHS alone (omitting any antibody) and incubating overnight at 4 °C, rocking (Figure 4B).
 - c. For identification of macrophages using CD68, substitute anti-CD68 primary antibody, diluted 1:100, for primary antibody against CD11b in this step (Table 2) (Figure 2A).
 - d. For identification of specific M2 macrophage populations, substitute anti-CD163 primary antibody, diluted 1:50, for anti-CD11b primary antibody in this step and continue with the staining protocol as written (Table 2).
 - e. For identification of satellite cell populations, anti-Pax7 antibody, diluted 1:100, should be substituted for primary antibody against CD11b at this step (Table 2).
21. Remove primary antibody from sections and wash (as outlined in Step C17 above) four times, 5 min with 1x PBS, rocking at RT.
22. Dump excess 1x PBS onto a paper towel, flick to remove remaining 1x PBS, wipe the back of the slide with a paper towel, return to the slide chamber.
23. Add the biotinylated goat anti-mouse IgG1, diluted 1:1,000 in 2.5% NHS, and incubate by rocking for 90 min at RT.
24. Remove the biotinylated antibody from the sections and repeat the wash in Steps C21 and C22.
25. Add the SA-HRP, diluted 1:500 in 1x PBS, and incubate for 60 min, rocking at RT.
26. Remove the SA-HRP from the sections and wash three times, 5 min with 1x PBS, rocking at RT.
27. After the third wash, repeat Step C22 then add TSA 488, diluted 1:500 in 1x PBS, and incubate for 20 min, rocking at RT.
28. Remove the TSA 488 and repeat the washes in Step C26.
29. Repeat Steps C15-C18 to block the streptavidin and biotin used to label CD11b and prevent false co-staining.
30. Add enough volume of 2.5% NHS to completely cover the sections and rock at RT for at least 60 min (sections can be left in 2.5% NHS longer if desired).

31. Remove 2.5% NHS, gently dab the PAP pen with a Kimwipe to re-establish the hydrophobic barrier, add the primary antibody for CD206, diluted 1:200 in 2.5% NHS, then return the slide chamber to the rocker at 4 °C overnight.
- To determine signal produced by background staining of the tissue specimen, a no primary antibody control should be included at this step also. Prepare the no primary antibody control by covering the section in 2.5% NHS alone (omitting any antibody) and incubating overnight at 4 °C, rocking (Figure 4C).
32. Remove CD206 antibody from sections and repeat Steps C21 and C22 to wash.
33. Add biotinylated rabbit anti-goat IgG, diluted 1:500 in 2.5% NHS, and incubate for 90 min at RT, rocking.
34. Remove the biotin from the sections and wash four times, 5 min with 1x PBS, rocking at RT.
35. Add the SA-594, diluted 1:200 in 1x PBS, and incubate for 60 min, rocking at RT.
36. Remove the SA-594 from the sections and wash three times, 5 min with 1x PBS, rocking at RT.
37. Incubate sections with DAPI, diluted 1:10,000 in 1x PBS, for 10 min at RT, rocking.
38. Remove DAPI from the sections and wash three times, 5 min with 1x PBS.
39. Dump excess 1x PBS onto a paper towel and add enough volume of PBS/Glycerol or Vectashield mounting medium to cover the sections (20-50 µl/slide).
40. Gently lower coverslip onto the slide, avoiding the formation of bubbles under the coverslip. Allow the mounting medium to spread by leaving the slides laying coverslip up for 5-10 min.
41. Drain excess mounting medium from slides by standing vertical on a paper towel for 5 min.
42. Image muscle sections or store slides at 4 °C protected from light until ready to image. Staining will last for several months if slides are stored properly at 4 °C, in the dark.

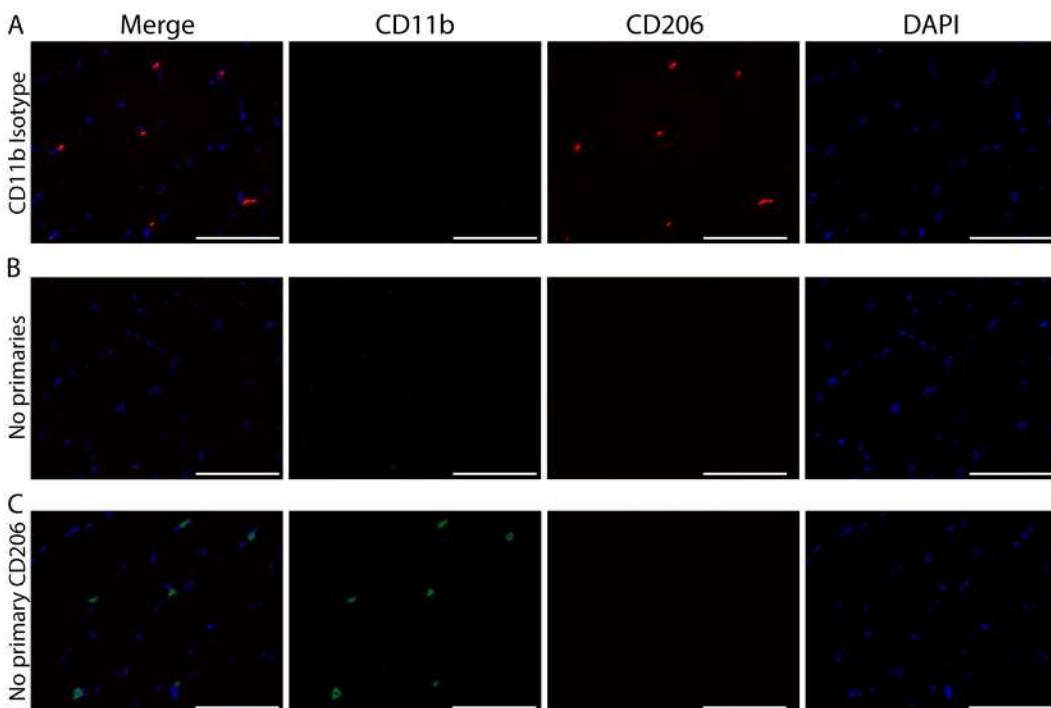


Figure 4. Immunohistochemical controls validating the specificity of CD11b and CD206 staining. Representative images of staining controls. A) No primary antibody for CD206, B) Isotype control antibody for CD11b and C) No primary antibody for either CD11b or CD206. CD11b (green), CD206 (red) and cell nuclei/DAPI (blue). Scale bars = 100 µm. All images were acquired on serial sections from the same sample with a 20x objective using the same exposure settings. The same display adjustment was applied across all images. A. Images showing a lack of CD206 staining when the primary antibody for CD206 is not applied to the sections. This control shows the specificity of the CD206 antibody; staining is not a product of cross reactivity with reagents used to amplify CD11b or due to non-specific tissue staining. B. Lack of CD11b+ staining when an isotype control antibody is applied. Similar to panel A, this control shows the specificity of the CD11b antibody since the isotype-matched control does not produce non-specific staining. Additionally, this control shows that CD206 staining does not result from cross reactivity to CD11b reagents since no CD11b staining is present but CD206+ cells are clearly identified. C. Images show very low non-specific background staining of skeletal muscle tissue sections from the use of amplification reagents; thus positive macrophage staining can clearly be distinguished from tissue background.

Data analysis

Quantification of skeletal muscle fiber number and macrophage abundance

1. General guidelines for skeletal muscle macrophage analysis
 - a. Sections to be analyzed for macrophage numbers should contain at least 50-100 skeletal muscle fibers.
 - b. Non-specific staining is common around the edges of skeletal muscle sections; therefore, exclude the edges when counting macrophages.
 - c. Do not include longitudinal skeletal muscle fibers in the area to be counted; this skews the number of macrophages/fiber.
 - d. Areas containing blood patches, edema or fibrosis will sometimes be observed in damaged tissues (Figure 5B). These areas are usually filled with macrophages and shouldn't be included in the same analysis as macrophages located in between skeletal muscle fibers (Figure 5A). Whether these macrophages should be quantified depends on the aim of the study.

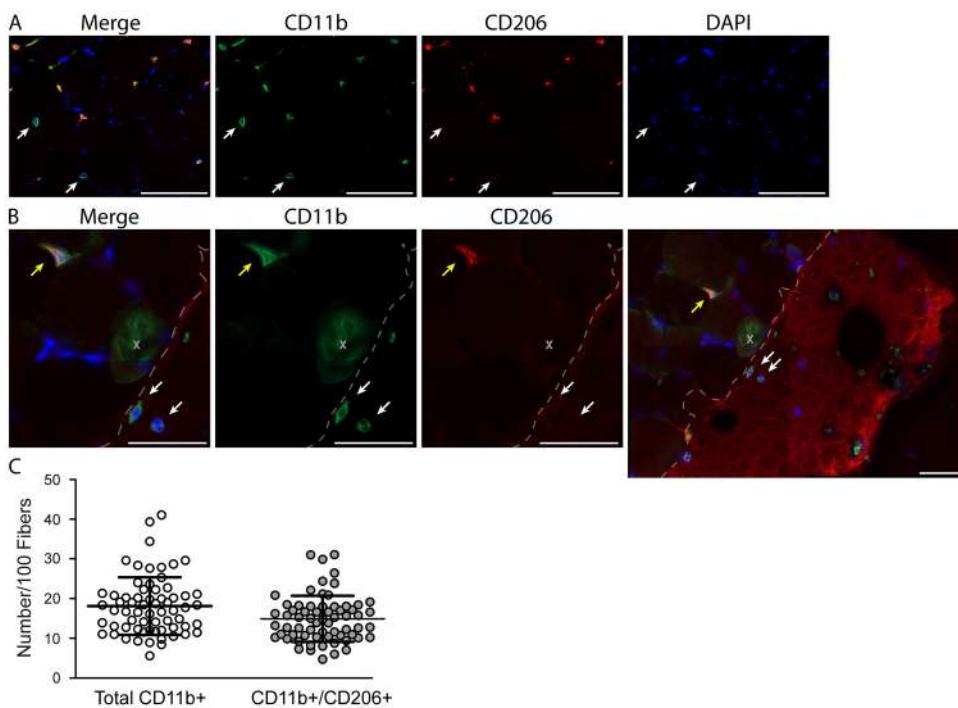


Figure 5. Resident macrophages largely co-express CD11b and CD206 in human vastus lateralis muscle. A. Representative images showing macrophage staining: CD11b (green), CD206 (red) and DAPI (cell nuclei, blue). The majority of macrophages are positive for both CD11b and CD206; however, CD11b+/CD206- macrophages can be observed (white arrows). Scale bars = 100 μ m. B. Muscle section from the vastus lateralis showing a CD11b+/CD206+ (yellow arrow) and two CD11b+/CD206- (white arrows) monocytes. Notice in the larger field of view (at right) the CD11b+/CD206- monocytes are located in a patch of blood at the edge of the muscle section, validating CD206 as a reliable marker of tissue resident macrophages in human skeletal muscle. The patch of blood can be identified by its dried, cracked appearance and high red background signal (border between blood patch and muscle fibers in the section denoted with a dashed line). The gray X is marking an area with green background signal produced by a bubble underneath the section (further outlined in [Figure S1](#)). Scale bars = 50 μ m. C. Bar graph quantification showing the number of total macrophages (all CD11b+) and CD11b+/CD206+ macrophages per 100 skeletal muscle fibers. Sixty-five muscle biopsies from the vastus lateralis were analyzed. Each dot represents macrophage counts from a single subject/section with overlap between Total CD11b+ and CD11b+/CD206+ groups, illustrating that the majority of macrophages (Total CD11b+) also express CD206 (CD11b+/CD206+). In skeletal muscle samples approximately 82% of the total macrophage population co-express CD11b and CD206.

2. Adjust the image display in any channel so that the background staining is visible and individual skeletal muscle fibers can be distinguished (Figures 6A-6B).

3. Manually count the number of total skeletal muscle fibers within the region of interest using the “event” tool in Zen image capture software to demarcate individual skeletal muscle fibers (Figures 6C-6D).

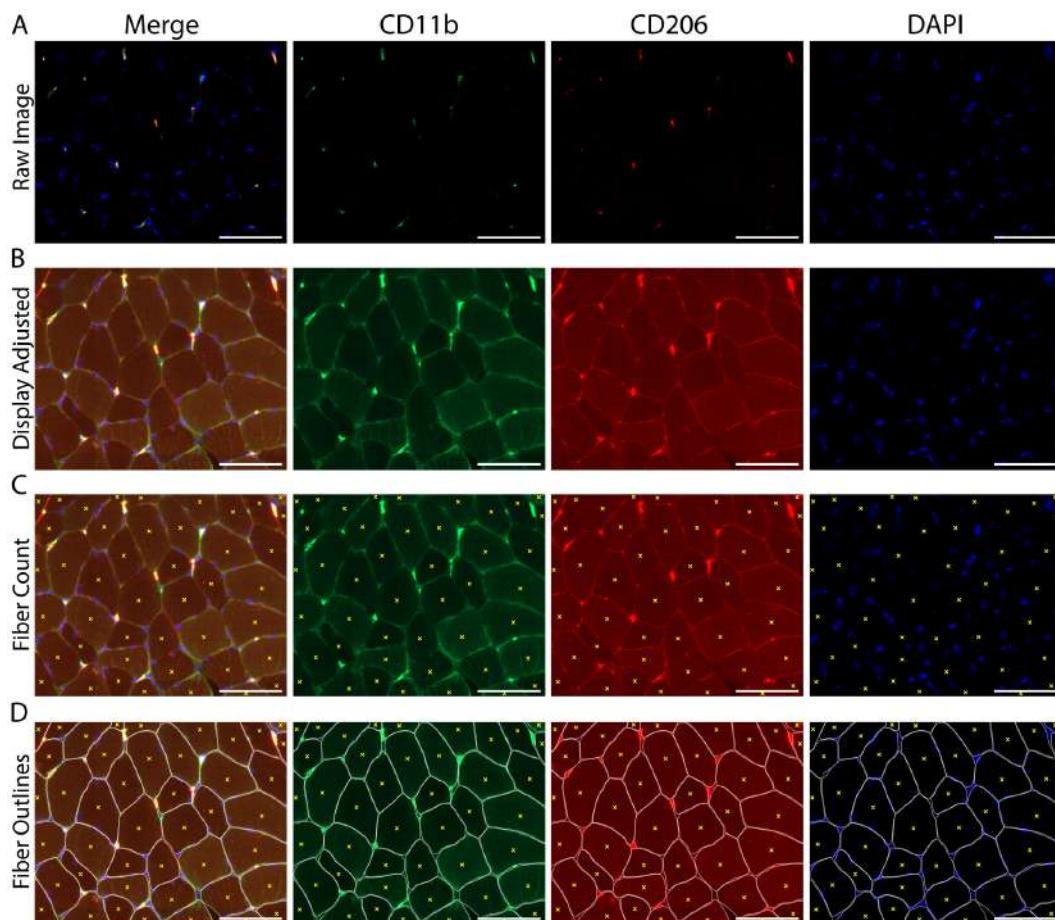


Figure 6. Image adjustment and counting of skeletal muscle fiber number within a cross-section. A. Original image, prior to manipulation of the display settings; B. The same image following adjustment of the display to increase the visible background staining, allowing for the identification and counting of skeletal muscle fibers; C. Count of individual muscle fibers, denoted by yellow Xs; D. Fibers were manually outlined to help demarcate individual skeletal muscle fiber borders (white) along with the fiber count from panel C (yellow Xs). A-D) CD11b (green), CD206 (red), cell nuclei/DAPI (blue). Scale bars = 100 μ m.

4. Adjust the image display so that CD11b+ macrophages (green) and DAPI+ cell nuclei (blue) can be clearly observed and cell shape/morphology is distinct (Figures 7A-7B).
 - a. Isotype-specific staining controls for antibody specificity should be used to help determine the appropriate display adjustment for identifying true positive staining (Figure 5A).
 - b. No primary antibody staining controls for non-specific binding of antibodies/amplification reagents can be used to aid in determining background tissue staining and display adjustment for counting (Figures 4B-4C).

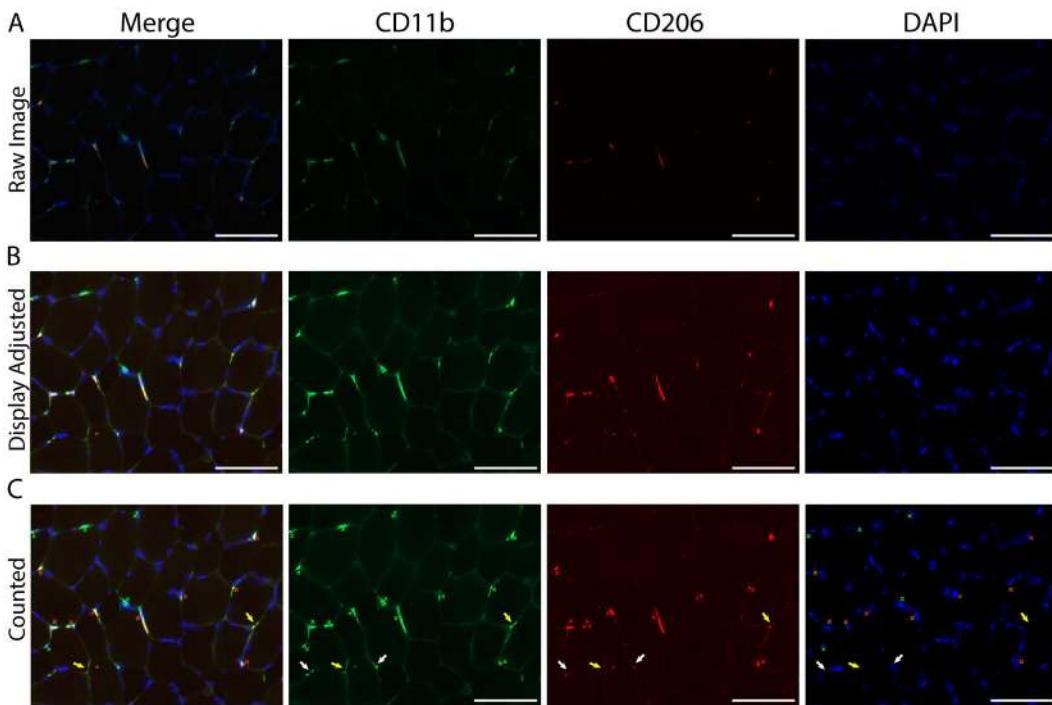


Figure 7. Identifying and quantifying macrophages within a skeletal muscle cross-section. A. Representative image of macrophage staining in human vastus lateralis: CD11b (green), CD206 (red) and cell nuclei/DAPI (blue). B. Display adjustment of the images in panel A showing enough tissue background staining to clearly identify macrophages from non-specific tissue staining. C. Macrophage count, demarcated with colored Xs: all CD11b+ (green Xs), all CD206+ (red Xs), CD11b+/CD206+ macrophages are marked with both a green and red X. White arrows indicate areas that appear to have positive staining and are near DAPI, but were not counted due to size and/or lack of distinct morphological features determinant of a cellular structure. Other areas were not counted due to a lack of DAPI staining (yellow arrows). Scale bars = 100 μm .

5. Using DAPI as a cell marker, manually identify and count the total number of CD11b+ macrophages located near DAPI (further explanation in the **Macrophage Counting Notes** section below) (Figure 7C).
 - a. Skeletal muscle macrophages are morphologically heterogeneous (Figures 8A-8D). For macrophage counting, we do not discriminate between different morphological groups (*i.e.*, all CD11b+/CD206+ macrophages are counted together regardless of morphology) (see General Note 4).
 - b. If DAPI is not present near the staining, we do not count the staining as a positive event, even if the morphology seems apparent.
 - c. Staining must be large enough in size to represent a cellular structure (at least the same size as an intact nucleus, $> 5 \mu\text{m}$) – small dots/patches are usually just non-specific staining of cellular debris by the secondary antibody.

- d. In patches where individual macrophages cannot be distinguished, manually count each DAPI+ nucleus that touches macrophage staining (Figures 9A-9D).

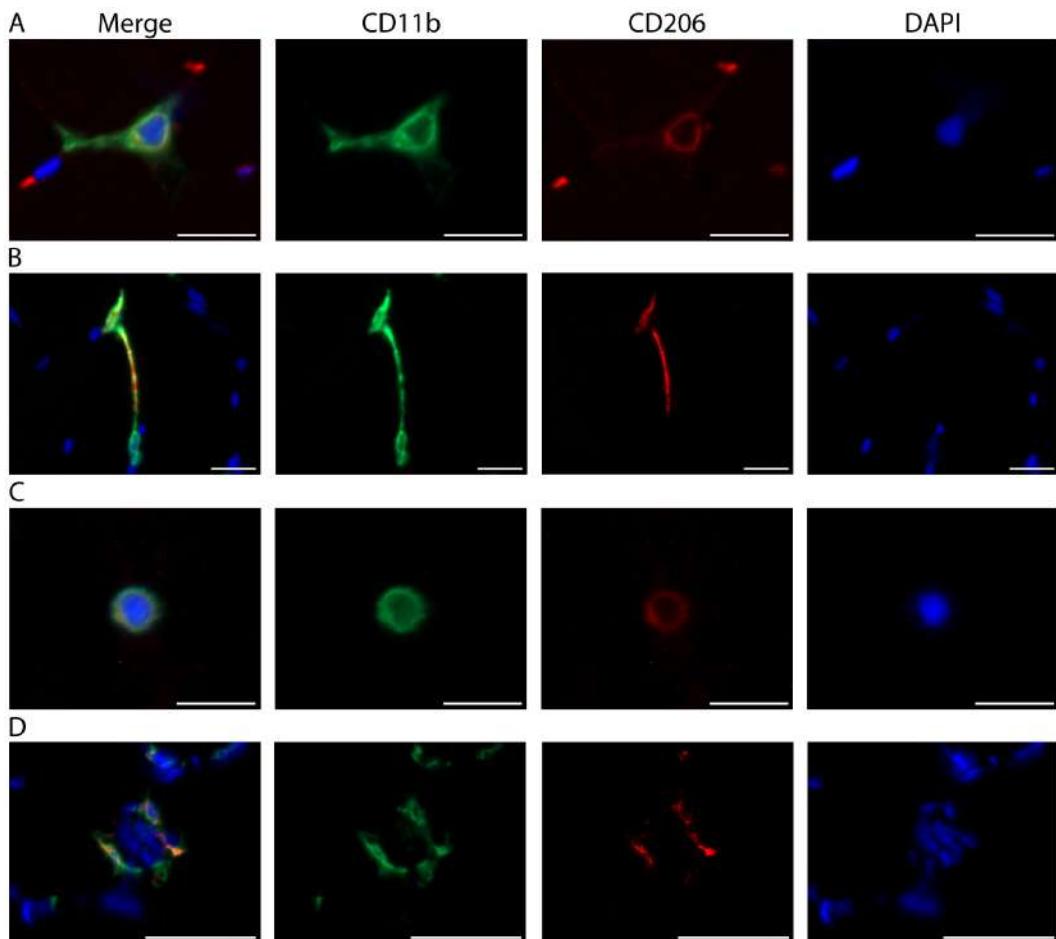


Figure 8. Distinct skeletal muscle macrophage morphologies. A. Representative image from the vastus lateralis muscle showing a macrophage with classic morphology. Described in other tissues, the outstretched processes of resting macrophages are thought to surveil the local environment (Olah *et al.*, 2011; Durafourt *et al.*, 2012). B. A CD11b+/CD206+ macrophage (top) stretching out toward a second CD11b+/CD206- macrophage (bottom). C. Round or amoeboid shaped macrophage. In other tissues, this morphology is thought to be indicative of activation and the production of inflammatory cytokines (Olah *et al.*, 2011; Durafourt *et al.*, 2012). A-C) Scale bars = 20 μ m. D. Macrophages surround a damaged skeletal muscle fiber (indicated by the presence of central nuclei). Scale bars = 50 μ m. A-D) Images were acquired as Z stacks using a 40x objective and cropped to enlarge. CD11b (green), CD206 (red), cell nuclei/DAPI (blue).

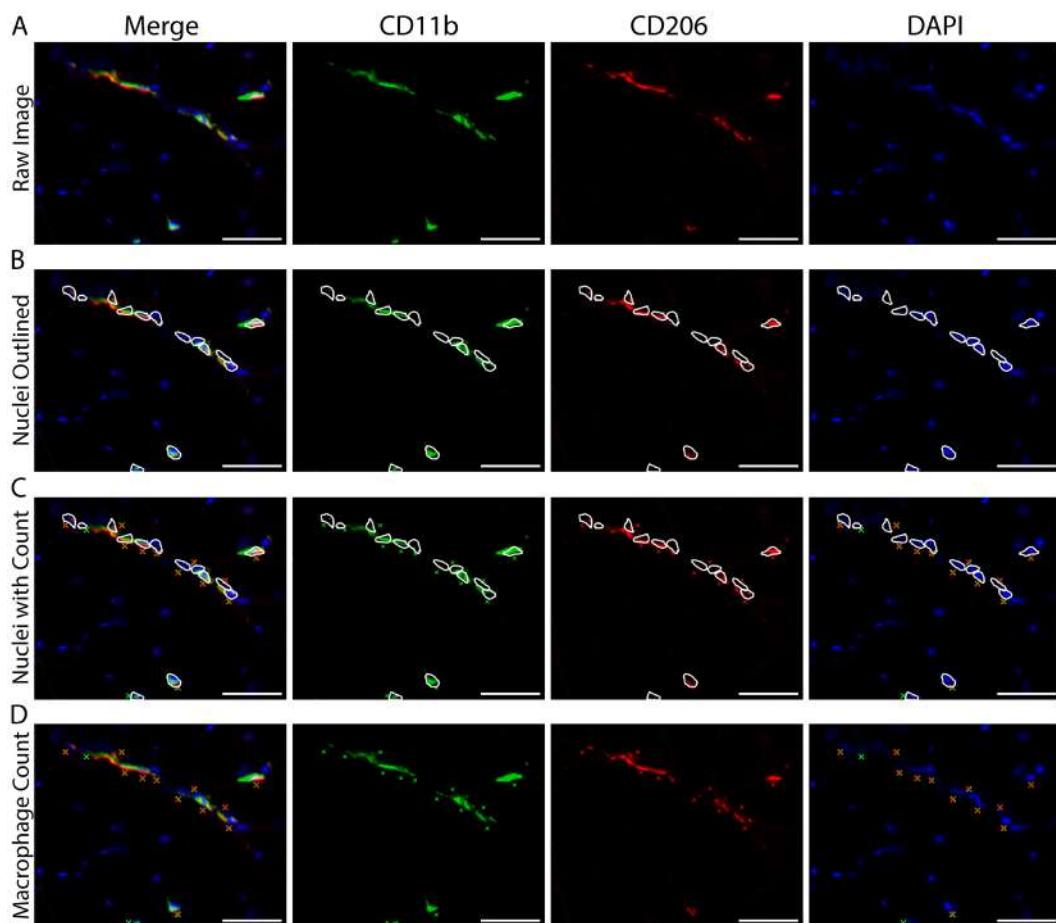


Figure 9. Counting macrophages when individual cells cluster together. A. Representative image showing an area of macrophage staining with multiple nuclei where individual macrophages cannot be distinguished. B. The image in panel A, with outlines demarcating DAPI+ nuclei touching areas of positive macrophage staining. C. Panel B images including Xs marking each nucleus counted as positive macrophage staining. D. Original image from panel A showing the Xs in panel C marking macrophage counts without the outlines demarcating positive nuclei. A-D) CD11b (green), CD206 (red) and cell nuclei/DAPI (blue). Scale bars = 50 μ m.

6. After counting total CD11b⁺ macrophages, go back and manually count the number of these macrophages that also express CD206.
 - a. CD11b+/CD206⁺ macrophages are the mixed M1/M2 skeletal muscle macrophage population.
 - b. CD11b+/CD206⁻ macrophages may represent the M1 population.
7. To determine the number of M1 macrophages, subtract the number of CD11b+/CD206⁺ macrophages from the total number of macrophages expressing CD11b+. This gives you the number of macrophages only expressing CD11b+ (CD11b+/CD206⁻).
8. For final numbers, divide each macrophage count by the total number of fibers in the region counted.

Notes**A. General Notes**

1. Collection of skeletal muscle biopsies was carried out in accordance with the Declaration of Helsinki. Skeletal muscle biopsies from three separate studies conducted at the University of Kentucky, the University of Alabama at Birmingham and the Geriatric Research Education and Clinical Center, Central Arkansas Veterans Healthcare System were used in this protocol. Subjects at the University of Kentucky provided their written informed consent from protocols approved by the Institutional Review Board and the University of Kentucky. Details regarding subject consent and protocol approval for the other two studies are outlined in the following publications: (Dennis *et al.*, 2015; Long *et al.*, 2017).
2. We have used this protocol to identify macrophage populations in subjects ranging from age 19 to 83 and spanning a wide range of activity levels. We have found the percentage of total CD11b+ macrophages that co-express CD206 (~82%) remains relatively consistent across demographics; however, the abundance (number/fiber) and phenotype may be affected by age, obesity and/or exercise.
3. We acknowledge that surface marker expression is not sufficient to infer function and further analyses should be employed to determine functional characteristics of macrophage populations.
4. Skeletal muscle macrophages display heterogeneous morphology; however, we do not currently include macrophage morphology as a variable in our analyses (Figure 8). Though morphology alone is likely not enough to distinguish macrophage populations, we believe macrophage morphology may be an important and telling variable for some studies and may provide further insight into macrophage phenotype beyond surface marker expression alone.
5. Post sectioning drying time depends on the size of the sample, but 3-4 h are usually sufficient for skeletal muscle biopsies. Following drying, slides can be stored at -20 °C if staining will be done at a later time.
6. If samples were stored at -20 °C, slides should be allowed to warm to RT for 15-20 min prior to acetone fixation.
7. Batch controls should be included if multiple sets of staining will be done at different time points within a single study and should include at least one control and one experimental sample.
8. For all reagents pipetted onto the slide, use enough volume to cover the sections and fill the area you created with the PAP pen, usually between 200 and 500 µl/slide.
9. During the incubation with 3% hydrogen peroxide, you may see bubbles form on the section indicating that the peroxide is working; a lack of bubbling will not affect the staining outcome but is indicative of poor tissue quality.
10. Following Step C26, slides can be coverslipped with PBS/Glycerol and staining can be checked prior to moving forward with the protocol. Incubate slides in a Coplin jar with 1x PBS to

remove the coverslips (they will fall off), wash 2 or 3 times with 1x PBS and continue with Step C27.

11. Draining slides post coverslip:
 - a. Slides should be drained enough that mounting medium is no longer leaking from under the coverslip and the coverslip tightly adheres to the slide.
 - b. Take care not to over-drain the slides; this will lead to the formation of air pockets underneath the coverslip.
 - c. For long-term storage, slides can be mounted with Vectashield mounting medium.
 - d. Slides can be sealed by painting the edges of the coverslip with nail polish to prevent the formation of air pockets underneath the coverslip over time.
12. Both CD11b and CD68 antibodies are a mouse IgG1 isotype and both require amplification using Superboost Tyramide signal amplification reagents. For these reasons, co-staining of CD11b and CD68 is not possible (false positive double staining occurred).
13. This protocol can be adapted to identify a subset of CD206+ macrophages expressing CD163 by substituting CD163 primary antibody (see Table 1) in place of CD11b primary antibody and following the remaining protocol as written.
14. Primary antibody for Pax7 (see Table 1) can be substituted for CD11b primary antibody to label satellite cells; the remaining protocol steps are the same (Figure 10).

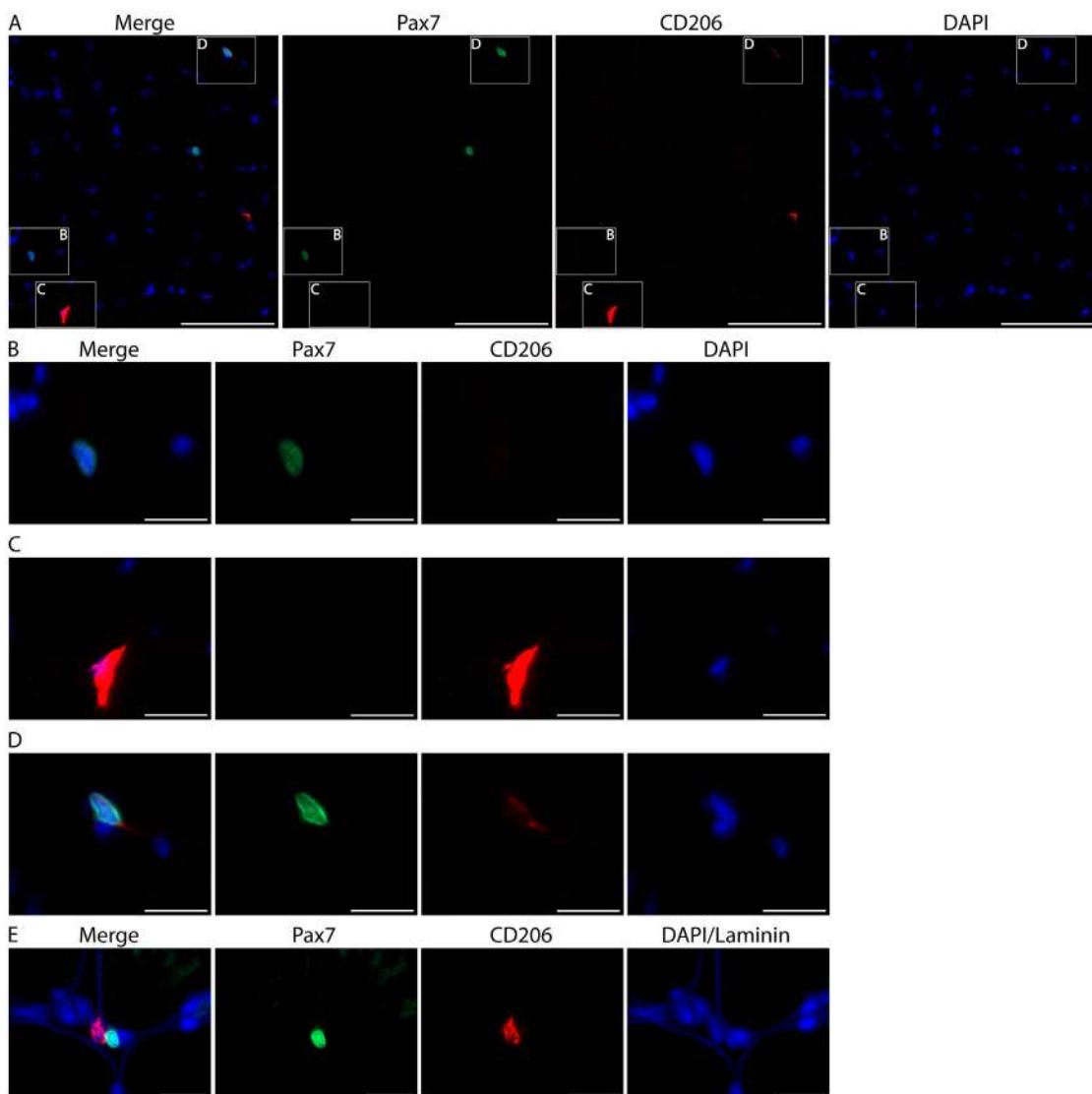


Figure 10. Skeletal muscle stem cells (satellite cells) can weakly express CD206 but are easily distinguished from macrophages. A. Representative image from human vastus lateralis showing a Pax7+ satellite cell (green, B), a CD206+ macrophage (red, C) and a Pax7+/CD206^{weak} satellite cell (D). Cell nuclei are stained with DAPI (blue). Scale bars = 100 μ m. B-D. Higher magnification of the boxes from panel A. Note the overexposure of CD206 macrophage staining (C) when the intensity is adjusted so that CD206 expression in satellite cells is visible (D). Images acquired as Z stacks with a 40x objective. Scale bars = 20 μ m. E. High magnification images from human vastus lateralis muscle showing a CD206+ muscle macrophage (red) in close proximity to a Pax7+ satellite cell (green). Cell nuclei are stained with DAPI and skeletal muscle fiber borders are stained with Laminin (both in blue). Images were acquired as Z stacks with a 100x oil objective. Scale bars = 20 μ m.

B. Macrophage Counting Notes

1. Image capture should be adjusted properly for easy identification of macrophages.

- a. Exposure times should be set so that macrophage and nuclear morphology is clear and discernable and background staining is as minimally visible as possible.
 - b. Images should be acquired with a 20x objective or higher magnification.
 - c. For stitched images:
 - i. Section boundaries are easiest to set using DAPI staining to find the edges of the tissue section.
 - ii. Focus points are best set using the FITC channel (CD11b staining), to ensure that macrophages are in focus with clear morphology.
2. Staining must be located near DAPI (within 5 μm), but does not have to be directly on top of DAPI staining. If the morphology is a macrophage and it is close but not touching DAPI, we will count the event as a macrophage (Figure 7).
 3. We have found that CD163+ macrophages make-up a subset of total CD206+ macrophages. Thus, if quantifying these populations, CD206+ macrophages should be counted as the ‘parent’ macrophage population (similar to CD11b above).
 4. Establishing a set of counting parameters prior to analyzing data sets is helpful when quantifying macrophages and also helps minimize variance between counters.
 5. Choose a handful of images as a ‘guide’ counting set and use these images to set up guidelines for how macrophages will be identified and to train new counters.
 - a. It is important to stay consistent between images, groups and studies with regard to the identification of macrophages.
 - b. Within one data set it is better to have the same blinded counter to analyze the entire data set due to variation between counters.
 - c. We have found that although absolute numbers vary between counters, overall trends with regard to increases or decreases in the abundance of macrophage populations remain consistent if basic guidelines are outlined and followed.

Recipes

1. 1x PBS (10 mM, pH 7.4)
 - a. Mix 69.68 g NaCl, 17.36 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2.08 g KH_2PO_4
 - b. Stir to dissolve in DI water
 - c. Dilute 10 N NaOH 1:5 with DI water to make a 2 N solution; dilute 6 N HCl 1:3 with DI water to make a 2 N solution
 - d. Adjust pH of 1x PBS with 2 N NaOH or HCl
 - e. Bring to a final volume of 8 L
 - f. 1x PBS can be kept at RT for up to 3 months
2. 3% hydrogen peroxide
Dilute 30% hydrogen peroxide 1:10 in 1x PBS
3. DAPI for staining cell nuclei

- a. Prepare a 5 mg/ml stock solution by diluting in 1x PBS
- b. Aliquot and store at -20 °C
- c. A working dilution of 1:10,000 in 1x PBS is used for labeling nuclei

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References

- 1 Ambarus, C. A., S. Krausz, M. van Eijk, J. Hamann, T. R. Radstake, K. A. Reedquist, P. P. Tak and D. L. Baeten (2012). [Systematic validation of specific phenotypic markers for *in vitro* polarized human macrophages](#). *J Immunol Methods* 375(1-2): 196-206.
- 2 Arnold, L., A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R. K. Gherardi and B. Chazaud (2007). [Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis](#). *J Exp Med* 204(5): 1057-1069.
- 3 Beaton, L. J., M. A. Tarnopolsky and S. M. Phillips (2002). [Contraction-induced muscle damage in humans following calcium channel blocker administration](#). *J Physiol* 544(Pt 3): 849-859.
- 4 Boyette, L. B., C. Macedo, K. Hadi, B. D. Elinoff, J. T. Walters, B. Ramaswami, G. Chalasani, J. M. Taboas, F. G. Lakkis and D. M. Metes (2017). [Phenotype, function, and differentiation potential of human monocyte subsets](#). *PLoS One* 12(4): e0176460.
- 5 Ceafalan, L. C., T. E. Fertig, A. C. Popescu, B. O. Popescu, M. E. Hinescu and M. Gherghiceanu (2017). [Skeletal muscle regeneration involves macrophage-myoblast bonding](#). *Cell Adh Migr* 1-8.
- 6 Chazaud, B., M. Brigitte, H. Yacoub-Youssef, L. Arnold, R. Gherardi, C. Sonnet, P. Lafuste and F. Chretien (2009). [Dual and beneficial roles of macrophages during skeletal muscle regeneration](#). *Exerc Sport Sci Rev* 37(1): 18-22.
- 7 Chistiakov, D. A., M. C. Killingsworth, V. A. Myasoedova, A. N. Orekhov and Y. V. Bobryshev (2017). [CD68/macrosialin: not just a histochemical marker](#). *Lab Invest* 97(1): 4-13.

