



Chapter 5

Detection of Mouse Eosinophils in Tissue by Flow Cytometry and Isolation by Fluorescence-Activated Cell Sorting (FACS)

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Abstract

Flow cytometry is a critical tool that can be employed to detect unique cells and to isolate cells from tissues based on their antigen profiles. While mouse eosinophils can be readily detected by one or more distinct antigen profiles, many of these strategies do not result in accurate eosinophil counts. We present here our basic protocol, which permits quantitative detection of eosinophils and isolation of eosinophils from bone marrow, spleen, and lung tissue of allergen-challenged wild-type and unchallenged *IL5* transgenic mice. With small protocol variations, eosinophils can be isolated from small intestines and muscle tissue, the latter from infiltrates characteristic of muscular dystrophy (mdx) mice.

Key words Inflammation, Cytokines, Flow cytometry

1 Introduction

Humans (*Homo sapiens sapiens*) and mice (*Mus musculus*) are both mammalian species within the animal kingdom; primates and rodents diverged as independent orders within this class only ~80 million years ago [1]. Genetically defined mouse strains have been a mainstay of medical research since the 1950s [2]. Mouse models have virtually overtaken in vivo modeling, most notably since the development and ready availability of transgenic and gene deletion technologies.

Eosinophils are present in bone marrow of both humans and mice at homeostasis, and transit to blood and tissues in similar numbers and in response to similar provocation in both organisms, including allergens and the eosinophil-active cytokine, interleukin (IL)-5 [3]. Nonetheless, those of us engaged in eosinophil research using mouse models face unique barriers to direct translation of research findings to the human condition, as mouse eosinophils are clearly distinct from their human counterparts. Among these

differences, mouse eosinophils maintain unique cell surface markers and secretory proteins, have only a limited propensity to degranulate, and have distinct responses to standard chemoattractants [4]. Most recently, mouse eosinophils expressing a human protein have been generated using the transgenic *eoCre* mouse strain [5].

In this chapter, we provide protocols for eosinophil detection and enumeration by flow cytometry and eosinophil isolation using fluorescence-activated cell sorting (FACS). The latter is a powerful technique that permits collection of eosinophils at high purity for protein and gene expression studies as well as functional assays.

2 Materials

2.1 Isolation of Lungs from Allergen-Challenged or *IL5tg* Mice

1. Wild-type allergen-challenged [6] or interleukin-5 transgenic [7, 8] mice.
2. 70% Ethanol: 70% vol/vol ethanol in distilled water.
3. Perfusion medium: 20 mL 0.5M EDTA to 1L in phosphate-buffered saline (PBS) filter sterilized and stored at 4 °C.
4. Digestion medium: 5 mL Heat-inactivated fetal bovine serum (FBS) to 100 mL with RPMI-1640 medium plus 100 µL DNase I stock solution and 5 mL collagenase D stock solution added just before use.
5. DNase I stock solution (20 mg/mL): 100 mg DNase I grade II (Roche) to 5 mL in glycerol/CaCl₂/Na-acetate solution (*see item 6* below), filter sterilized. Store in 250 µL aliquots at −20 °C.
6. Glycerol/CaCl₂/Na-acetate solution: 50 µL of 1 M CaCl₂, 83 µL of 3 M Na-acetate, 25 mL glycerol, to 50 mL with deionized water.
7. Collagenase D stock solution (40 mg/mL): 2.5 g Collagenase D (Roche) in 62.5 mL PBS. Filter sterilize and store in 1.5 mL aliquots at −20 °C.

2.2 Preparation of Single-Cell Suspensions

1. ACK lysis buffer.
2. 70-µm cell strainer.
3. Wuerzburg buffer: 1.5 g BSA, 5 mL 0.5 M EDTA pH 8.0 with PBS to 500 mL. Store buffer at 4 °C. Just prior to use add DNase I stock (prepared as above) and 100 µL sock solution to 100 mL buffer.
4. Hanks' balanced salt solution (HBSS).
5. 0.1% BSA in PBS: 0.1% Bovine serum albumin (BSA) in PBS.
6. Trypan blue solution.

7. 0.5 M Ethylenediaminetetraacetate (EDTA), pH 8.0.
8. Freezing medium: 10% Dimethyl sulfoxide in fetal bovine serum (FBS; serum heat inactivated for 1 h at 56 °C).
9. Paraformaldehyde (4% vol/vol in PBS).