- 8 Crameri, R. M., P. Aagaard, K. Qvortrup, H. Langberg, J. Olesen and M. Kjaer (2007). [Myofibre damage in human skeletal muscle: effects of electrical stimulation versus voluntary contraction.](#) *J Physiol* 583(Pt 1): 365-380.
- 9 Crameri, R. M., H. Langberg, B. Teisner, P. Magnusson, H. D. Schroder, J. L. Olesen, C. H. Jensen, S. Koskinen, C. Suetta and M. Kjaer (2004). [Enhanced procollagen processing in skeletal muscle after a single bout of eccentric loading in humans.](#) *Matrix Biol* 23(4): 259-264.
- 10 Dennis, R. A., U. Ponnappan, R. L. Kodell, K. K. Garner, C. M. Parkes, M. M. Bopp, K. P. Padala, C. A. Peterson, P. R. Padala and D. H. Sullivan (2015). [Immune function and muscle adaptations to resistance exercise in older adults: study protocol for a randomized controlled trial of a nutritional supplement.](#) *Trials* 16: 121.
- 11 DiPasquale, D. M., M. Cheng, W. Billich, S. A. Huang, N. van Rooijen, T. A. Hornberger and T. J. Koh (2007). [Urokinase-type plasminogen activator and macrophages are required for skeletal muscle hypertrophy in mice.](#) *Am J Physiol Cell Physiol* 293(4): C1278-1285.
- 12 Du, H., C. H. Shih, M. N. Wosczyna, A. A. Mueller, J. Cho, A. Aggarwal, T. A. Rando and B. J. Feldman (2017). [Macrophage-released ADAMTS1 promotes muscle stem cell activation.](#) *Nat Commun* 8(1): 669.
- 13 Dumont, N. A. and J. Frenette (2013). [Macrophage colony-stimulating factor-induced macrophage differentiation promotes regrowth in atrophied skeletal muscles and C2C12 myotubes.](#) *Am J Pathol* 182(2): 505-515.
- 14 Durafourt, B. A., C. S. Moore, D. A. Zammit, T. A. Johnson, F. Zaguia, M. C. Guiot, A. Bar-Or and J. P. Antel (2012). [Comparison of polarization properties of human adult microglia and blood-derived macrophages.](#) *Glia* 60(5): 717-727.
- 15 Fink, L. N., S. R. Costford, Y. S. Lee, T. E. Jensen, P. J. Bilan, A. Oberbach, M. Bluher, J. M. Olefsky, A. Sams and A. Klip (2014). [Pro-inflammatory macrophages increase in skeletal muscle of high fat-fed mice and correlate with metabolic risk markers in humans.](#) *Obesity (Silver Spring)* 22(3): 747-757.
- 16 Gordon, S., A. Pluddemann and F. Martinez Estrada (2014). [Macrophage heterogeneity in tissues: phenotypic diversity and functions.](#) *Immunol Rev* 262(1): 36-55.
- 17 Gottfried, E., L. A. Kunz-Schughart, A. Weber, M. Rehli, A. Peuker, A. Muller, M. Kastenberger, G. Brockhoff, R. Andreesen and M. Kreutz (2008). [Expression of CD68 in non-myeloid cell types.](#) *Scand J Immunol* 67(5): 453-463.
- 18 Hong, E. G., H. J. Ko, Y. R. Cho, H. J. Kim, Z. Ma, T. Y. Yu, R. H. Friedline, E. Kurt-Jones, R. Finberg, M. A. Fischer, E. L. Granger, C. C. Norbury, S. D. Hauschka, W. M. Philbrick, C. G. Lee, J. A. Elias and J. K. Kim (2009). [Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle.](#) *Diabetes* 58(11): 2525-2535.
- 19 Ikeda, S., Y. Tamura, S. Kakehi, K. Takeno, M. Kawaguchi, T. Watanabe, F. Sato, T. Ogihara, A. Kanazawa, Y. Fujitani, R. Kawamori and H. Watada (2013). [Exercise-induced enhancement of](#)

- [insulin sensitivity is associated with accumulation of M2-polarized macrophages in mouse skeletal muscle.](#) *Biochem Biophys Res Commun* 441(1): 36-41.
- 20 Iqbal, S. a. K., A (2015). [Characterization of *in vitro* generated human polarized macrophages.](#) *J Clin Cell Immunol* 6(380).
- 21 Italiani, P. and D. Boraschi (2014). [From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation.](#) *Front Immunol* 5: 514.
- 22 Jansen, K. M. and G. K. Pavlath (2006). [Mannose receptor regulates myoblast motility and muscle growth.](#) *J Cell Biol* 174(3): 403-413.
- 23 Kharraz, Y., J. Guerra, C. J. Mann, A. L. Serrano and P. Munoz-Canoves (2013). [Macrophage plasticity and the role of inflammation in skeletal muscle repair.](#) *Mediators Inflamm* 2013: 491497.
- 24 Kunisch, E., R. Fuhrmann, A. Roth, R. Winter, W. Lungershausen and R. W. Kinne (2004). [Macrophage specificity of three anti-CD68 monoclonal antibodies \(KP1, EBM11, and PGM1\) widely used for immunohistochemistry and flow cytometry.](#) *Ann Rheum Dis* 63(7): 774-784.
- 25 Liu, L., T. H. Cheung, G. W. Charville and T. A. Rando (2015). [Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting.](#) *Nat Protoc* 10(10): 1612-1624.
- 26 Lolmede, K., L. Campana, M. Vezzoli, L. Bosurgi, R. Tonlorenzi, E. Clementi, M. E. Bianchi, G. Cossu, A. A. Manfredi, S. Brunelli and P. Rovere-Querini (2009). [Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways.](#) *J Leukoc Biol* 85(5): 779-787.
- 27 Long, D. E., B. D. Peck, J. L. Martz, S. C. Tuggle, H. M. Bush, G. McGwin, P. A. Kern, M. M. Bamman and C. A. Peterson (2017). [Metformin to Augment Strength Training Effective Response in Seniors \(MASTERS\): study protocol for a randomized controlled trial.](#) *Trials* 18(1): 192.
- 28 Mackey, A. L. and M. Kjaer (2017). [The breaking and making of healthy adult human skeletal muscle *in vivo*.](#) *Skelet Muscle* 7(1): 24.
- 29 MacNeil, L. G., S. K. Baker, I. Stevic and M. A. Tarnopolsky (2011). [17 \$\beta\$ -estradiol attenuates exercise-induced neutrophil infiltration in men.](#) *Am J Physiol Regul Integr Comp Physiol* 300(6): R1443-1451.
- 30 Mahoney, D. J., A. Safdar, G. Parise, S. Melov, M. Fu, L. MacNeil, J. Kaczor, E. T. Payne and M. A. Tarnopolsky (2008). [Gene expression profiling in human skeletal muscle during recovery from eccentric exercise.](#) *Am J Physiol Regul Integr Comp Physiol* 294(6): R1901-1910.
- 31 Martinez, F. O. and S. Gordon (2014). [The M1 and M2 paradigm of macrophage activation: time for reassessment.](#) *F1000Prime Rep* 6: 13.
- 32 Martinez, F. O., S. Gordon, M. Locati and A. Mantovani (2006). [Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.](#) *J Immunol* 177(10): 7303-7311.
- 33 McWhorter, F. Y., T. Wang, P. Nguyen, T. Chung and W. F. Liu (2013). [Modulation of macrophage phenotype by cell shape.](#) *Proc Natl Acad Sci U S A* 110(43): 17253-17258.

- 34 Melton, D. W., A. C. Roberts, H. Wang, Z. Sarwar, M. D. Wetzel, J. T. Wells, L. Porter, M. T. Berton, L. M. McManus and P. K. Shireman (2016). [Absence of CCR2 results in an inflamming environment in young mice with age-independent impairments in muscle regeneration](#). *J Leukoc Biol* 100(5): 1011-1025.
- 35 Mikkelsen, U. R., H. Langberg, I. C. Helmark, D. Skovgaard, L. L. Andersen, M. Kjaer and A. L. Mackey (2009). [Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise](#). *J Appl Physiol* (1985) 107(5): 1600-1611.
- 36 Mosser, D. M. and J. P. Edwards (2008). [Exploring the full spectrum of macrophage activation](#). *Nat Rev Immunol* 8(12): 958-969.
- 37 Murray, P. J., J. E. Allen, S. K. Biswas, E. A. Fisher, D. W. Gilroy, S. Goerdt, S. Gordon, J. A. Hamilton, L. B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F. O. Martinez, J. L. Mege, D. M. Mosser, G. Natoli, J. P. Saeij, J. L. Schultze, K. A. Shirey, A. Sica, J. Suttles, I. Udalova, J. A. van Ginderachter, S. N. Vogel and T. A. Wynn (2014). [Macrophage activation and polarization: nomenclature and experimental guidelines](#). *Immunity* 41(1): 14-20.
- 38 Novak, M. L. and T. J. Koh (2013). [Phenotypic transitions of macrophages orchestrate tissue repair](#). *Am J Pathol* 183(5): 1352-1363.
- 39 Olah, M., K. Biber, J. Vinet and H. W. Boddeke (2011). [Microglia phenotype diversity](#). *CNS Neurol Disord Drug Targets* 10(1): 108-118.
- 40 Paulsen, G., R. Crameri, H. B. Benestad, J. G. Fjeld, L. Morkrid, J. Hallen and T. Raastad (2010a). [Time course of leukocyte accumulation in human muscle after eccentric exercise](#). *Med Sci Sports Exerc* 42(1): 75-85.
- 41 Paulsen, G., I. Egner, T. Raastad, F. Reinholt, S. Owe, F. Lauritzen, S. H. Brorson and S. Koskinen (2013). [Inflammatory markers CD11b, CD16, CD66b, CD68, myeloperoxidase and neutrophil elastase in eccentric exercised human skeletal muscles](#). *Histochem Cell Biol* 139(5): 691-715.
- 42 Paulsen, G., I. M. Egner, M. Drange, H. Langberg, H. B. Benestad, J. G. Fjeld, J. Hallen and T. Raastad (2010b). [A COX-2 inhibitor reduces muscle soreness, but does not influence recovery and adaptation after eccentric exercise](#). *Scand J Med Sci Sports* 20(1): e195-207.
- 43 Peterson, J. M., T. A. Trappe, E. Mylona, F. White, C. P. Lambert, W. J. Evans and F. X. Pizza (2003). [Ibuprofen and acetaminophen: effect on muscle inflammation after eccentric exercise](#). *Med Sci Sports Exerc* 35(6): 892-896.
- 44 Przybyla, B., C. Gurley, J. F. Harvey, E. Bearden, P. Kortebain, W. J. Evans, D. H. Sullivan, C. A. Peterson and R. A. Dennis (2006). [Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise](#). *Exp Gerontol* 41(3): 320-327.
- 45 Reidy, P. T., C. C. Lindsay, A. I. McKenzie, C. S. Fry, M. A. Supiano, R. L. Marcus, P. C. LaStayo and M. J. Drummond (2017). [Aging-related effects of bed rest followed by eccentric exercise rehabilitation on skeletal muscle macrophages and insulin sensitivity](#). *Exp Gerontol*.
- 46 Rigamonti, E., P. Zordan, C. Sciorati, P. Rovere-Querini and S. Brunelli (2014). [Macrophage plasticity in skeletal muscle repair](#). *Biomed Res Int* 2014: 560629.

- 47 Roszer, T. (2015). [Understanding the mysterious M2 macrophage through activation markers and effector mechanisms](#). *Mediators Inflamm* 2015: 816460.
- 48 Saclier, M., S. Cuvellier, M. Magnan, R. Mounier and B. Chazaud (2013a). [Monocyte/macrophage interactions with myogenic precursor cells during skeletal muscle regeneration](#). *FEBS J* 280(17): 4118-4130.
- 49 Saclier, M., H. Yacoub-Youssef, A. L. Mackey, L. Arnold, H. Ardjoune, M. Magnan, F. Sailhan, J. Chelly, G. K. Pavlath, R. Mounier, M. Kjaer and B. Chazaud (2013b). [Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration](#). *Stem Cells* 31(2): 384-396.
- 50 Sciorati, C., E. Rigamonti, A. A. Manfredi and P. Rovere-Querini (2016). [Cell death, clearance and immunity in the skeletal muscle](#). *Cell Death Differ* 23(6): 927-937.
- 51 Shanely, R. A., K. A. Zwetsloot, N. T. Triplett, M. P. Meaney, G. E. Farris and D. C. Nieman (2014). [Human skeletal muscle biopsy procedures using the modified Bergstrom technique](#). *J Vis Exp*(91): 51812.
- 52 Smith, C., M. J. Kruger, R. M. Smith and K. H. Myburgh (2008). [The inflammatory response to skeletal muscle injury: illuminating complexities](#). *Sports Med* 38(11): 947-969.
- 53 Sprangers, S., T. J. de Vries and V. Everts (2016). [Monocyte heterogeneity: consequences for monocyte-derived immune cells](#). *J Immunol Res* 2016: 1475435.
- 54 Stupka, N., M. A. Tarnopolsky, N. J. Yardley and S. M. Phillips (2001). [Cellular adaptation to repeated eccentric exercise-induced muscle damage](#). *J Appl Physiol* (1985) 91(4): 1669-1678.
- 55 Tam, C. S., L. M. Sparks, D. L. Johannsen, J. D. Covington, T. S. Church and E. Ravussin (2012). [Low macrophage accumulation in skeletal muscle of obese type 2 diabetics and elderly subjects](#). *Obesity (Silver Spring)* 20(7): 1530-1533.
- 56 Tarnopolsky, M. A., E. Pearce, K. Smith and B. Lach (2011). [Suction-modified Bergstrom muscle biopsy technique: experience with 13,500 procedures](#). *Muscle Nerve* 43(5): 717-725.
- 57 Tidball, J. G., K. Dorshkind and M. Wehling-Henricks (2014). [Shared signaling systems in myeloid cell-mediated muscle regeneration](#). *Development* 141(6): 1184-1196.
- 58 Tidball, J. G. and S. A. Villalta (2010). [Regulatory interactions between muscle and the immune system during muscle regeneration](#). *Am J Physiol Regul Integr Comp Physiol* 298(5): R1173-1187.
- 59 Varga, T., R. Mounier, A. Horvath, S. Cuvellier, F. Dumont, S. Poliska, H. Ardjoune, G. Juban, L. Nagy and B. Chazaud (2016). [Highly dynamic transcriptional signature of distinct macrophage subsets during sterile inflammation, resolution, and tissue repair](#). *J Immunol* 196(11): 4771-4782.
- 60 Varma, V., A. Yao-Borengasser, N. Rasouli, G. T. Nolen, B. Phanavanh, T. Starks, C. Gurley, P. Simpson, R. E. McGehee, Jr., P. A. Kern and C. A. Peterson (2009). [Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action](#). *Am J Physiol Endocrinol Metab* 296(6): E1300-1310.

- 61 Wang, H., D. W. Melton, L. Porter, Z. U. Sarwar, L. M. McManus and P. K. Shireman (2014). [Altered macrophage phenotype transition impairs skeletal muscle regeneration.](#) *Am J Pathol* 184(4): 1167-1184.
- 62 Wehling-Henricks, M., S. S. Welc, G. Samengo, C. Rinaldi, C. Lindsey, Y. Wang, J. Lee, O. M. Kuro and J. G. Tidball (2018). [Macrophages escape Klotho gene silencing in the mdx mouse model of Duchenne muscular dystrophy and promote muscle growth and increase satellite cell numbers through a Klotho-mediated pathway.](#) *Hum Mol Genet* 27(1): 14-29.

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Measurement of TLR4 and CD14 Receptor Endocytosis Using Flow Cytometry

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[Abstract] After recognizing extracellular bacterial lipopolysaccharide (LPS), the toll-like receptor 4 (TLR4)-CD14 signaling complex initiates two distinct signaling pathways—one from the plasma membrane and the other from the signaling endosomes (Kagan *et al.*, 2008). Understanding the early stages of TLR4 signal transduction therefore requires a robust and quantitative method to measure LPS-triggered TLR4 and CD14 receptor endocytosis, one of the earliest events of LPS detection. Here, we describe a flow cytometry-based method that we used recently to study the role of the ion channel TRPM7 in TLR4 endocytosis (Schappe *et al.*, 2018). The assay relies on stimulating the cells with LPS and measuring the cell surface levels of TLR4 (or CD14) at various time points using flow cytometry. Although we detail the method specifically for TLR4 and CD14 from murine bone marrow-derived macrophages, it can be readily adapted to evaluate receptor endocytosis in a variety of other signaling contexts.

Keywords: Toll-like receptor, TLR, TLR4, CD14, Endocytosis, Macrophage, BMDM, Innate immunity, LPS

[Background] Innate immune cells, including macrophages and dendritic cells, employ a variety of pattern recognition receptors (PRRs) to survey their environments for danger- and pathogen-associated molecular patterns. Trafficking and signaling of PRRs from various subcellular compartments enables wider immune surveillance and has emerged as an important design principle of innate immunity (Brubaker *et al.*, 2015). The detection of the bacterial endotoxin LPS is highly dependent on TLR4 and its co-receptor CD14. The endocytosis of the TLR4 complex requires CD14 and is essential for LPS-induced macrophage activation (Zanoni *et al.*, 2011; Tan *et al.*, 2015). Endocytosis of TLR4 is essential to activate secondary signaling complexes at the newly-formed ‘signaling endosome,’ which promotes interferon regulatory factor 3-dependent transcription through the signaling adaptor TIR-domain containing adapter-inducing interferon-β (TRIF) (Kagan *et al.*, 2008). TLR4 endocytosis has been observed in macrophages, dendritic cells, and epithelial cells (Roy *et al.*, 2014). Understanding the underlying mechanisms of this critical step in macrophage activation requires a robust and quantitative method to measure LPS-triggered TLR4 endocytosis. Here, we describe a version of a flow cytometry-based method that was initially reported by Kagan and colleagues (Kagan *et al.*, 2008), and used by others, to monitor TLR4 endocytosis. We have used the method recently to study the role of transient receptor potential melastatin-like 7 (TRPM7), an ion channel, in TLR4

endocytosis (Schappe *et al.*, 2018). The experimental logic of this method relies on measuring the loss of TLR4 and CD14 staining at the cell surface after LPS treatment. We stain LPS-treated cells with an anti-TLR4 (or anti-CD14) fluorophore-conjugated antibody without permeabilization. The fluorescence intensity acquired using flow cytometry reports the relative quantity of receptor resident in the plasma membrane (Figure 1). Although specific for TLR4 and CD14, the assay can be readily adapted to evaluate receptor endocytosis in a variety of other signaling contexts.

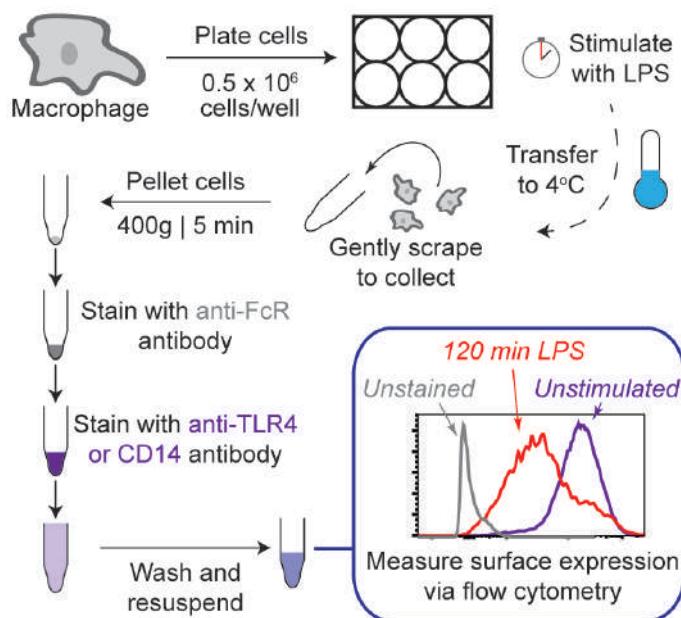


Figure 1. Schematic of TLR4 and CD14 endocytosis protocol. Experimental workflow described in protocol “Procedure”.

Materials and Reagents

A. Materials

1. Pipette tips
2. 5 ml round, disposable round-bottom tube (FACS Tube) (Corning, Falcon[®], catalog number: 352052)
3. Aluminum foil (Genesee Scientific, catalog number: 88-101)
4. 0.2 µm bottle filter (Thermo Fisher Scientific, NalgeneTM, catalog number: 566-0020)
5. 6-well non-treated culture plates (Corning, catalog number: 3736)
6. Sterile cell scrapers (Fisher Scientific, FisherbrandTM, catalog number: 08-100-240)
7. Sterile individually packaged serological pipette (10 ml) (Greiner Bio One International, catalog number: 607160)
8. Sterile individually packaged serological pipette (5 ml) (Greiner Bio One International, catalog number: 606160)

9. 1.7 ml microfuge Eppendorf tubes (Genesee Scientific, Olympus Plastics, catalog number: 24-281)
10. Nunc™ TripleFlask™ Treated Cell Culture Flasks (Thermo Fisher Scientific, catalog number: 132867)
11. Falcon® 50 ml Conical Centrifuge Tube (Corning, catalog number: 352098)

B. Cell line

1. L-929 cells (ATCC, catalog number: CCL-1)

C. Reagents

1. LPS EB-Ultrapure (lipopolysaccharide from *E. coli* O111:B4, InvivoGen, catalog number: tlrl-3pelps)
2. PBS (Thermo Fisher Scientific, Gibco™, catalog number: 10010023)
3. Mouse TruStain fcX™ (anti-CD16/32) (BioLegend, catalog number: 101320)
4. TLR4 [anti-mouse CD284] (PE) (clone: SA15-21; isotype: Rat IgG2a, κ) (BioLegend, catalog number: 145404)
5. CD14 [anti-mouse] (APC) (clone: Sa2-8; isotype: Rat IgG2a, κ) (Thermo Fisher Scientific, eBioscience™, catalog number: 17-0141-81)
6. RPMI 1640 (Thermo Fisher Scientific, Gibco™, catalog number: 11875093)
7. Fetal bovine serum (heat-inactivated), certified, USA origin (Thermo Fisher Scientific, Gibco™, catalog number: 10082147)
8. Trypan blue (Thermo Fisher Scientific, Gibco™, catalog number: 15250061)
9. HBSS, no calcium, no magnesium (Thermo Fisher Scientific, Gibco™, catalog number: 14170112)
10. BSA (Bovine serum albumin) (Roche Molecular Systems, catalog number: 3116956001)
11. DMEM, high glucose (Thermo Fisher Scientific, Gibco™, catalog number: 11965092)
12. BMDM Media (see Recipes)
13. Culture Media (see Recipes)
14. Treatment Media (see Recipes)
15. FACS Buffer (see Recipes)
16. L929-conditioned media (see Recipes)

Equipment

1. TC20 Automated cell counter (Bio-Rad Laboratories, catalog number: 1450102)
2. Pipet-aid Pipette Controller (Drummond Scientific, catalog number: 4-000-101)
3. 4 °C Cold Room
4. 4 °C Benchtop centrifuge
5. 37 °C Cell Culture Incubator with CO₂ control

6. Sterile cell culture hood
7. Flow Cytometer (BD, model: FACSCanto™ II, or equivalent)

Software

1. GraphPad Prism 7 (Graph Pad Software; La Jolla, CA USA)

Procedure

1. Day 1, Cell culture
 - a. Collect cultured bone marrow-derived macrophages (BMDMs) by gentle scraping. Disperse the cells into a single-cell suspension by repeatedly running the pipetted cell suspension along the test tube walls. Centrifuge cells ($400 \times g$, 5 min, 23 °C), aspirate supernatant, and resuspend cell pellet in BMDM media. Count live cells via trypan blue exclusion assay.
 - b. Plate 0.5×10^6 cells/well in a 6-well, non-treated, tissue culture plate. Culture the cells overnight in 2 ml/well of BMDM media. After 16 h of incubation (37 °C, 5% CO₂), cells should be adherent and ready for the experiment.
2. Day 0, Preparation before LPS stimulation
 - a. Prepare Culture and Treatment Media as described in Recipes. Warm the LPS-containing treatment media to 37 °C prior to use.
 - b. Chill sterile PBS, 1.5 ml Eppendorf tubes, and FACS buffer to 4 °C, prior to use. After LPS treatment, the cells will be collected using these solutions and tubes.
3. Day 0, LPS stimulation of cells
 - a. Aspirate BMDM media and wash 3 x with 3 ml of HBSS (room temperature) to remove dead cells and debris from each well. Add HBSS down the wall of the culture well and gently swirl plate to wash.
 - b. Gently add 2 ml of Culture Media to wells labeled “Unstained BMDMs” and “ $t = 0$ min/Untreated” treatment groups.
 - c. Gently add 2 ml of Treatment Media to each well by pipetting the media along the side of the wells. Gently swirl the plate to ensure that the media is evenly distributed in the wells.
 - d. Incubate at 37 °C for desired time points. Repeat Steps 3a to 3c as necessary for remaining LPS-treatment groups. Stagger the LPS treatment such that all samples are harvested at the same time.
4. Day 2, Cell collection and antibody staining

Note: All reagents should be cold and the procedure should be performed at 4 °C (cold room).

 - a. Transfer plates treated in Step 3 to 4 °C for 5 min prior to collection – this is required to arrest endocytosis.
 - b. Aspirate media from each well. Wash 2 x each with 2 ml of sterile, pre-chilled PBS.

- c. Add 1 ml of sterile, pre-chilled PBS to each well. Gently scrape to detach cells and pipette-mix to disperse the cells into a single cell suspension.
- d. Transfer the cell suspension to 1.5 ml Eppendorf tubes and centrifuge (400 \times g, 5 min) to pellet the cells.
- e. After aspirating and discarding the supernatant, resuspend the cell pellet in 50 μ l of cold FACS Buffer premixed with TruStain fcX™ antibody (1 μ g/ml), for 10 min.
- f. Add 50 μ l of the 2x-concentrate of antibody (anti-TLR4 or anti-CD14; see Table 1) in FACS Buffer to the cell suspension. Add 50 μ l of FACS Buffer to “Unstained BMDMs” samples.

Table 1. Antibodies used for measuring TLR4 and CD14 endocytosis

Antibody	Clone	Suggested Final Concentration	Notes
Anti-TLR4	SA15-21 (anti-mouse)	0.2 μ g/ml	Recognizes TLR4 independent of LPS-receptor complex formation
Anti-CD14	Sa2-8 (anti-mouse)	0.4 μ g/ml	Recognizes CD14

- g. Pipette gently to mix and stain for 20 min in the dark.
- h. Add 1 ml of FACS Buffer, collect cells by centrifugation (400 \times g, 5 min) and aspirate supernatant to remove excess antibody.
- i. Resuspend the cell pellet in 200 μ l of FACS Buffer and transfer the cell suspension to FACS tubes. Keep samples on ice and in the dark (e.g., cover with aluminum foil) prior to measurement by flow cytometry.
- j. Analyze samples via flow cytometry within 1 h.

Data analysis

1. Flow cytometry analysis
 - a. For flow cytometry analysis, collect > 100,000 events for each sample.
 - b. For analysis, the events are gated on FSC-A and SSC-A bivariate cytographs; the low FSC-SSC events comprising of dead cells and cellular debris are excluded from analysis. Cells are then gated on FSC-A and FSC-H to gate on single cells.
 - c. These cells can then be visualized for the intensity of the antibody stain as a histogram. Fluorescent intensity of the antibody stain on the gated population is recorded as the geometric mean of the cell population, or mean fluorescent intensity (MFI).
2. Data analysis for the measurement of TLR4 and CD14 Endocytosis
 - a. For data analysis, the MFI of “Unstained cells” can be used for background subtraction from all samples [“Background Subtracted MFI”]. Divide the “background subtracted MFI” value for a given time point by the “Unstimulated [t = 0 min]” sample; “t = 0 min” value

should be 1.00. Repeat this for all subsequent experimental samples to determine “Relative % of surface expression” relative to the “Unstimulated [t = 0 min]” sample. All sample values reflect the ratio of MFI from stimulated to unstimulated cells at desired time points.

- b. We convert the “Relative % of surface expression” value to a percentage; Thus, the “Untreated” or “Time = 0 min” sample should equal ‘100% percentage of surface expression’. One expects to see a steady reduction in this value at various time points after LPS stimulation.
- c. Since MFI values are sensitive to variations in flow cytometry calibrations, we recommend that data analysis be confined to each independent experiment and each condition run in technical triplicate. “Percentage of surface expression” should be reproducible across independent experiments and therefore amenable to statistical analysis of multiple experiments. Results from a typical experiment are shown in Figure 2. The original data presentation and additional information are available in our manuscript, which originally utilized the protocol described herein [PubMed](#).

A.

Relative percentage of surface receptor at given timepoint x :

$$\text{TLR4 or CD14 receptor endocytosis : } \frac{\text{MFI}_{x \text{ time}} - \text{MFI}_{\text{Unstained}}}{\text{MFI}_{0 \text{ min}} - \text{MFI}_{\text{Unstained}}} \times 100\%$$

B.

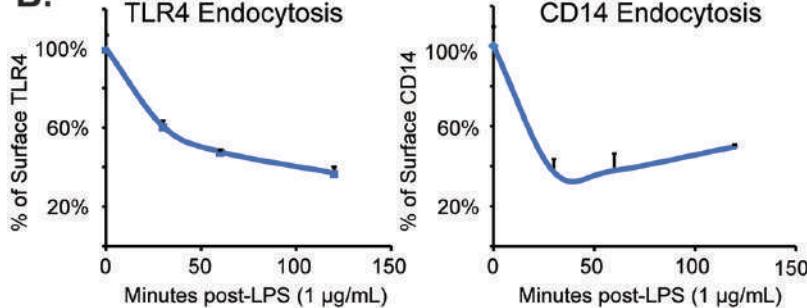


Figure 2. TLR4 and CD14 receptor endocytosis data analysis and suggested presentation. A. “Data analysis” calculation described as a formula. B. Characteristic TLR4 and CD14 endocytosis measured over time in bone-marrow derived macrophages. Data was modified from its original presentation in Schappe et al., 2018 with author permission.

3. For experimental and statistical analysis, we use GraphPad Prism. To compare two experimental groups, we use a Student’s *t*-test. For comparison of three or more data groups, other statistical analysis, such as a one-way ANOVA, are necessary.

Notes

1. The murine macrophage RAW 264.7 cell line also exhibits characteristic TLR4 endocytosis—it can be used to establish the method and for experiments.
2. Although non-treated culture plates may permit detachment with trypsin, the enzymatic detachment may alter macrophage epitope expression at the plasma membrane.
3. Performing “Procedure” Step 4 in a walk-in 4 °C cold room greatly improves the quality of data. Although chilling materials and reagents on ice may be convenient, variations in temperature between the ice, samples, and ambient laboratory air may inadvertently warm samples above 4 °C, thereby permitting endocytosis to proceed.
4. Although procedural steps after “Procedure” Step 3d are not performed under sterile conditions, using sterile reagents minimizes inadvertent contamination with ligands that may promote TLR4 or CD14 endocytosis.
5. Although spectrally non-overlapping fluorophores are available, we advise staining with a single anti-TLR4 or CD14 antibody for each experiment.
6. With careful spectral consideration, fluorescent live/dead dyes can be included in this assay to enrich for live cell populations. If the experimenter includes these dyes, we advise that only spectrally-compatible nucleic acid binding dyes, which can rapidly label dead cells during the final suspension be used. Some viability dyes and staining methods (such as “live/dead fixable dyes” or Annexin V-based staining kits) require additional staining steps that may compromise the time and temperature-sensitivity of this assay.
7. Titration of antibodies, including new batches of the same antibody clone, is essential. Although recommendations are provided in Table 1, improper staining will limit signal-to-noise ratio (SNR) in the assay and lower data quality in terms of sensitivity and consistency.
8. Avoid sample groups larger than 24 samples to minimize sample processing time prior to flow cytometry analysis.
9. The majority of our data were collected on the BD FACSCanto II flow cytometer.

Recipes

1. BMDM Media
 - a. RPMI 1640 + 10% FBS + 20% L929-conditioned media
 - b. Store at 4 °C for up to 1 month
2. Culture Media
 - a. RPMI 1640 + 10% FBS
 - b. Store at 4 °C for up to 1 month
3. Treatment Media
 - a. RPMI 1640 + 10% FBS + 1 µg/ml LPS

- b. Prepare fresh for each experiment
4. FACS Buffer
 - a. PBS + 1% BSA
 - b. Sterile filter buffer through 0.22 µm filter prior to use
 - c. Store at 4 °C for up to 1 week
5. L929-conditioned Media

Note: Generated from the culture of L-929 cells (available from ATCC). Cells are passaged according to the vendor's instructions and cultured in DMEM, high glucose + 10% FBS.

 - a. To generate L929-conditioned media, add 150 ml of DMEM, high glucose + 10% FBS to a T-150 TripleFlask. Add 0.72×10^6 L-929 cells and carefully mix by equilibrating the media volume at the hole in the corner of the flask. Culture for 7 days at 37 °C, 5% CO₂
 - b. On Day 7, collect media, sterile filter through a 0.22 µm filter into a flask, and store at -20 °C ["Week 1 media"]; add 150 ml of DMEM, high glucose + 10% FBS to TripleFlask to replace collected media
 - c. On Day 14, collect media from the flask and sterile filter through a 0.22 µm filter ["Week 2 media"]. Thaw Week 1 media at 23 °C
 - d. Combine Week 1 and Week 2 media and aliquot into 50 ml tubes. L929-conditioned media is stored at -20 °C for up to 6 months

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The authors declare that they have no conflicts of interest to report.

References

1. Brubaker, S. W., Bonham, K. S., Zanoni, I. and Kagan, J. C. (2015). [Innate immune pattern recognition: a cell biological perspective](#). *Annu Rev Immunol* 33: 257-290.
2. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S. and Medzhitov, R. (2008). [TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-β](#). *Nat Immunol* 9(4): 361-368.
3. Roy, S., Karmakar, M. and Pearlman, E. (2014). [CD14 mediates Toll-like receptor 4 \(TLR4\) endocytosis and spleen tyrosine kinase \(Syk\) and interferon regulatory transcription factor 3 \(IRF3\) activation in epithelial cells and impairs neutrophil infiltration and *Pseudomonas aeruginosa* killing in vivo](#). *J Biol Chem* 289(2): 1174-1182.

4. Schappe, M. S., Szteyn, K., Stremska, M. E., Mendu, S. K., Downs, T. K., Seegren, P. V., Mahoney, M. A., Dixit, S., Krupa, J. K., Stipes, E. J., Rogers, J. S., Adamson, S. E., Leitinger, N. and Desai, B. N. (2018). [Chanzyme TRPM7 mediates the Ca²⁺ influx essential for lipopolysaccharide-induced toll-like receptor 4 endocytosis and macrophage activation.](#) *Immunity* 48(1): 59-74 e55.
5. Tan, Y., Zanoni, I., Cullen, T. W., Goodman, A. L. and Kagan, J. C. (2015). [Mechanisms of toll-like receptor 4 endocytosis reveal a common immune-evasion strategy used by pathogenic and commensal bacteria.](#) *Immunity* 43(5): 909-922.
6. Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F. and Kagan, J. C. (2011). [CD14 controls the LPS-induced endocytosis of Toll-like receptor 4.](#) *Cell* 147(4): 868-880.

Platelet Migration and Bacterial Trapping Assay under Flow

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[Abstract] Blood platelets are critical for hemostasis and thrombosis, but also play diverse roles during immune responses. We have recently reported that platelets migrate at sites of infection *in vitro* and *in vivo*. Importantly, platelets use their ability to migrate to collect and bundle fibrin (ogen)-bound bacteria accomplishing efficient intravascular bacterial trapping. Here, we describe a method that allows analyzing platelet migration *in vitro*, focusing on their ability to collect bacteria and trap bacteria under flow.

Keywords: Platelets, Cell migration, Bacteria, Shear flow, Fibrinogen, *E. coli*

[Background] Platelets are small, anucleate cellular fragments released from megakaryocytes that reside within the bone marrow of mammalian organisms (Machlus and Italiano, 2013). Approximately 750 billion platelets circulate in human blood, constantly scanning the vasculature for damage of the endothelial surface. Upon encountering endothelial injury, platelets are immediately recruited in a well-characterized cascade of events including initial platelet tethering and rolling, followed by platelet activation, adhesion and spreading, eventually leading to fibrin (ogen)-dependent aggregation and subsequent thrombus retraction (Jackson, 2007). Platelet plug formation is a major step in physiological hemostasis, but also in pathological thrombosis after atherosclerotic plaque rupture, triggering myocardial infarction or stroke (Jackson, 2011).

In addition to their well-established role in hemostasis and thrombosis, platelets also evolved diverse immunologic functions (Semple *et al.*, 2011). Being among the first cells recruited to sites of inflammation and infection, platelets play an essential role in initiating intravascular immune responses (Wong *et al.*, 2013). Accordingly, platelets coordinate the recruitment of a variety of immune cells and instruct them with their effector programs (Sreeramkumar *et al.*, 2014). Platelets also have the ability to directly fight pathogens by releasing anti-microbial mediators and/or physically trapping and encapsulating invaders, thus preventing dissemination with the blood flow (Yeaman, 2014).

We recently identified platelet migration as an autonomous platelet function and showed that migration of single platelets provides a mechanism of controlling their interaction with pathogenic bacteria within the microcirculation (Gaertner *et al.*, 2017). Once adhering to immobilized fibrin (ogen), activated platelets use $\alpha IIb\beta 3$ integrins to probe the resistance of their local microenvironment. When actomyosin-dependent traction forces overcome substrate resistance platelets eventually polarize and migrate thereby removing and accumulating platelet-bound ligands. As a prominent example, migrating

platelets collect and bundle fibrin (ogen)-bound bacteria accomplishing efficient intravascular bacterial trapping. In contrast to phagocytes like neutrophils, platelets behave like “covercytes” that do not internalize collected bacteria but rather accumulate them on their surface within invaginations of the plasma membrane (White, 2005). When exposed to shear stress *in vitro* platelets strongly bind to piled-up bacteria.

Here we provide a detailed protocol for the isolation of platelets from human blood and microscopic observation of platelet migration and trapping of *Escherichia coli* (*E. coli*) under flow.

Materials and Reagents

1. Pipette tips 0.1-10 µl (Eppendorf, epT.I.P.S.® Reloads, catalog number: 022491504)
2. Pipette tips 0.5-20 µl (Eppendorf, epT.I.P.S.® Reloads, catalog number: 022491521)
3. Pipette tips 2-200 µl (Eppendorf, epT.I.P.S.® Reloads, catalog number: 022491539)
4. Pipette tips 50-1,000 µl (Eppendorf, epT.I.P.S.® Reloads, catalog number: 022491555)
5. 15 ml Falcon tube (Corning, catalog number: 352096)
6. Tygon 3350 silicone tube (Saint-Gobain Performance Plastics, catalog number: ABW00002)
7. Safety-Multifly-Needle 21G (SARSTEDT, catalog number: 85.1638.935)
8. Membrane adaptor (SARSTEDT, catalog number: 14.1112)
9. 5 ml syringes (BD, Discardit II™, catalog number: 301285)
10. 50 ml syringes (BD, perfusion, catalog number: 300136)
11. Bottomless 6 channel sticky slide (IBIDI, sticky slides VI^{0.4}, catalog number: 80608)
12. Glass coverslips 24 mm x 24 mm (SCHOTT, NEXTERION®, No. 1.5, catalog number: D263T)
13. Cuvette (Eppendorf, catalog number: 952010069)
14. Human blood (taken from male and female healthy donors with the age of 25-40 years old)
15. Plasmid ptdTomato (Takara Bio, catalog number: 632531)
16. *E. coli* strains (DH12S, Invitrogen)

E. coli was transformed with plasmid ptdTomato. TdTomato is encoded within the *lac* operon and transcription is induced in the presence of isopropylthio-β-galactoside (IPTG) (IPTG binds to the *lac* repressor and releases *lac* repressor from the *lac* operator). The plasmid carries the bla-gene for ampicillin resistance.

Note: *E. coli* stock was made in LB with 7% dimethyl sulfoxide (v/v) and kept at -80 °C.

17. Distilled water (ddH₂O) (Millipore)
18. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2438)
19. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: D8537)
20. Recombinant human albumin (rHSA) (Sigma-Aldrich, catalog number: A9731)

Note: Dissolve rHSA in ddH₂O to 8% (w/v) solution and store the aliquots at -20 °C.

21. Prostaglandin I₂ sodium salt (PGI₂) (Abcam, catalog number: ab120912)

Note: Dissolve it in DMSO to 10 mg/ml and store the aliquots at -20 °C.