2.3 Antibodies for Flow Cytometry and Fluorescence- Activated Cell Sorting (FACS)

1. Live/Dead Aqua (Invitrogen).
2. Anti-CD16/32 (BD Pharmingen).
3. Anti-CD45-eF450 and isotype control (eBioscience).
4. Anti-CD11c-AF700 and isotype control (eBioscience).
5. Anti-Ly-6G/anti-Ly6C-APC (anti-Gr1) and isotype control (BD Pharmingen).
6. Anti-Siglec F-PE and isotype control (BD Pharmingen).
7. Anti-MHC II-PE-Cy7 and isotype control (eBioscience).
8. UltraComp eBeads (eBioscience).
9. Arc Amine Reactive Compensation Bead Kit (Invitrogen).

2.4 Equipment/ Computer Resources

1. Dissection tools and board.
2. Straight razors.
3. 6-Well plates.
4. 27-gauge ½ inch needles.
5. 12 mL Syringes.
6. Petri dishes.
7. Tube rotator.
8. Tabletop centrifuge.
9. Light microscope.
10. [BD](#) LSRII benchtop flow cytometer, 12 parameters (10 fluorescent colors and 2 light scatters), 4 lasers: 488 nm argon, 633 nm HeNe, 407 nm solid state, and 325 nm HeCad with fixed-alignment dyes capable of measuring 488 nm laser dyes: FITC, PE, PE-Cy5, and PE-Cy7; 325 nm laser dyes: Indo-1, Hoechst, and DAPI; 633 nm laser dyes: APC or Cy5 and APC-Cy7; and 407 nm laser dyes: Cascade Blue, Cascade Yellow, Alexa 430, and CFP.
11. [FACS Aria](#) 13 Colors, 4 lasers: 405 nm, 488 nm, 561 nm, 640 nm 4-way sorting (4 populations simultaneously); sorting into 15 mL tubes enclosed in Class II Biosafety Cabinet.
12. FlowJo 10.4.2, operating system: Windows 10, Java version: 1.8.0_161-b12, build number: 10.4.

3 Methods

3.1 Isolation of Lungs from Allergen-Challenged or IL5tg Mouse

1. Euthanize mouse using the method recommended by the Institutional Animal Care and Use Committee.
2. Lay the mouse supine on a clean surface of absorbent paper. Rinse mouse with 70% ethanol. The wet fur aids with dissection.
3. With scissors, open the abdominal skin layer and secure with pins to Styrofoam board. Cut open the diaphragm and cut the blood vessels that enter the diaphragm. Open the thorax to visualize the lungs and the heart.
4. Perfuse the lung by gently injecting 8 mL of perfusion medium into the heart (right ventricle, 12 mL syringe, 27-gauge ½ inch needle) to remove circulating blood cells. The lungs will appear white after this step.
5. Remove the lungs and place them into one well of a 6-well plate with 3 mL digestion medium; hold on ice until all samples are collected.

3.2 Preparation of Single-Cell Suspensions

1. Transfer lungs from the 6-well plate to a Petri dish; mince the lung tissue with a straight razor blade until pieces of tissue are about 3 × 3 mm. Transfer minced tissue to a 15 mL screw-cap tube, and hold on ice until all samples are complete.
2. Rinse the Petri dish and blade with 3 mL of digestion medium from the 6-well plate and transfer the remaining material to the 15 mL screw-cap conical tube containing lung tissue; repeat × 1. If lung tissue is pooled, use maximum two lungs/tube.
3. Incubate tubes at 37 °C on a tube rotator at 10 revolutions per minute for a total of 90 min. After 45 min of incubation, add 3 mL of fresh digestion medium and pipet up and down.
4. Place the tube containing digested lung at room temperature.
5. Add cold 0.5 M EDTA to each tube to a final concentration of 10 mM (50-fold dilution-180 µL in 9 mL). Pipette up and down.
6. Incubate for 5 min at room temperature. Pipette up and down.
7. Set a 70-µm cell strainer on a 50 mL screw-cap conical tube. Pour cell suspension on to the filter. Add 10 mL cold Wuerzburg buffer to the empty tube to wash out any residual cells and rinse over filter.
8. Centrifuge cells in 50 mL tube for 5 min at 300 × *g* at 4 °C to pellet cells.

9. Decant the supernatant. Add 1–2 mL of ACK lysis buffer. Incubate for 2 min at room temperature.
10. Add 10 mL of Wuerzburg buffer. Mix the cell suspension end over end and transfer to a 15 mL screw-cap conical tube.
11. Centrifuge for 5 min at $300 \times g$ at 4°C to pellet cells.
12. Decant the supernatant, and resuspend pellet in 10 mL 0.1% BSA in PBS.
13. Count the remaining cells with trypan blue dye exclusion to evaluate viability, which is typically $>90\%$. Each set of lungs from a naïve wild-type mouse will yield $\sim 1.5 \times 10^6$ cells; lungs from an allergen-challenged wild-type mouse will yield $\sim 8 \times 10^6$ cells, and lungs from an IL5 transgenic mouse will yield $\sim 35 \times 10^6$ cells.
14. Cells to be analyzed by flow cytometry can be frozen in freezing medium at 10^6 – 10^7 cells per mL and stored at -80°C . NB: Freshly prepared, not frozen, and defrosted cells should be used in FACS protocols (*see* Subheading 3.4).

3.3 Flow Cytometric Analysis

This protocol was designed for use on the Becton Dickinson LSRII. It is critical to recognize that each flow cytometer has distinct features and that all protocols require optimization; the images included with the text are meant as general guidelines.

The information included below assumes a strong basic understanding of flow cytometry (*see* **Note 1**) and access to an appropriate flow cytometer. While initially designed to identify eosinophils from mouse lung tissue, specific protocol variations have facilitated identification and enumeration of eosinophils from bone marrow, muscle tissue, and small intestines as detailed in **Notes 2–4**.

1. When ready to analyze, thaw frozen cells in a 37°C water bath.
2. Resuspend thawed cells at 10^6 cells/mL in PBS in 5 mL polystyrene round-bottom tubes and centrifuge at 4°C , $300 \times g$, for 5 min. Decant supernatant and tap the bottom of the tubes to resuspend the cells in the minimal volume that remains.
3. Incubate cells with Live/Dead Aqua reagent for 30 min at 4°C in the dark following the manufacturer's guidelines. Be sure to include critical controls: cells with Live/Dead Aqua only, without any of the other fluorophore-conjugated antibodies to follow and fluorescence minus one (FMO), which includes all five antibodies minus Live/Dead Aqua and one tube for each of the five antibodies that includes the Live/Dead Aqua and only four of the fluorophore-conjugated antibodies. These controls are critical for ascertaining whether fluorescence detected in a given channel is specifically and definitively from the conjugated antibody bound to its cognate antigen on target cells. *See* more on controls below (*see* **step 10**).

4. Wash cells by adding 0.1% BSA in PBS at 3 mL per tube followed by centrifugation at 4 °C, $300 \times g$, for 5 min. Decant the supernatant and tap the bottom of the tubes to resuspend the cells in the minimal volume that remains.
5. To each tube, add 0.5 µg of anti-CD16/CD32 (FcR blocking Ab), followed by a master mix containing anti-CD45-eF450, anti-CD11c-AF700, Gr1-APC, anti-Siglec F-PE, and anti-MHCII-PE-CY7 each at 0.2 µg per 10^6 cells in a final volume of 100 µL.
6. Incubate for 30 min at 4 °C in the dark.
7. Wash and resuspend cells as above. Leave a minimal amount of volume in each tube after decanting supernatants (~100 µL).
8. If planning to evaluate samples sometime later, fix cells with 500 µL of 4% paraformaldehyde and store for up to 2–3 days at 4 °C in the dark. Wash fixed cells with 3 mL 0.1% BSA in PBS when ready for analysis.
9. Analyze samples using a BD LSR II Flow Cytometer, collecting at least 10^5 events per sample.
10. Unstained cells are used to control for any background fluorescence. To select gate placement (*see step 11*) the following controls are necessary for each antibody: (1) single color—cells with one of each of the fluorophore-conjugated antibodies, (2) single color—cells with one of each of the antibodies corresponding to the fluorophore-conjugated isotype controls, and (3) fluorescence minus ones (FMOs)—the complete set minus a single fluorophore-conjugated antibody. As indicated above, in this panel, which includes 5 fluorophore-conjugated antibodies, one would need 15 tubes of cells for these 3 sets of controls, as well as the controls discussed above for the Live/Dead Aqua (*see step 3*).
11. In the gating example shown in Fig. 1a, the first panel demonstrates the use of scatter properties to identify whole cells and eliminate debris; in the second panel (Fig. 1b) single cells were identified for further analysis, and in the panel to follow, the (dead) cells staining with the Live/Dead reagent are eliminated (Fig. 1c). From this point, leukocyte (CD45⁺) cells were selected (Fig. 1d), followed by high forward/high side scatter myeloid cells (Fig. 1e) and CD11c[−]/Gr1^{−/lo} cells (Fig. 1e) followed by MHCII[−], Siglec F⁺ cells, which are the eosinophils (Fig. 1f).