22. HEPES solution (Sigma-Aldrich, catalog number: H0887)

23. 20% HNO₃ (Carl Roth, catalog number: 4337.2)
24. Hexamethyldisilazane (HMDS) (Sigma-Aldrich, catalog number: 440191)
25. Fibrinogen from human plasma (Sigma-Aldrich, catalog number: F3879)
Note: Dissolve it in 0.9% NaCl to 2 mg/ml and store the aliquots at -20 °C.
26. Fibrinogen from human plasma, Alexa Fluor 488 conjugate (Thermo Fisher Scientific, Life technology, catalog number: F13191)
Note: Dissolve it in 0.1 M NaHCO₃ (pH 8.3) to 1.5 mg/ml and store the aliquots at -20 °C.
27. U46619 (Enzo Life Sciences, catalog number: BML-PG023-0001)
Note: Dissolve it in DMSO to 28.8 mM and store the aliquots at -20 °C.
28. Adenosine 5'-diphosphate sodium salt (ADP) (Sigma-Aldrich, catalog number: A2754)
Note: Dissolve it in PBS to 20 mM and store the aliquots at -20 °C.
29. Thrombin from bovine plasma (Sigma-Aldrich, catalog number: T4648)
Note: Dissolve it in 0.1% (w/v) bovine serum albumin (BSA) to 100 U/ml and store the aliquots at -20 °C.
30. LB broth (Sigma-Aldrich, catalog number: L3022)
Note: 20 g LB powder was dissolved in 1 L ddH₂O and autoclaved.
31. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, catalog number: I6758)
Note: Dissolve IPTG in ddH₂O to 100 mM and store the aliquots at -20 °C.
32. Ampicillin sodium salt (Sigma-Aldrich, catalog number: A9518)
Note: Dissolve ampicillin in ddH₂O to 50 mg/ml and store the aliquots at -20 °C.
33. Sodium citrate tribasic dehydrate (C₆H₅Na₃O₇·2H₂O) (Sigma-Aldrich, catalog number: S4641)
34. Citric acid monohydrate(C₆H₈O₇·H₂O) (Sigma-Aldrich, catalog number: C1909)
35. Glucose (Merck, Calbiochem, catalog number: 346351)
36. Sodium chloride (NaCl) (Carl Roth, catalog number: 3957.3)
37. Sodium bicarbonate (NaHCO₃) (Merck, catalog number: 106329)
38. Potassium chloride (KCl) (Carl Roth, catalog number: 6781.3)
39. Calcium chloride (CaCl₂) (Sigma-Aldrich, catalog number: C8106)
Note: CaCl₂·2H₂O was dissolved in ddH₂O to make 100 mM stock solution and stored at 4 °C.
40. Acid-citrate-dextrose (ACD) buffer, pH 4.7 (see Recipes)
41. Modified Tyrode's buffer (see Recipes)
42. Fibrin buffer (see Recipes)

Equipment

1. Pipette 0.1-2.5 µl (e.g., Eppendorf, Research plus: O18485E)
2. Pipette 0.5-10 µl (e.g., Eppendorf, Research plus: O30964E)
3. Pipette 10-100 µl (e.g., Eppendorf, Research plus: O30379E)
4. Pipette 100-1,000 µl (e.g., Eppendorf, Research plus: O29260E)
5. Orbi-Shaker (e.g., Benchmark)

6. pH meter (e.g., Mettler Toledo)
7. BioPhotometer (e.g., Eppendorf, model: BioPhotometer 6131)
8. Bunsen burner (e.g., Campingaz)
9. Centrifuge (e.g., Eppendorf, model: 5804)
10. Cell culture incubator (e.g., Binder)
11. Hematology counter (e.g., Horiba Medical)
12. KLM spin coater (e.g., Schaefer)
13. Inverted phase contrast and fluorescent microscope (e.g., OLYMPUS, model: IX83)
14. Syringe pump (e.g., KD Scientific, model: KDS 100)

Software

1. Live cell imaging software that was connected to the microscope (e.g., CellSense)
2. Fiji software [National Institutes of Health (NIH)]

Procedure

A. Isolation of washed platelets

1. Take human blood from cubital vein by safety multify-needle on volunteers. Discard the first 1 ml then take another 5 ml into a syringe with 714 µl ACD buffer (see Recipe 1) inside.

Notes:

- a. *The volunteers should not take any drugs that affect platelets in the last two weeks.*
- b. *Draw blood smoothly to avoid platelets activation.*

2. Mix the blood with an equal volume of modified Tyrode's buffer (pH 6.5) (see Recipe 2) in a 15 ml falcon tube.
3. Centrifuge the mixture in Step A2 at 70 \times g for 35 min without brake at room temperature (RT) to obtain platelet-rich plasma (PRP) retrieved from the upper layer.
4. Mingle 3 ml PRP with 7 ml modified Tyrode's buffer (pH 6.5) in the presence of 0.1% rHSA (w/v) and 100 ng/mg PGI₂ in a 15 ml Falcon tube.
5. Centrifuge the diluted PRP in Step A4 at 1,200 \times g for 10 min at RT.
6. Discard the supernatant and suspend the pellet carefully in 1 ml Tyrode's buffer with pH 6.5.
7. Measure the platelet concentration on an automated hematology counter.
8. Store the purified platelets at room temperature until use.

Notes:

- a. *Platelets cannot be cultured, so they should be prepared daily from freshly drawn blood.*
- b. *The whole process of platelets preparation should be gentle enough to prevent platelets activation.*

B. Bacteria preparation

1. Inoculate *E. coli* from a frozen stock in LB-broth medium with 100 µg/ml Ampicillin.
*Note: Conduct *E. coli* inoculation at the Bunsen burner.*
2. Culture the inoculated *E. coli* at 37 °C with 260 rpm shaking overnight.
3. On the following day, dilute saturated culture of *E. coli* in LB-broth medium with 1 mM IPTG and 100 µg/ml Ampicillin (1:500 in a volume of 5 ml).
4. Culture the diluted *E. coli* in Step B3 at 37 °C with 260 rpm for 2.5 h to allow growth to mid-log phase.
5. Determine the colony forming units (CFU)/ml of cultured *E. coli* at OD_{600nm}=1 (1, Determine viability of bacteria as a function of OD_{600nm} by plating bacteria and counting colony forming units at various time points with increasing OD_{600nm}. 2, Based on obtained growth curves determine CFUs/ml at OD_{600nm}=1, we obtained 2.8 × 10⁸ CFU/ml at OD_{600nm} = 1.0).
6. Measure the *E. coli* concentration by optical density at OD_{600nm} (expected OD_{600nm} ~0.5-0.6).
7. Adjust the cultured *E. coli* to OD_{600nm} equal to 0.25 in LB medium (0.7 × 10⁸ CFU/ml; 1 ml), centrifuge at 1,200 × g for 5 min and discard the supernatant.
8. Suspend the bacteria pellet in equal volume of PBS (1 ml) and keep them on ice under light protection until use.

Note: Perform the bacteria preparation according to the safety regulations of your laboratory.

C. Coverslip coating

1. Immerse glass coverslips in 20% HNO₃ for 1 h on a shaker with the speed set at 60 rpm, then wash with ddH₂O four times.
2. Soak acid-washed coverslips in ddH₂O for another 1 h on the same shaker, and rinse again with ddH₂O twice.
3. Dry the freshly cleaned coverslips at 90 rps for 10 sec by a KLM spin coater.
4. Silanize air-dried coverslips with HMDS at 80 rps for 30 sec using a spin coater to ensure homogeneous silanization.

Note: HMDS is volatile and inflammable, so coat the HMDS in a ventilation hood.

5. Cut the 6 channel bottomless Ibidi sticky-slide into single channel slides (Figure 1), and mount to the silanized coverslip.

Note: Mount coverslips and sticky-slide with some pressure until the bottom is sealed, otherwise the buffer in the channel will leak out.



Figure 1. A photo of Ibidi sticky slides. The upper is the 6-channel slide; the lower is the 6-cutted single slides.

6. Seed 120 μl prepared *E. coli* (8.4×10^6 CFUs) in PBS on silanized coverslip in the Ibidi channel at 37 °C for 30 min.
7. Coat 120 μl fibrin (see Recipe 4) on the *E. coli* seeded coverslip for 15 min at RT.
8. Wash the fibrin-coated slides 5 times with modified Tyrode's buffer (pH 7.4; 120 μl) (Figures 2 and 3).

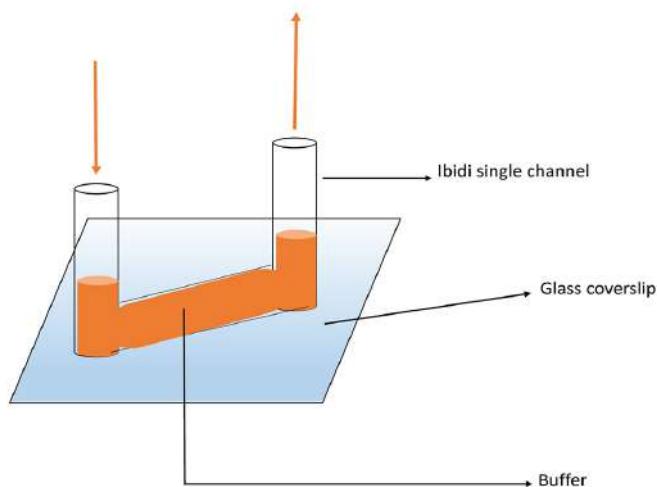


Figure 2. Diagram of the experimental setup. The single Ibidi sticky slide channel is mounted on a silanized glass coverslip. The buffer is pipetted as indicated by the red arrows.

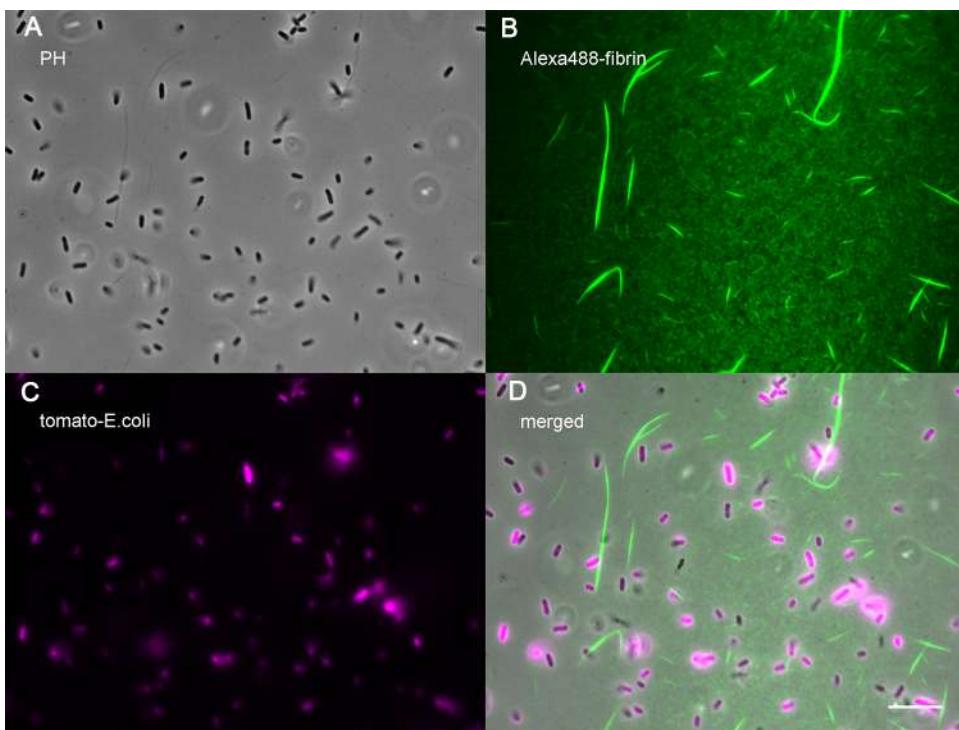


Figure 3. A representative coverslip coated with fibrin and bacteria. Freshly cleaned and silanized glass coverslip was seeded with *E. coli* and incubated at 37 °C for 30 min, then coated with fibrin for 15 min at RT, and finally washed with Tyrode's buffer. A. Phase contrast image of the coated channel. B. Fluorescent fibrin coating of the channel. C. Fluorescent *E. coli* bound to the channel. D. Merged channels. Scale bar = 10 µm.

D. Microscopic observation of migrating platelets and bacterial capture

1. Supplement modified Tyrode's buffer pH 7.4 in a total volume of 240 µl with 10⁴/µl human platelets, 1,000 µg/ml rHSA, 200 µM CaCl₂, 2 µM U46619 and 4 µM ADP and add to above prepared channels coated with fibrin and live *E. coli*.

Note: ADP, U46619 and CaCl₂ synergistically trigger platelets activation and spreading. Albumin is an anti-adhesive protein that lowers substrate adhesiveness.

2. Incubate the channel in Step D1 at 37 °C for 1 h and 20 min (Figure 4).

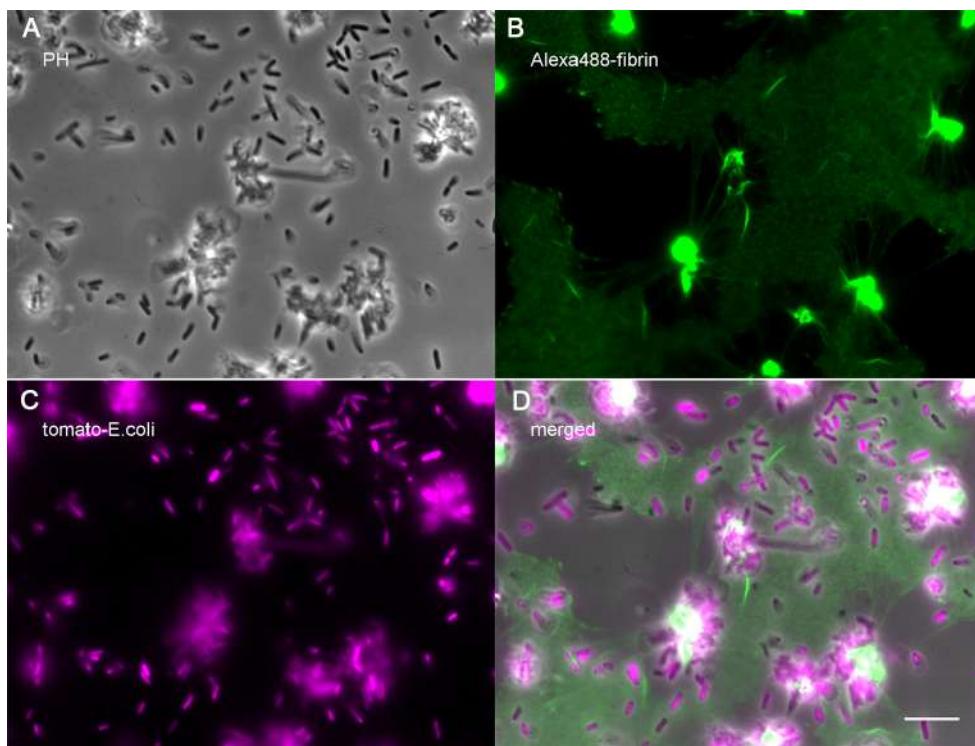


Figure 4. A representative image of migrating platelets collecting fibrin-bound *E. coli*.

Fibrin and live *E. coli* coated channel was incubated with $10^4/\mu\text{l}$ human platelets in the presence of 1,000 $\mu\text{g}/\text{ml}$ rHSA, 200 μM CaCl_2 , 2 μM U46619 and 4 μM ADP in modified Tyrode's buffer at 37 °C for 1 h and 20 min. Images show phase contrast (A) and epifluorescent micrographs (B and C). The black area in the fibrin image (B) indicates the area cleared by migrating platelets. Scale bar = 10 μm . *E. coli* accumulated on platelets and bacteria density is reduced in areas where platelets migrated (D).

3. Assemble the Ibidi channel with a fluid tube connected to the syringe pump and place it on an inverted microscope with a heated stage.

Note: Always carefully rule out air bubbles in the syringe and tube before the perfusion.

4. Perfusion the channel with pre-warmed (37 °C) modified Tyrode's buffer (pH 7.4) at 0.5 dyne/cm² for 1 min to remove non-adhering bacteria and take phase contrast and epifluorescent images.
Note: The flow rate Φ (ml/min) of the syringe pump is calculated by the following formulas provided by the instruction of sticky sides from Ibidi:

$$\tau = \eta \times 97.1 \times \Phi$$

τ: Shear stress (dyne/cm²)

η: Dynamical viscosity (dyn s/cm²)

The dynamical viscosity of cell culture medium at 37 °C is ~0.007.

5. Perfusion the channel with modified Tyrode's buffer (pH 7.4) at 37 °C at 5 dyne/cm² for 5 min and take images (Figure 5).

6. Image acquisition: Filtercubes (Excitation/Emission): fibrin (Alexa 488) (494/528 nm); *E. coli* (tdTomato) (590/617 nm).

Note: Deal with the waste of the perfusion buffer that contains bacteria properly according to the safety regulations.

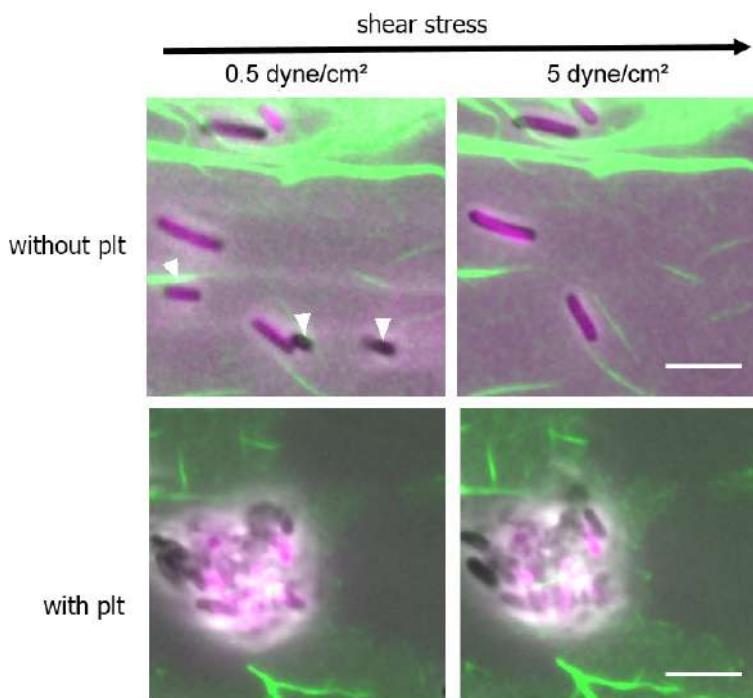


Figure 5. Representative images of bacteria trapped without platelets and with platelets after 0.5 dyne/cm² and 5 dyne/cm². The micrographs show the merged phase contrast and fluorescent images. Scale bars = 5 μ m.

*Note: In the absence of platelets, the number of *E. coli* (magenta) on the fibrin (green) was reduced after perfusion. The white arrows indicate the *E. coli* that were flushed away. In contrast, the bacteria bundle collected by migrating platelets is resistant to shear stress.*

Data analysis

1. Open the acquired images in FIJI software (Schindelin et al., 2012).
2. Adjust brightness and contrast of each channel separately to clearly identify bacteria and fibrin fibers, respectively.
3. Manually count fibrin-bound bacteria aggregates (in the presence and absence of platelets) before and after perfusion.
4. Calculate the fraction of bacteria resistant to perfusion for each experiment by dividing the number of bacteria aggregates after perfusion by the number of bacteria before perfusion. As an example, the bar plot in Figure 6 shows flow resistance of bacterial aggregates in the presence and absence of platelets.

5. Test the statistical significance of your results. In the example shown in Figure 6 we repeated experiments 6 times and performed a Wilcox rank sum test to test for statistical significance (R software [R Development Core Team 2011]).

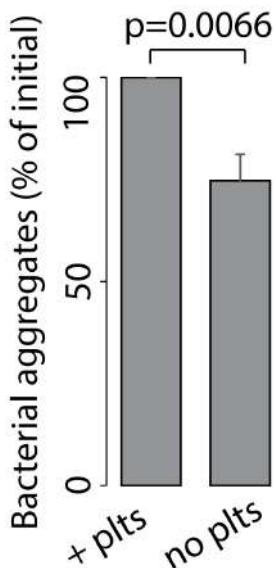


Figure 6. Bar plots showing surface-bound bacterial aggregates after perfusion (5 dyne/cm²) as fraction of initially bound bacteria (in %). Bacteria aggregates bound to platelets are more resistant to shear stress than bacteria aggregates bound to fibrin only. n = 6; Mean ± SEM; statistical significance was tested using the Wilcox rank sum test.

Notes

E. coli (DH 12)-strain used in this protocol requires safety level 1 (S1) laboratories.

Recipes

1. ACD buffer, pH 4.7
 - 85 mM Sodium citrate tribasic dehydrate
 - 65 mM citric acid monohydrate
 - 111 mM glucose
2. Modified Tyrode's buffer
 - 136.9 mM NaCl
 - 12.1 mM NaHCO₃
 - 2.6 mM KCl
 - 5.5 mM glucose
 - 10 mM HEPES

Note: Prepare 10x Tyrode's buffer (1.37 mol NaCl, 121 mM NaHCO₃, 26 mM KCl) and store at

4 °C. Dilute the stock Tyrode's buffer with ddH₂O and add glucose as well as HEPES buffer for daily use. Then adjust the pH to 6.5 for platelets isolation and 7.4 for platelet migration.

3. Fibrin buffer

Modified Tyrode's buffer (pH 7.4) was supplemented with 300 µg/ml fibrinogen (1:10 mixed with Alexa488 conjugate fibrinogen), 12 mg/ml rHSA, 2 U/ml thrombin, and 1 mM CaCl₂

Note: Prepare the fibrin buffer just before use.

Acknowledgments

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Competing interests

The authors declare that there is no conflict of interest or competing interest regarding the publication of this article.

References

1. Gaertner, F., Ahmad, Z., Rosenberger, G., Fan, S., Nicolai, L., Busch, B., Yavuz, G., Luckner, M., Ishikawa-Ankerhold, H., Hennel, R., Benechet, A., Lorenz, M., Chandraratne, S., Schubert, I., Helmer, S., Striednig, B., Stark, K., Janko, M., Böttcher, R. T., Verschoor, A., Leon, C., Gachet, C., Gudermann, T., Mederos, Y. S. M., Pincus, Z., Iannacone, M., Haas, R., Wanner, G., Lauber, K., Sixt, M. and Massberg, S. (2017). [Migrating platelets are mechano-scavengers that collect and bundle bacteria](#). *Cell* 171(6): 1368-1382 e1323.
2. Jackson, S. P. (2007). [The growing complexity of platelet aggregation](#). *Blood* 109(12): 5087-5095.
3. Jackson, S. P. (2011). [Arterial thrombosis--insidious, unpredictable and deadly](#). *Nat Med* 17(11): 1423-1436.
4. Machlus, K. R. and Italiano, J. E., Jr. (2013). [The incredible journey: From megakaryocyte development to platelet formation](#). *J Cell Biol* 201(6): 785-796.
5. R Development Core Team (2011). [R: A Language and Environment for Statistical Computing](#). Vienna, Austria: the R Foundation for Statistical Computing.
6. 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.
7. Semple, J. W., Italiano, J. E., Jr. and Freedman, J. (2011). [Platelets and the immune continuum](#). *Nat Rev Immunol* 11(4): 264-274.
8. Sreeramkumar, V., Adrover, J. M., Ballesteros, I., Cuartero, M. I., Rossaint, J., Bilbao, I., Nacher, M., Pitaval, C., Radovanovic, I., Fukui, Y., McEver, R. P., Filippi, M. D., Lizasoain, I., Ruiz-Cabello, J., Zarbock, A., Moro, M. A. and Hidalgo, A. (2014). [Neutrophils scan for activated platelets to initiate inflammation](#). *Science* 346(6214): 1234-1238.
9. White, J. G. (2005). [Platelets are covercytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system](#). *Platelets* 16(2): 121-131.
10. Wong, C. H., Jenne, C. N., Petri, B., Chrobok, N. L. and Kubes, P. (2013). [Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance](#). *Nat Immunol* 14(8): 785-792
11. Yeaman, M. R. (2014). [Platelets: at the nexus of antimicrobial defence](#). *Nat Rev Microbiol* 12(6): 426-437.

Isolation and Culture of Mouse Lung ILC2s

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[Abstract] Group 2 Innate Lymphoid Cells (ILC2) play an important role in immune responses at barrier surfaces, notably in the lung during airway allergic inflammation or asthma. Several studies have described methods to isolate ILC2s from wild-type naive mice, most of them using cell sorting to obtain a pure population. Here, we describe in detail, a simple, efficient method for isolation and culture of lung mouse ILC2s. Lungs from *Rag2^{-/-}* mice pretreated with IL-33 are collected and processed into single cell suspensions. Lymphoid cells are then recovered by density gradient separation. Lin⁻CD45⁺ cells are selected by depletion of lineage positive cells followed by positive selection of CD45⁺ cells. Culture of the isolated cells for several days results in a highly purified ILC2 population expressing typical cell surface markers (CD90.2, Sca1, CD25, CD127, and IL-33R). These cells can be expanded in culture for up to 10 days and used for diverse *ex vivo* assays or *in vivo* adoptive transfer experiments.

Keywords: Innate immunity, Allergic inflammation, ILC2, IL-33, Type 2 cytokine, Lung, Culture

[Background] Group 2 Innate Lymphoid Cells (ILC2) are tissue resident cells that play crucial roles in anti-parasitic innate immunity as well as in the development of allergic inflammation. They respond to epithelial cell-derived cytokines such as interleukin-33 (IL-33) by producing large amounts of type 2 cytokines IL-5 and IL-13, which in turn induce eosinophilia and mucus production (Cayrol and Girard, 2018). In order to better characterize the function and regulation of these cells, numerous groups have sorted ILC2s from the lung of wild-type mice (WT) by fluorescence-activated cell sorting (FACS). Due to the low number of the ILC2s present in the lungs at steady state, this method results in a low yield of purified cells (1×10^4 per mouse) (for a review, see Moro *et al.*, 2015). In the present protocol, we pretreat mice with IL-33, which triggers the *in vivo* expansion of lung ILC2s, resulting in a better yield of purified cells (1.3×10^6 per mouse). Moreover, we use *Rag2^{-/-}* mice instead of WT mice because 1) the absence of B and T cells in these mice facilitates the purification of ILC2s, and 2) the number of ILC2s is greater in these mice. Culture of the isolated Lin⁻CD45⁺ cell population for a couple of days *ex vivo* provides highly purified lung ILC2s without the need to use a cell sorter. In summary, the procedure we describe is highly reproducible and provides abundant highly purified mouse lung ILC2s.

Materials and Reagents

1. Sterile disposable scalpel (LABELIANS, Nahita™, catalog number: SCMEC24)
2. 96-Well polystyrene conical bottom MicroWell™ Plates (Thermo Fisher Scientific, catalog number: 249570)
3. 15 ml and 50 ml conical centrifuge polypropylene tubes (Corning, Falcon®, catalog numbers: 352096 and 352070 respectively)
4. 5 ml round-bottom polystyrene tubes (Corning, Falcon®, catalog number: 352054)
5. Cell culture 60 x 15 mm Petri dishes (Thermo Fisher Scientific, catalog number: 150288)
6. 1 ml tuberculin syringes (with 25 G x 16 mm disposable) (Terumo, catalog number: SS-01T)
7. BD Micro-Fine+™ Insulin Syringes 0.3 ml; 30 G x 8 mm needle (BD, catalog number: 324826)
8. 70 µm cell strainer (Corning, Falcon®, catalog number: 352350)
9. Plunger of 2.5 ml syringes (Terumo, catalog number: SS*02SE1)
10. Hypodermic needles (Terumo, catalog number: NN-2516R)
11. Non cottoned open Pasteur pipettes (150 mm 2 ml) (Hilgenberg, catalog number: 3150102)
12. MS columns (Miltenyi Biotec, catalog number: 130-042-201)
13. 6-well polystyrene (PS) multidish (Thermo Fisher Scientific, catalog number: 140675)
14. 1.2 ml Cluster Tubes loose (Thermo Fisher Scientific, Abgene®, catalog number: AB-0672)
15. *Rag2*^{-/-} mice on a C57BL/6J background (*B6.129-Rag2^{tm1Fwa}*) (European Mouse Mutant Archive [EMMA])
16. Human recombinant Interleukin-33 (rIL-3395-270), natural form (home-made; previously described in Lefrançais *et al.*, 2014)
Note: Alternatively, recombinant IL-33 can be purchased from R&D Systems (R&D Systems, catalog number: 3625-IL).
17. Ice
18. Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX™ Supplement, pyruvate) (Thermo Fisher Scientific, Gibco™, catalog number: 31966021)
19. Dulbecco's Phosphate-Buffered Saline (DPBS, no calcium, no magnesium) (Thermo Fisher Scientific, Gibco™, catalog number: 14190169)
20. RPMI 1640 with high glucose, L-Glutamine, HEPES (ATCC, catalog number: 30-2001)
21. Penicillin/Streptomycin 100x liquid (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
22. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
23. Recombinant mouse IL-2 (Cys 160 Ser) Protein (R&D Systems, catalog number: 1150-ML-020; Reconstitute at 100 µg/ml in sterile DPBS containing 0.1% bovine serum albumin. Store stock solution at -70 °C)
24. Collagenase Type IV (Thermo Fisher Scientific, Gibco™, catalog number: 17104019)
25. DNase I, from bovine pancreas (Roche Diagnostics, catalog number: 11284932001)

26. Fetal Bovine Serum (Thermo Fisher Scientific, Gibco™, catalog number: 10270-106)
27. Normal mouse serum (Thermo Fisher Scientific, catalog number: 10410)
28. Lympholyte®-M (CEDARLANE, catalog number: CL5035)
29. EasySep™ mouse hematopoietic progenitor cell isolation kit (STEMCELL Technologies, catalog number: 19856)
30. CD45 MicroBeads, mouse (Miltenyi Biotec, catalog number: 130-052-301)
31. FACS reagents

Note: Dilutions have to be determined for each lot of reagent.

- a. Fixable Viability Dye eFluor 506 (dilution: 1/1,000) (Thermo Fisher Scientific, eBioscience™, catalog number: 65-0866-14)
- b. Streptavidin, PE-Cy™ 7 conjugated (dilution: 1/100) (BD Pharmingen™, catalog number: 557598)

32. FACS Monoclonal Antibodies

Note: Dilutions have to be determined for each lot of antibody.

- a. Rat anti-Mouse CD16/CD32 (mouse BD Fc Block™) (clone 2.4G2, dilution: 1/200) (BD Pharmingen™, catalog number: 553142)
- b. Rat Anti-Mouse CD4, FITC conjugated (clone GK1.5, dilution: 1/2,000) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-0041-85)
- c. Rat Anti-Mouse CD3, FITC conjugated (clone 17A2, dilution: 1/600) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-0032-80)
- d. Rat Anti-Mouse CD19, FITC conjugated (clone 1D3, dilution: 1/2,000) (BD Pharmingen™, catalog number: 553785)
- e. Rat Anti-Mouse CD45R/B220, FITC conjugated (clone RA3-6B2, dilution: 1/1,000) (BD Pharmingen™, catalog number: 553088)
- f. Hamster Anti-Mouse CD11c, FITC conjugated (clone N418, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-0114-82)
- g. Rat Anti-Mouse CD11b, FITC conjugated (clone M1/70, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-0112-85)
- h. Rat Anti-Mouse Ter119, FITC conjugated (clone Ter119, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-5921-85)
- i. Rat Anti-Mouse Ly-6G/Ly-6C, FITC conjugated (clone RB6-8C5, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-5931-85)
- j. Hamster Anti-Mouse FceR1α, FITC conjugated (clone MAR-1, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-5898-85)
- k. Mouse Anti-Mouse NK1.1, FITC conjugated (clone PK136, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 11-5941-85)
- l. Rat Anti-Mouse CD45, PerCP conjugated (clone 30-F11, dilution: 1/1,000) (BD Pharmingen™, catalog number: 557235)
- m. Rat Anti-Mouse CD90.2, APC-Cy™7 conjugated (clone 53-2.1, dilution: 1/600) (BD

Pharmingen™, catalog number: 561641)

- n. Rat Anti-Mouse CD25, eFluor 450 conjugated (clone PC61.5, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 48-0251-82)
- o. Rat Anti-Mouse CD127, PE conjugated (clone A7R34, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 12-1271-83)
- p. Rat Anti-Mouse Ly-6A/E (Sca-1), APC conjugated (clone D7, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 17-5981-83)
- q. Rat Anti-Mouse T1/ST2 (IL-33R), biotinylated (clone DJ8, dilution: 1/100) (MD Biosciences, catalog number: 101001B)

33. FACS Isotype controls

Note: Isotype controls are used at the same concentration as the specific antibody. So, dilutions have to be determined according to the concentration of the matched antibody.

- a. Rat IgG1 κ Isotype Control, eFluor 450 (clone eBRG1, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 48-4301-80)
- b. Rat IgG2a κ Isotype Control, APC (clone eBR2a, dilution: 1/300) (Thermo Fisher Scientific, eBioscience™, catalog number: 17-4321-81)
- c. Rat IgG2a, κ Isotype Control, PE (clone eBR2a, dilution: 1/100) (Thermo Fisher Scientific, eBioscience™, catalog number: 12-4321-82)
- d. Rat IgG2a, κ Isotype Control, APC-Cy™ 7 (Clone R35-95, dilution: 1/600) (BD Pharmingen™, catalog number: 552770)
- e. Rat IgG1, κ Isotype Control, Biotin (Clone R3-34, dilution: 1/50) (BD Pharmingen™, catalog number: 553923)

34. Lung digestion solution (see Recipes)

35. PEF buffer (see Recipes)

36. PEB buffer (see Recipes)

37. ILC2 culture medium (see Recipes)

38. FACS buffer (see Recipes)

39. FACS staining buffer (see Recipes)

Equipment

1. MiniMACS™ separator (Miltenyi Biotec, catalog number: 130-042-102)
2. EasySep™ Magnet (STEMCELL Technologies, catalog number: 18000)
3. Malassez Hemocytometer
4. Water bath
5. Centrifuge (Eppendorf, model: 5804 R)
6. 4 °C refrigerator
7. Flow cytometer (BD, model: LSR II)

Software

1. FlowJo software (Tree Star)
2. BD FACSDiva™ software (BD Biosciences)

Procedure

Note: In order to get enough ILC2 cells to perform various experiments, we generally purify ILC2s from 4 or 5 mice.

A. In vivo treatment of mice

1. Prepare purified human recombinant IL-33₉₅₋₂₇₀ as previously described by Lefrançais *et al.* (2014). Human IL-33 is a potent activator of mouse ST2⁺ cells (Cayrol *et al.*, 2018).
2. For each mouse, inject intraperitoneally 4 µg of human recombinant IL-33₉₅₋₂₇₀ into a *Rag2*^{-/-} mouse (diluted in DPBS; V = 100 µl/mouse), using an insulin syringe (0.3 ml; 30 G), five times at days 0, 1, 4, 5 and 6 (see Figure 1).

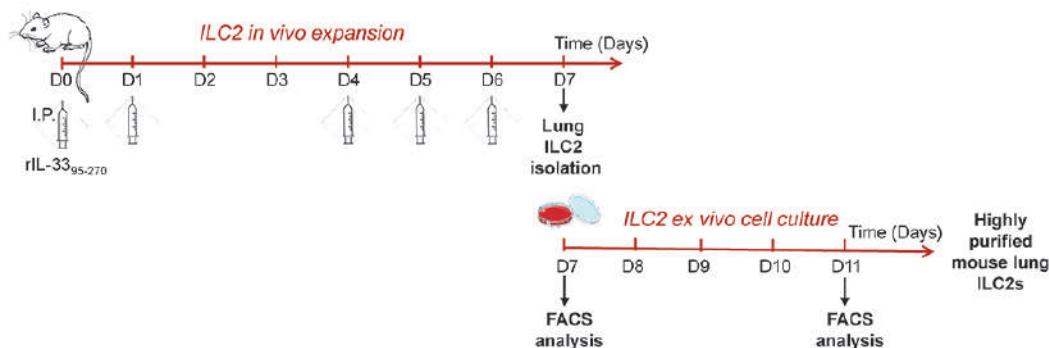


Figure 1. A schematic view of the whole procedure. *Rag2*^{-/-} mice were first treated with rIL-33₉₅₋₂₇₀ to expand ILC2s *in vivo* (see Procedure A). Lin⁻CD45⁺ ILC2s are then isolated (see Procedure B) and cultured for several days (see Procedure C), before being used for subsequent ex vivo or *in vivo* experiments.

B. ILC2 Isolation

Notes:

- a. *Lympholyte®-M is a density separation medium. Put it at room temperature for at least 20 min before use, because the density of Lympholyte®-M is temperature dependent (Step B2).*
- b. *At different steps during this procedure, 100 µl of cell suspensions is collected in a 96-well plate for subsequent FACS analysis. Keep that plate on ice, until Step B5.*

1. Preparing lung single-cell suspension

For each mouse:

- a. Sacrifice the mouse on Day 7, remove the lungs and place them into a 15 ml Falcon® tube filled with 5 ml DMEM.
- b. Freshly prepare lung digestion solution (2 ml per mouse; see Recipes).
- c. Transfer the lungs into a Petri dish containing 500 µl of lung digestion solution and smoothly inject 500 µl of lung digestion solution into the lobes of the lung, using a 25 G needle mounted on a 1 ml syringe. The lung must swell (see Video 1)



Video 1. Procedure of lung digestion. The video shows the injection of digestion solution into the lung.

- d. Cut the lungs into small pieces using a scalpel and transfer the crushed lungs into a Falcon® 50 ml tube. Rinse the Petri dish with 1 ml of lung digestion solution and transfer to the 50 ml tube.
 - e. Incubate the 50 ml tube containing the sample in a water bath at 37 °C for 1 h.
 - f. Put a 70 µm cell strainer on a 50 ml tube; apply the sample on it and rinse the tube used for the digestion with 2 ml DPBS.
 - g. In order to obtain a uniform single-cell suspension from tissue, grind the remaining tissue on the strainer with the plunger of a 2.5 ml syringe and wash the strainer with additional 2 ml DPBS.
2. Enrichment of leucocyte population by density gradient separation
 - a. Discard the cell strainer and transfer the cell suspension into a Falcon® 15 ml tube. Centrifuge at 400 x g at room temperature for 5 min.
 - b. Remove the supernatant from the samples and resuspend the cell pellet in 5 ml DPBS.
 - c. Using Pasteur pipette, slowly add 5 ml Lympholyte®-M under the cell suspension (see Figure 2A).
 - d. Centrifuge at 1,300 x g at room temperature for 20 min without any brake.
 - e. For each sample, collect the cells of interest from the Lympholyte®-M/DPBS interface with a Pasteur pipette (see Figure 2B). Combine all samples in a single 15 ml Falcon® conical

tube filled with 5 ml DPBS. This step enables the recovery of lymphoid cells and eliminates erythrocytes, dead cells and debris.

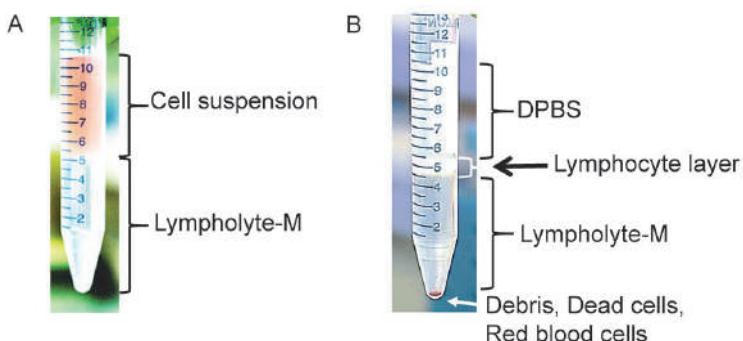


Figure 2. Density gradient separation procedure. A. Before centrifugation, lymphoid cells are located in the cell suspension phase above the Lympholyte®-M. B. After centrifugation, lymphoid cells are concentrated in the interface between the DPBS buffer and the Lympholyte®-M. Red blood cells, dead cells and debris are found at the bottom of the tube.

3. Enrichment of lineage negative population

- Centrifuge at $400 \times g$ at 4°C for 5 min to pellet the cells.

Note: Work under sterile conditions (hood) from this step.

- Remove the supernatant and resuspend the cell pellet in 10 ml PEF buffer.

Note: Take 100 μl of cell suspension for subsequent FACS analysis (see Figure 3A for gating strategy and Figure 3B-before purification).

- Count the cells using a hemocytometer. The typical recovered cell number is 20×10^6 per mouse.

- Centrifuge again at $400 \times g$ at 4°C for 5 min.

- Proceed with negative selection: deplete lineage positive cells using The EasySep™ Mouse Hematopoietic Cell Isolation Kit. This method uses biotinylated antibodies directed against non-hematopoietic stem cells and non-progenitor cells (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119) and streptavidin-coated magnetic particles. Follow the kit protocol to separate unlabeled cells (Lin^-) using an EasySep™ magnet. At the end of this step, the cells of interest (Lin^-) are recovered in a 15 ml tube.

- Repeat the depletion step with the EasySep™ magnet using the negative fraction containing Lin-ILC2s

Note: Take 100 μl of each cellular fraction for subsequent FACS analysis (see Figure 3B-lineage depletion; first and second depletion).

4. Selection of CD45⁺ population

- Centrifuge at $400 \times g$ at 4°C for 5 min.

- Remove the supernatant, resuspend the cell pellet in 5 ml of PEB buffer and determine cell number using a hemocytometer. The typical recovered cell number is 3.7×10^6 per

mouse.

- c. Proceed with CD45 positive selection: select CD45⁺ cells by using magnetic beads conjugated to anti-mouse CD45 monoclonal antibody, MS columns, MiniMACS™ separator and follow the recommendations of the manufacturer (Miltenyi Biotech). At the end of this step, the cells of interest (Lin⁻CD45⁺) are recovered.
Note: Take 100 µl of cell suspension for subsequent FACS analysis (see Figure 3B-CD45 selection).
 - d. Centrifuge at 400 x g at 4 °C for 5 min.
 - e. Remove the supernatant, resuspend cell pellet in 1 ml of ILC2 culture medium (see Recipes).
 - f. Count cells by using a hemocytometer. Typically, we obtain 1.3 x 10⁶ ILC2s per mouse.
5. Proceed with ILC2s staining for FACS analysis
 - a. Fill wells of the 96-well plate containing cells from subsequent isolation steps with DPBS.
 - b. Centrifuge the plate at 400 x g at 4 °C for 5 min and remove the supernatant.
 - c. Perform the viability staining: resuspend cell pellets with 100 µl/well of Viability Dye-eFluor506 diluted to 1/1,000 in DPBS. Incubate for 30 min at 4 °C.
 - d. Wash the cells: complete wells with FACS buffer (see Recipes), centrifuge the plate at 400 x g at 4 °C for 5 min and remove the supernatant.
 - e. Resuspend cell pellets in 50 µl/well of the different antibodies (listed in Materials and Reagents #31-33) diluted in FACS staining buffer (containing Fc block and mouse serum). Incubate for 30 min at 4 °C.
 - f. Repeat the washing step.
 - g. Resuspend cell pellets in 100 µl of FACS buffer, transfer each sample to annotated FACS tubes and add 200 µl of FACS buffer (300 µl total volume).
 - h. Proceed to FACS analyses (on a LSR II for example). We generally obtain an ILC2 population with about 90% of cells expressing typical ILC2s markers (Lin⁻CD45⁺ CD25⁺ CD90.2⁺ CD127⁺ Sca1⁺ ST2⁺).

Note: Cell surface markers analysis after ILC2 isolation—see an example in Figures 3C and 3D.

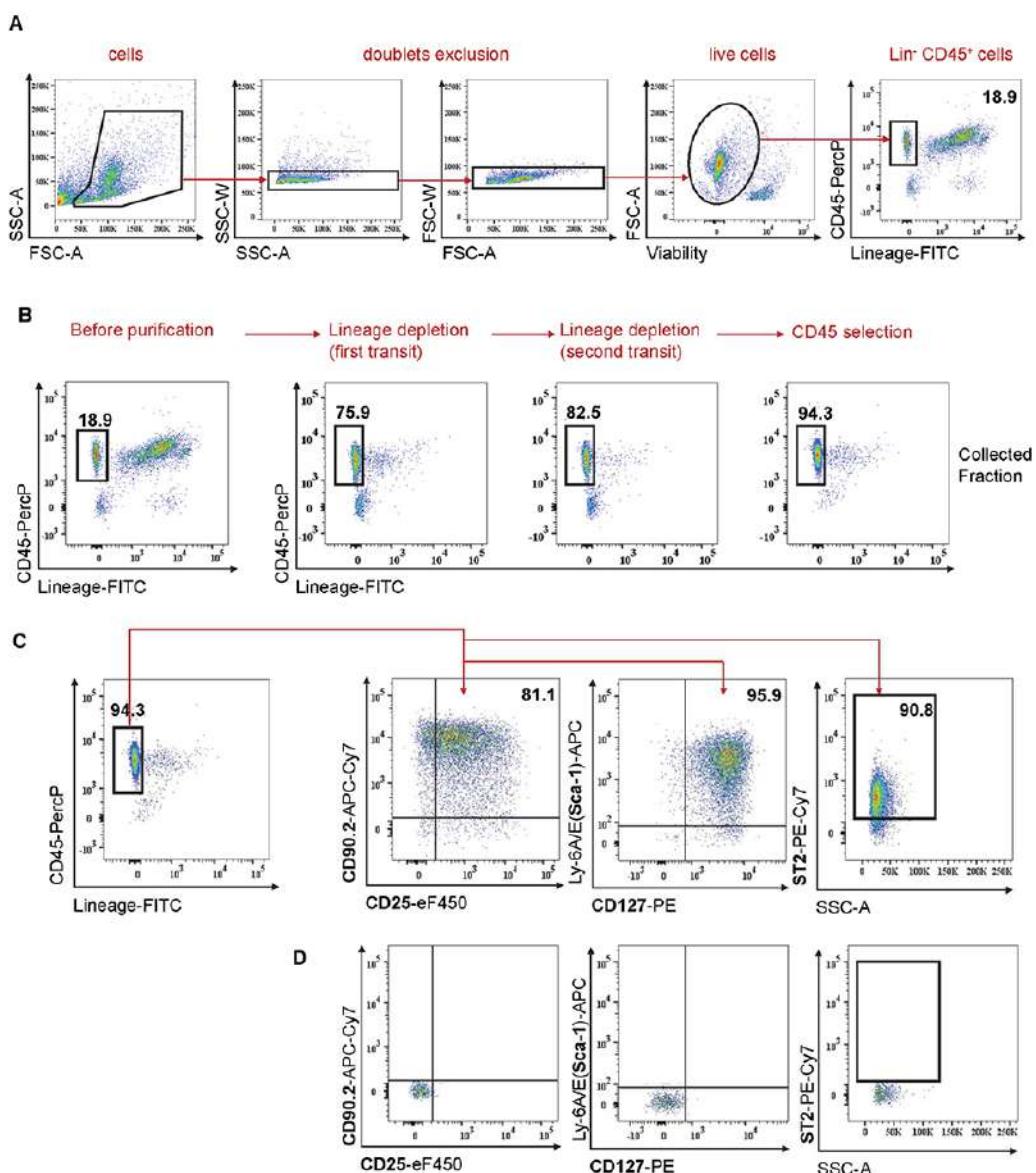


Figure 3. Isolation of mouse lung ILC2s. A. Gating strategy used to identify ILC2s. Doublet cells and dead cells are first excluded. ILC2s lack lineage markers and express CD45 surface marker. Number indicates % among live cells. B. ILC2 purification is analyzed by FACS at different steps: before purification, after depletion of lineage + cells, and after CD45 positive selection. Cells are gated on live single cells. Numbers indicate % among live cells. C. Analysis of cell surface markers after ILC2 isolation (Day 7). Cells are gated on live single cells. Numbers indicate % among Lin⁻CD45⁺ cells. D. Isotype controls are shown.

C. ILC2 culture

This step of ILC2 culture leads to increased numbers of ILC2s and a better degree of cell purity.

1. Dilute cell suspension (from Step B4) in order to get 0.3×10^6 cells/ml.
2. Distribute 3 ml of ILC2 cell suspension/well in a 6-well plate (Day 7).
3. The day after (Day 8), in the morning, add another 3 ml ILC2 medium to each well. In the

evening, pipet the cells up and down, and split them into 3 wells and add again 3 ml medium per well (total volume per well = 5 ml).

4. At Day 11, collect cells by pipetting into 15 ml Falcon® tube, centrifuge at 400 \times g for 5 min.
5. Count cells with a hemocytometer. ILC2s are ready to be used for *ex vivo* or *in vivo* experiments. They can also be maintained in culture for up to 10 days at 0.4 \times 10⁶ cells/ml.

Note: Take 100 μ l of ILC2 cell suspension for subsequent FACS analysis (see Figure 4).

6. Proceed to FACS analysis as described above (Step B5). We generally obtain an ILC2 population (13.6 \times 10⁶ cells per mouse) with > 95% of cells expressing typical ILC2s markers (Lin⁻CD45⁺ CD25⁺ CD90.2⁺ CD127⁺ Sca1⁺ ST2⁺).

Note: See an example in Figure 4—Cell surface markers analysis after ILC2 isolation and culture.

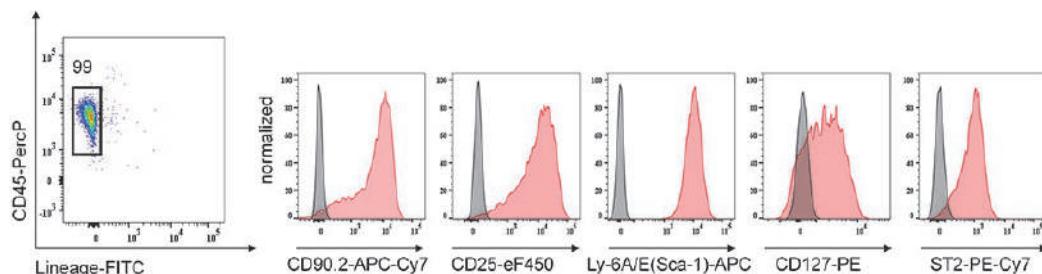


Figure 4. Analysis of ILC2 cell surface markers by flow cytometry after 4 days in culture (Day 11). Representative histograms of CD90.2, CD25, Sca-1, CD127 and ST2 markers at the surface of purified ILC2s. Number indicates % among live cells. Isotype controls are shown (grey histograms).

Data analysis

Samples are acquired on a LSR II flow cytometer using FACSDiVa™ software, before (see Figure 3) and after (see Figure 4) ILC2 cell culture. Flow cytometry data are analyzed using FlowJo to gate, quantify, and analyze ILC2 population.

Recipes

1. Lung digestion solution

Freshly prepare 2 ml/sample of DMEM containing 2 mg/ml collagenase IV and 0.1 mg/ml DNase I

2. PEF buffer

DPBS containing 2% filtered FBS and 1 mM EDTA

Keep sterile and cold during the experiment and store at 4 °C

3. PEB buffer

DPBS containing 0.5% BSA and 2 mM EDTA

- Keep sterile and cold during the experiment and store at 4 °C
4. ILC2 culture medium
RPMI-1640 medium supplemented with 10% filtered FBS, 1% penicillin/streptomycin, 50 µM 2-mercaptoethanol and 20 ng/ml recombinant mouse IL-2
Prepare freshly, keep sterile and store at 4 °C during the time of the experiment only
 5. FACS buffer
DPBS containing 5% filtered FBS and 5 mM EDTA
Store at 4 °C
 6. FACS staining buffer
FACS buffer containing 5% mouse serum and 1/200 anti-mouse CD16/CD32 (Fc block)
Store at 4 °C

Acknowledgments

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Competing interests

The authors declare no conflict of interest.

Ethics

All mice experiments were handled according to institutional guidelines under protocols approved by the French Ministry of Research and the FRBT (C2EA-01) animal care committee (projects 00663.02 and APAFIS#3873-2016020116301837v3).

References

1. Cayrol, C., Duval, A., Schmitt, P., Roga, S., Camus, M., Stella, A., Burlet-Schiltz, O., Gonzalez-de-Peredo, A. and Girard, J. P. (2018). [Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33](#). *Nat Immunol* 19(4): 375-385.
2. Cayrol, C. and Girard, J. P. (2018). [Interleukin-33 \(IL-33\): A nuclear cytokine from the IL-1 family](#). *Immunol Rev* 281(1): 154-168.
3. Lefrançais, E., Duval, A., Mirey, E., Roga, S., Espinosa, E., Cayrol, C. and Girard, J. P. (2014).

- [Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells.](#) *Proc Natl Acad Sci U S A* 111(43): 15502-15507.
4. Moro, K., Ealey, K. N., Kabata, H. and Koyasu, S. (2015). [Isolation and analysis of group 2 innate lymphoid cells in mice.](#) *Nat Protoc* 10(5): 792-806.

H1N1 Virus Production and Infection

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[Abstract] Influenza A virus is a member of orthomyxoviridae family causing wide-spread infections in human respiratory tract. Mouse infection model is widely used in antiviral research and pathogenesis study against influenza A virus. Here, we report a protocol in infected mice with different virus doses and strains to explore how an inhibitor of lysine-specific demethylase (LSD1) impacts disease progression.

Keywords: Influenza A virus, Infected mouse model, H1N1, Virus production, Plaque assay

[Background] Influenza A virus, a member of the family Orthomyxoviridae, is a negative-sense RNA virus with eight segmented genomes of single-stranded viral RNAs (vRNAs) that encode more than 10 proteins. During the past 100 years, outbreaks of influenza-virus strains regularly appeared in human populations, including “Spanish flu” in 1918 caused by the H1N1 subtype, “Asian flu” in 1957 by H2N2, “Hong Kong flu” in 1968 by H3N2, “Russian flu” in 1977 by H1N1, and “swine flu” in 2009 by H1N1 (Smith *et al.*, 2009; Lim and Mahmood, 2011; Kumar *et al.*, 2018). Seasonal influenza A viruses also circulate worldwide, spread easily from person to person, and result in the hospitalization of three to five million individuals worldwide annually (Molinari *et al.*, 2007). The seasonal influenza infections are responsible for 290,000-650,000 deaths annually, mainly among young children, elderly adults, and critically ill patients (Kumar *et al.*, 2018).

Animal models are used in influenza virus research not only to elucidate the viral and host factors that affect disease outcomes and spread among susceptible hosts but also to evaluate interventions designed to prevent or reduce influenza morbidity and mortality (Thangavel and Bouvier, 2014). In this paper, we use two strains of influenza A virus, A/WSN/33(H1N1) (WSN) which is a commonly-used lab strain, and A/Sichuan/01/2009 (SC09) which is a natural isolate to infect mice. This experiment aims to explore how Trans-2-phenylcyclopropylamine hydrochloride (TCP) (a chemical inhibitor against LSD1 [Shan *et al.*, 2017]) impacts disease progress. Moreover, we applied different doses of virus to infect the mice for different purposes to reveal the function of TCP.

Materials and Reagents

1. 1.5 ml Eppendorf tubes (Eppendorf)
2. 10 µl, 200 µl, and 1 ml pipette tips (FUKAEKASEI and WATSON, catalog number: 1201-705C)
3. 24-well plates (Corning, catalog number: 3526)
4. 6-well plates(Corning, catalog number: 353046)
5. 6 cm dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 150288)
6. Syringes
7. 75 cm flask (T75 flask) (Corning, catalog number: 430641U)
8. 0.22 µm filters (Axiva Sichem Biotech, catalog number: SFPV13 R)
9. Pencil
10. BALB/c mice (6-8W, female)
11. 293T cells (ATCC, catalog number: CRL-3216)
12. MDCK cells (ATCC, catalog number: CCL-34)
13. A/WSN/33(H1N1) (WSN) virus (Hoffmann *et al.*, 2000)
14. A/Sichuan/1/2009 (H1N1) (SC09) (Kindly provided by Prof. Yuelong Shu in China CDC)
15. TCP (Santa Cruz Biotechnology, catalog number: sc-208452)
16. 1x PBS (Lonza, catalog number: 17-516Q)
17. Isoflurane (Abbott, catalog number: 5260-04-05)
18. Picric acid (Fisher Scientific, catalog number: 13205) (Used for labeling the mice)
19. DMEM (Thermo Fisher Scientific, Gibco™, catalog number: 11965092)
20. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148)
21. True Blue substrate (KPL, catalog number: 50-78-02)
22. anti-nucleoprotein antibody (Antibody Research Centre, Shanghai Institute of Biological Science)
23. Anti-rabbit IgG secondary antibody (Antibody Research Centre, Shanghai Institute of Biological Science)
24. Avicel (FMC BioPolymer, catalog number: CL 611)
25. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A3912)
26. 2x DMEM (Thermo Fisher Scientific, catalog number: 12800017)
27. Trypsin (TPCK) (Sigma-Aldrich, catalog number: 4370285)
28. Fetal bovine serum heat inactivated (FBS) (Sigma-Aldrich, catalog number: F9665)
29. Bacto™ agar (BD, Bacto™, catalog number: 214010)
30. 18 MΩ H₂O
31. Overlay medium (DMEM + 2% FBS + 0.9% Bacto™-Agar)
32. Infection medium (DMEM + 1 µg/ml TPCK)
33. Trans-2-phenylcyclopropylamine hydrochloride (TCP) (1 mg/ml) (see Recipes)
34. 2.4% Avicel (see Recipes)
35. 6% BSA (see Recipes)

36. Overlay medium (see Recipes)

Equipment

1. Pipettes (Thermo Fisher Scientific, catalog number: 1156-6963)
2. Dissection equipment (forceps, tweezers, scissors)
3. Anesthesia machine (Parkland Scientific, catalog number: V3000PK)
4. Eppendorf centrifuge
5. Class II biological safety hood (Thermo Fisher Scientific)
6. Incubator
7. Freezer (4 °C, -20 °C and -80 °C)
8. Ultraviolet (UV) light

Software

1. GraphPad Prism (<https://www.graphpad.com/>)
2. Statistical Product and Service Solutions (SPSS)
(<https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software>)

Procedure

A. Virus production

Generate WSN virus by reverse genetics as described previously in biosafety level 2 hood (Hoffmann *et al.*, 2000). A/Sichuan/1/2009 [H1N1] [SC09] virus was kindly provided by Prof. Yuelong Shu in China CDC.

1. Transfection and transduction
 - a. Transfect monolayer of 293T cells in a 6 cm dish with 1 µg each of eight plasmids of pHW2000-WSN-segment1-8 (kindly provided by Prof. Hans Klenk, Germany) by following the manufacturer's protocol.
 - b. Twenty-four hours post-transfection, change the medium to 4 ml DMEM with 2% FBS, and culture at 37 °C for another 48 h.
 - c. Transfer all the supernatant from the transfected 293T cells to the monolayer of MDCK cells in the 6 cm dish, fill with fresh DMEM with 2% FBS if the whole volume is less than 4 ml.
 - d. Culture the MDCK cells for 48-96 h till CPE can be observed. Collect the MDCK supernatant and make a stock.
2. Plaque purification
 - a. Dilute the stock supernatant in serial 10-times dilution in PBS and apply 300 µl of each dilution onto the indicated wells of monolayer of MDCK cells plated in 6-well plate for 0.5 h.

- b. After this, discard the residual medium and wash the cells in PBS for 2 times. Then add 2 ml of overlay medium (DMEM + 2% FBS + 0.9% BactoTM-Agar) on the cells and culture the cells for 2-3 days to observe plaques.
- c. Encircle the plaque from beneath with a pencil and pick one plaque from the top with a 1 ml-pipette-tip, and then resuspend the plaque in 1 ml PBS at 4 °C for 16 h. This 1 ml PBS containing viruses will be used as the first-round plaque to repeat plaque purification for a second time.
- d. Sequence the resulted virus and multiply in MDCK cells in a 75 cm flask to make a virus stock.

B. Virus amplification and virus titer determinations**1. Virus amplification**

- a. Seed monolayers of MDCK cells in a T75 flask, after 12 h cells should be 80% confluent. Then infect cells with virus at MOI = 0.001 in 15 ml infection medium (DMEM + 1 µg/ml TPCK).
- b. Collect virus supernatant when cells are almost dead (about 3 days). Centrifuge at 1,000 x g for 10 min, sub-pack the supernatant and keep them in -80 °C.

2. Determine viral titer by plaque assay (Matrosovich *et al.*, 2016)

- a. Seed monolayers of MDCK cells in 24-well plates, after 12 h cells should be 100% confluent.
- b. Then infect cells with 200 µl virus supernatants which are in ten-fold serial dilution by DMEM.
- c. After a 1 h incubation at 37 °C in 5% CO₂, remove the medium and wash once with 1x PBS.
- d. Then add 2 ml of overlay medium to cover the MDCK cells and incubate for 2 days, subsequently fix the cells in 4% paraformaldehyde and exposure to ultraviolet (UV) light for 30 min.
- e. Perform immunostaining using an anti-nucleoprotein polyclonal primary antibody for 1 h at room temperature and an HRP-conjugated anti-rabbit IgG secondary antibody for 2 h at room temperature.
- f. Finally, add 100 µl True Blue substrate to visualize the plaques.

C. The measurement of median lethal dose

1. Transfer the mice (BALB/c mice aged 6-8 weeks) to ABSL2 for adaptive feeding for 3-7 days. Divide the mice (BALB/c mice aged 6-8 weeks) randomly into four groups to infect different concentrations of influenza A virus, 4 mice for each group.
2. Dilute virus in 50 µl PBS at different concentrations (4×10^4 pfu/mouse, 4×10^3 pfu/mouse, 4×10^2 pfu/mouse, 4×10^1 pfu/mouse) for WSN and (9×10^5 pfu/mouse, 1.3×10^5 pfu/mouse, 1.3×10^4 pfu/mouse, 1.3×10^3 pfu/mouse) for SC09 (H1N1) strains.

3. Anesthetize the mice with isoflurane and infect the mice intranasally by 50 μ l droplets containing different concentrations of viruses diluted in PBS.
4. Monitor the weight and death of the mice throughout the infection time course from Day 0 to Day 14 (Figure 1).
5. Calculate the median lethal dose by Statistical Product and Service Solutions (SPSS)

To run the probit analysis in SPSS, we follow the steps as follow: Firstly, input a minimum of three columns into the Data Editor (Number of individuals per container that responded, Total of individuals per container, Concentrations). Secondly, after columns are set, go to analyze, and press regression, then press probit. Thirdly, set your number responded column as the “Response Frequency”, the total number per container as the “Total Observed”, and the concentrations as the “Covariates”. Don’t forget to select the log base 10 to transform your concentrations. Press “OK” and then get the LD50 results.

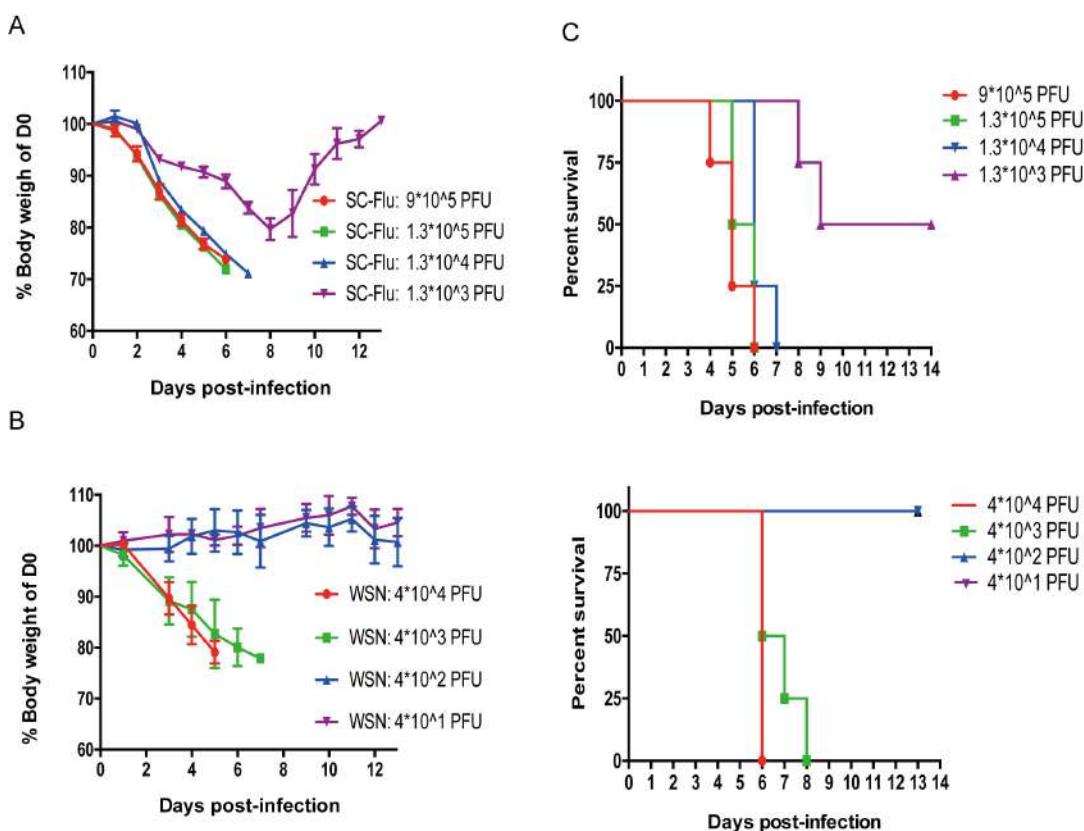


Figure 1. Exploration of median lethal dose of WSN and SC09. Mice were divided into four groups randomly and different doses of H1N1 were used to infect mice. The body weights of mice were monitored throughout the infection time course from Day 0 to Day 14 (A and C). The survival curve of mice is shown in B and D.

D. Mice infection with different doses of virus for different purposes

To explore the impact of TCP on mice body-weight loss and mortality, we infected mice with a lethal dose of viruses to see if treatment of TCP can rescue or worsen the disease. For this, we infected

the mice with a dose of 10,000 pfu of WSN (about 5LD50) virus to guarantee a lethal infection. (Figures 4A and 4B of Shan *et al.*, 2017)

1. Transfer the mice (BALB/c mice aged 6-8 weeks) to ABSL2 for adaptive feeding for 3-7 days.
2. Divide the mice (BALB/c mice aged 6-8 weeks) randomly into four groups: PBS-mock group, TCP-mock group, PBS-WSN treated group, and TCP-WSN treated group.
3. Dilute 10,000 pfu of WSN in 50 μ l PBS.
4. Anesthetize mice with isoflurane and infect the mice intranasally by 50 μ l droplets containing different concentrations of viruses diluted in PBS.
5. Inject 1 mg/ml TCP (100 μ l/mouse) and PBS (100 μ l/mouse) intraperitoneally once a day for 10 days.
6. Monitor the weight and death of the mice throughout the infection time course from Day 0 to Day 14 (Figures 4A and 4B of Shan *et al.*, 2017).

After we know that TCP accelerate the disease, we then decide to infect the mice with a sublethal dose of virus, 500 pfu of WSN virus (0.25LD50) (Figure 2) or 300 pfu of SC09 (0.25LD50), wherein the impact of TCP can be observed more clearly (Figures 4C and 4D of Shan *et al.*, 2017). For sublethal infection of WSN virus, all the groups of animals could survive till at least Day 9 post-infection (Figure 2), so that infected tissues can be collected for histological analyses (Figure 5 of Shan *et al.*, 2017). The procedure is almost as above except that the mice are sacrificed at Day 9 for further analysis.

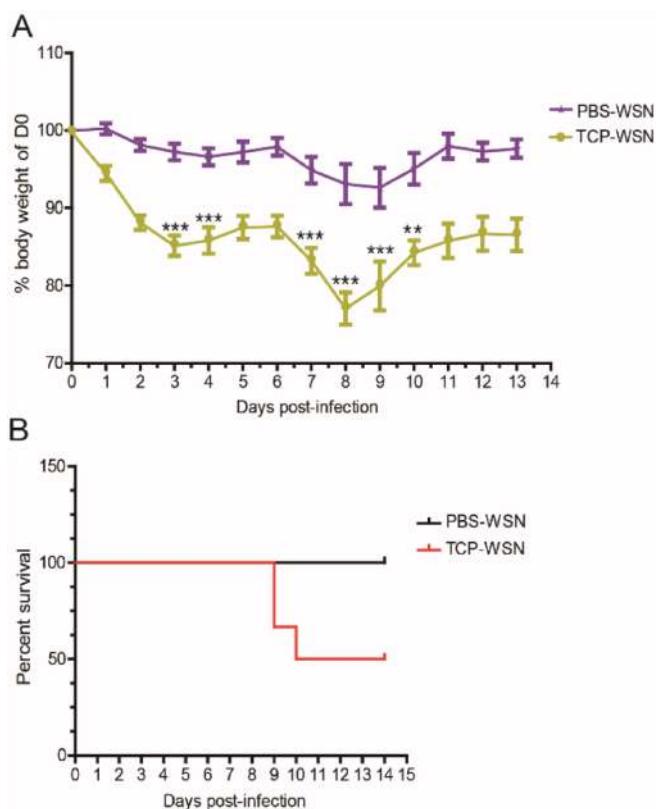


Figure 2. Low doses of viruses were used to guarantee mice of all groups could survive

till at least Day 9 post-infection. The body weights of mice were monitored throughout the infection time course from Day 0 to Day 14 (A). The survival curve of mice is shown in B.

Data analysis

The median lethal dose is calculated with Bliss algorithm using Statistical Product and Service Solutions (SPSS). Survival curve and weight change curve are analyzed using GraphPad Prism. Data are analyzed by Student's *t*-test. *P* value of < 0.05 is considered to be statistically significant difference. *ns*, *P* > 0.05; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Statistical analyses are done using GraphPad Prism.

Recipes

1. 1 mg/ml Trans-2-phenylcyclopropylamine hydrochloride TCP
Dissolve 50 mg of TCP in 50 ml PBS
Once reconstituted, the solution should be stored at 4 °C, protected from light
2. 2.4% Avicel
Dissolve 1.2 g of Avicel in 50 ml 18 MΩ H₂O
Once reconstituted, the solution is autoclaved and then stored at 4 °C
3. 6% BSA
Dissolve 0.6 g of BSA in 100 ml PBS
Once reconstituted, the solution is filtered with 0.22 μm filters and then stored at -20 °C
4. Overlay medium
1x DMEM
1.2% (w/v) Avicel
0.3% (w/v) BSA

Acknowledgments

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Competing interests

The authors have declared that no competing interests exist.

Ethics

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Institut Pasteur of Shanghai, Chinese Academy of Sciences (Animal protocol #A2015006). All animal care and use protocols were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

References

1. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. and Webster, R. G. (2000). [A DNA transfection system for generation of influenza A virus from eight plasmids](#). *Proc Natl Acad Sci U S A* 97(11): 6108-6113.
2. Kumar, A., Meldgaard, T. S. and Bertholet, S. (2018). [Novel Platforms for the Development of a Universal Influenza Vaccine](#). *Front Immunol* 9: 600.
3. Lim, B. H. and Mahmood, T. A. (2011). [Influenza A H1N1 2009 \(Swine Flu\) and Pregnancy](#). *J Obstet Gynaecol India* 61(4): 386-393.
4. Matrosovich, M., Matrosovich, T., Garten, W. and Klenk, H. D. (2006). [New low-viscosity overlay medium for viral plaque assays](#). *Virology J* 3: 63.
5. Molinari, N. A., Ortega-Sanchez, I. R., Messonnier, M. L., Thompson, W. W., Wortley, P. M., Weintraub, E. and Bridges, C. B. (2007). [The annual impact of seasonal influenza in the US: measuring disease burden and costs](#). *Vaccine* 25(27): 5086-5096.
6. Shan, J., Zhao, B., Shan, Z., Nie, J., Deng, R., Xiong, R., Tsun, A., Pan, W., Zhao, H., Chen, L., Jin, Y., Qian, Z., Lui, K., Liang, R., Li, D., Sun, B., Lavillette, D., Xu, K. and Li, B. (2017). [Histone demethylase LSD1 restricts influenza A virus infection by erasing IFITM3-K88 monomethylation](#). *PLoS Pathog* 13(12): e1006773.
7. Smith, G. J., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghwan, J., Bhatt, S., Peiris, J. S., Guan, Y. and Rambaut, A. (2009). [Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic](#). *Nature* 459(7250): 1122-1125.
8. Thangavel, R. R. and Bouvier, N. M. (2014). [Animal models for influenza virus pathogenesis, transmission, and immunology](#). *J Immunol Methods* 410: 60-79.

Induction and Analysis of Anti-CD40-induced Colitis in Mice

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[Abstract] Colon inflammation or colitis affects more than 1 million people worldwide. Several pre-clinical models, including chemical-induced (*i.e.*, DSS, TNBS) or pathogen-induced (*i.e.*, *Citrobacter rodentium*) have been used to study mechanisms involved in the development and regulation of colitis. Anti-CD40 induced colitis model has gained acceptance to study the roles of innate immune cells during acute intestinal inflammation. Here we describe a rapid, robust and reproducible protocol to induce and analyze anti-CD40 mediated colitis in mice.

Keywords: Anti-CD40, Colitis, IBD, Innate immunity, Inflammation, IL-23, IL-22

[Background] Inflammatory Bowel Disease (IBD), including Crohn's disease and Ulcerative colitis, affects about 1.5 million people in the United States (Ng *et al.*, 2017). To better understand the mechanisms involved in the development and progression of IBD, a number of pre-clinical models (*i.e.*, DSS, TNBS, anti-CD40, etc.) have been developed to address various aspects of immune response during tissue injury over the last two decades. CD40 is highly expressed by the colon lamina propria antigen presenting cells. We have demonstrated that activation of CD40 signaling using an agonist anti-CD40 antibody can trigger colitis in T and B cells deficient mice (here referred as *Rag*^{-/-} mice) driven by excessive production of IL-23, IL-1 β and IL-12 by myeloid cells (Uhlig *et al.*, 2006). Anti-CD40 model is a unique model of colitis driven by IL-23-producing gut resident CX3CR1⁺ macrophages and IL-22-producing group 3 innate lymphoid cells (ILC3) (Bauche *et al.*, 2018). This model of colitis is restricted to the proximal colon and is a potent model to study the role of innate immunity in colon inflammation. Here, we describe a robust and reproducible method to induce and analyze anti-CD40-induced colitis in mice. Anti-CD40-treated *Rag*^{2 --} mice lose up to 20% of their initial weight within three days post injection, and then return to their initial weight by Day 7 post induction (Figure 1A). Elevated levels of pro-inflammatory cytokines can be detected in the proximal colon as soon as Day 1 post induction (Cayatte *et al.*, 2012) (Figure 1B) but maximal disease—characterized by massive infiltration of innate immune cells, loss of goblet cells and development of mitotic figures—is observed in the proximal colon at day 7 post disease induction (Figure 1C). Immune cell infiltration in the proximal colon and cytokine production, such as IL-22, by innate lymphoid cells can be measured by flow cytometry (Figure 1D).

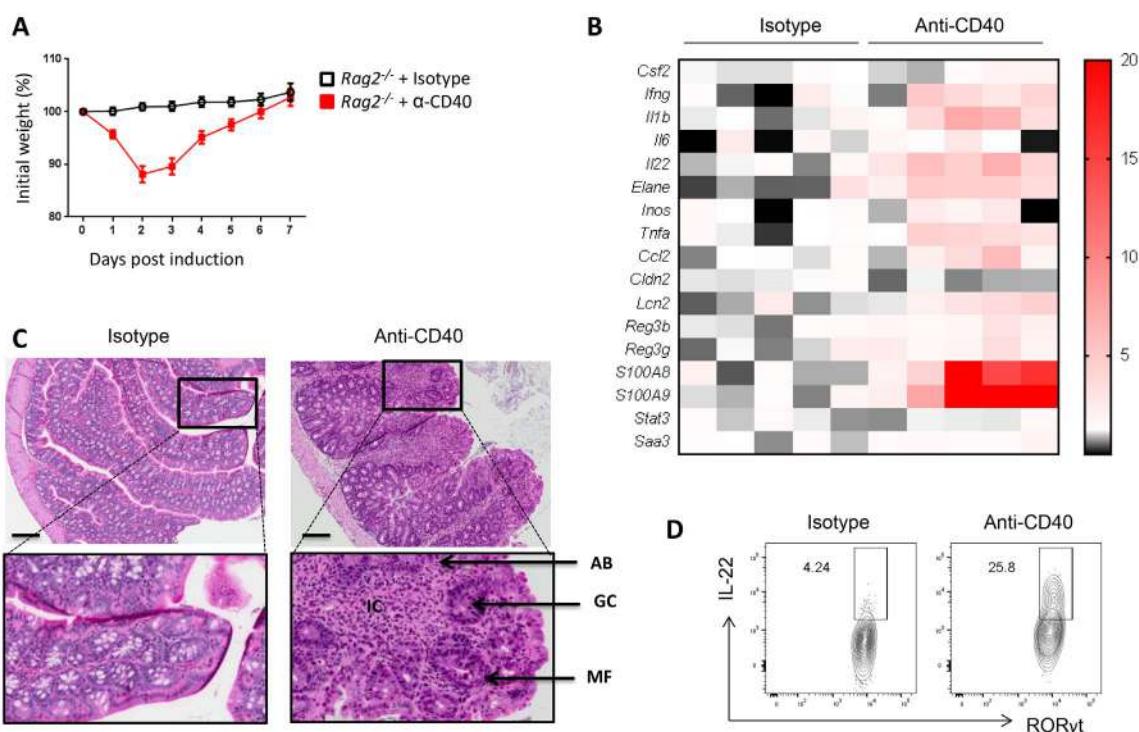


Figure 1. Induction and analysis of the anti-CD40 colitis mouse model. A. Percentage of initial weight over a 7 days period. Initial weight is measured right before injection of isotype or anti-CD40 antibodies. B. Gene expression profile of the proximal colon at Day 7 post treatment. Data shows relative fold change over the isotype control. C. Representative photomicrographs of H&E stained colon section 7 days after injection of isotype (left) or anti-CD40 antibodies (right). IC: Immune cell infiltration; GC: Loss of Goblet cells; AB: Apoptotic body; MF: Mitotic figures. Scale bars = 250 µm. D. Representative dot plot of IL-22 production by proximal colon lamina propria ILC3 (gated on lineage-, CD90^{high}, CD45^{int}, RORyt⁺ cells) at Day 2 post treatment.

Materials and Reagents

A. Materials

1. Pipette tips (Thermo Scientific)
2. Eppendorf tubes, 1.5 ml (Eppendorf, catalog number: 05-402-5)
3. 1 ml sterile sub-Q syringe 26 G (BD, catalog number: 3095971)
4. Sterile cell strainer 70 µm Nylon mesh (Fisher Scientific, catalog number: 22363548)
5. 50 ml centrifuge tube (Fisher Scientific, catalog number: 05-539-13)
6. 15 ml conical centrifuge tube (Fisher Scientific, catalog number: 339650)
7. 15 ml sterile plastic tube (Fisher Scientific, catalog number: 14-956-1D)
8. 5 ml Serological pipettes (Falcon, catalog number: 357543)
9. 25 ml Serological pipettes (Fisher Brand, catalog number: 13-678-11)
10. Petri dishes (Falcon, catalog number: 351029)
11. Serum or plasma separation tubes (SARSTEDT, catalog number: 41.1378.005)

12. Parafilm (Pechiney, catalog number: PM-996)
13. V bottom 96-well plate (Costar, catalog number: 3894)
14. 70 µm cell strainer

B. Animals

Eight to twelve weeks old *Rag2^{-/-}* mice (Taconic, catalog number: RAGN12) were used to study anti-CD40 mediated colitis.

C. Reagents

1. Liquid nitrogen
2. Anti-mouse CD45 APC-eFluor780 antibody (eBioscience, catalog number: 47-0451-82)
3. Anti-mouse CD90.2 BV786 antibody (BD Bioscience, catalog number: 564365)
4. Anti-mouse RORyt PerCP-eFluor710 antibody (eBioscience, catalog number: 46-6981-82)
5. Anti-mouse NK1.1 Alexa Fluor 700 antibody (BD Bioscience, catalog number: 560515)
6. Anti-mouse CD11c PE-Cy7 antibody (BD Bioscience, catalog number: 561022)
7. Anti-mouse CD11b FITC antibody (BD Bioscience, catalog number: 557396)
8. Anti-mouse IL-22 APC antibody (eBioscience, catalog number: 17-7222-82)
9. Endotoxin-free Anti-CD40 antibody (Clone FKG45, BioXCell, catalog number: BE0016-2)
10. Anti-Rat IgG2a isotype control (Clone 2A3, BioXCell, catalog number: BP0089)
11. HyClone Phosphate Buffered Saline (DPBS), 1x (GE Healthcare, catalog number: SH30028.02)
12. Hank's buffered salt solution (HBSS)
13. 10% Neutral Buffered Formalin (Thermo Fisher Scientific, catalog number: 245-685)
14. Penicillin/streptomycin solution 100x (Corning, catalog number: 30-002C1)
15. Sodium pyruvate 100 mM (Gibco, catalog number: 11360070)
16. 2-Mercaptoethanol 55 mM (Gibco, catalog number: 21985023)
17. Ultra Pure 0.5 µM EDTA pH 8.0 (Gibco, catalog number: 15575-038)
18. HEPES buffer solution 1 M (Gibco, catalog number: 15630-080)
19. Hyclone Standard Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, catalog number: SH30088.03)
20. Collagenase type VIII (Sigma-Aldrich, catalog number: C2139)
21. DNase I (Sigma-Aldrich, catalog number: D5025)
22. Dispase (Corning, catalog number: 354235)
23. Percoll (GE Healthcare, catalog number: 17-0891-01)
24. IMDM, GlutaMAX supplement (Thermo Fisher Scientific, catalog number: 31980097)
25. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, catalog number: P8139)
26. Ionomycin (Sigma-Aldrich, catalog number: I0634)
27. Brefeldin A (BD Bioscience, catalog number: 555029)
28. Stain Buffer (BD, Pharmingen, catalog number: 554656)

29. Transcription Factor Buffer Set (BD, Pharmingen, catalog number: 562725)
30. Fixable viability stain 510 (BD, Horizon, catalog number: 564406)
31. Mouse BD Fc Block (BD, Pharmingen, catalog number 553141)
32. Ethanol 70% (Fisher Scientific, catalog number: HC1500)
33. Ethanol 95% (Fisher Scientific, catalog number: HC1300)
34. Ethanol 100% (Fisher Scientific, catalog number: HC600)
35. Xylene (Fisher Scientific, catalog number: HC700)
36. Paraffin (Fisher Scientific, catalog number: 23-0210-400)
37. Hematoxylin (MasterTech Scientific, catalog number: HXHHEGAL)
38. Eosin (Thermo Scientific, catalog number: 71311)
39. Collagenase D (Sigma, catalog number: C5138-1G)
40. Epithelial cell dissociation solution (see Recipes)
41. Enzyme digestion mix (see Recipes)
42. Complete IMDM media (see Recipes)

Equipment

1. Scalpel (Southmedic, catalog number: SMI1/73-0121)
2. Pipettes (Thermo Scientific)
3. Forceps and Scissors (Fine Science Tools)
4. Sterile cell culture hood
5. Centrifuge (Thermo Fisher Scientific)
6. Shaking water bath (Precision)
7. Brightfield Microscope
8. LSRII Flow cytometer (BD Biosciences)
9. Incubator
10. Vortex (Scientific Industries)

Software

1. FlowJo_V10 (FlowJo, LLC, <https://www.flowjo.com>)
2. GraphPad Prism 7 (GraphPad, <https://www.graphpad.com>)
3. Microsoft Excel (Microsoft)

Procedure

A. Induction of colitis in *Rag2^{-/-}* mice

1. Dilute the anti-CD40 antibody or isotype control to 1 mg/ml in sterile 1x PBS.
2. Inject 100 µg of anti-CD40 or isotype control antibody intraperitoneal (i.p.) or 50 µg intravenous injection (i.v.)—based on researcher’s technical expertise—with a 1 ml sterile sub-Q syringe 26 G. Each batch of anti-CD40 antibody should be verified to be endotoxin-free (by checking with the manufacturer) to avoid systemic immune activation.