3.4 Fluorescence-Activated Cell Sorting (FACS)

This protocol was designed for use on the [FACS Aria](#) 13 Colors, 4 lasers: 405 nm, 488 nm, 561 nm, and 640 nm which permits 4-way sorting (4 populations simultaneously). As noted in Sub-heading 3.3 above, each protocol requires individual and specific optimization.

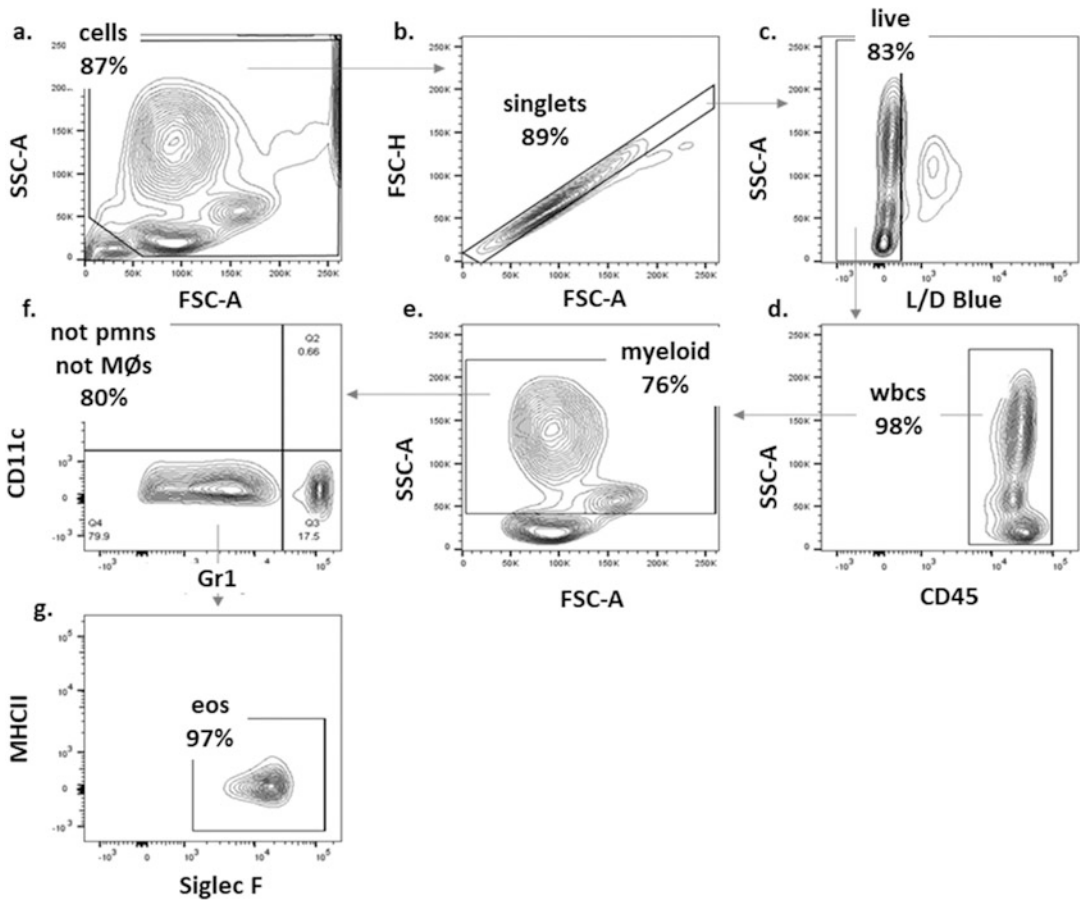


Fig. 1 Identification of eosinophils in lung tissue of interleukin-5 transgenic mice. Gating strategy used to isolate eosinophils (live CD45⁺CD11c⁻Gr1⁻MHCIi^{lo}SiglecF⁺ cells) as shown in panels a–g

1. Better results are obtained using freshly prepared, never frozen cells.
2. Resuspend live cells at 10^7 per mL in 0.1% BSA in PBS. Retain 10^6 cells to use as unstained compensation controls.
3. To block F_c receptors, add 10 μ L of anti-CD16/32 per mL of cells; no Live/Dead reagent is needed. Add 0.2 μ g of each antibody for every 100 μ L of cells (10^6 cells per 100 μ L) as in Subheading 3.3, step 5. Incubate for 30 min at 4 °C in covered tubes (protected from light).
4. After incubation with antibodies, collect cells by centrifugation at $500 \times g$ for 5 min at 4 °C, wash with 0.1% BSA in PBS, and repeat centrifugation.
5. Resuspend cells to a concentration of 10^7 cells per mL for sorting.

6. The profiling is much the same as shown in Fig. 1. Compensation is performed using unstained cells (*see* **step 2**, above) and commercial compensation beads are used for each antibody in the panel (*see* Subheading 3.3, **step 10**).
7. Eosinophils are isolated by sorting into cold 0.1% BSA in PBS and held on ice, protected from light. Lungs of allergen-challenged wild-type mice yield about 10^6 eosinophils per mouse and lungs of unchallenged *IL5*tg mice, 15×10^6 eosinophils per mouse.

We have designed a variant of this FACS protocol that eliminates the need for anti-Siglec F, an antibody that may induce eosinophil apoptosis (*see* **Note 5**).

4 Notes

1. Flow cytometry is best learned hands-on with an instructor and an instrument to use. Some good books that also provide insight and instructions include the following: Givan, AL (2001) *Flow Cytometry, First Principles*, second edition; Wiley-Liss ISBN#0-471-38,224-8 and Shapiro HM (2003) *Practical Flow Cytometry*, Fourth Edition; and Wiley-Liss ISBN# 0-471-41,125-6.
2. Eosinophils can be isolated from bone marrow of wild-type and *IL5*tg mice using the fluorophore-conjugated antibody panel used here. Briefly, mouse bone marrow leukocytes were isolated as described [9]; mature eosinophils (~5% of total leukocytes) were determined to be $CD45^+MHCII^{low}CD11c^-SiglecF^+$. Of interest, the neutrophil surface antigen Ly6G (a component of the Gr1 monoclonal antibody) was identified on a substantial fraction of Siglec F⁺ eosinophils isolated from mouse bone marrow, with a higher proportion of Ly6G cells detected in mice that overexpress the eosinophil-active cytokine, IL5 [10].
3. Eosinophils can also be isolated from dense tissue infiltrates, such as skeletal muscle of mdx (muscular dystrophy) mice [11]. Single-cell suspensions were prepared by enzymatic digestion as described by Liu and colleagues [12]. Eosinophils in the muscle tissue were identified as $CD45^+CD11c^-Gr1^-MHCII^{lo}SiglecF^+$ cells; *see* Fig. 2.
4. We have also isolated eosinophils from the small intestine. We used strategy for generating single-cell suspensions published by Berek and colleagues [13] together with our standard FACS protocols. Eosinophils isolated were $CD45^+CD11c^+MHCII^-Gr1^-SiglecF^+$; $13 \pm 4\%$ of these cells were also $NOD1^+$ (Percopo, Kraemer & Rosenberg, unpublished findings).