Notes:

- a. Every animal facility will have a specific microbial environment, so it is crucial to determine the dose of anti-CD40 empirically by performing an initial dose-titration (25-100 µg) (Figure 2).
 - b. A dose that is too high will result in death of the animals while an insufficient dose will lead to weak disease induction (characterized by inconsistent or poor colon inflammation) and variability in colitis development.
 - c. Mice are weighed every day for seven consecutive days and % weight loss is calculated in comparison to the initial weight on Day 0. Mice that lose more than 20% of initial body weight or demonstrate shaking or severe systemic responses are euthanized within 24 h.
 - d. Weight loss kinetics of colitis may vary between i.p. and i.v. injection type. In our hands, mice recover weight more quickly after i.p. delivery. However, the route of injection does not alter the timing or severity of colitis development.
3. At Days 2-7 post induction, euthanize mice by carbon dioxide asphyxiation according to the approved ethical protocol.
 4. Collect serum via cardiac puncture for the detection of systemic cytokines levels.

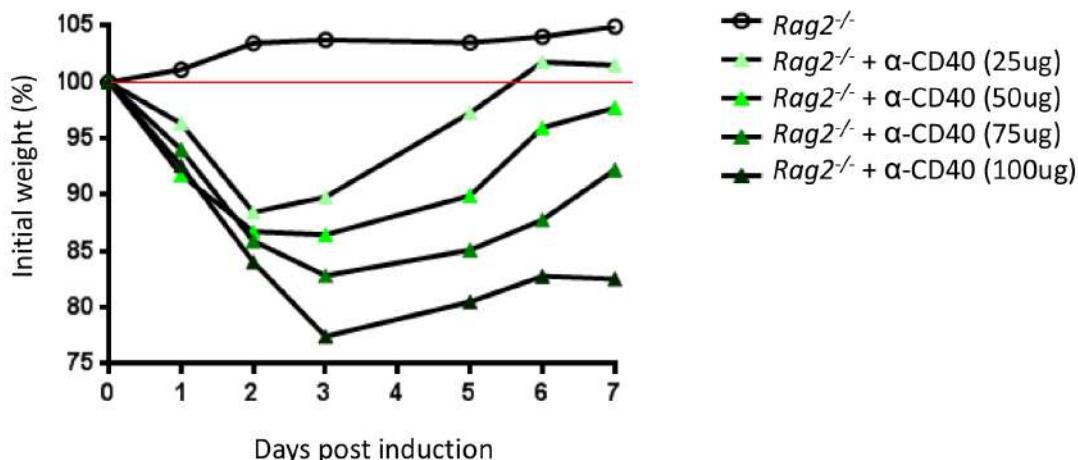


Figure 2. Titration of the anti-CD40 antibody. *Rag2^{-/-}* mice received a single dose of anti-CD40 antibody i.v. as indicated. Percentage of initial weight over a 7 days period. Initial weight is measured right before injection of anti-CD40 antibody.

B. Isolation and preparation of colon tissue/cells for endpoint analysis

1. Histopathological analysis by H&E staining
 - a. Harvest colons and remove fecal content by gently dislodging from colon with forceps. Roll colon into a tissue embedding cassette with proximal end in the center and fix in 10% neutral buffered formalin overnight (the cassette needs to be submerged).
 - b. Transfer the cassettes to 70% ethanol, embed in paraffin, section at 4-5 µm and then stain with hematoxylin for 10 min and eosin as previously described (Wang *et al.*, 2017).
 - c. Wash with running water until the water is clear.
 - d. Transfer the slides to the Eosin solution and incubate for 3 min.
 - e. Successively transfer the slides into 70% ethanol for 20 s, 90% ethanol for 20 s, 100% ethanol for 1 min and xylene for 3 min.
 - f. Take out slides from xylene and dry them out in a fume hood.
 - g. Mount and cover the slides.
 2. Gene expression profiling of the proximal colon
 - a. At the desired time point after induction of colitis, mice are euthanized by carbon dioxide asphyxiation according to approved ethical protocol.

Note: For analysis of genes encoding cytokines produced by the innate immune cells, we recommend euthanizing mice on Days 1 to 3 post induction. For analysis of genes expressed by epithelial cells in response to inflammation, we recommend euthanizing the mice on Day 7 post induction when the disease is maximal.
 - b. Harvest a piece of proximal colon (about 0.5 cm long), remove the fecal content by gently dislodging it from colon with forceps, transfer into a sterile polypropylene tube and snap freeze in liquid nitrogen (Figure 3B). Since inflammatory infiltrate can be variable, it is important to cut a vertical section of the colon starting from the base of the cecum and descending into the proximal colon to ensure consistent molecular signature.

Note: To avoid RNA degradation, samples must be snap frozen in liquid nitrogen within 2 min post euthanasia. Frozen proximal colons can be stored at -80 °C for at least 6 months.
 - c. Extract RNA and run RT-PCR protocols as described in Bauche *et al.*, 2018.
 3. Isolation of proximal colon lamina propria cells, ex-vivo stimulation and flow cytometry
- Isolation of proximal colon lamina propria cells***
- a. At 2-7 days post induction, mice are killed by carbon dioxide asphyxiation according to approved ethical protocol.
 - b. Harvest and collect the proximal colon in a 15 ml Falcon tube filled with 5 ml of 1x HBSS.
 - c. Remove the fat (Figure 3A).
 - d. Transfer the colon into a Petri dish.
 - e. Cut colon open lengthwise and wash by submerging and gently shaking in 50 ml of 1x HBSS until the tissue is clear of fecal matter.
 - f. Cut into 0.5 cm segments.

- g. Place into a 50 ml Falcon tube with 20 ml of epithelial cell dissociation solution.
- h. Wrap in parafilm, place sideways in a water bath at 37 °C for 20 min with medium shaking.
- i. Vortex well for 15 s.
- j. Filter through a 70 µm strainer, collect and transfer the undigested piece of colon tissues in a new 50 ml Falcon tube.
- k. Rinse fragments with 20 ml 1x HBSS by gently vortexing for 10 s.
- l. Transfer colon fragments into a petri dish and chop into tiny pieces with a scalpel.
- m. Using a 25 ml Serological pipettes, transfer into a new 50 ml tube with 10 ml of enzyme digestion mix then incubate for 20 min in a water bath at 37 °C with shaking as above.
- n. Vortex for 15 s.
- o. Collect sup by filtering through a 70 µm cell strainer and put on ice. Pellet cells for 5 min at 375 x g, 4 °C.
- p. Prepare 100% Percoll solution (9 parts Percoll + 1 part 10x PBS).
- q. Prepare 40% and 80% Percoll solutions by diluting 100% Percoll with 1x PBS.
- r. In a 15 ml tube resuspend the pellet in 5 ml of 40% Percoll, using a 5 ml Serological pipettes, and underlay with 5 ml of 80% Percoll for each colon.
- s. Spin 670 x g for 20 min at 20 °C with no acceleration and no brake.
- t. Collect interface (white ring) between and the two layers of percoll into a 15 ml tube, wash with 10 ml of complete IMDM media and spin for 5 min at 375 x g, 4 °C.
- u. Resuspend in 1 ml of complete IMDM and cells are ready to go.

Note: The interface is characterized by a white ring and contained mainly colon lamina propria immune cells. Cell number and viability should be measured at this step.

Ex-vivo stimulation

- a. For cytokines analysis, stimulate 2×10^6 viable cells/well in a 96-well plate for 4 h at 37 °C, 5% CO₂ with 50 ng/ml PMA, 500 ng/ml Ionomycin and Brefeldin A (1/1,000) in 200 µl of complete IMDM.
- b. Spin for 5 min at 375 x g, 4 °C and proceed to flow cytometry staining.

Flow cytometry staining

- a. Incubate cells in 100 µl of 1x PBS with 0.3 µl of live/dead fixable dead cell stains and 5 µl of mouse BD Fc block per well for 25 min at 4 °C in the dark.
- b. Wash with 100 µl of 1x PBS per well and spin for 5 min at 375 x g.

For surface staining:

- a. Incubate cell suspension with 0.2 µg of anti-mouse conjugated antibodies (*i.e.*, CD45, CD3, CD4) in 100 µl of BD stain buffer per well for 25 min at 4 °C in the dark.
- b. Wash with 100 µl of BD stain buffer and spin for 5 min at 375 x g.

For intracellular staining:

- a. Resuspend cell suspension in 100 µl of 1x Fix/perm buffer (BD) and incubate for 45 min at

- 4 °C in the dark.
- b. Wash with 1x Perm/Wash buffer and spin for 5 min at 375 x g.
 - c. Incubate cell suspension with 0.2 µg of anti-mouse conjugated antibodies (*i.e.*, ROR γ t, IL-22) in 100 µl of 1x Perm/Wash buffer per well for 45 min at 4 °C in the dark.
 - d. Wash with 1x Perm/Wash buffer and spin for 5 min at 375 x g.
 - e. Resuspend cells in 300 µl of 1x Perm/Wash buffer. Analyze and acquire data using a flow cytometer (we used LSRII flow cytometer from BD and analyzed data on FlowJo). Samples can be stored overnight at 4 °C in the dark.

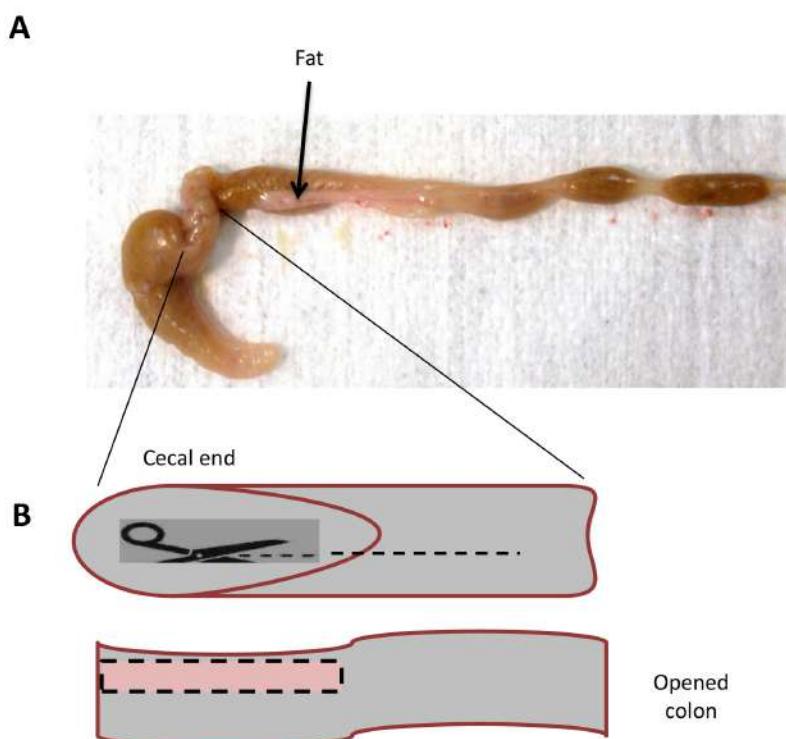


Figure 3. Preparation of colon tissue. A. Attached fat tissue (here shown by arrows) must be removed from the colon as fat can decrease the viability of isolated colon lamina propria. B. Taqman Sampling: The colon is separated from the cecum and carefully dissected from host by removing all the fat and connective tissue. The colon is cut from the proximal (cecal) end, down the midline and until the distal end. Flatten out the tissue, cut a 2-3 cm strip (pink square) and immediately snap freeze using polypropylene tubes in liquid nitrogen.

Data analysis

1. Tissues are scored for severity of disease by a pathologist according to three criteria (each criterion is scored from 0 to 3, 0 = negative, 1 = mild, 2 = moderate and 3 = severe):
Inflammation: when present is characterized by infiltration of large numbers (60%-70%) of mononuclear cells (macrophages and lymphocytes) and 30%-40% of neutrophils and band

cells. The scoring of inflammation includes severity of infiltration, loss of glands, erosion, and dilatation of glandular lumina, presence of crypt abscess and degeneration of epithelial cells.

Apoptosis: The prevalence of apoptotic bodies is scored on a scale of 0-3: 0 = negative; 1 = low; 2 = moderate; 3 = high.

Regeneration: Regenerative changes assessed include scoring the prevalence of mitotic figures in the upper 1/3 of the mucosa, nuclear density (nuclear crowding) within individual glandular structures, regularity of the surface epithelium. Regeneration is scored on a scale of 0-3: 0 = negative; 1 = low; 2 = moderate; 3 = high.

2. For gene expression profiling, average the normalized values from isotypes

Calculate the fold change over the isotype control using the following formula:

$$\text{fold change} = \frac{\text{Normalized value of the sample}}{\text{Average normalized value of the isotype control group}}$$

3. For flow cytometry analysis, acquire at least 5×10^5 colon lamina propria cells
 - a. The events are gated on FSC-A and SSC-A, then gated on FSC-A and FSC-H to remove doublets.
 - b. Exclude cellular debris and dead cells (using LIVE/DEAD fixable stain).
 - c. To study the phenotype of infiltrating immune cells, gate on CD45⁺ cells.
 - d. For analysis of IL-22-producing ILC3, gate on live cells, lineage (CD11c, CD11b, NK1.1)⁻, CD90.2^{high}, CD45^{int}, ROR γ T⁺, IL-22⁺ cells. For further details, gating strategy can be found in previous publication (Bauche *et al.*, 2018).
4. Statistical analysis was performed using Prism 7. Unpaired *t*-test is used as statistical test. A *P* value < 0.05 is considered statistically significant.

Recipes

1. Epithelial cell dissociation solution

1x HBSS

Penicillin/streptomycin (1,000 U/ml)

5 mM EDTA

10 mM HEPES

2. Enzyme digestion mix

1x HBSS

Penicillin/streptomycin (1,000 U/ml)

10% FBS

2 mg/ml collagenase type VIII

30 U/ml of DNase I

50 U/ml of Dispase

Alternately, 1 mg/ml of collagenase D (Sigma) can be used to preserve cell surface molecules when analyzing by FACS

3. Complete IMDM media, 500 ml
 - 450 ml of IMDM, GlutaMAX supplement
 - 1,000 U/ml of Penicillin/streptomycin
 - 1 mM of Sodium pyruvate
 - 0.55 mM of 2-Mercaptoethanol
 - 50 ml of Heat inactivated FBS

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Competing interests

The authors declare no competing financial interests.

Ethics

Mice were maintained under specific pathogen-free conditions and kept in microisolators with filtered water at Merck Research Laboratories (MRL) animal facility, Palo Alto. All animal procedures were approved by the Institutional Animal Care and Use Committee of MRL in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC).

References

1. Bauche, D., Joyce-Shaikh, B., Jain, R., Grein, J., Ku, K. S., Blumenschein, W. M., Ganal-Vonarburg, S. C., Wilson, D. C., McClanahan, T. K., Malefyt, R. W., Macpherson, A. J., Annamalai, L., Yearley, J. H. and Cua, D. J. (2018). [LAG3⁺ regulatory t cells restrain interleukin-23-producing cx3cr1⁺ gut-resident macrophages during group 3 innate lymphoid cell-driven colitis.](#) *Immunity* 49(2): 342-352: e345.
2. Cayatte, C., Joyce-Shaikh, B., Vega, F., Boniface, K., Grein, J., Murphy, E., Blumenschein, W. M., Chen, S., Malinao, M. C., Basham, B., Pierce, R. H., Bowman, E. P., McKenzie, B. S., Elson, C. O., Faubion, W. A., Malefyt Rde, W., Kastelein, R. A., Cua, D., McClanahan, T. K. and Beaumont, M. (2012). [Biomarkers of therapeutic response in the il-23 pathway in inflammatory bowel disease.](#) *Clin Transl Gastroenterol* 3: e10.

3. Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., Panaccione, R., Ghosh, S., Wu, J. C. Y., Chan, F. K. L., Sung, J. J. Y. and Kaplan, G. G. (2017). [Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies](#). *Lancet* 390(10114): 2769-2778.
4. Uhlig, H. H., McKenzie, B. S., Hue, S., Thompson, C., Joyce-Shaikh, B., Stepankova, R., Robinson, N., Buonocore, S., Tlaskalova-Hogenova, H., Cua, D. J. and Powrie, F. (2006). [Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology](#). *Immunity* 25(2): 309-318.
5. Wang, C., Yue, F. and Kuang, S. (2017). [Muscle histology characterization using H&E staining and muscle fiber type classification using immunofluorescence staining](#). *Bio-protocol* 7(10): e2279.

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Induced Germinal Center B Cell Culture System

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[Abstract] The germinal center (GC) is the site where B cells undergo clonal expansion, affinity-based selection, and differentiation into memory B cells or plasma cells. It has been difficult to elucidate regulatory mechanisms for the dynamic GC B cell maturation and differentiation, partly because experimental manipulation of GC B cells *in vivo* has been limited and no *in vitro* system has been available that resembles B cell reaction in GC. Here we describe the protocol for a culture system named “induced GC B (iGB) culture system” which can induce massive expansion of B cells that exhibit GC B cell-like phenotype, and thus it mimics the GC reaction. This protocol can be useful to elucidate the molecular mechanisms of GC B cell differentiation.

Keywords: iGB cell, 40LB cell, CD40L, BAFF, Germinal center, Memory B cell, B cell culture

[Background] In the field of T cell immunology, *in vitro* culture systems that allow differentiation of naive T cells toward specific effector T cell subsets have been widely used to study molecular mechanisms for the T-cell differentiation. However, there has been no practical culture system to effectively induce expansion of B cells with high viability and differentiation towards GC B cells. To study the GC B cell biology *in vitro*, we have developed a novel B-cell culture system that mimics GC B-cell reaction (Nojima *et al.*, 2011). For this system, we generated a feeder cell line named 40LB, a Balb/c 3T3 cell line expressing exogenous CD40-ligand (CD40L) and B-cell activating factor (BAFF) that together promote B cell proliferation and survival. When naive B cells are cultured with IL-4 for the first 4 days and then with IL-21 for the second 4 days on the feeder cells, B cells proliferate exponentially up to 10,000 fold, efficiently undergo isotype switching to IgG1 and IgE, the Th2 related isotypes, and express GC B-cell markers such as GL7 and Fas. Thus, we termed the B cells propagated in this system *in-vitro*-induced GC B (iGB) cells, and this system the iGB cell culture system. Compared to simply stimulating B cells with anti-CD40 antibody and BAFF with IL-4, which can induce only a 20-fold expansion of B cells in 6 days and less efficient isotype switching, iGB cell culture system is very useful. The iGB cell culture system have been widely used to study B cell biology, such as GC B cell expansion and differentiation, signaling from various receptors, immunoglobulin gene class-switch recombination, and so on (Fukao *et al.*, 2014; Haniuda *et al.*, 2016). The iGB cells not only phenotypically resemble genuine GC B cells but they functionally mimic some aspects of GC B cells: the iGB cells after the primary culture with IL-4 differentiate *in vivo* into memory-like B cells when transferred into mice, whereas those after the secondary culture with IL-21 differentiate *in vivo* into plasma cells that are relatively long-lived (Nojima *et al.*, 2011; Moutai *et al.*, 2014). Hence the iGB cell

culture system is also applicable for the studies of molecular mechanisms for their differentiation into memory B cells or long-lived plasma cells, and their responses (Takatsuka *et al.*, 2018). Here, we describe a detailed protocol for the iGB cell culture system.

Materials and Reagents

1. 50 ml tube (Thermo, catalog number: 339652)
2. 15 ml tube (Thermo, catalog number: 339650)
3. 5 ml round-bottom tube (Falcon, catalog number: 352054)
4. 10 cm dish (Falcon, catalog number: 353003)
5. 6-well plate (Costar, catalog number: 3516)
6. 12-well plate (Costar, catalog number: 3513)
7. 26 G needle (Terumo, catalog number: NN-2613S)
8. 10 ml syringe (Terumo, catalog number: SS-10SZ)
9. Cryovial (Thermo, catalog number: 375418)
10. Frosted glass slides (Matsunami, catalog number: S2226)
11. MACS LS Columns (Miltenyi Biotec, catalog number: 130-042-401)
12. 100 µm mesh (TGK, catalog number: N—No.200HD)
13. 40 µm mesh (TGK, catalog number: N—No.355T)
14. 40LB cells: BALB/c3T3 cells (provided by RIKEN BRC) stably expressing mouse CD40L and mouse BAFF (Nojima *et al.*, 2011)
15. D-MEM (high glucose, WAKO, catalog number: 044-29765)
16. Fetal bovine serum (FBS, see Notes)
17. Trypsin-EDTA (WAKO, catalog number: 204-16935)
18. Penicillin-streptomycin (Gibco, catalog number: 15140-122)
19. RPMI-1640 (WAKO, catalog number: 189-02025)
20. HEPES (Dojindo, catalog number: 346-08235)
21. 2-mercaptoethanol (2-ME, Gibco, catalog number: 21985-023)
22. NaCl (WAKO, catalog number: 195-01663)
23. KCl (WAKO, catalog number: 163-03545)
24. Na₂HPO₄ (WAKO, catalog number: 196-02835)
25. KH₂PO₄ (WAKO, catalog number: 169-04245)
26. EDTA (Dojindo, catalog number: 345-01865)
27. Biotinylated anti-CD43 (BD Pharmingen, clone: S7, catalog number: 553269)
28. Biotinylated anti-Ter119 (Biolegend, clone: TER-119, catalog number: 116204)
29. Biotinylated anti-CD4 (Biolegend, clone: GK1.5, catalog number: 100404)
30. Biotinylated anti-CD8 (Biolegend, clone: 53-6.7, catalog number: 100704)
31. Biotinylated anti-CD49b (Biolegend, clone: DX5, catalog number: 108904)
32. Biotinylated anti-H-2K^d (Biolegend, clone: SF1-1.1, catalog number: 116604)

33. Biotinylated anti-CD138 (Biolegend, clone: 281-2, catalog number: 142512)
34. Streptavidin Particles Plus-DM (BD, IMag, catalog number: 557812)
35. Recombinant mouse IL-4 (PeproTech, catalog number: 214-14)
36. Recombinant mouse IL-21 (PeproTech, catalog number: 210-21)
37. 3T3 medium (see Recipes)
38. B cell medium (BCM) (see Recipes)
39. Phosphate-buffered saline (PBS) (see Recipes)
40. MACS buffer (see Recipes)
41. Antibody cocktail for B cell isolation (for one spleen) (see Recipes)
42. Antibody cocktail for feeder cell depletion (for cells) (see Recipes)

Equipment

1. Pipettes
2. Autoclave
3. 37 °C water bath
4. CO₂ incubator (Panasonic, catalog number: MCO-170AIC-PJ)
5. Laminar flow hood (SANYO, Bio clean bench)
6. Mr. Frosty (Thermo, catalog number: 5100-0001)
7. -80 °C freezer
8. Liquid nitrogen storage tank
9. Cell separation Magnet (BD IMag, catalog number: 552311)
10. Centrifuge (Kubota, 4000, 5911, 6200)
11. MACS MultiStand (Miltenyi Biotec, catalog number: 130-042-303)
12. MidiMACS Separator (Miltenyi Biotec, catalog number: 130-042-302)

Procedure

A schematic overview of the experimental procedure is shown in Figure 1.

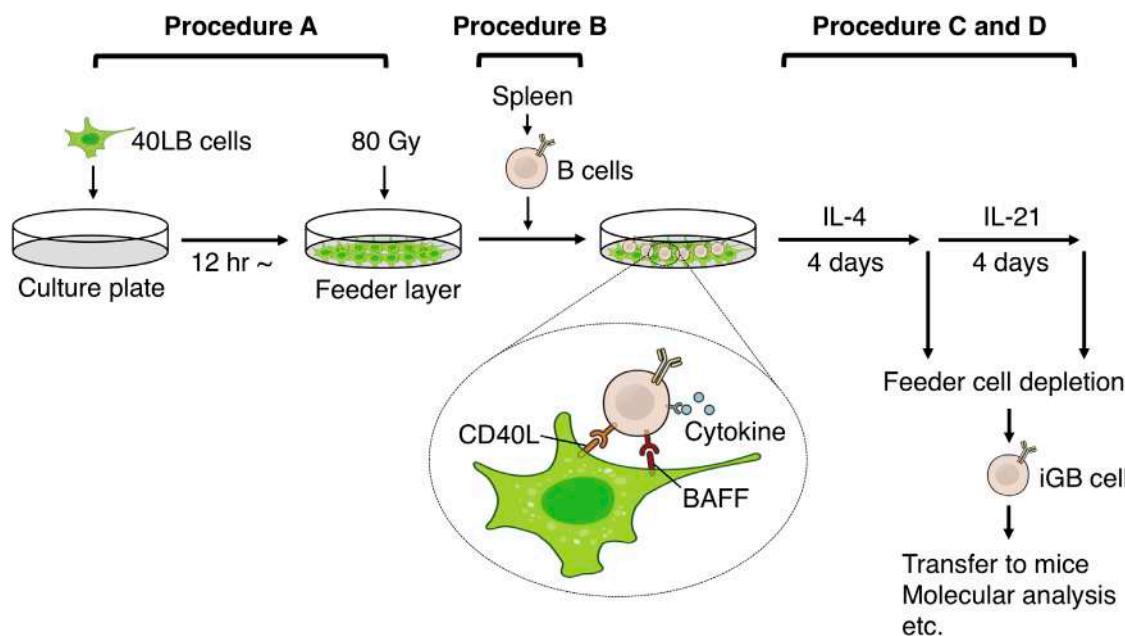


Figure 1. A schematic illustration of the procedure for iGB cell culture system

A. Propagation of 40LB cells and preparation of feeder layer of 40LB cells

40LB feeder cells are typically cultured and maintained with 10 ml of 3T3 medium in a 10 cm dish at 37 °C, 5% CO₂. Passage the 40LB cells every 3 days with 6-fold dilutions and keep in culture up to one month, as described below. After one month or so, restart the culture from a frozen stock. The following protocol is for the culture using 10 cm dishes. Change the volume of solutions in proportion to the bottom area of the plate (well) to use, as shown by examples in Table 1.

1. Thaw a frozen 1 ml 40LB cell stock and dilute the content with 12 ml of 3T3 medium (see Recipes).
2. Centrifuge cells at 270 × g for 5 min at 4 °C.
3. Discard the supernatant and resuspend the cells in 10 ml of 3T3 medium by pipetting, and seed all cells into a 10 cm culture dish.
4. Culture cells at 37 °C, 5% CO₂ for 1-2 days until they reach 70%-80% confluence.
5. Remove the medium from the cell culture dish, and rinse it with 2 ml of PBS (see Recipes).
6. Discard the PBS, add 1 ml of Trypsin-EDTA and leave the dish at room temperature until cells get dispersed (it takes about 1-3 min).
7. Add 3 ml of 3T3 medium and gently pipette up and down to completely detach the cells.
8. Transfer the cell suspension to a tube and centrifuge at 270 × g for 5 min at 4 °C.
9. Discard the supernatant and resuspend the cells in the desired volume of 3T3 medium.
10. For passage, dilute cell suspension 6-fold from Step A9, seed cells into 10 cm culture dishes and culture cells at 37 °C, 5% CO₂ for 3 days.
11. For irradiation, seed an appropriate number of 40LB cells from Step A9 to the desired plate (see Table 1), and allow cells at least 12 h to attach the plate. Alternatively, irradiate the

harvested 40LB cells in a tube, then seed them onto plates, and culture them for at least 12 h until the 40LB cells attach to the plates and completely spread out.

12. Irradiate the cells with 80 Gy of γ -ray to stop proliferation.
13. For cell stock, resuspend the cell pellet from Step A9 in ice-cold 20% FBS and 10% DMSO ($\sim 2 \times 10^6$ cells per ml), add 1 ml of cell suspension to each cryovial, place the vials in a Mr. Frosty, then place the cells in a -80 °C deep freezer for 24 h. After 24 h, transfer the vials to a liquid nitrogen storage tank.

Table 1. Plate type, cell number to seed and volume of solutions

Plate type	10 cm	6-well	12-well
No. of 40LB cells to seed ($\times 10^4$)	300	50	20
Vol. of PBS to rinse a dish or a well (ml)	2	0.5	0.25
Vol. of Trypsin-EDTA to harvest cells (ml)	1	0.5	0.25
Vol. of 3T3 medium to inactivate trypsin (ml)	3	1	0.5
No. of B cells to seed ($\times 10^4$)	50	5	2
Vol. of BCM to seed (ml)	40	8	4

B. Isolation and purification of B cells from mouse spleen for the iGB cell culture

Keep cells on ice or 4 °C, and use pre-chilled solutions. The following is the protocol for one spleen.

1. Take a spleen from a C57BL/6 mouse and put the spleen in a 10 cm dish.
2. Inject 10 ml of MACS buffer (see Recipes) inside the spleen using a 26 G needle and 10 ml syringe, in order to extract splenocytes, and then gently disrupt the remaining spleen capsular using a pair of frosted glass slides.
3. Transfer the cell suspension with a pipette to a tube through a 100 μ m mesh.
4. Centrifuge at 370 $\times g$ for 6 min.
5. Discard the supernatants, resuspend the cells with 10 ml of MACS buffer and count the cell number.
6. Discard the supernatant, add 100 μ l of antibody cocktail for B cell isolation (see Recipes) per 10^8 cells and gently pipette up and down to suspend the cells completely.
7. Incubate for 20 min on ice.
8. Wash the cells twice by adding 10 ml of MACS buffer with centrifugation (370 $\times g$ for 6 min) at each step.
9. Discard the supernatants, add 50 μ l of Streptavidin Particles Plus-DM per 10^8 cells and gently pipette up and down to suspend the cells completely.
10. Incubate the cells for 20 min on ice.
11. Place two 5 ml round-bottom tubes onto the IMag cell separation Magnet.
12. Resuspend cells with 2 ml of MACS buffer and transfer into the first tube on the magnet.

13. Wait for 5 min until the labeled cells attach to the wall of the tube beside the magnet.
14. Collect unbound cells from the first tube and transfer it to the second tube.
15. Detach the first tube and resuspend the attached cells in the first tube with a new 2 ml of MACS buffer, and then place the tube back onto the magnet and leave it for 5 min.
16. Collect unbound cells from the second tube and transfer it to the collection tube.
17. Repeat Steps B13-B15 once more.
18. Collect unbound cells from the first tube, transfer it to the second tube and leave it for 5 min.
19. Collect unbound cells from the second tube and transfer it to the collection tube.
20. Centrifuge the whole collected cells at $370 \times g$ for 6 min.
21. Place an LS column onto a MidiMACS Separator attached to MACS MultiStand, and place 40 μm mesh on the top of the LS column.
22. Rinse the column with 2 ml of MACS buffer.
23. Discard the supernatants from Step B19 and resuspend the cells with 1 ml of MACS buffer.
24. Apply the cell suspension onto the LS column through the 40 μm mesh and allow all the suspension to pass through the column.
25. Wash the column 3 times with 1 ml of MACS buffer each.
26. Wash the column once with 3 ml of MACS buffer.
27. Collect all the effluent and centrifuge at $370 \times g$ for 6 min.
28. Discard the supernatants, resuspend the cells with 10 ml of B cell medium (BCM, see Recipes) and count the cell number.

Note: The expected B cell number from one spleen is 2×10^7 - 5×10^7 cells.

29. Check the purity of isolated B cells using flow cytometry. Use fluorochrome-conjugated antibodies against B cell markers such as CD19 and B220. The purity of B cells is usually more than 97%.

Note: Alternatively, positively sorted B cells using MACS or FACS can be used for the culture. B cells from peripheral blood or other lymphoid organs such as lymph nodes can also be used.

C. iGB cell culture

Use pre-warmed buffer and medium. The following protocol is for the culture using a 10 cm dish.

Note: After the B cells start to proliferate and become 'iGB cells', the iGB cells should be treated at room temperature during all procedures such as centrifugation, antibody staining, iMag, MACS or flow cytometry, etc. iGB cells tend to die on ice.

1. Remove culture medium of irradiated feeder cells from the 10 cm dish and add 35 ml of BCM supplemented with IL-4 (final concentration is 1 ng/ml).
2. Add 5 ml of BCM containing an appropriate number of the isolated B cells (see Table 1) and culture at 37°C , 5% CO_2 for 4 days (Figure 2).

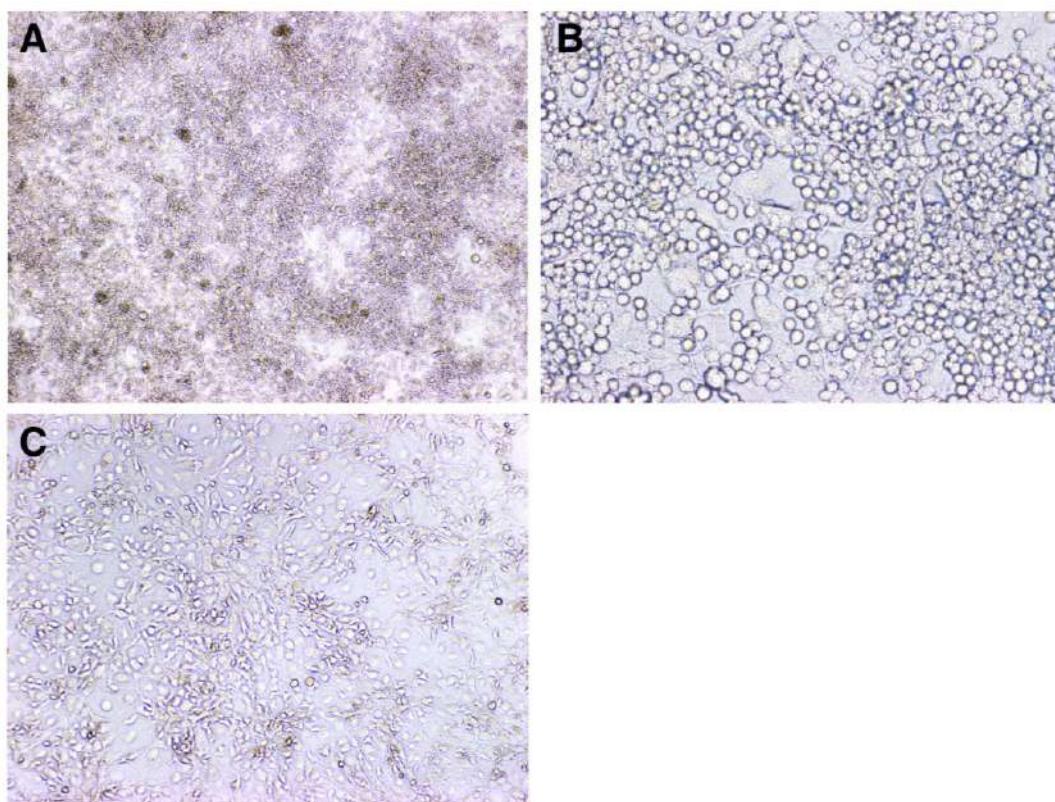


Figure 2. Bright-field microscopy of the iGB cells and a 40LB layer. A and B. iGB cells on a feeder layer after culturing with IL-4 for 4 days. (A) 4x magnification, (B) 20x magnification. C. 40LB feeder layer after 80 Gy irradiation at 4x magnification.

3. To harvest cells, carefully remove medium from the surface down to the level about 0.5 cm above the bottom and transfer the remaining medium to a collection tube (the volume of remaining medium is about 10 ml).
4. Add 4 ml of MACS buffer and leave dish at room temperature until cells begin to get dispersed (it takes about 3-5 min).
5. Gently shake the dish to detach the cells and collect them to the tube.
6. Rinse the plate with 5 ml of MACS buffer and collect the medium into the tube. Repeat once more.
7. Centrifuge all the collected cells at $370 \times g$ for 5 min at room temperature.
8. Discard the supernatants and disrupt the cell pellet by gentle shaking. Resuspend the cell pellet with 10 ml of B cell medium and count the cell number. iGB cells are distinguishable as the cells smaller than the 40LB cells.
Note: The expected iGB cell number from a 10 cm dish is 2×10^7 - 4×10^7 , and the viability of iGB cells exceeds 90%.
9. To culture the cells sequentially, seed appropriate number of the iGB cells onto a new irradiated feeder layer and culture them as described in Step C1 with an appropriate cytokine.
Note: Primary cultured iGB cells can be re-expanded on the new feeder layer with 10 ng/ml

IL-21 (or IL-4 which will promote further switching to IgE), as described in the original article (Nojima et al., 2011). For this, put the whole harvested cells including the feeder cells onto the new feeder layer, adjusting the number of iGB cells as desired. For example, primary iGB cells on Day 4 can be seeded, to culture with IL-21, at 5×10^5 (for 3 more days) or 5×10^4 (for 4 more days) per 10 cm dish. In the secondary culture with IL-21, the growth rate of iGB cells is difficult to predict, thus change the medium when it begins to turn yellowish. In our experience, the expected expansion factor of iGB cell during the secondary culture is 100-300 fold (in 4 days), and the viability of iGB cells always exceeds 70%. The secondary culture can be continued for at least 6 days without feeder renewal.

D. Feeder cell depletion

It is desirable to remove feeder cells from harvested cells of the iGB cell culture when they are transferred into mice or analyzed at a molecular level. This depletion protocol is for cells from one 10 cm dish. Use pre-warmed buffer and medium.

1. Harvest cells as in Steps C3-C7.
2. Discard the supernatants and disrupt the cell pellet with gentle shaking. Add 260 μ l of an antibody cocktail for feeder cell depletion (see Recipes) and gently pipette up and down to suspend the cells completely. For cells from 1 well of a 6-well or 12-well plate, use 1/6 or 1/12 volume, respectively, of diluted antibody with the same concentration.
3. Incubate for 20 min at room temperature.
4. Wash the cells twice by adding 10 ml of MACS buffer with centrifugation (370 $\times g$ for 5 min at room temperature) at each step.
5. Discard the supernatant and disrupt the cell pellet with gentle shaking. Add 50 μ l of Streptavidin Particles Plus-DM and 100 μ l of MACS buffer, and then gently pipette up and down to suspend the cells completely.
6. Incubate for 20 min at room temperature.
7. Apply to iMag and MACS procedure as described in Steps B10-B24.
8. Collect all the effluent and centrifuge at 370 $\times g$ for 5 min at room temperature.
9. Resuspend the collected cells in 10 ml of an appropriate medium or buffer and count the cell number.

Notes

An FBS lot check is required, as lot-specific differences in FBS influence the cell growth, class switching and the generation of the iGB cells. Indeed, only ~30% of the lots we tested worked well. When we select the FBS lot for iGB cell culture, we usually use the following parameters: 1) Cell expansion factor during the primary culture with IL-4 (more than 60-fold in 96 h) and the secondary culture with IL-21 (more than 100-fold in 96 h). 2) FACS profiles after the primary and the secondary culture: efficient class switching to IgG1 and IgE (but no more than 50% IgE⁺ cells) and

homogeneous expression of GL7 and Fas, and minimal differentiation to CD138⁺ cells after the primary culture. Most of the FBS lots can support the expansion and survival of 40LB cells.

Recipes

Note: When preparing the following solutions, mix all of components in a sterile hood.

1. 3T3 medium

D-MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS)

100 U/ml penicillin

100 µg/ml streptomycin

Store at 4 °C , Shelf life: 2 months from the date of manufacture

Note: FBS can be heat-inactivated by incubation at 56 °C for 30 min.

2. B cell medium (BCM)

RPMI-1640 supplemented with 10% heat-inactivated FBS

10 mM HEPES-NaOH pH 7.5

5.5 × 10⁻⁵ M 2-ME

100 U/ml penicillin

100 µg/ml streptomycin

Store at 4 °C, Shelf life: 2 months from the date of manufacture

3. Phosphate-buffered saline (PBS)

a. Stock (20x): dissolve 160 g NaCl, 4 g KCl, 23 g Na₂HPO₄ and 4 g KH₂PO₄ in MilliQ water

b. Fill up to 1 L with MilliQ water

c. Dilute 1:20 with MilliQ water and autoclave

Store at 4 °C, Shelf life: 1 year from the date of manufacture

4. MACS buffer

a. Make 500 ml of PBS supplemented with 2 mM EDTA and autoclave

b. Add 12.5 ml of 0.22 µm-filtered 20% BSA in PBS

Store at 4 °C, Shelf life: 6 months from the date of manufacture

5. Antibody cocktail for B cell isolation

100 µl of MACS buffer containing 3 µg/ml of biotinylated anti-CD43

2.5 µg/ml of biotinylated anti-Ter119

1.5 µg/ml of biotinylated anti-CD4

1.5 µg/ml of biotinylated anti-CD8

1.5 µg/ml of biotinylated anti-CD49b

Make the cocktail just before use

Note: Anti-CD43 antibody can be used for depletion of various immune cells except for B cells, e.g., macrophages, granulocytes, dendritic cells, and T cells. The anti-CD49b antibody recognizes NK and NKT cells. Addition of both antibodies improves the purity of B cells.

6. Antibody cocktail for feeder cell depletion (for cells from one 10 cm dish)
260 µl of MACS buffer containing 2.89 µg/ml of biotinylated anti-H-2K^d
To deplete plasma cells simultaneously, add biotinylated anti-CD138 at 1.15 µg/ml to the cocktail
Make the cocktail just before use

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Competing interests

The authors declare no conflict of interest.

References

1. Fukao, S., Haniuda, K., Nojima, T., Takai, T. and Kitamura, D. (2014). [gp49B-mediated negative regulation of antibody production by memory and marginal zone B cells](#). *J Immunol* 193(2): 635-644.
2. Haniuda, K., Fukao, S., Kodama, T., Hasegawa, H. and Kitamura, D. (2016). [Autonomous membrane IgE signaling prevents IgE-memory formation](#). *Nat Immunol* 17(9): 1109-1117.
3. Moutai, T., Yamana, H., Nojima, T. and Kitamura, D. (2014). [A novel and effective cancer immunotherapy mouse model using antigen-specific B cells selected *in vitro*](#). *PLoS One* 9(3): e92732.
4. Nojima, T., Haniuda, K., Moutai, T., Matsudaira, M., Mizokawa, S., Shiratori, I., Azuma, T. and Kitamura, D. (2011). [In-vitro derived germinal centre B cells differentially generate memory B or plasma cells *in vivo*](#). *Nat Commun* 2: 465.
5. Takatsuka, S., Yamada, H., Haniuda, K., Saruwatari, H., Ichihashi, M., Renauld, J. C. and Kitamura, D. (2018). [IL-9 receptor signaling in memory B cells regulates humoral recall responses](#). *Nat Immunol* 19(9): 1025-1034.

Characterization of Mouse Adult Testicular Macrophage Populations by Immunofluorescence Imaging and Flow Cytometry

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[Abstract] Testicular macrophages (tMΦ) are the most abundant immune cells residing in the testis, an immune-privileged organ. tMΦ are known to exhibit different functions, such as protecting spermatozoa from auto-immune attack by producing immunosuppressive cytokines and trophic roles in supporting spermatogenesis and male sex hormone production. They also contribute to fetal testicular development. Recently, we characterized two distinct tMΦ populations based on their morphology, localization, cell surface markers, and gene expression profiling. Here, we focus and describe in detail the phenotypical distinction of these two tMΦ populations by fluorescence-activated cell sorting (FACS) using multicolor panel antibodies combining with high-resolution immunofluorescence (IF) imaging. These two techniques enable to classify two tMΦ populations: interstitial tMΦ and peritubular tMΦ.

Keywords: Macrophage, Testis, FACS, IF, Spermatogenesis, Immune-privilege organ

[Background] The testis is the male reproductive organ where spermatogenesis and testosterone production occurs. tMΦ might be considered as “guardians of fertility” by their immuno-suppressive function to assure the immune-privilege status of the testis and their trophic roles in spermatogenesis and male hormone production, but the distinct functions of these macrophage populations are only starting to be elucidated (Mossadegh-Keller and Sieweke, 2018). Tissue-resident macrophages exhibit tissue-specific functions and gene expression patterns depending of their organs of residency (Gentek *et al.*, 2014; Lavin *et al.*, 2014) but also share common core tissue-resident macrophage markers as F4/80, CD11b, CD64, M-CSFR and for some tissues CX3CR1 (Gordon, 2002; Gautier *et al.*, 2012; Yona *et al.*, 2013; Gentek *et al.*, 2014). Recently, two testicular macrophages (tMΦ) populations have been characterized using IF imaging and FACS (DeFalco *et al.*, 2015; Mossadegh-Keller *et al.*, 2017). Interstitial tMΦ can be identified by microscopy by their rounded morphology and localization in the interstitial space of the testis in close contact with testosterone-producing Leydig cells (Smith and Walker, 2014; DeFalco *et al.*, 2015; Mossadegh-Keller *et al.*, 2017). In contrast, the peritubular tMΦ exhibit an elongated morphology and surround the seminiferous tubules housing spermatogonial stem cells (SSC) (DeFalco *et al.*, 2015; Mossadegh-Keller *et al.*, 2017). Confirming previous observations (DeFalco *et al.*, 2015), we observed that both tMΦ were positive for CX3CR1 and F4/80 by IF. Interstitial tMΦ can be distinguished by the strong expression of M-CSFR from peritubular tMΦ that

selectively express high levels of MHCII (Figure 2) (Mossadegh-Keller *et al.*, 2017). We further analyzed in depth the interstitial and peritubular tMΦ populations by FACS. We established an extended antibody panel going beyond previous protocols (DeFalco *et al.*, 2015), excluding monocytes and dendritic cells and including CD64 as a key tissue-resident macrophage (Gautier *et al.*, 2012; Mossadegh-Keller *et al.*, 2017). Within the tMΦ CD45⁺Ly6C⁻CD11c^{lo}F4/80⁺CD11b⁺ fraction, we defined interstitial tMΦ as M-CSFR⁺CD64^{hi}MHCII⁻ cells and peritubular tMΦ as M-CSFR^{lo}CD64^{lo}MHCII⁺ cells (Figure 3).

Here, we describe IF and FACS protocols that will be instrumental to define and isolate these two tMΦ populations for further phenotypic and functional characterization.

Materials and Reagents

1. Pipette tips
2. Microscope slides (SuperFrost Plus, VWR, catalog number: 631-0108)
3. Cover glass (VWR, catalog number: 470820)
4. Hydrophobic pen, Mini PAP Pen (Life Technologies, catalog number: 008877)
5. Simport Scientific Disposable Base Molds (Fisher Scientific, catalog number: 11670990)
6. Eppendorf tube 1.5 ml (Sigma-Aldrich, catalog number: Z606340-1000EA)
7. Sterilin tube (Thermo Fisher Scientific, catalog number: 129A)
8. 5 ml Falcon Polystyrene Round-Bottom Tube (Fisher Scientific, Corning, catalog number: 352008)
9. Filter 50 µm Filcon, Sterile, Syringe-type (Becton, catalog number: 340601)
10. Wild-type C57BL/6J mice from Janvier labs
11. CX3CR1GFP/+ mice (Jung *et al.*, 2000)
12. Ice
13. DPBS 1x (Gibco, Life Technologies, catalog number: 14190-094)
14. HBSS 1x (Gibco, Life Technologies, catalog number: 14025-050)
15. BSA (Sigma-Aldrich, catalog number: A2153)
16. Fetal Calf Serum (Biosera, catalog number: FB-1001/500)
17. Saponin (Sigma-Aldrich, catalog number: 47036)
18. Antigenfix (Diapath, catalog number: P0014)
19. Optimum Cutting Temperature (O.C.T.) Tissue-Tek (VWR, catalog number: 256008-930)
20. Isopentane (VWR, catalog number: 24872-323)
21. Antibodies:
 - a. Anti-CD16/32 (clone 2.4G2, BD Biosciences, catalog number: 553142, working dilution 1/200)
 - b. Anti-CD45-BV421 (clone 30F11, BD Biosciences, catalog number: 560501, working dilution 1/200)

- c. Anti-F4/80-BV785 (clone BM8, BioLegend, catalog number: 123141, working dilution 1/200)
 - d. Anti-CD11b-BV605 (clone M1/70, BD Biosciences, catalog number: 563015, working dilution 1/800)
 - e. Anti-CD64-PerCP/Cy5.5 (clone X54-5/7.1, BioLegend, catalog number: 139308, working dilution 1/300)
 - f. Anti-CD11c-BV711 (clone N418, BioLegend, catalog number: 117349, working dilution 1/300)
 - g. Anti-CD115-PE (clone AFS98, eBiosciences, catalog number: 112-1152-82, working dilution 1/200)
 - h. Anti-Ly6C-APC/Cy7 (clone HK1.4, BioLegend, catalog number: 128026, working dilution 1/300)
 - i. Anti-I-A/I-E (MHCII)-PE/Cy7 (clone M5/114.15.2, BioLegend, catalog number: 107630, working dilution 1/300)
 - j. Anti-F4/80-Alexa Fluor647 (clone BM8, BioLegend, catalog number: 123122, working dilution 1/200)
 - k. Anti-MCII-eFluor450 (clone M5/114.15.2, eBiosciences, catalog number: 48-5321-82, working dilution 1/500)
 - l. Anti-M-CSFR (clone C-20, Santa Cruz, catalog number: sc-692, working dilution 1/800)
 - m. Anti-rabbit-Alexa Fluor 594 (Jackson ImmunoResearch, catalog number: 711-585-152, working dilution 1/500)
22. Prolong Gold antifade reagent (Life Technologies, catalog number: P36930)
23. Zombie Violet fixable live/dead staining (BioLegend, catalog number: BLE423113, working dilution 1/1,000)
24. 30% sucrose (Sigma-Aldrich, catalog number: S9378) (see Recipes)
25. Blocking Buffer for IF (see Recipes)
26. Wash Buffer for IF (see Recipes)
27. Prolong containing Sytox blue dye (Life Technologies, catalog number: S34857) (see Recipes)
28. Enzymatic digestion Mix (see Recipes)
- a. Collagenase II (Worthington Biochemicals/Serlabo Technologies, catalog number: LS004174, 125 U/mg dry weight)
 - b. DNase I (Roche, catalog number: 10104159001)
 - c. Working enzymatic digestion (500 µl/testis sample)

Equipment

1. Pipettes
2. Microscope slide holder/box (Heathrow Scientific, catalog number: 15994G)
3. Vertical plastic staining rack

4. Dissection scissors (Harvard Apparatus, catalog number: 72-8422)
5. Milligramme balance (Mettler Toledo, catalog number: 30029085)
6. SnapFrost80 (Alphelys)
7. Cryostat (Leica, model: CM3050S)
8. Confocal microscope (ZEISS, model: LSM 780)
9. 40x/1.4 oil differential interference contrast objective (Plan-Apochromat)
10. FACS LSRII instrument (BD)
11. Thermomixer comfort (Eppendorf, catalog number: 5382000015)
12. Rocking agitator (VWR, catalog number: 444-0756)
13. Centrifuge
14. Vortexer
15. 4 °C refrigerator
16. -80 °C freezer

Software

1. FlowJo (Version 10.0.8)
2. ImageJ (Version 1.49s, National Institutes of Health)
3. Adobe Illustrator CS6 (Version 16.0.3)

Procedure

- A. Preparing tissue testis sections for IF (Figure 1)
 1. Euthanize mice by cervical dislocation.
 2. Excise the whole testis organ and place it directly in a Sterilin tube with 1 ml cold Antigenfix. Incubate on a rocking agitator for 3 h at 4 °C.
 3. Wash the fixed testis in cold PBS 1x at 4 °C for 20 min.
 4. Incubate the testis organ in 5 ml of 30% sucrose solution overnight at 4 °C, until the testis sinks completely to the bottom of the tube.
 5. Transfer the testis into disposable base molds in a coronal plane and place immediately into cold (-80 °C) isopentane inside the SnapFrost80. After 2 min, remove the frozen molds and store at -80 °C.
 6. Section the mouse testis at 20 µm-thick with the cryostat on SuperFrost slides and store at -20 °C.

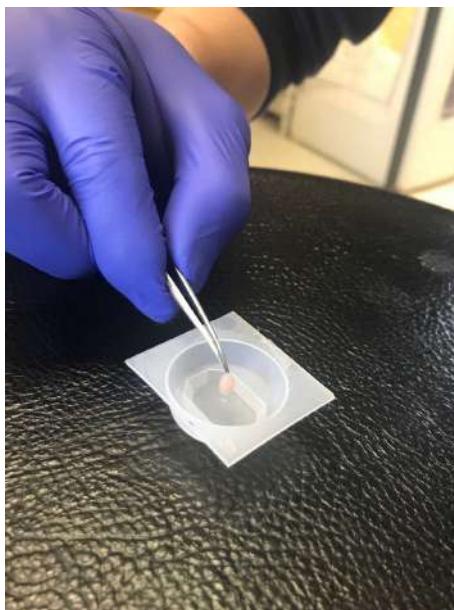


Figure 1. Coronal testis plane preparation. The testis is placed into disposable base molds with OCT in a coronal plane.

B. Immunofluorescence (Figure 2)

1. Place slides in a microscope slide holder/box, delimit the testis section with a hydrophobic pen and add 100 µl PBS 1x per section for 10 min at room temperature (RT).
2. Incubate in blocking buffer for 1 h at RT.
3. Wash 3 times with the wash buffer solution for 5 min at RT by moving the slide rack up and down between each wash.
4. Place wet tissues into the slide holder/box in order to make a humid chamber for the staining.
5. Incubate with antibodies (100 µl per section) in PBS/0.05% saponin overnight at 4 °C at the following dilutions:
 - a. Anti-F4/80-Alexa Fluor647 dilution 1/200
 - b. Anti-MCII-efluor450 dilution 1/500
 - c. Anti-M-CSFR dilution 1/800
6. After three washes using the wash buffer for 5 min each at RT, incubate for 1 h at RT with anti-rabbit-Alexa Fluor 594, dilution 1/500, for anti-MCSFR antibody detection.
7. Wash slides three times with PBS/0.05% saponin for 5 min each at RT by moving the slide rack up and down several times. Place slides on a vertical plastic staining rack, protected from the light, in order to dry the slides.
8. Mount slides in Prolong containing Sytox blue dye (see Recipes).

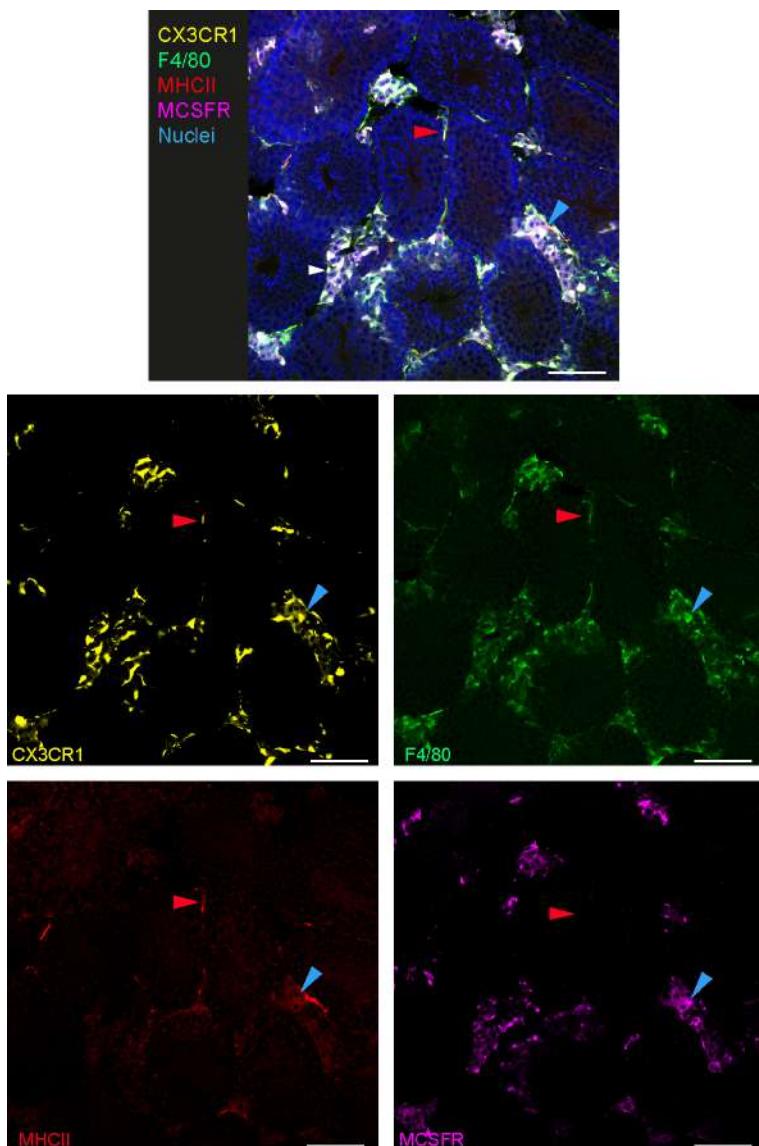


Figure 2. Phenotypic characterization of interstitial and peritubular macrophages by IF.
IF imaging of CX3CR1GFP/+ adult mouse testis is revealing morphology, localization, F4/80, MHCII and M-CSFR staining of interstitial and peritubular testicular macrophages. One example of interstitial macrophages is indicated with a blue arrow showing a cell with round morphology and expressing CX3CR1, F4/80 and M-CSFR. One example of peritubular macrophages is indicated with a red arrow showing a cell with elongated morphology and expressing CX3CR1, F4/80 and MHCII. Scale bars = 40 μ m.

C. Fluorescence-Activated Cell Sorting (FACS) (Figure 3)

1. Euthanize mice by cervical dislocation.
2. Collect the whole testis organ and place it directly in a 1.5 ml Eppendorf tube in cold PBS containing 1 mg/ml Collagenase II/0.15 mg/ml DNase I. Mechanically dissociate the testis by mincing with scissors directly inside the tube.
3. Place the minced tissue in a thermomixer at 37 °C for 40 min by shaking for enzymatic

digestion.

4. Transfer the digested testis through a 50 μm filter placed on top of a 5 ml Falcon tube and filter using 1 ml PBS.
5. Centrifuge the digested tissue in a refrigerated centrifuge for 5 min at 300 $\times g$ at 4 °C.
6. Resuspend the pellet of cells in 200 μl of cold PBS and incubate with blocking anti-CD16/32 antibody, dilution 1/200, leaving it on ice for 15 min.
7. Wash in 1 ml cold PBS and centrifuge for 5 min at 300 $\times g$ at 4 °C.
8. Incubate single-cell suspensions in cold PBS and stain for expression of surface antigens for 20 min on ice. The following antibodies are used in the same mix preparation:
 - a. Anti-F4/80-BV785 dilution 1/200
 - b. Anti-CD11b-BV605 dilution 1/800
 - c. Anti-CD64-PerCP/Cy5.5 dilution 1/300
 - d. Anti-CD11c-BV711 dilution 1/300
 - e. Anti-M-CSFR-PE dilution 1/200
 - f. Anti-Ly6C-APC/Cy7 dilution 1/300
 - g. Anti-I-A/I-E (MHCII)-PE/Cy7 dilution 1/300
 - h. Anti-CD45-BV421 dilution 1/200
 - i. Zombie Violet fixable live/dead cell dye as a viability marker dilution 1/1,000
9. Wash in 1 ml cold PBS and centrifuge for 5 min at 300 $\times g$ at 4 °C. Take up single cell suspension in 800 μl of PBS and pass the solution through a 50 μm filter. The sample is ready for the analysis on FACS.

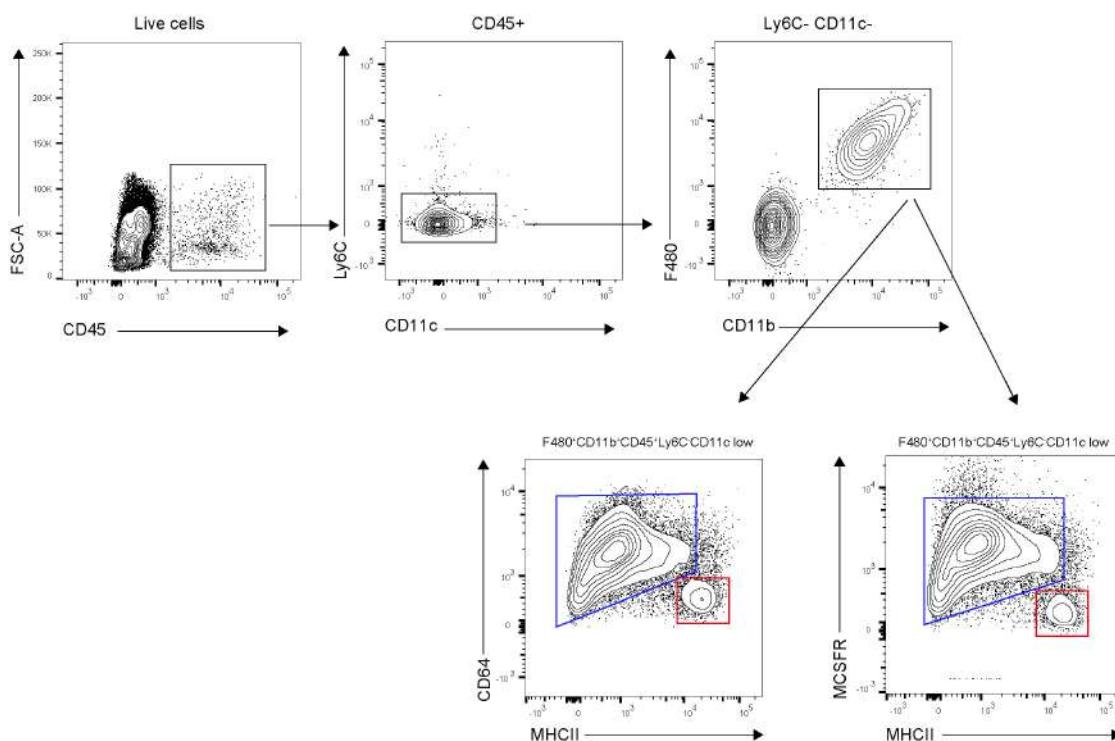


Figure 3. Phenotypic characterization of interstitial and peritubular macrophages by FACS.

Gating strategy describing in CD45⁺Ly6C⁻CD11c⁺F4/80⁺CD11b⁺ adult mouse testis fraction, the distinction of interstitial M-CSFR⁺CD64⁺MHCII⁻ and peritubular M-CSFR^{lo}CD64^{lo}MHCII⁺ population, respectively represented by blue and red gates.

Data analysis

Confocal microscopy acquisitions were performed on a confocal microscope (LSM780; ZEISS) at room temperature, and slides were imaged with a 40x/1.4 oil differential interference contrast objective (Plan-Apochromat). Different lasers were used (405 nm, 488 nm, 56 nm, and 633 nm) to excite the fluorophores (Sytox blue, eFluor 450, Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647). Fluorescence was recorded in individual channels acquired in a sequential mode to avoid cross-talk using a highly sensitive 32-channel gallium arsenide phosphide detector. The pinhole was set to 1 airy unit. Image processing was done with ImageJ (National Institutes of Health). Only a median filter was applied to the images to remove salt and pepper noise.

FACS data were acquired on an LSR II instrument (BD) using violet laser 405 nm, blue laser 488 nm, green laser 561 nm, and red laser 633 nm. FACS data were analyzed using FlowJo software (V10.0.8). The FACS gating strategy to distinguish Interstitial CD64^{hi}MHCII⁻ tMΦ and peritubular CD64^{lo}MHCII⁺ tMΦ of adult mouse testis and a representative FACS profile is shown in Figure 2. This can be used to quantify the proportion of these two tMΦ populations and the details regarding the analysis can be found in the original article (Mossadegh-Keller *et al.*, 2017; e.g., Figures 2A and 2B).

Recipes

1. 30% sucrose
30 g sucrose
Bring the volume to 100 ml with PBS
Store at 4 °C
2. Blocking Buffer for IF
2 g BSA
1 ml FCS
0.1 g saponin
Bring the volume to 100 ml with PBS
Store at 4 °C
3. Wash Buffer for IF
0.05 g saponin
Bring the volume to 100 ml with PBS
Store at 4 °C

4. Prolong containing Sytox blue dye
 - 1 ml of Prolong without DAPI
 - Add 3 µl of Sytox blue dye
 - Vortex 3 min
 - Add 1 ml Prolong without DAPI
 - Store at -20 °C
5. Enzymatic digestion Mix
 - a. Collagenase 50 mg/ml stock solution
 - Resuspend 1 g of collagenase in 20 ml of HBSS
 - Make aliquots of 1 ml
 - Store at -20 °C
 - b. DNase I 10 mg/ml stock solution
 - Resuspend 100 mg of DNase in 10 ml of HBSS
 - Make aliquots of 500 µl
 - Store at -20 °C
 - c. Working enzymatic digestion (500 µl/testis sample)
 - 10 µl of collagenase stock solution
 - 7.5 µl of DNase I stock solution, bring the volume to 500 µl with PBS

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Competing interests

The authors declare no competing financial interests.

Ethics

In vivo procedures were performed under specific pathogen-free conditions following protocols approved by the Ethics Committee of Marseille in accordance with institutional, national, and European regulations (approval nos. APAFIS 3292-2015 1221 09359224 and APAFIS 10545-2017 0710 08253541).

References

1. DeFalco, T., Potter, S. J., Williams, A. V., Waller, B., Kan, M. J. and Capel, B. (2015). [Macrophages contribute to the spermatogonial niche in the adult testis.](#) *Cell Rep* 12(7): 1107-1119.
2. Gautier, E. L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A., Elpek, K. G., Gordonov, S., Mazloom, A. R., Ma'ayan, A., Chua, W. J., Hansen, T. H., Turley, S. J., Merad, M., Randolph, G. J. and Immunological Genome, C. (2012). [Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages.](#) *Nat Immunol* 13(11): 1118-1128.
3. Gentek, R., Molawi, K. and Sieweke, M. H. (2014). [Tissue macrophage identity and self-renewal.](#) *Immunol Rev* 262(1): 56-73.
4. Gordon, S. (2002). [Pattern recognition receptors: Doubling up for the innate immune response.](#) *Cell* 111(7): 927-930.
5. Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A. and Littman, D. R. (2000). [Analysis of fractalkine receptor CX\(3\)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion.](#) *Mol Cell Biol* 20(11): 4106-4114.
6. Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S. and Amit, I. (2014). [Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment.](#) *Cell* 159(6): 1312-1326.
7. Mossadegh-Keller, N. and Sieweke, M. H. (2018). [Testicular macrophages: Guardians of fertility.](#) *Cell Immunol* 330: 120-125.
8. Mossadegh-Keller, N., Gentek, R., Gimenez, G., Bigot, S., Mailfert, S. and Sieweke, M. H. (2017). [Developmental origin and maintenance of distinct testicular macrophage populations.](#) *J Exp Med* 214(10): 2829-2841.
9. Smith, L. B. and Walker, W. H. (2014). [The regulation of spermatogenesis by androgens.](#) *Semin Cell Dev Biol* 30: 2-13.
10. Yona, S., Kim, K. W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D. A., Perlman, H., Malissen, B., Zelzer, E. and Jung, S. (2013). [Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.](#) *Immunity* 38(1): 79-91.

Gentamicin Protection Assay to Determine the Number of Intracellular Bacteria during Infection of Human TC7 Intestinal Epithelial Cells by *Shigella flexneri*

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[Abstract] *Shigella flexneri* is an intracellular bacterial pathogen that gains access to the gut epithelium using a specialized Type III Secretion System (T3SS). Various determinants mediating this invasive infection have been experimentally verified using the classical gentamicin protection assay presented here. In this assay epithelial cell lines are infected by bacteria *in vitro* and the extracellular bacteria are killed by gentamicin. The internalized bacteria, which are protected from the bactericidal action of gentamicin, are recovered by lysing the epithelial cells and enumerated by determining the colonies formed on solid medium. Various techniques based on light microscopy, such as immunofluorescence and bacteria expressing fluorescent proteins, are also used for studying intracellular bacteria. However, these techniques are not only labor intensive and require sophisticated equipment, but mostly are also not quantitative. Despite being an easy quantitative method to study invasiveness of bacteria, the gentamicin protection assay cannot distinguish between the survival and multiplication of the internalized bacteria over longer incubation periods. To alleviate the complications created by multiplication and dissemination of internalized bacteria, complementary assays like plaque formation assays are required. This protocol presents an easy and cost-effective method to determine the invasiveness and the capacity to establish an infection of *Shigella* under different conditions.

Keywords: *Shigella*, Intracellular pathogen, Invasion, Type III Secretion System, Intestinal epithelial cells

[Background] *Shigella* infects about 160 million people leading to about 600,000 deaths every year (Reference 8). The clinical manifestations of *Shigella* infection, or shigellosis, arise only after the bacterium enters the epithelium, where it multiplies and spreads to adjacent cells causing cell death and tissue necrosis. Thus, the entry into the epithelial cells is a critical step in the infectious life cycle of *Shigella* (Ashida *et al.*, 2015). Most of the determinants mediating this step have been ascribed to a large virulence plasmid that encodes a T3SS responsible for injecting bacterial proteins into the host cell via a needle-like projection (Puhr and Sansonetti, 2014).

The gentamicin protection assay is a classical method that is used to assess the invasiveness of *Shigella* and has led to the identification of a number of mediators of invasion by mutational analysis and comparisons with the wild-type bacteria. In a typical experiment, the bacteria are allowed to infect the intestinal cells in a synchronized manner followed by removal of external bacteria by gentamicin

treatment. The invasiveness is assessed by determining the number of surviving bacteria (protected from gentamicin being intracellular) from the lysates of infected cells. A lower number of surviving bacteria with respect to the wild-type indicates a defect in invasion or intracellular survival. If short infection times are used after the gentamicin treatment (typically 1h), the gentamicin protection assay allows to compare the bacterial capacity to enter host cells only. However, if longer infection times are used after gentamicin treatment (2 h or more), the assay will also reflect the bacterial capacity to survive and multiply within the target cell besides the capacity to invade. Hence, if the gentamicin protection assay is employed to study the capacity to survive and multiply by allowing long infection times, these experiments should always be complemented with tests carried out at short infection times to ensure correct interpretation of the results. The gentamicin protection assay, however, is not suitable to assess the extent of intercellular spread of bacteria throughout the monolayer, which is conveniently determined by a plaque formation assay. Although the invasiveness of bacterial pathogens can also be studied by fluorescence microscopy-based techniques such as immunofluorescence and FACS; it requires the use of costly reagents like labeled probes, special reporter strains and sophisticated equipment. The gentamicin protection assay is not only relatively easy to perform and cheap, it is fairly rapid and less labor intensive making it amenable to higher throughput.

The protocol presented here reprises the classical gentamicin protection assay, which has been optimized for use with *Shigella flexneri* and TC7 intestinal epithelial cells but can be modified to be used with other intracellular pathogens and other host cell types.

Materials and Reagents

1. Conical flask (Duran, catalog number: 2121628)
2. Microfuge tubes (Eppendorf, Safe-Lock tubes, catalog numbers: 0030120086, 0030120094)
3. Centrifuge tubes (Sarstedt, catalog numbers: 62.554.502, 62.547.254)
4. Culture tubes (TPP, catalog number: 91016)
5. Pipette tips (VWR, catalog numbers: 89041-404, 89041-412, 89041-400)
6. Serological pipettes (VWR, catalog numbers: 612-3702, 612-3700, 612-3698)
7. 6-well tissue culture test plates (TPP, catalog number: 92406)
8. TC7 cells (human cell line, a derivative of Caco-2 colon adenocarcinoma cells) (Chantret *et al.*, 1994)
9. *Shigella flexneri* M90T (Sansonetti *et al.*, 1982)
10. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, catalog number: 21885-025)
11. Penicillin-Streptomycin solution (10,000 U/ml) (Gibco, catalog number: 15140122)
12. Minimum Essential Medium Non-Essential Amino Acids solution (100x) (Gibco, catalog number: 11140-035)
13. Dulbecco's Phosphate Buffered Saline (PBS) solution (Gibco, catalog number: 14190-144)
14. Fetal Bovine Serum (Gibco, catalog number: 10500056)
15. HEPES 1 M solution (Gibco, catalog number: 15630-080)

16. Trypsin-EDTA (0.05%), phenol red (Gibco, catalog number: 25300-054)
17. Trypan Blue Stain (0.4%) (Thermo Scientific, catalog number: T10282)
18. Agarose (VWR Life Science, catalog number: 35-1020)
19. Tryptic Soy Broth (TSB) ready to use powder (Merck, catalog number: 105459)
20. Tryptic Soy Agar (TSA) ready to use powder (Merck, catalog number: 105458)
21. Ethanol (VWR chemicals, catalog number: 20821.558)
22. Congo red (Sigma, catalog number: C6277)
23. Sodium deoxycholate (Sigma, catalog number: 30970)
24. Gentamicin sulfate (Sigma, catalog number: G1264)
25. Sterile disposable petriplates (Sigma, catalog number: P5606-400EA)
26. Congo red solution (see Recipes)
27. Growth medium (see Recipes)
28. Infection buffer (see Recipes)
29. Sodium Deoxycholate solution (see Recipes)
30. Gentamicin solution (see Recipes)

Equipment

1. Biosafety cabinet (Thermo Scientific, HERAsafe KS 18)
2. Spectrophotometer (Amersham, Utrospec 2100 pro)
3. Pipette controller (VWR, Accurvette)
4. CO₂ Incubator (Thermo Scientific, Heracell VIOS 160i)
5. Benchtop centrifuge (VWR, Micro Star 17R)
6. Centrifuge (Eppendorf, 5810R with Rotor A-4-81 for plates)
7. Water bath (Grant, JBA 12)
8. Pipettes (Eppendorf, Research plus)
9. Inverted microscope (Motic, AE2000 Binocular)
10. Cell counter (Thermo Scientific, Countess II FL)
11. Cell counter slides (Thermo Scientific, Countess Cell Counting Chamber Slides, C10228)
12. Orbital shaker (Edmund Bühler, Swip SM25)
13. Vacuum pump (VWR, Mini diaphragm vacuum pump VP 86)

Software

1. Prism 7 (GraphPad, <https://www.graphpad.com>)

Procedure

Note: The experiment should be carried out in a biosafety level 2 lab.

A. Preparation of bacteria

Refer to Procedure A of the Protocol—Plaque Assay to Determine Invasion and Intercellular Dissemination of *Shigella flexneri* in TC7 human Intestinal Epithelial Cells (Sharma and Puhar, 2019).

B. Preparation of TC7 cells

Refer to Procedure B of the Protocol—Plaque Assay to Determine Invasion and Intercellular Dissemination of *Shigella flexneri* in TC7 human Intestinal Epithelial Cells (Sharma and Puhar, 2019).

C. Infection and cell lysis

Day 3

1. On Day 3, when the bacteria reach $OD_{600nm} = 0.3\text{-}0.4$, pipette 1-1.5 ml culture into a microcentrifuge tube.
2. Spin the tube at $3,000 \times g$ for 5 min. Aspirate the supernatant. Add an equal volume of PBS at room temperature and resuspend the pellet by tapping the microcentrifuge tube or by gentle vortexing.
3. Repeat Step C2 at least twice and finally resuspend the pellet in infection buffer (see Recipes) at room temperature. Record the OD_{600nm} of the bacterial suspension.

The recommended multiplicity of infection (MOI) for this experiment is 5. Since there are 1×10^6 cells in each well, the number of bacteria required per well is 5×10^6 .

The number of *Shigella* cells at $OD_{600nm} = 1$ is $0.5 \times 10^9/\text{ml}$; hence at $OD = z$, the number of bacterial cells = $z \times 0.5 \times 10^9$. Calculate the volume of bacterial suspension required for infecting each well using the following formula:

$$\text{Volume of bacterial suspension required (in ml)} = 5 \times 10^6 / z \times 0.5 \times 10^9$$

4. Aspirate the medium from the cells and add 2 ml of sterile PBS at room temperature to each well.
5. Swirl the plate gently and carefully aspirate all the liquid. After at least 2 washings, add 2 ml of infection buffer (see Recipes) at room temperature to each well.
6. Add the desired volume of bacterial suspension (typically about 10 μl , calculated in Step C3) to each well of the 6-well plate. Centrifuge the plate at $180 \times g$ for 10 min at room temperature.
7. Incubate the plate at 37°C , 10% CO_2 , for the desired infection time, typically 1 h. When *Shigella* comes in contact with the cells at the incubation temperature of 37°C (35°C and

above) the secretion of effector proteins through the T3SS is triggered which mediates the bacterial invasion.

8. After incubation, wash the wells with sterile PBS three times as in Steps C4 and C5.
9. Add 2 ml of gentamicin solution (see Recipes) to each well and incubate the plate for one more hour at 37 °C and 10% CO₂. If intracellular bacterial survival and multiplication is assessed, this incubation time can be extended.
10. After incubation with gentamicin, wash the wells thrice with PBS (as in Steps C4 and C5).
11. Aspirate the PBS from the wells and add 1 ml of sodium deoxycholate solution (see Recipes) to lyse the TC7 cells (the bacteria resist deoxycholate).
12. Using a 1 ml pipette, scrape the cells off the surface and pipette up and down to lyse the cells effectively and homogenize the lysate.

D. Plating

1. Prepare log serial dilutions of this lysate in sterile PBS as follows.
2. Dispense 450 µl of sterile PBS in 1.5 ml microcentrifuge tubes.
3. Add 50 µl of cell lysate to one tube and mark it 10⁻¹ dilution (as in Figure 1). Vortex the tube for a few seconds and transfer 50 µl of suspension from this tube to the next tube containing 450 µl of sterile PBS. Mark this new tube as 10⁻² dilution. Repeat this procedure to obtain further dilutions of the lysate (Figure 1).
4. For shorter infection time points, a lower dilution will provide significant results. For example, if infection time is 1 h, 10⁻¹ dilution is enough; but if the infection time is increased to 2 h, 10⁻² dilution would be more appropriate owing to bacterial multiplication. Similarly, consider higher dilutions for even longer infection times.

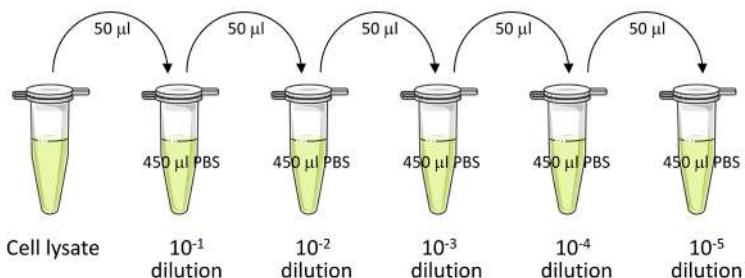


Figure 1. Schematic representation of logarithmic serial dilution of the cell lysate

5. Mark sectors on the agar plates (on the bottom of the plate) as in Figure 2.
6. Put a 10 µl drop of diluted suspension (10⁻¹/10⁻²/10⁻³/10⁻⁴ as labeled on the plate in Figure 2) on one of the sectors on the TSA plate. Let the drop dry.
7. Put at least three drops of each dilution (R1, R2 and R3 in Figure 2; representing technical replicates).

8. On another plate, mark sectors in a similar way (on the bottom of the plate) and put drops of suspension prepared from the lysate of a duplicate well. This plate serves as another technical replicate.
9. Incubate the plates overnight at 37 °C.
10. Count the colonies in the drop. Only consider the dilutions in which the number of colonies is within the countable range (3-30 colonies).
11. In parallel, make log serial dilutions of the starting bacterial suspension and spot 10 µl drops on TSA plates in a similar way as described above.
12. Incubate the plates overnight at 37 °C, along with the other plates, and count the colonies in the drop similar to Step D10. This colony count of the inoculum serves as a verification of the calculation performed in Step C3.

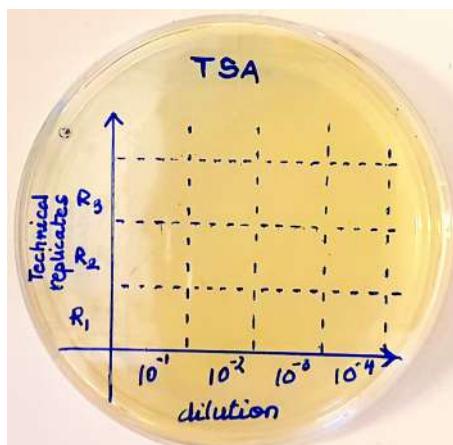


Figure 2. Sectors on a TSA plate for putting drops of up to four different dilutions in triplicate

Data analysis

1. From the raw colony count, determine the CFU/ml using the following formula:

$$\text{CFU/ml} = \text{No. of colonies} \times \text{dilution factor} \times 100$$

For example, if 15 colonies were counted in 10^{-2} dilution, CFU/ml would be calculated using the aforementioned formula as follows:

$$\text{CFU/ml} = 15 \text{ (No. of colonies)} \times 100 \text{ (dilution factor, from } 10^{-2}) \times 100 = 150,000 \text{ or } 1.5 \times 10^5$$

2. Using Prism software plot the number of bacteria expressed in CFU/ml against time for every strain to be analyzed and perform statistical analysis using the same software (Figure 3). For comparing only two sample values at a fixed time point, use Student's *t*-test. However, for

studying the statistical significance of multiple samples across multiple time points, use two-way ANOVA with Tukey's post-hoc test.

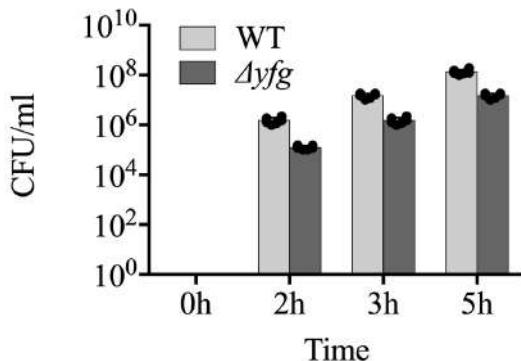


Figure 3. An example plot of the number of intracellular bacteria in CFU/ml at different infection times for two different strains. Each dot represents an independent value obtained from a replicate.