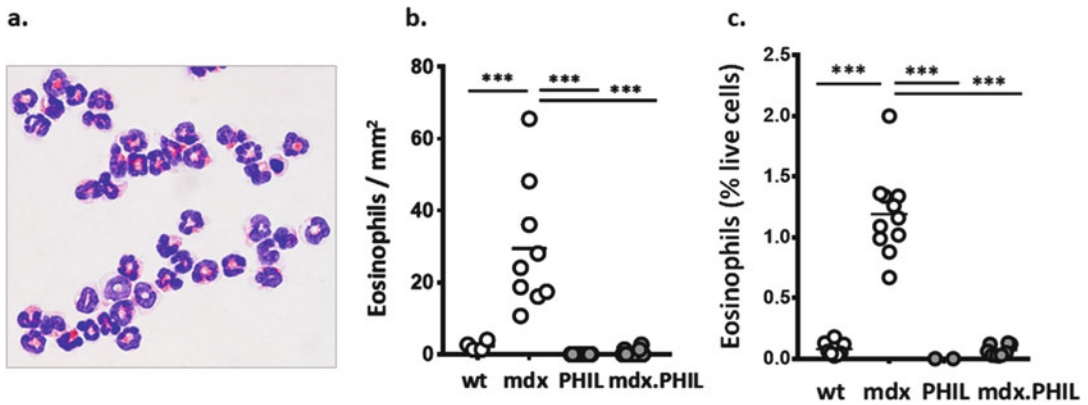


Fig. 2 Eosinophils isolated from quadriceps muscle infiltrate. (a) Eosinophils (CD45⁺CD11c⁻Gr1⁻MHCII⁻SiglecF⁺ cells) isolated from quadriceps muscle by fluorescence-activated cell sorting (FACS) and stained with modified Giemsa. (b) Eosinophils per mm² determined by visual inspection of anti-major-basic protein-1-stained tissue. (c) Eosinophils as % of live cells determined by flow cytometry, ****p* < 0.001 one-way ANOVA. Reprinted with permission from reference [11]

- Flow cytometry protocols designed to identify mouse eosinophils typically target Siglec F. However, ligation of this antigen induces eosinophil apoptosis, which limits its usefulness for sorting and isolations that are to be followed by functional studies. To address this problem, we devised a method to isolate eosinophils from tissues that avoid Siglec F (yield $\sim 4 \times 10^6$ CD11c⁻Gr1⁻/MHCII⁻ cells per mouse, >98% pure eosinophils [14]). We found this method to be useful for eosinophil-enriched lung tissue; it may be useful for other target tissues as well.

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References

- Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York
- Silver LM (2008) Mouse genetics: concepts and applications. Oxford University Press, Oxford. <http://www.informatics.jax.org/silver/>
- Rosenberg HF, Phipps S, Foster PS (2007) Eosinophil trafficking in allergy and asthma. *J Allergy Clin Immunol* 119:1303–1310
- Lee JJ, Jacobsen EA, Ochkur SI et al (2012) Human versus mouse eosinophils: “that which we call an eosinophil, by any other name would stain as red”. *J Allergy Clin Immunol* 130:572–584
- O’Sullivan JA, Wei Y, Carroll DJ, Moreno-Vinasco L, Cao Y, Zhang F, Lee JJ, Zhu Z, Bochner BS (2018) Frontline science: characterization of a novel mouse strain expressing

- human Siglec-8 only on eosinophils. *J Leukoc Biol* 104:11–19
6. Percopo CM, Brenner TA, Ma M, Kraemer LS, Hakeem RM, Lee JJ, Rosenberg HF (2017) SiglecF⁺Gr1^{hi} eosinophils are a distinct subpopulation within the lungs of allergen-challenged mice. *J Leukoc Biol* 101:321–328
 7. Dent LA, Strath M, Mellor AL, Sanderson CJ (1990) Eosinophilia in transgenic mice expressing interleukin 5. *J Exp Med* 172:1425–1431
 8. Lee NA, McGarry MP, Larson KA, Horton MA, Kristensen AB, Lee JJ (1997) Expression of IL-5 in thymocytes / T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis and unique histopathologies. *J Immunol* 158:1332–1344
 9. Byström J, Wynn TA, Domachowske JB, Rosenberg HF (2004) Gene microarray analysis reveals interleukin-5 dependent transcriptional targets in mouse bone marrow. *Blood* 103:868–877
 10. Limkar AR, Mai E, Sek AC, Percopo CM, Rosenberg HF (2020) Frontline science: cytokine-mediated developmental phenotype of mouse eosinophils: IL-5-associated expression of the Ly6G/Gr1 surface antigen. *J Leuk Biol* 107(3):367–377
 11. Sek AC, Moore IN, Smelkinson MG, Pak K, Minai M, Smith R, Ma M, Percopo CM, Rosenberg HF (2019) Eosinophils do not drive acute muscle pathology in the mdx mouse model of Duchenne Muscular Dystrophy. *J Immunol* 203:476–484
 12. Liu L, Cheung TH, Charville GW, Rando TA (2015) Isolation of skeletal muscle stem cells by fluorescence activated cell sorting. *Nat Protoc* 10:1612–1624
 13. Berek C, Beller A, Chu VT (2016) Isolation of eosinophils from the lamina propria of the murine small intestine. *Methods Mol Biol* 1422:213–221
 14. Geslewitz WE, Percopo CM, Rosenberg HF (2018) FACS isolation of live mouse eosinophils at high purity via a protocol that does not target Siglec F. *J Immunol Methods* 454:27–31