Notes

1. To prepare glycerol stocks of *Shigella flexneri*, dilute the overnight culture 200 times in fresh TSB and incubate at 37 °C with 200 rpm shaking. Monitor the OD₆₀₀ of the culture periodically. At OD₆₀₀ = 0.6, withdraw 1 ml of culture in a 2 ml cryovial and add sterile glycerol to a concentration of 15-50% (v/v). Mix gently and store the tube at -80 °C. The frozen stocks are stable for years at -80 °C, however, multiple freeze-thaw cycles may reduce the shelf life.
2. Carefully select only the red colonies on Congo red agar for the experiment. The off-white colonies have lost the virulence plasmid and are invasion-deficient. If plates are not used immediately after overnight culture, but kept at 4 °C (which is possible for up to 3 weeks), white colonies need to be marked because they will turn red due to non-specific binding of Congo red. Red staining of invasive colonies only develops during growth above 35 °C.
3. Congo red is able to induce secretion via the Type III Secretion System (T3SS) in *Shigella*. The dye binds to the bacterial colonies that have an active T3SS making them appear red in color. It is a simple and quick screening method to differentiate virulent *Shigella* colonies from the avirulent ones (due to curing of the virulence plasmid). Unfortunately, the mechanism of action of Congo red is not understood.
4. The TC7 cells are cultured and maintained in presence of Penicillin and Streptomycin (100 U/ml). However, infections are carried out in infection buffer that do not contain any antibiotics (which may inhibit the bacterial growth). This also highlights the importance of washing the cell monolayers before starting the infections.
5. The 6-well plates are centrifuged after addition of bacteria to aid binding, as *Shigella* lacks adhesion factors.

6. Keep all the buffers and reagents that are required at initiation of invasion and thereafter, warmed up to 37 °C before using. The secretion of T3SS effectors mediating invasion is temperature dependent. It is active above 35 °C, but inactive at room temperature. In order to synchronize the invasion, it is important to keep the bacteria in buffers at room temperature before invasion.
7. The medium containing gentamicin should be prepared on the day of use.
8. Even during longer incubations, gentamicin won't be able to penetrate the cells and kill the intracellular bacteria. The intracellular bacteria remain 'protected' all the times.
9. Deoxycholate is a detergent and therefore TC7 cell lysates should be made with little pipetting to avoid bubbles.
10. Use a dedicated incubator for infections.
11. When using the pipette controller, gently dispense the solutions with gravity ("G" setting in Accuripipette) or at the lowest flow speed into the 6-well plate to avoid detaching the cell monolayer.
12. Be careful while swirling the plate, washing the cells and during centrifugation, because the confluent cell monolayer detaches easily.
13. The TC7 cells can survive for about 6 h in a medium devoid of serum. Therefore, additional serum is not required for infection times up to about 6 h. However, for longer incubation times, serum may be added after gentamicin treatment.
14. For further reading and details on basic methods that have not been detailed in this manuscript (like bacterial inoculation, pouring the plates, etc.) the reader is suggested to go through the following books: A Laboratory Manual (Cappuccino and Welsh, 2017) and Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications (Freshney, 2010).

Recipes

1. Congo red solution

1% (w/v) Congo red in water

Weigh 1 g of Congo red dye and dissolve it in 70 ml of water. Bring the volume of the solution to 100 ml and filter sterilize the solution, using 0.2 µm membrane filters. The Congo red solution is stable for a long time at room temperature

2. Growth medium

DMEM

1x amino acid solution

100 U/ml Pen-Strep solution

10% heat inactivated FBS

To 500 ml DMEM, add 5 ml of amino acid solution, 5 ml of Pen-Strep solution and 50 ml of FBS (inactivated by incubation at 55 °C for 30 min)

Note: The growth medium must be stored at 4 °C and can be used for 3-4 weeks.

3. Infection buffer

DMEM (serum free)

20 mM HEPES, pH 7.4

To 50 ml of serum free DMEM, add 1 ml of 1 M sterile HEPES pH 7.4

Note: Infection buffer is stable at 4 °C for months.

4. Sodium Deoxycholate solution

0.5% sodium deoxycholate in PBS

To 40 ml of PBS, add 200 mg of sodium deoxycholate and filter-sterilize

Note: The solution is stable at room temperature for several months.

5. Gentamicin solution

50 µg/ml gentamicin in infection buffer

To 15 ml of infection buffer, add 25 µl of gentamicin from a stock solution of 30 mg/ml (prepared in water)

Note: The stock solution of gentamicin must be stored at -20 °C in small aliquots. Although the frozen stock solution is stable for several months, repeated freeze-thaw cycles may render the antibiotic inefficient. The working gentamicin solution should not be stored and always be freshly prepared.

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Competing interests

The authors declare no conflict of interest.

References

1. Ashida, H., Mimuro, H. and Sasakawa, C. (2015). [Shigella manipulates host immune responses by delivering effector proteins with specific roles.](#) *Front Immunol* 6: 219.
2. Cappuccino, J. G. and Welsh, C.T (2017). *Microbiology: A Laboratory Manual, 11th Edition.* ISBN: 978-0134298672.
3. Chantret, I., Rodolosse, A., Barbat, A., Dussault, E., Brot-Laroche, E., Zweibaum, A. and Rousset, M. (1994). [Differential expression of sucrase-isomaltase in clones isolated from early](#)

[and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation.](#)
J Cell Sci 107 (Pt 1): 213-225.

4. Freshney, R. I. (2010). *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications. Sixth Edition.* ISBN: 9780470528129.
5. Puher, A. and Sansonetti, P. J. (2014). [Type III secretion system.](#) *Curr Biol* 24(17): R784-791.
6. Puher, A., Tronchere, H., Payrastre, B., Nhieu, G. T. and Sansonetti, P. J. (2013). [A *Shigella* effector dampens inflammation by regulating epithelial release of danger signal ATP through production of the lipid mediator PtdIns5P.](#) *Immunity* 39(6): 1121-1131.
7. Sansonetti, P. J., Kopecko, D. J. and Formal, S. B. (1982). [Involvement of a plasmid in the invasive ability of *Shigella flexneri*.](#) *Infect Immun* 35(3): 852-860.
8. Shigellosis—Chapter 3—2018 Yellow Book | Travelers' Health | CDC. Available at: <https://wwwnc.cdc.gov/travel/yellowbook/2018/infectious-diseases-related-to-travel/shigellosis>.

Isolation and Long-term Cultivation of Mouse Alveolar Macrophages

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[Abstract] Alveolar macrophages (AM) are tissue-resident macrophages that colonize the lung around birth and can self-maintain long-term in an adult organism without contribution of monocytes. AM are located in the pulmonary alveoli and can be harvested by washing the lungs using the method of bronchoalveolar lavage (BAL). Here, we compared different conditions of BAL to obtain high yields of murine AM for *in vitro* culture and expansion of AM. In addition, we describe specific culture conditions, under which AM proliferate long-term in liquid culture in the presence of granulocyte-macrophage colony-stimulating factor. This method can be used to obtain large numbers of AM for *in vivo* transplantation or for *in vitro* experiments with primary mouse macrophages.

Keywords: Macrophage, Alveolar, Lungs, Self-renewal, Bronchoalveolar lavage, Primary cell culture

[Background] AM are resident tissue macrophages of the lungs with critical importance for immune regulation and surfactant homeostasis (Kopf *et al.*, 2015). Due to their localization in the airspace of the alveoli, AM are directly exposed to inhaled air and pathogens or other aerosolized particles. Consequently, AM play a crucial role in the initiation or suppression of inflammatory responses and are the subject of investigation in numerous studies that explore mechanisms of pulmonary diseases (Hodge *et al.*, 2007; Sun and Metzger, 2008; Happle *et al.*, 2014; Schneider *et al.*, 2014a; Machiels *et al.*, 2017; Yu *et al.*, 2017). Interestingly, AM in the mouse originate from fetal monocytes and are able to self-maintain their numbers *in vivo* without contribution of bone-marrow-derived monocytes under steady state conditions (Guilliams *et al.*, 2013; Hashimoto *et al.*, 2013). AM are unique in that they reside outside of the body surface and are directly exposed to the external environment. They can therefore be isolated with minimal tissue disturbance using bronchoalveolar lavage (BAL). We previously demonstrated that the self-renewal property of AM harvested by BAL is maintained in culture by growing AM long-term in liquid media or serially re-plating AM in semi-solid media (Soucie *et al.*, 2016; Imperatore *et al.*, 2017). Here, we describe the methodological advancements in BAL and specific culturing conditions optimized for long-term culture and high AM yields.

To obtain sufficient quality and numbers of AM, we tested different cell harvesting conditions and developed a culture method that allows long-term maintenance of AM *in vitro*. Our BAL method was based on earlier studies using pre-warmed PBS and EDTA for detaching AM from lung alveoli (Steele

et al., 2003; Zhang et al., 2008). Additionally, serum was added for cellular protection during the isolation period. Importantly, both omitting EDTA in the lavage buffer and using pre-cooled PBS for lavage resulted in lower AM yield in our hands. Our comparisons demonstrated that the variable with the largest effect on yield was the temperature of the BAL buffer (Figure 1). Whereas many earlier studies obtain BAL cell numbers below 2×10^5 per wild-type mouse (Table 1), our comparative analysis indicated that BAL cell numbers can be considerably increased by these optimizations. We generally obtained up to 5×10^5 - 7×10^5 live (Trypan-Blue-negative) cells per mouse using this method (Figure 1). FACS analysis revealed that more than 98% of cells were alive at the time of recording using the Zombie Violet Fixable Viability Kit, independent of whether the BAL was performed with 4 °C PBS or 37 °C PBS/EDTA/FBS. Our experience has shown that when performing dozens of BALs on the same day long waiting times on ice will result in higher cell death unless low amounts of FBS are added to the BAL buffer (typically 0.5%, although we have good experience with up to 2%).

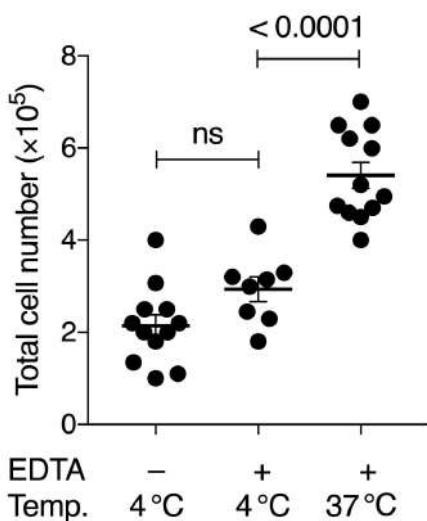


Figure 3. Comparison of BAL conditions using pre-cooled or pre-warmed PBS with or without 2 mM EDTA. Either 4 °C PBS without EDTA, 4 °C PBS with 2 mM EDTA and 0.5% FBS, or 37 °C PBS with 2 mM EDTA and 0.5% FBS was used. Numbers show the total amount of living cells (Trypan-Blue-negative) per BAL treatment per mouse. Each symbol denotes the mean cell count of 3 technical replicates of an individual mouse; horizontal lines indicate the mean, error bars show standard error of the mean (SEM); one-way ANOVA with Tukey's multiple comparisons test; ns, non-significant.

Table 1. Comparison of the efficiency of different BAL protocols. Total cell number in BAL fluid obtained in cited studies. Only counts from WT animals (or comparable conditions, such as control-treated WT animals) were considered. WT denotes wild-type mouse.

Mouse strain	Total cell number	Volume	Buffer conditions	References
WT (4 weeks)	20,000	2 x 0.4 ml	0.5 mM EDTA	Yu <i>et al.</i> , 2017
WT (control)	40,000	2 x 0.8 ml		Yuan <i>et al.</i> , 2015
WT (floxed)	30-40,000	3 x 0.4 ml		Schneider <i>et al.</i> , 2014b
WT (control)	50,000	2 x 1 ml	EDTA, 4 °C	Machiels <i>et al.</i> , 2017
WT	60,000	3 x 0.4 ml		Schneider <i>et al.</i> , 2014a
WT (6 weeks)	50-150,000	3 x 1 ml	0.5 mM EDTA	van de Laar <i>et al.</i> , 2016
WT (1 year)	100-200,000	3 x 1 ml	0.5 mM EDTA	van de Laar <i>et al.</i> , 2016
WT (control)	250,000	5 x 1 ml	5 mM EDTA	Qian <i>et al.</i> , 2018
WT (1 year)	250-300,000	5 x 1 ml		Suzuki <i>et al.</i> , 2014
WT	300-500,000	3 x 2 ml	0.5 mM EDTA, 37 °C	Zhang <i>et al.</i> , 2008
WT	400-500,000	3 x 1 ml	2% BSA, 2 mM EDTA	Dong <i>et al.</i> , 2018
WT (6-8 weeks)	500-700,000	10 x 1 ml	0.5% FBS, 2 mM EDTA, 37 °C	Soucie <i>et al.</i> , 2016, Imperatore <i>et al.</i> , 2017 and this study

Increased efficiency in harvesting BAL cells is advantageous for *in vivo* reconstitution of multiple recipient animals such as strains devoid of endogenous AM, such as GM-CSF-receptor-deficient mice (Guilliams *et al.*, 2013; van de Laar *et al.*, 2016). High starting cell numbers and viability of harvested cells will accelerate establishment of long-term AM cultures and improve cellular yield. We also noticed several culture conditions outlined in the detailed protocol that affect the quality, yield, doubling time and durability of the cultures. In order to avoid cell activation or death, several parameters need to be controlled. Firstly, we used exclusively sterile supplies and applied sterile handling techniques to avoid activation of AM. Secondly, we use non-treated plastic ware (not tissue-culture treated plates or dishes) and a gentle detachment protocol. Together, these technical improvements will be helpful for starting and maintaining a long-term AM culture.

Biochemical and genetic manipulations of macrophages that require a large number of cells could so far only be done in cell lines, such as RAW 264.7 or J774A.1 cells (Ralph and Nakoinz, 1975; Raschke *et al.*, 1978), oncogene-transformed cells, for example Myc (Baumbach *et al.*, 1986; Baumbach *et al.*, 1987) or SV40 transformed macrophages like IC-21 (Walker and Demus, 1975) or MH-S cells (Mbawuike and Herscowitz, 1989), macrophages differentiated from progenitors (Zhang *et al.*, 2008; Feijer *et al.*, 2013) or non-transformed but genetically modified macrophages, such as Maf-DKO macrophages (Aziz *et al.*, 2009). The ability to obtain large numbers of normal unmodified AM in culture allows such experiments in primary resident macrophages. Our studies on macrophage self-renewal mechanisms serve as an example (Soucie *et al.*, 2016; Imperatore *et al.*, 2017).

Materials and Reagents

1. 15-ml conical tubes (Corning, catalog number: 352196)

2. Bottle-top vacuum filter with 0.22 µm membrane (Corning, catalog number: 431161)
3. Plastic storage bottle (Corning, catalog number: 430281)
4. 70-µm sterile cell strainer (BD, catalog number: 340633)
5. 1-ml syringe (Braun, catalog number: 9161406V)
6. 18-G cannula (Braun, catalog number: 4667123)
7. Petri dish 94/16 mm (Greiner Bio-one, catalog number: 633181)
8. Non-treated 6-well plate (Nunc™, catalog number: 150239)
9. C57BL/6 mice (aged 6–10 weeks)
10. PBS, pH 7.2 (Thermo Fisher Scientific, Gibco™, catalog number: 20012019)
11. EDTA stock solution (e.g., 0.5 M, pH 8.0)
12. Hemolysis buffer (self-made or commercial, e.g., Morphisto, catalog number: 12146)
13. Trypan Blue solution 0.4% (Sigma-Aldrich, catalog number: T8154)
14. RPMI 1640 Medium, no glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 31870025)
15. Fetal bovine serum (Testing of different batches is recommended)
16. Gentamicin sulphate 50 mg/ml in aqueous solution (Lonza, catalog number: BE02-012E)
17. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
18. Sodium Pyruvate (100 mM) (Thermo Fisher Scientific, Gibco™, catalog number: 11360070)
19. GlutaMAX™ Supplement (Thermo Fisher Scientific, Gibco™, catalog number: 35050038)
20. Conditioned medium from J558L cell line transfected with murine GM-CSF cDNA as a source for GM-CSF (Zal *et al.*, 1994; Stockinger *et al.*, 1996; Rayasam, 2015)
21. ESGRO Complete Accutase (Merck, catalog number: SF006)
22. EGTA stock solution (e.g., 0.5 M, pH 8.0)
23. UltraComp eBeads™ Compensation Beads (Thermo Fisher Scientific, Invitrogen, catalog number: 01-2222-41)
24. Zombie Violet Fixable Viability Kit (BioLegend, catalog number: 423113)
25. FACS antibodies (as indicated in Table 2)
26. BAL buffer (see Recipes)
27. Complete medium (see Recipes)
28. AM culture medium (see Recipes)
29. Detachment medium (see Recipes)

Equipment

1. Pipettes
2. Mouse dissection tools (scissors, forceps)
3. Water bath set to 37 °C
4. Refrigerated benchtop centrifuge for spinning conical tubes

5. Hemocytometer (Roth, catalog number: T729.1)
6. Incubator (37 °C, 5% CO₂)
7. Inverse microscope

Procedure

A. Harvest alveolar macrophages by bronchoalveolar lavage (BAL)

1. For each mouse, prepare a 15-ml conical tube filled with 3 ml complete medium (see Recipes).
2. Warm-up BAL buffer (see Recipes) to 37 °C in a water bath. Keep warm during the whole procedure.
3. Euthanize the mouse by cervical dislocation without rupturing the jugular vein or the trachea to avoid exposing AM to CO₂ or isoflurane, which could affect functional properties of AM.
4. Using dissection tools, remove the skin, ribcage and muscles to expose both lungs and trachea. Avoid cutting or rupturing blood vessels.
Note: Since methods for the surgical exposure of lungs and the trachea have been published previously in this journal, the reader is referred to those protocols for instructions (Han and Ziegler, 2013; Tibbitt and Coquet, 2016; Jhingran et al., 2016; Sun et al., 2017).
5. Use a fine scissor to make a small incision in the upper part of the trachea just below the larynx. The part of the trachea facing downwards (away from the experimenter) should remain intact, do not cut through the whole trachea.
6. Use the incision to insert a slightly blunted 18-G cannula and direct the cannula 5 mm deeper into the trachea towards the lungs. Take care not to damage lung tissue.
7. Attach a 1 ml syringe filled with 1 ml warm BAL buffer onto the inserted cannula.
8. Inject 1 ml buffer while fixating the cannula position with the other hand.
9. Pull the plunger to collect BAL fluid in the syringe. About 800-900 µl can be recovered. Observe that the pressure should not be too high, otherwise the alveoli will burst and BAL fluid will be lost. Upon injection and collection, the lungs should visibly inflate and deflate.
10. Filter collected BAL fluid through a 70 µm cell strainer into the 15-ml tube with 3 ml complete medium from Step A1.
11. Repeat Steps A6-A10 for 9 more times each time with fresh warm BAL buffer. Pool cells into the same 15-ml tube.
12. Collect cells by centrifugation at 300 × g, 5 min at 4 °C. Remove supernatant. The cell pellet should be white. A red/pink color indicates that blood was accidentally collected during the BAL.
13. Add 1 ml hemolysis buffer for 2 min incubation at room temperature to lyse residual erythrocytes. Fill up tube with complete medium to stop lysis and collect cells by centrifugation as before. Remove supernatant. The color of the cell pellet should be white now.
14. Resuspend cell pellet in 500 µl BAL buffer and take a sample for counting using a hemocytometer chamber after staining with Trypan Blue to exclude dead cells. Count only live (Trypan-Blue negative) cells.

15. Calculate the total cell number per BAL. Typically, 5×10^5 - 7×10^5 live cells per adult wild-type mouse aged 6-8 weeks can be recovered when using pre-warmed BAL buffer, containing PBS with 2 mM EDTA and 0.5% serum.
16. Proceed to cell staining and flow cytometry analysis or *in vitro* cultivation.

B. Flow cytometric analysis of alveolar macrophages

1. Block unspecific binding sites on cells with TruStain fcX and concomitantly stain with Zombie Violet in 200 μ l cold PBS (without FBS) at 4 °C in the dark for 15 min (see Table 2).
2. Wash cells with cold BAL buffer by centrifugation at 300 $\times g$ for 5 min at 4 °C.
3. Stain cells in a volume of 100 μ l per 1 million cells according to Table 2 for 30 min at 4 °C in the dark using BAL buffer.

Table 2. FACS reagents used for staining BAL AM

	Conjugate	Clone	Dilution	Company, Cat. No.
TruStain fcX	–	93	1:200	BioLegend, 101320
ZombieViolet	BV421	–	1:400	BioLegend, 423113
SiglecF	PE	E50-2440	1:200	BD, 552126
CD11c	PE/Cy7	N418	1:400	BioLegend, 117318

4. Prepare compensation beads for each antibody conjugate.
5. Wash cells with BAL buffer, resuspend in 200 μ l BAL buffer for recording.
6. Record cells by flow cytometry after acquiring the compensation beads. AM are double-positive for SiglecF and CD11c (Figures 2A-2C), and > 98% viable (Figure 2D).

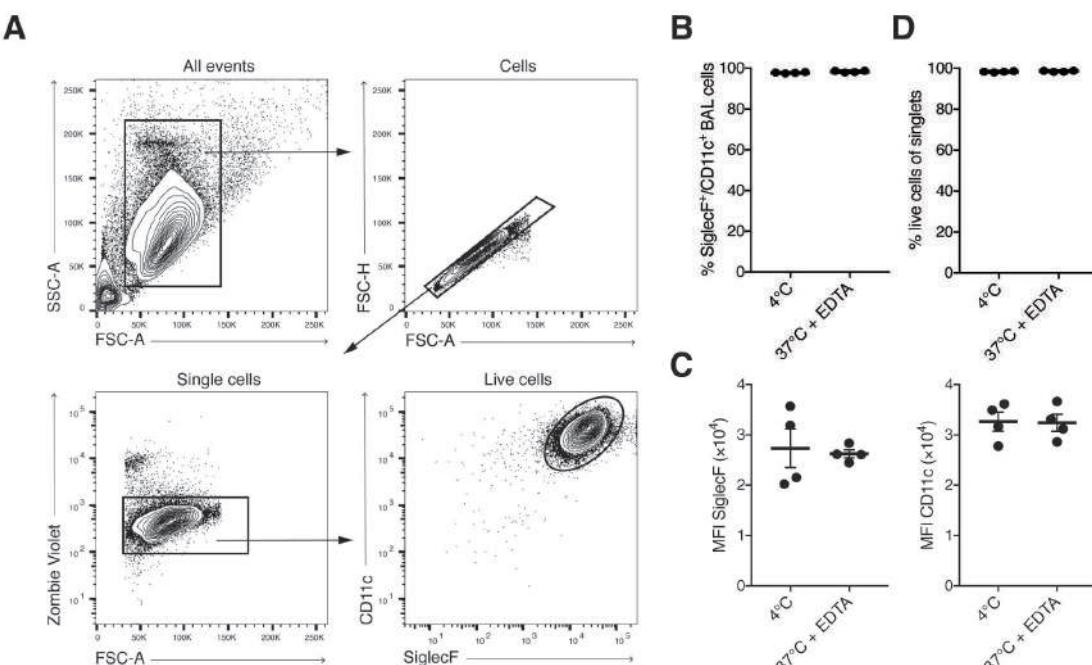


Figure 4. FACS analysis of BAL AM. A. Simple gating strategy for exclusion of doublets, dead cells. AM are SiglecF- and CD11c-positive. B-C. BAL cells harvested with pre-warmed BAL buffer containing EDTA are phenotypically not different from BAL cells harvested using pre-cooled PBS. Each symbol denotes 1 mouse. Typically, > 95% of BAL cells are AM. D. Viability analysis of BAL singlet cells assessed by staining with Zombie Violet Fixable Dye.

C. Cultivation of alveolar macrophages

1. Collect cells by centrifugation as before. Remove supernatant.
2. Plate 3×10^5 - 4×10^5 cells per well of a non-treated 6-well plate in 3 ml pre-warmed AM culture medium (see Recipes).

Note: Typically, 3×10^5 - 4×10^5 cells are plated in 1 well of a 6-well plate. If BAL cells of several mice are pooled, 1.1-1.2 million cells can be plated in a non-treated 94 mm Petri dish in 10 ml pre-warmed AM culture medium.

3. Add gentamicin to the AM culture (1:1,000).

Note: Gentamicin is omitted after the first medium change.

4. Incubate at 37 °C, 5% CO₂.
5. Replace culture supernatant after 6-18 h with fresh AM culture medium and discard the supernatant.

Note: Cells will adhere fully within a few hours after the first plating and we do not keep cells in suspension at the first medium exchange. However, for subsequent medium exchanges, the cells in the supernatant are collected as well since a typical AM culture consists of both adherent and suspended cells (see also Notes section).

6. Change medium every 2 days until the cell culture reaches confluence.
7. To change medium, transfer the medium and suspension cells into a 15 ml-tube. Add 2 ml warm AM culture medium to the well with adherent cells to prevent drying-out. Collect the suspension

cells using centrifugation at $300 \times g$ 5 min. Resuspend the pelleted cells in 1 ml warm AM culture medium and combine with adherent cells.

Note: Freshly harvested primary AM will double every 7-10 days (Soucie et al., 2016). If the majority of AM appear stretched (spindle-like) and activated, increasing the amount of conditioned medium or adding recombinant GM-CSF might help; however, proliferative capacity will be limited and it might advisable to start a new culture (see Figure 3 for exemplary images of early AM culture).

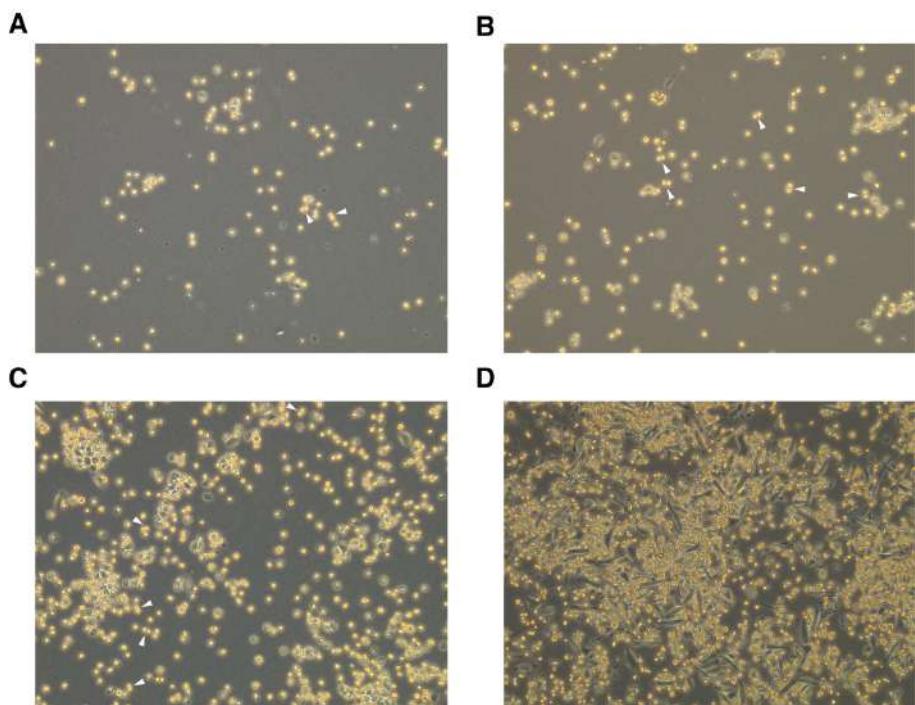


Figure 3. Representative images of AM culture within the first days after plating the cells.

A. AM culture with predominantly round-shaped cells that are partly floating and partly adherent on Day 1 after plating. B. Same culture as (A) on Day 2. C. Same culture as (A) on Day 4. D. Example for an AM culture with a large fraction of elongated, dark cells on Day 4 after plating. Arrowheads indicate dividing cells, 100 \times magnification.

8. To detach cells from a confluent well, collect suspension cells into a 15-ml tube.
9. Add 750 μ l detachment medium (see Recipes) to 1 well of a 6-well plate (or 3 ml to a 94 mm Petri dish) and incubate for 10-30 min at 37 °C.

Note: AM are very adherent and prone to rupture when using too harsh detachment procedures. Thus, the use of non-treated plastic ware and proper detachment medium is important (see Recipes). Ruptured cells in the culture medium might affect both activation status and proliferative capacity of AM.

10. Detachment of cells can be supported by pipetting on the plastic bottom gently to avoid cellular damage (see Notes).
11. Pool detached cells with cells in suspension and centrifuge cells at $300 \times g$ for 5 min.

12. Resuspend cell pellet in 1 ml warm AM cultured medium and take a sample for counting using a hemocytometer chamber after staining with Trypan Blue to exclude dead cells. Count only live (Trypan-Blue negative) cells.
13. If the cell number has doubled, add 5 ml warm AM culture medium and split into 2 wells of a 6-well plate (or correspondingly to 2x 94 mm Petri dishes). In general, the cell number plated is maintained around the values indicated above in the note to Step C2.

Note: Earlier, we could show that AM culture remains proliferative for at least 10 passages (Soucie et al., 2016). Since then, we have experience with AM cultures that remain proliferative even beyond 20 passages with no indication of a decline in proliferative capacity.

Data analysis

Harvested cells were counted manually using a hemocytometer and considering only Trypan-Blue-negative cells. Stained cells were recorded on a BD LSRII with 5 lasers using BD FACSDiva software and analyzed using FlowJo v10. Microscope images were acquired on an inverse microscope (Leica DMI1) equipped with a digital camera (MC120). Gating was performed as indicated in Figure 2A. To test for statistically significant differences between the means of three groups (Figure 1), one-way ANOVA with Tukey's multiple comparisons test was performed using GraphPad Prism 7. No data points were excluded.

Notes

1. Proliferative AM are round-shaped and semi-adherent. Re-plating of suspension AM will result in part of the cells attaching to the new well, while the other part remains in suspension. Take care to not lose the suspended cells when changing medium as this will reduce the number of proliferative cells and slow the expansion of the culture.
2. When detaching cells, do not pipet the cell suspension up and down extensively, this might affect the viability of the culture; if cells do not detach readily, collect detachment medium containing already detached cells and perform another round of incubation with fresh detachment medium and/or increase the incubation time. Late-passage cells require shorter incubation times (~ 5 min) than early-passage cultures (up to 30 min).
3. The percentage of conditioned medium should be titrated after preparation of each batch by testing the growth of AM in the presence of various amounts of conditioned medium (e.g., 1, 2, 5, 10% in complete medium). In our batches, we use typically 1%-3% conditioned medium diluted into complete medium (e.g., 100 µl in 10 ml), which corresponds roughly to 2-5 ng/ml purified recombinant mouse GM-CSF.
4. We successfully replaced conditioned medium with 20 ng/ml recombinant GM-CSF (Peprotech) for long-term culture. Lower GM-CSF concentrations might be sufficient but have not been tested.

Recipes

1. BAL buffer
 - PBS
 - 2 mM EDTA (dilute 1:250 from 0.5 M EDTA stock solution)
 - 0.5% Fetal bovine serum (FBS)
 - Sterile-filter using vacuum filtration and keep at 4 °C until use
2. Complete medium
 - RPMI 1640
 - 1x GlutaMAX
 - 1x Pyruvate
 - 1x Penicillin/Streptomycin
 - 10% FBS
 - Sterile-filter using vacuum filtration and keep at 4 °C until use
3. AM culture medium
 - Supplement complete medium with 1-5% conditioned medium containing mouse GM-CSF (needs to be titrated)
 - Pre-warm an aliquot in a water bath to 37 °C before use
4. Detachment medium
 - ESGRO Complete Accutase
 - 1 mM EGTA (dilute 1:500 from 0.5 M EGTA stock solution)
 - Aliquot and freeze at -20 °C. Pre-warm an aliquot in the water bath to 37 °C before use

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Competing interests

The authors declare no competing financial interests.

Ethics

Animal husbandry and mouse work were conducted in accordance with the German Animal Welfare legislation, after the approval by the Landesamt für Gesundheit und Soziales (for work in Berlin, following the guidelines of the Institutional Animal Care and Use Committee of the Max Delbrück Centrum für Molekulare Medizin) and after the approval by the Landesdirektion Sachsen (for work in Dresden).

References

1. Aziz, A., Soucie, E., Sarrazin, S. and Sieweke, M. H. (2009). [MafB/c-Maf deficiency enables self-renewal of differentiated functional macrophages](#). *Science* 326(5954): 867-871.
2. Baumbach, W. R., Keath, E. J. and Cole, M. D. (1986). [A mouse c-myc retrovirus transforms established fibroblast lines *in vitro* and induces monocyte-macrophage tumors *in vivo*](#). *J Virol* 59(2): 276-283.
3. Baumbach, W. R., Stanley, E. R. and Cole, M. D. (1987). [Induction of clonal monocyte-macrophage tumors *in vivo* by a mouse c-myc retrovirus: rearrangement of the CSF-1 gene as a secondary transforming event](#). *Mol Cell Biol* 7(2): 664-671.
4. Dong, Y., Poon, G. F. T., Arif, A. A., Lee-Sayer, S. S. M., Dosanjh, M. and Johnson, P. (2018). [The survival of fetal and bone marrow monocyte-derived alveolar macrophages is promoted by CD44 and its interaction with hyaluronan](#). *Mucosal Immunol* 11(3): 601-614.
5. Fejer, G., Wegner, M. D., Gyory, I., Cohen, I., Engelhard, P., Voronov, E., Manke, T., Ruzsics, Z., Dolken, L., Prazeres da Costa, O., Branzk, N., Huber, M., Prasse, A., Schneider, R., Apte, R. N., Galanos, C. and Freudenberg, M. A. (2013). [Nontransformed, GM-CSF-dependent macrophage lines are a unique model to study tissue macrophage functions](#). *Proc Natl Acad Sci U S A* 110(24): E2191-2198.
6. Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., Deswarte, K., Malissen, B., Hammad, H. and Lambrecht, B. N. (2013). [Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF](#). *J Exp Med* 210(10): 1977-1992.
7. Han, H. and Ziegler, S. F. (2013). [Bronchoalveolar lavage and lung tissue digestion](#). *Bio-protocol* 3(16): e859.
8. Happle, C., Lachmann, N., Skuljec, J., Wetzke, M., Ackermann, M., Brennig, S., Mucci, A., Jirmo, A. C., Groos, S., Mirenska, A., Hennig, C., Rodt, T., Bankstahl, J. P., Schwerk, N., Moritz, T. and Hansen, G. (2014). [Pulmonary transplantation of macrophage progenitors as](#)

- [effective and long-lasting therapy for hereditary pulmonary alveolar proteinosis.](#) *Sci Transl Med* 6(250): 250ra113.
9. Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M. B., Leboeuf, M., Becker, C. D., See, P., Price, J., Lucas, D., Greter, M., Mortha, A., Boyer, S. W., Forsberg, E. C., Tanaka, M., van Rooijen, N., Garcia-Sastre, A., Stanley, E. R., Ginhoux, F., Frenette, P. S. and Merad, M. (2013). [Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes.](#) *Immunity* 38(4): 792-804.
10. Hodge, S., Hodge, G., Ahern, J., Jersmann, H., Holmes, M. and Reynolds, P. N. (2007). [Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease.](#) *Am J Respir Cell Mol Biol* 37(6): 748-755.
11. Imperatore, F., Maurizio, J., Vargas Aguilar, S., Busch, C. J., Favret, J., Kowenz-Leutz, E., Cathou, W., Gentek, R., Perrin, P., Leutz, A., Berruyer, C. and Sieweke, M. H. (2017). [SIRT1 regulates macrophage self-renewal.](#) *EMBO J* 36(16): 2353-2372.
12. Jhingran, A., Kasahara, S. and Hohl, T. M. (2016). [Flow cytometry of lung and bronchoalveolar lavage fluid cells from mice challenged with fluorescent Aspergillus reporter \(FLARE\) conidia.](#) *Bio-protocol* 6(18): e1927.
13. Kopf, M., Schneider, C. and Nobs, S. P. (2015). [The development and function of lung-resident macrophages and dendritic cells.](#) *Nat Immunol* 16(1): 36-44.
14. Machiels, B., Dourcy, M., Xiao, X., Javaux, J., Mesnil, C., Sabatel, C., Desmecht, D., Lallemand, F., Martinive, P., Hammad, H., Guilliams, M., Dewals, B., Vanderpasschen, A., Lambrecht, B. N., Bureau, F. and Gillet, L. (2017). [A gammaherpesvirus provides protection against allergic asthma by inducing the replacement of resident alveolar macrophages with regulatory monocytes.](#) *Nat Immunol* 18(12): 1310-1320.
15. Mbawuike, I. N. and Herscowitz, H. B. (1989). [MH-S, a murine alveolar macrophage cell line: morphological, cytochemical, and functional characteristics.](#) *J Leukoc Biol* 46(2): 119-127.
16. Qian, G., Jiang, W., Zou, B., Feng, J., Cheng, X., Gu, J., Chu, T., Niu, C., He, R., Chu, Y. and Lu, M. (2018). [LPS inactivation by a host lipase allows lung epithelial cell sensitization for allergic asthma.](#) *J Exp Med* 215(9): 2397-2412.
17. Ralph, P. and Nakoinz, I. (1975). [Phagocytosis and cytology by a macrophage tumour and its cloned cell line.](#) *Nature* 257(5525): 393-394.
18. Raschke, W. C., Baird, S., Ralph, P. and Nakoinz, I. (1978). [Functional macrophage cell lines transformed by Abelson leukemia virus.](#) *Cell* 15(1): 261-267.
19. Rayasam, A. (2015). [Isolating and culturing dendritic cells \(Dendritic Cell J558 Protocol\).](#) *Protoc Exch* 22-24.
20. Schneider, C., Nobs, S. P., Heer, A. K., Kurrer, M., Klinke, G., van Rooijen, N., Vogel, J. and Kopf, M. (2014a). [Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection.](#) *PLoS Pathog* 10(4): e1004053.
21. Schneider, C., Nobs, S. P., Kurrer, M., Rehrauer, H., Thiele, C. and Kopf, M. (2014b). [Induction of the nuclear receptor PPAR-γ by the cytokine GM-CSF is critical for the differentiation of fetal](#)

- [monocytes into alveolar macrophages.](#) *Nat Immunol* 15(11): 1026-1037.
22. Soucie, E. L., Weng, Z., Geirsdottir, L., Molawi, K., Maurizio, J., Fenouil, R., Mossadegh-Keller, N., Gimenez, G., VanHille, L., Beniazza, M., Favret, J., Berruyer, C., Perrin, P., Hacohen, N., Andrau, J. C., Ferrier, P., Dubreuil, P., Sidow, A. and Sieweke, M. H. (2016). [Lineage-specific enhancers activate self-renewal genes in macrophages and embryonic stem cells.](#) *Science* 351(6274): aad5510.
23. Steele, C., Marrero, L., Swain, S., Harmsen, A. G., Zheng, M., Brown, G. D., Gordon, S., Shellito, J. E. and Kolls, J. K. (2003). [Alveolar macrophage-mediated killing of *Pneumocystis carinii* f. sp. *muris* involves molecular recognition by the Dectin-1 β-glucan receptor.](#) *J Exp Med* 198(11): 1677-1688.
24. Stockinger, B., Zal, T., Zal, A. and Gray, D. (1996). [B cells solicit their own help from T cells.](#) *J Exp Med* 183: 891-899.
25. Sun, F., Xiao, G. and Qu, Z. (2017). [Murine bronchoalveolar lavage.](#) *Bio-protocol* 7(10): e2287.
26. Sun, K. and Metzger, D. W. (2008). [Inhibition of pulmonary antibacterial defense by interferon-γ during recovery from influenza infection.](#) *Nat Med* 14(5): 558-564.
27. Suzuki, T., Arumugam, P., Sakagami, T., Lachmann, N., Chalk, C., Sallese, A., Abe, S., Trapnell, C., Carey, B., Moritz, T., Malik, P., Lutzko, C., Wood, R. E. and Trapnell, B. C. (2014). [Pulmonary macrophage transplantation therapy.](#) *Nature* 514(7523): 450-454.
28. Tibbitt, C. and Coquet, J. M. (2016). [House dust mite extract and cytokine instillation of mouse airways and subsequent cellular analysis.](#) *Bio-protocol* 6(14): e1875.
29. van de Laar, L., Saelens, W., De Prijck, S., Martens, L., Scott, C. L., Van Isterdael, G., Hoffmann, E., Beyaert, R., Saeyns, Y., Lambrecht, B. N. and Guilliams, M. (2016). [Yolk sac macrophages, fetal liver, and adult monocytes can colonize an empty niche and develop into functional tissue-resident macrophages.](#) *Immunity* 44(4): 755-768.
30. Walker, W. S. and Demus, A. (1975). [Antibody-dependent cytolysis of chicken erythrocytes by an *in vitro*-established line of mouse peritoneal macrophages.](#) *J Immunol* 114(2 pt 2): 765-769.
31. Yu, X., Buttigereit, A., Lelios, I., Utz, S. G., Cansever, D., Becher, B. and Greter, M. (2017). [The cytokine TGF-β promotes the development and homeostasis of alveolar macrophages.](#) *Immunity* 47: 903-912.e4.
32. Yuan, X., Shan, M., You, R., Frazier, M. V., Hong, M. J., Wetsel, R. A., Drouin, S., Seryshev, A., Song, L. Z., Cornwell, L., Rossen, R. D., Corry, D. B. and Kheradmand, F. (2015). [Activation of C3a receptor is required in cigarette smoke-mediated emphysema.](#) *Mucosal Immunol* 8(4): 874-885.
33. Zal, T., Volkmann, A. and Stockinger, B. (1994). [Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen.](#) *J Exp Med* 180(6): 2089-2099.
34. Zhang, X., Goncalves, R. and Mosser, D. M. (2008). [The isolation and characterization of murine macrophages.](#) *Curr Protoc Immunol* Chapter 14: Unit 14.1.

Quantitation of TLR4 Internalization in Response to LPS in Thioglycollate Elicited Peritoneal Mouse Macrophages by Flow Cytometry

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[Abstract] Internalization of cell surface Toll-like Receptor 4 (TLR4) is a convenient read-out to measure LPS dependent activation of the TRIF adaptor pathway. We here provide a protocol to quantify the LPS dependent internalization of TLR4 using thioglycollate-elicited peritoneal macrophages by flow cytometry.

Keywords: TLR4, LPS, TRIF, CD14, Endocytosis, Peritoneal macrophages

[Background] Toll-like Receptor 4 (TLR4) is unique among the Toll-like receptors in that it can deliver qualitatively different signals from two distinct cellular locations (reviewed in Takeda and Akira, 2004). Cell surface TLR4/MD2 is initially ligated by extracellular bacterial lipopolysaccharide (LPS) and engages the intracellular adaptor MAL and MyD88. Subsequently a fraction of cell surface LPS bound TLR4/MD2 will undergo CD14 and clathrin dependent internalization and translocation to an early endosomal compartment (Zanoni *et al.*, 2011). In this endosomal compartment TLR4, engages the signaling adaptors TRAM and TRIF. Much of the regulation of TLR4 internalization and transport remains to be discovered. A flow cytometric method for quantifying loss of TLR4 surface expression, as a metric for endosomal translocation was first described in Bone Marrow Derived Macrophages (BMDMs) by Jonathan Kagan and colleagues (Kagan *et al.*, 2008). Here we describe a modified version of this protocol to enable quantitation of TLR4 internalization in thioglycollate-elicited peritoneal macrophages on which we have published (Rajaiah *et al.*, 2015; Perkins *et al.*, 2018).

Materials and Reagents

A. Consumables

1. Pipette tips
2. Ice buckets
3. Pipettes
4. 10 ml syringes (BD, catalog number: 309604)
5. 18 G x 1 needles (BD, catalog number: 305195)
6. 50 ml conical tubes (Denville, catalog number: C1062-P)
7. FACS tubes (round), sterile, with caps (Corning, Falcon, catalog number: 352054)
8. FACS tubes (round), non-sterile, without caps
9. Cell strainers, 70 µm (Fisherbrand, catalog number: 22363548)

10. 0.22 µm filters for filtering culture media and FACS buffer (Sarstedt, catalog number: 83.1823.001)

B. Reagents

1. Ultra pure LPS, purified from *E. coli* K235 by the hot phenol method as previously described (McIntire *et al.*, 1967)
2. Unconjugated rat IgG2a, λ anti-mouse CD16/32 (FC receptor) antibody, clone 93 (Biolegend, catalog number: 101302)
3. PE-conjugated anti-mouse CD284 (TLR4) antibody, clone SA15-21 (Biolegend, catalog number: 145404)
4. PE-conjugated Isotype control antibody, Rat IgG2a, κ, clone RTK2758 (Biolegend, catalog number: 400507)
5. Optional: other antibodies for cellular identification (e.g. Alexafluor-conjugated anti mouse F480; Biolegend, catalog number: 123119)
6. Optional: paraformaldehyde solution (PFA), 4% in PBS (Affymetrix, catalog number: 19943)
7. Sterile Saline (Baxter, catalog number: 0338-0048-03)
8. Optional: ACK lysis buffer (Quality Biological, Inc., catalog number: 118-156-721)
9. Sterile Phosphate Buffered Saline (PBS) (Corning, catalog number: 21-040-CV)
10. Culture Media (see Recipes)
 - a. RPMI 1640 (Corning, catalog number: 15-040-CV)
 - b. Fetal Bovine Serum (FBS) (Atlanta Biologicals, catalog number: S11550)
 - c. Penicillin/Streptomycin (Gibco, catalog number: 15140-122)
 - d. L-glutamine (Gibco, catalog number: 25030-081)
11. FACS buffer (see Recipes)
 - a. Sterile Phosphate Buffered Saline (PBS) (Corning, catalog number: 21-040-CV)
 - b. EDTA, 0.5 M (Quality Biological, Inc., catalog number: 351-027-101)
 - c. Fetal Bovine Serum (FBS) (Atlanta Biologicals, catalog number: S11550)

Equipment

1. Light microscope with hemocytometer slide
2. 37 °C incubator with CO₂ control designated for cell culture work (no bacterial/viral work)
3. Biosafety Cabinet designated for cell culture work (no bacterial/viral work) Sterile Culture Hood
4. Vortex Genie
5. Refrigerated Swing Bucket Centrifuge with inserts for 50 ml conical tubes and flow cytometry tubes
6. Flow Cytometer (e.g., Becton Dickinson LSR II)
7. Computer for analysis

Software

1. FCS Express 6.0 Flow (De Novo Software; Glendale, CA USA)
2. GraphPad Prism 7 (Graph Pad Software; La Jolla, CA USA)

Procedure

Day 1

1. Collect macrophages from previously thioglycollate injected animals by peritoneal lavage using 2 x 10 ml of sterile saline, using the 10 ml syringe with an 18 G needle attached. We usually pool peritoneal exudates from 2-5 littermates of the same gender to ensure sufficient yield for all controls and time points. A typical protocol can be found here: <https://www.jove.com/video/1488/isolation-of-mouse-peritoneal-cavity-cells>.
2. Pellet cells by centrifugation at 93-218 $\times g$ (1,000 rpm on a Thermo Scientific TX-750 4 rotor) for 10 min, and resuspend in culture media.
3. Count cells using a hemocytometer (or cell counter) and add 1×10^6 - 1.5×10^6 Thioglycollate elicited peritoneal macrophages (TEPMs) to each sterile FACS tube in 1.5 ml culture media (see Recipes below). A typical experiment includes three time points in unstimulated and three timepoints in stimulated macrophages. For each timepoint, you can set up tubes as following: 1) A tube for unstained control, 2) A tube for isotype control, 3) A tube for unstimulated control with TLR4 antibody stain, 4) A tube for LPS stimulation with TLR4 antibody stain, 5) single-color controls if cells are to be co-stained with other cell markers, and 6) A tube for dual color stain if using. Lightly recap FACS tubes to allow gas exchange in the incubator.
4. Allow TEPM to rest overnight in a 37 °C incubator with 5% CO₂.

Day 2

5. The following morning, add 100 ng/ml ultrapure *E. coli* K235 LPS to each tube to be used to measure TLR4 internalization (excepting unstimulated controls). Lightly recap FACS tubes and return to the 37 °C incubator during stimulation.
6. At appropriate time points, transfer each tube of control or LPS stimulated TEPMs to an ice bath and add two volumes ice-cold culture media or FACS buffer (see Recipes below) to arrest TLR4 internalization.
7. Pellet cells in a pre-cooled swing bucket by centrifuging at 112-264 $\times g$ (1,100 rpm on TX-750 4 rotor) for 5-10 min.

Note: For this and all subsequent washes: decant media, resuspend cells by gently running the flow tubes over an empty tube rack, and immediately return to ice bucket. If more time points will be collected, resuspend these cells in ice-cold culture media and keep on ice until the last samples are removed from the incubator.

8. Wash cells twice more with ice-cold FACS buffer. Resuspend in 100 μ l final volume of ice-cold FACS buffer.
9. Add 2 μ g α FC CD16/32—(approx. 4 μ l of Biolegend #101302) Ab and incubate for 15 min on ice. This prevents F_c mediated nonspecific antibody binding.
10. Add 0.5 μ g PE labeled anti-TLR4 Ab or Isotype control (optional: co-stain with other antibodies to aid in identification of cellular subsets) and incubate for 30 min on ice.
11. Wash cells 3 x with ice-cold FACS buffer.
12. Wash 1 x with ice-cold PBS.
13. Either proceed to Flow Cytometry run immediately or fix cells using 1% PFA (final concentration) in 100 μ l PBS. Resuspend cells in 0.5-1.0 ml PBS and pass samples through a 70 μ m cell strainer into a clean flow tube (not required to be sterile) to remove clumps just prior to running on a flow cytometer.

Data analysis

1. Flow cytometry analysis
 - a. For flow cytometry analysis, select single cells by forward scatter (FSC) profile (Height and Area of peaks). Set “stop gate” to \geq 20,000 single events for each sample. Leave “record gate” to record all events.
 - b. For analysis, gate events on FSC-A and SSC-A profile to exclude dead cells and cellular debris.
 - c. TLR level can be visualized in a histogram for PE intensity.
 - d. Data (all events) are exported as FCS3.0 files.
2. Data analysis for the measurement of TLR4 endocytosis.
 - a. Open data files in FCS Express 6.0 Flow to fine tune “single cell” and “live macrophage” gates.
 - b. Record the median fluorescence intensity (MFI) for each sample.
 - c. Subtract the MFI of the unstained cells (autofluorescence) from the MFI of each sample.
 - d. Average the MFI of the “0 min time point” TLR4-stained samples (do this for each genotype, if multiple strains of mice were used)—and normalize the MFI data to the average (Time 0 min = 100%).
 - e. The internalization rate is calculated as the % of TLR4 lost from the surface over time.
3. Expected results: In wild type mice, the level of TLR4 surface expression in unstimulated cells should remain constant over the course of the experiment, and be well above the level of the isotype control-stained cells. LPS-stimulated cells should lose surface expression at a steady rate (at least over the first 90 min). For example please see Figure 1.

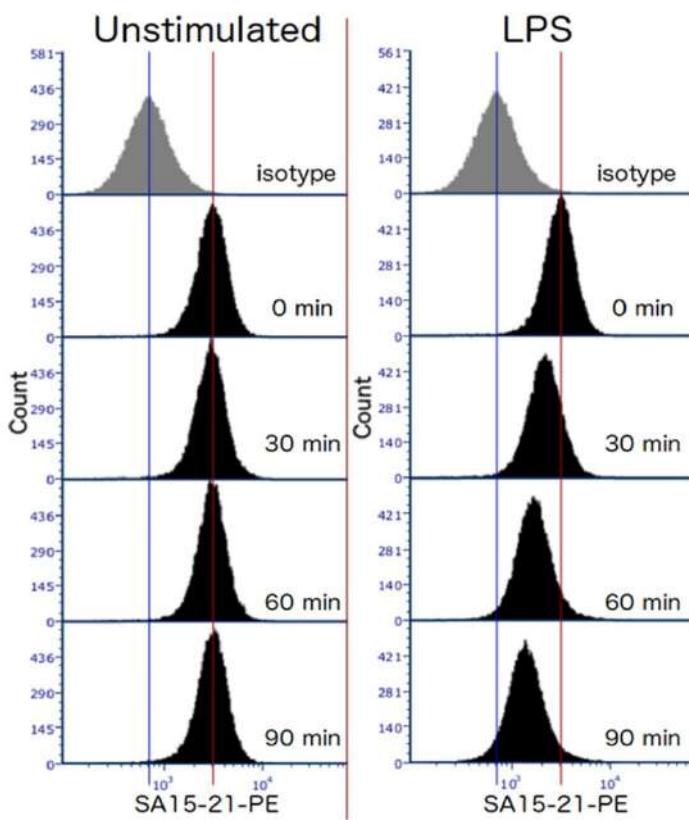


Figure 1. Representative data showing TLR4 internalization in response to 100 ng/ml *E. coli* LPS in TEPMs at the indicated time points. TEPMs were stained with anti-mouse TLR4 antibody (clone SA15-21) and analyzed by flow cytometry.

Notes

1. We strongly recommend testing different lots of thioglycollate before starting these experiments, as endotoxin contamination is common and will pre-activate macrophages.
2. The yield of thioglycollate-elicited macrophages can vary based not only on the lot of thioglycollate, but also on the experience level of the lab member performing the peritoneal lavage, and the age and strain of the mice. The following table (Table 1) shows the range of peritoneal lavage cell yields from our laboratory for 6-8 weeks old mice of commonly used strains.

Table 1. Typical yields of thioglycollate elicited macrophages. For each mouse genotype, expected yields of macrophages obtained following peritoneal lavage are provided. For C57BL/6J animals, average yields obtained by new investigators are also indicated.

Strain	Novice	Experienced
C57BL/6J	0.8-2.1 x 10 ⁷	1.4-9.2 x 10 ⁷ Median: 4.1 x 10 ⁷
TLR4 ^{-/-}	N/A	0.7-3.2 x 10 ⁷ Median: 2.1 x 10 ⁷
Ticam1 ^{-/-}	N/A	2.2-3.2 x 10 ⁷
Ifnb ^{-/-}	N/A	1.6-2.8 x 10 ⁷

3. If the cell pellets from the peritoneal lavage look bloody, we perform red blood cell lysis using ACK buffer, followed by two washes with sterile PBS before resuspending in culture media.
4. It is important to allow the macrophages to rest overnight after isolation in order to achieve consistent kinetics of internalization from experiment to experiment.
5. Inspect each tube before adding LPS. Any tubes that have acidified or become cloudy in the overnight incubation are probably contaminated and should be discarded.
6. The success of this experiment depends on keeping the cells cold to prevent further internalization of the TLR4 complex. At cold temperatures, the cell membranes change physical properties from fluid to gel (Thewalt and Bloom, 1992; Spink *et al.*, 1996). Protein complexes required for internalization will still form inside the membrane and as soon as membranes liquidize, internalization will proceed. Therefore, please be sure to pre-cool centrifuges and have an ice bucket and ice-cold FACS buffer or media available for rapid-cooling of cells at the time they are removed from the incubator. Minimize the time that the lid of the centrifuge is open, avoid touching the bottom of the flow cytometry tubes, and return tubes to the ice bucket immediately when removing from centrifuge. Without touching the bottom, you can hold up the tubes against a light to visually inspect the tubes after their centrifugation: Pellets of cold cells have higher opacity. Experiments can also be performed in a cold-room.
7. This assay is compatible with surface staining of other cell markers. If intracellular staining is required, we recommend setting up a parallel set of samples for permeabilization.

Recipes

1. Culture Media
 - a. RPMI 1640 + 2% FBS + 2 mM L-glutamine + 1% Pen/Strep
 - b. Filter sterilize through 0.22 µm filter
 - c. Store at 4 °C for up to three weeks
2. FACS buffer

- a. Ice-cold PBS + 0.5% FBS + 2 mM EDTA
- b. Store at 4 °C for up to three weeks

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Competing interests

We declare there are no competing interests related to this work.

References

1. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S. and Medzhitov, R. (2008). [TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-β](#). *Nat Immunol* 9(4): 361-368.
2. McIntire, F. C., Sievert, H. W., Barlow, G. H., Finley, R. A. and Lee, A. Y. (1967). [Chemical, physical, biological properties of a lipopolysaccharide from Escherichia coli K-235](#). *Biochemistry* 6(8): 2363-2372.
3. Perkins, D. J., Richard, K., Hansen, A. M., Lai, W., Nallar, S., Koller, B. and Vogel, S. N. (2018). [Autocrine-paracrine prostaglandin E2 signaling restricts TLR4 internalization and TRIF signaling](#). *Nat Immunol* 19(12): 1309-1318.
4. Rajaiah, R., Perkins, D. J., Ireland, D. D. and Vogel, S. N. (2015). [CD14 dependence of TLR4 endocytosis and TRIF signaling displays ligand specificity and is dissociable in endotoxin tolerance](#). *Proc Natl Acad Sci U S A* 112(27): 8391-8396.
5. Spink, C. H., Manley, S. and Breed, M. (1996). [Thermodynamics of transfer of cholesterol from gel to fluid phases of phospholipid bilayers](#). *Biochim Biophys Acta* 1279(2): 190-196.
6. Takeda, K. and Akira, S. (2004). [TLR signaling pathways](#). *Semin Immunol* 16(1): 3-9.
7. Thewalt, J. L. and Bloom, M. (1992). [Phosphatidylcholine: cholesterol phase diagrams](#). *Biophys J* 63(4): 1176-1181.
8. Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F. and Kagan, J. C. (2011). [CD14 controls the LPS-induced endocytosis of Toll-like receptor 4](#). *Cell* 147(4): 868-880.

Lysosome Targeting RedGreen-assay: Selective Autophagy Sensing Assay for Mammalian Cells

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[Abstract] The process of autophagy is an essential cellular mechanism, required to maintain general cell health through the removal of dysfunctional organelles, such as the ER, peroxisomes and mitochondria, as well as protein aggregates, and bacteria. Autophagy is an extremely dynamic process, and tools are constantly being developed to study the various steps of this process. This protocol details a method to study the end steps of autophagy-lysosomal fusion and the formation of the autolysosome. Many techniques have been used to study the various steps of the autophagy process. Here we describe the RedGreen-assay (RG-assay), an immunofluorescence-based technique used to visualize the targeting of substrates to the autolysosome in live cells. This technique takes advantage of the low lysosomal pH and over-expression of a tandem GFP-mCherry tagged protein targeted to an organelle of interest. While in the neutral cytosol or autophagosome, both GFP and RFP will fluoresce. However, within the autolysosome, the GFP signal is quenched due to the low pH environment and the RFP emission signal will predominate. This technique is readily quantifiable and amenable to high throughput experiments. Additionally, by tagging the GFP-RFP tandem fluorescent protein with organelle specific targeting sequences, it can be used to measure a wide range of substrates of autophagy.

Keywords: Autophagy, Pexophagy, Mitophagy, Lysosome, Autolysosome, Microscopy, Live-cell imaging

[Background] Macroautophagy (herein referred to as autophagy) is an essential cellular process, required for the removal of large cellular material. It can be both general and selective, either removing bulk material or specific cytoplasmic material, respectively. The method described in this protocol can be used in the study of either process, but the focus here will be on selective autophagy. In brief, selective autophagy in mammalian cells begins with the tagging of damaged organelles or other cellular material, generally with the small protein ubiquitin. The collection of ubiquitinated proteins on the substrate allows the recruitment of autophagy receptors which interact with the ubiquitin protein and with the ATG8 family of autophagic factors on developing phagophores through their LC3 interacting region (LIR). This interaction results in the engulfment of the cargo by the phagophore, producing an autophagosome. Finally, the autophagosome fuses with the lysosome, producing an autolysosome and degrading the cargo within. Selective autophagy is responsible for degrading cargo such as

peroxisomes (pexophagy), mitochondria (mitophagy), protein aggregates (aggrephagy) and bacteria (xenophagy). For a review, refer to Lahiri *et al.*, 2019 and Morishita and Mizushima, 2019. As autophagy proceeds in many steps, different assays have been developed to study the various stages. Historically, the most popular assay to detect changes in autophagy induction is the activation of the ATG8 family of proteins, particularly LC3B. Another common assay for detecting targeting of substrate to the autolysosome is the co-localization between the cargo with either the autophagosome marker LC3, or with various lysosomal markers such as Lamp1. Co-localization studies are technically easy to perform, can be performed in a high throughout manner and can detect specific cargo within the lysosome easily through the use of fluorescent tagging. However, it is limited by the microscope resolution, and the cargo can be difficult to detect due to degradation. For a review describing these and other methods for detecting autophagy, refer to Klionsky *et al.*, 2016.

The protocol described here, lysosome targeting Red-Green (RG) assay, can be used to study the end stages of autophagy, namely the localization of the cargo to the autolysosome. This assay takes advantage of the acidic pH of the lysosome (~4.6) and its ability to quench GFP ($pK_a \sim 6$) fluorescence without affecting RFP ($pK_a = 4.5$) fluorescence. Through the use of a tandem RFP-GFP fusion protein targeted to an organelle of choice, cargo that is cytosolic or within an autophagosome, and therefore exposed to neutral pH, will appear yellow when using pseudo colors green for GFP and red for RFP due to the fluorescence of both GFP and RFP. Upon lysosomal fusion, the labeled cargo will be exposed to an acidic pH, quenching GFP fluorescence and resulting in mainly RFP fluorescence (Figure 1). Again, this can be detected by microscopy and the ratio of cargo with RFP-only fluorescence can be compared to the total RFP fluorescence, to obtain a measure of autophagy. This method was first used to study aggrephagy (Pankiv *et al.*, 2007). By tagging LC3 with a tandem GFP-mCherry, autophagic vesicles and autolysosomes were able to be readily visualized and distinguished. We have adopted the principle used with LC3 to visualize the targeting of peroxisomes to autolysosomes. This is done by tagging the peroxisome targeting transmembrane domain of PEX26 with the tandem fluorophore, allowing it to be localized to the cytosolic face of the peroxisomal membrane (Deosaran *et al.*, 2013; Riccio *et al.*, 2019). This allows for the visualization of pexophagy specifically. Others have also used a similar technique to study pexophagy, where the fluorescent proteins were targeted to the peroxisome matrix through the use of a matrix targeting sequence (Nazarko *et al.*, 2014). Importantly, our method, targeting the fluorophore to the cytosolic face of the peroxisome membrane, allows for increased sensitivity of the tandem fluorophore to the acidic environment of the lysosome. Additionally, our quantification method differs, taking into account the relative GFP and mCherry fluorescence in order to determine the quantity of peroxisomes targeted to the lysosome. In addition to our use of the tandem fluorophore in pexophagy, we and others have used this method to quantify mitophagy (Allen *et al.*, 2013; Kim *et al.*, 2014; Wang *et al.*, 2015).

We have shown that the use of the tandem fluorophore is a reproducible method for observing and quantifying selective autophagy. As displayed above, the tandem mCherry-GFP lysosome targeting assay is extremely flexible, and can be used to measure various forms of selective autophagy, as well as general autophagy. Through the pH sensitivity of the fluorophores, it is a reliable method to detect

lysosomal targeting. Additionally, because GFP and mCherry are expressed at equal levels, comparing the fluorescent intensities of mCherry to GFP allows for a reproducible method to quantify pexophagy.

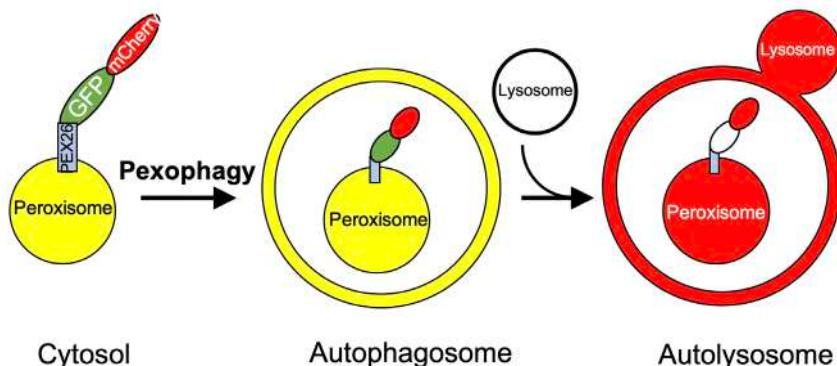


Figure 1. Schematic of the lysosome targeting RG assay. Peroxisomes expressing the PEX26TM-RG protein will appear yellow within the cytosol or the autophagosome due to fluorescence of both GFP and mCherry. When targeted to the lysosome, GFP fluorescence will be quenched and the peroxisome will appear red.

Materials and Reagents

1. Stericup Quick Release Millipore Express PLUS 0.22 µm PES filter, 500 ml (Millipore Sigma, catalog number: S2GPU05RE)
2. T25 flask (Corning Life Sciences, catalog number: C353109)
3. 4-well NuncTM Lab-TekTM Chambered Coverglass (Thermo Fisher Scientific, catalog number: 155383)
4. Lipofectamine 2000 (Life Technologies, catalog number: 11668-019)
5. E-64 (Enzo Life Sciences, catalog number: BML-PI107-0001)
6. Leupeptin (Bioshop, catalog number: LEU001)
7. HeLa human cervical cancer cell line (ATCC CCL-2)
8. GFP-mCherry (RG)-PEX26TM plasmid
9. Dulbecco's Modified Eagle's Medium (Hyclone, catalog number: SH3008101)
10. L-Glutamine (Hyclone, catalog number: SH30034.01)
11. Fetal Bovine Serum (Life Technologies, catalog number: A12617)
12. OptiMEM Reduced Serum Medium (Thermo Fisher, catalog number: 31985062)
13. Trypsin-EDTA (0.05%), phenol red (Thermo Fisher, catalog number: 25300062)
14. CO₂ independent medium (Life Technologies, catalog number: 18045)
15. Hank's Balanced Salt Solution (HBSS) (Thermo Fisher, catalog number: 14025092)

Equipment

1. Zeiss LSM710 equipped with a 63x 1.4 NA oil immersion objective and the appropriate lasers (488 nm on a tuneable Argon 458/477/488/514 nm at 30 mW with a 493-565 nm bandpass filter and 561 nm DPSS laser with a 600-700 nm bandpass)
2. Forma Steri-Cycle CO₂ Incubator (Thermo Fisher)

Software

1. Velocity (Perkin Elmer, version 6.3)
2. Zeiss Zen (Zeiss, <https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html>)
3. Microsoft® Office Excel (Microsoft Office 365)

Procedure

A. Gene cloning

GFP-mCherry-PEX26™ was constructed in several steps as described in Deosaran *et al.*, 2013. First, the PEX26 ORF from pSPORT-PEX26 (Invitrogen) was cloned into the EcoRI and Sall sites of pmEGFP-C1, to insert the entire ORF of PEX26 at the C-terminus of GFP. Next, the entire PEX26 but its C-terminus tail-anchor domain (TM) was removed by PCR using two primers containing a BgIII site. Finally, primers containing a BgIII or BamHI sites were used to PCR amplified mCherry. Following digestion with BgIII and BamHI, the PCR product was inserted into the BgIII site on the pmEGFP-PEX26™ vector to generate the GFP-mCherry-PEX26™ (RG-PEX26™) vector. Refer to Figure 2 for a schematic.

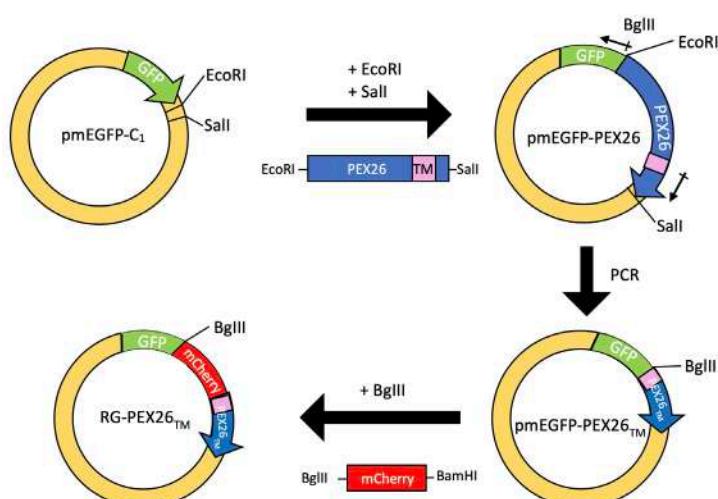


Figure 2. Schematic diagram of the construction of the RG-PEX26™ construct.

Restriction enzymes used are indicated. Small arrows indicate the location of primer used.

B. Cell seeding

Here we will describe work done on HeLa cells which were grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and 5% CO₂. Media was filter sterilized with FBS. However, this protocol can be adapted to most transfectable cell lines. We have tested this protocol in a number of transfectable cells such as MEFs, Cos7, U2OS, Hek293 and MDCKs.

1. To seed cells, first warm sterile media and PBS to 37 °C. Thaw trypsin and warm to room temperature.
2. Remove cells from the incubator and aspirate growth media. Wash with PBS (about 5 ml) and aspirate. Add trypsin to the flask at an appropriate volume to ensure cells will not dry (*i.e.*, 1 ml trypsin in a T25 flask).
3. Incubate cells in trypsin at 37 °C for 3-5 min.
4. Once cells are no longer adhered to the flask, add media to dilute and inactivate the trypsin (about 5 ml). Collect media containing cells in a 15 ml tube and centrifuge at 1,000 *x g* for 5 min to pellet cells.
5. Following centrifugation, aspirate media and resuspend the cells in fresh media.
6. Count the cells using a hemocytometer or other cell counting device.
7. Seed cells in a 4-well Lab-Tek chamber (or equivalent coverslip system that allows for live cell imaging) at a density of 25,000 cells/well. Total volume within the well should be 0.5 ml. For other cell types or longer procedures, seed cells at an appropriate density for transfection.
8. Allow cells to adhere and rest for 24 h before transfection in the 37 °C incubator.

C. Cell transfection

1. On Day 2, transfect with the RG-PEX26™ construct (and any other construct required for the experiment). Use 1-2 µg of DNA per well and Lipofectamine2000 transfection reagent according to the manufacturer's instructions. In brief, combine 0.5 µl of Lipofectamine2000 with 50 µl of Opti-mem medium in a 1.5 ml tube. Allow it to sit for 5 min. In another 1.5 ml tube, combine 50 µl of Opti-mem medium and DNA. Combine the lipofectamine and DNA containing mixtures and let sit for 20 min. Do this for each transfected well. Add complete mixture (100 µl) to each well.
2. Let cells grow for 24-36 h.
3. Change media to DMEM + FBS or HBSS without FBS. HBSS induces mTORC1 related pexophagy due to amino acid starvation. Both are supplemented with 2 µM E-64 and 0.5 mM leupeptin to prevent degradation of protein inside lysosomes and allow detection of mCherry signal. Incubate at 37 °C for 4-24 h. Incubation at 37 °C is accomplished through the use of a CO₂ dependent incubator.

D. Image acquisition

1. For imaging, the cells should be imaged under the appropriate conditions (*i.e.*, 37 °C and 5% CO₂). If using a microscope system without CO₂, the media should be replaced with appropriate CO₂ independent media supplemented with 0.5 mM leupeptin and 2 µM E-64.
2. Ensure microscope is powered on and warmed up to 37 °C before starting.
3. For each set of experiments, calibrate settings such that the mean GFP and mCherry fluorescence intensities ($I_{G/R}$) are approximately equal (*i.e.*, $I_{GFP}/I_{mCherry} = 1$). Calibration will depend on the microscope used, however in short, the laser, gain and speed should be set to have the background close to zero intensity while most of the signal for the fluorophores are not saturated. The ratio of the mean intensity for RFP over GFP for each cell should be close to 1 for the control cells. Ensuring that GFP and mCherry signals are approximately equal allows for optimal detection of changes in the fluorescence intensity ratio upon pexophagy induction. For each trial, all conditions should be imaged on the same day. Either 3D z-sectioning or a single slice can be used for quantification but ensure to keep the imaging method consistent throughout trials. Refer to Figure 3 for sample images.

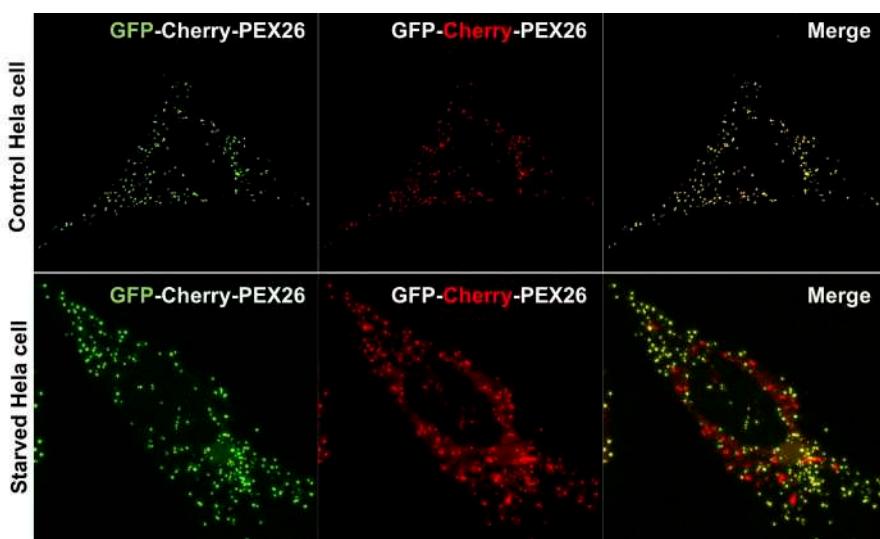


Figure 3. HeLa cells transfected with RG construct. Control cells have been incubated in DMEM supplemented with E-64 and leupeptin as above. Starved cells were grown in HBSS for 12 h before imaging, also supplemented with E-64 and Leupeptin. Both cells are expressing GFP-mCherry-PEX26™. Control cell (Top) shows almost equal GFP and mCherry pixel intensities. Cells where pexophagy has been induced by amino acid starvation (Bottom) show increased ‘red-only’ signal indicating peroxisomes have been targeted to lysosomes. Note, an increase in ‘red-only’ signal is more easily observable as a decrease in green fluorescent intensity. Red fluorescent intensity should not change. Scale bar = 10 µm.

Data analysis

Note: Please refer to Figure 3 for a detailed spreadsheet.

1. Using Volocity software or its equivalent, first determine the cell area by drawing a region of interest (ROI) around an individual cell expressing the RG construct (Figure 4A).
2. Next, identify peroxisomes by using the mCherry signal only (Figure 4B, column 1). In Volocity, this can be done in several different ways, however for most images with very high signal to noise in the ROI is the easiest. Most other imaging analysis software with similar algorithms can be used to find objects within Z-stack images. Measure the mean fluorescent intensity of GFP and mCherry signal per puncta as well as the volume of each puncta, and input into a spreadsheet (Figure 4B, columns 2-4).
3. Next, identify the proportion of lysosomally targeted peroxisomes (“red only” peroxisomes) by calculating the ratio of mCherry to GFP fluorescence per puncta (Figure 4B, column 5). A puncta is considered ‘red-only’ if the mean mCherry fluorescent signal is at least 3 times higher than the mean GFP fluorescent signal. This is denoted in the spreadsheet as a value of 1 (lysosomally targeted) or 0 (not lysosomally targeted) (Figure 4B, column 6A). Using this information, the volume of lysosomally targeted peroxisomes per cell can be tabulated efficiently by Excel (Figure 4B, column 6B).
4. Add the volumes of all lysosomally targeted peroxisomes per cell (Figure 4B, column 7). Identify the area of all peroxisomes in the cell by adding the volume of all mCherry positive puncta (Figure 4B, column 8). To identify the proportion of lysosomally targeted peroxisomes per cell, divide the volume of the lysosomally targeted peroxisomes by the total peroxisome volume (Figure 4B, column 9).
5. Repeat this process for every cell, quantifying at least 50 cells per trial. Calculate an average of proportion of lysosomally targeted puncta per tested condition and plot on a graph.
6. Once a minimum of 3 independent trials has been completed, perform statistical analysis and graph. For a sample graph, refer to Figure 4C.

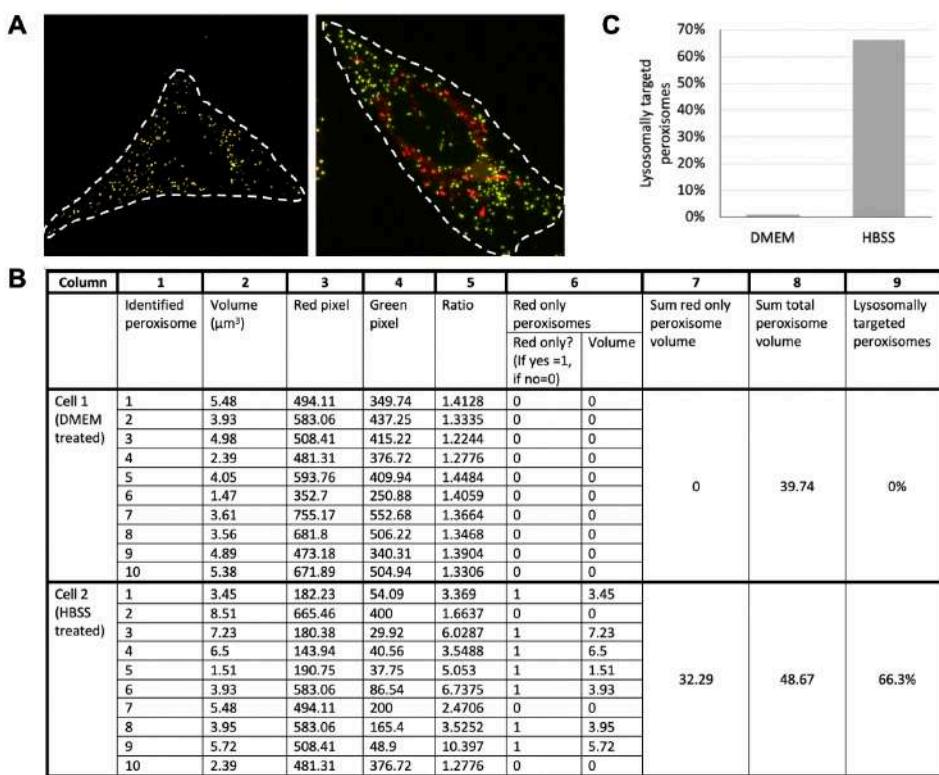


Figure 4. Sample cell ROI generation and calculation spreadsheet. A. ROI generation for cells imaged in Figure 2 (left—cell incubated for 12 h in DMEM, right—cell incubated for 12 h in HBSS). ROI is denoted by the white dotted line. Merged images are shown. B. Sample calculation spreadsheet. Quantification of 10 peroxisomes from each cell imaged in A is shown. Cell 1 was incubated in DMEM (control), while Cell 2 was incubated in HBSS (pexophagy induced) for 12 h. Please refer to the protocol for detailed column explanations. Note, red only peroxisomes (column 6) are those peroxisomes which have a mean red fluorescent intensity at least 3 times greater than the mean green fluorescent intensity. C. Sample data from B graphed.

Notes

1. This experiment must be imaged live, as fixation may alter the mCherry and GFP pixel intensity.
2. If additional staining is required, for example to determine if a construct is expressed, cells may be fixed following imaging, stained normally and re-imaged, to determine what proportion of cells expressing the RG construct are also expressing your construct of interest.
3. Laser intensity or gain must not be altered between images or samples within the same trial.

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Competing interests

There are no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

References

1. Allen, G. F., Toth, R., James, J. and Ganley, I. G. (2013). [Loss of iron triggers PINK1/Parkin-independent mitophagy](#). *EMBO Rep* 14(12): 1127-1135.
2. Deosaran, E., Larsen, K. B., Hua, R., Sargent, G., Wang, Y., Kim, S., Lamark, T., Jauregui M., Law, K., Lippincott-Schwartz, J., Brech, A., Johansen, T. and Kim, P. K. (2013). [NBR1 acts as an autophagy receptor for peroxisomes](#). *J Cell Sci* 126(Pt 4): 939-952.
3. Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., *et al.* (2016). [Guidelines for the use and interpretation of assays for monitoring autophagy \(3rd edition\)](#). *Autophagy* 12(1): 1-222.
4. Kim, S. J., Syed, G. H., Khan, M., Chiu, W. W., Sohail, M. A., Gish, R. G. and Siddiqui, A. (2014). [Hepatitis C virus triggers mitochondrial fission and attenuates apoptosis to promote viral persistence](#). *Proc Natl Acad Sci USA* 111(17): 6413-6418.
5. Lahiri, V., Hawkins, W. D. and Klionsky, D. J. (2019). [Watch what you \(self-\) eat: Autophagic mechanisms that modulate metabolism](#). *Cell Metab* 29(4): 803-826.
6. Morishita, H. and Mizushima, N. (2019). [Diverse cellular roles of autophagy](#). *Annu Rev Cell Dev Biol* 35: 453-475.
7. Nazarko, T. Y., Ozeki, K., Till, A., Ramakrishnan, G., Lotfi, P., Yan, M. and Subramani, S. (2014). [Peroxisomal Atg37 binds Atg30 or palmitoyl-CoA to regulate phagophore formation during pexophagy](#). *J Cell Biol* 204(4): 541-557.
8. Pankiv, S., Clausen, T. H., Lamark, T., Brench, A., Bruun, J. A., Outzen, H., Overvatn, A., Bjorkoy, G. and Johansen, T. (2007). [p62/SQSTM1 binds directly to ATG8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy](#). *J Biol Chem* 282(33): 24131-24145.
9. Riccio, V., Demers, N., Hua, R., Vissa, M., Cheng, D. T., Strilchuk, A. W., Wang, Y., McQuibban, G. A. and Kim, P. K. (2019). [Deubiquitinating enzyme USP30 maintains basal peroxisome abundance by regulating pexophagy](#). *J Cell Biol* 218(3): 798-807.
10. Wang, Y., Serricchio, M., Jauregui, M., Shanbhag, R., Stoltz, T., Di Paolo, C. T., Kim, P. K. and McQuibban, G. A. (2015). [Deubiquitinating enzymes regulate PARK2-mediated mitophagy](#).

Autophagy 11(4): 595-606.

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