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Improved Macrophage Isolation from Mouse Skeletal Muscle

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[Abstract] Macrophages are a heterogeneous class of innate immune cells that offer a primary line of defense to the body by phagocytizing pathogens, digesting them, and presenting the antigens to T and B cells to initiate adaptive immunity. Through specialized pro-inflammatory or anti-inflammatory activities, macrophages also directly contribute to the clearance of infections and the repair of tissue injury. Macrophages are distributed throughout the body and largely carry out tissue-specific functions. In skeletal muscle, macrophages regulate tissue repair and regeneration; however, the characteristics of these macrophages are not yet fully understood, and their involvement in skeletal muscle aging remains to be elucidated. To investigate these functions, it is critical to be able to efficiently isolate macrophages from skeletal muscle with sufficient purity and yield for various downstream analyses. Here, we describe in detail an optimized method to isolate skeletal muscle macrophages from mice. This method has allowed the isolation of high-purity $CD45^+/CD11b^+$ macrophages from young and old mice, which can be further used for flow cytometry analysis, fluorescence-activated cell sorting (FACS), and single-cell RNA sequencing.

Keywords: Macrophage, CD11b, Skeletal muscle, Aging, Senescence

[Background] Macrophages were discovered by Metchnikoff and colleagues more than a century ago as 'professional' phagocytes (Underhill *et al.*, 2016). Later studies revealed that macrophages constitute a heterogenous class of cells that exert diverse functions in tissues throughout the body (Wynn *et al.*, 2013). Macrophages can be divided into two major types: tissue-resident and non-tissue-resident macrophages (Ginhoux and Guilliams, 2016). Tissue-resident macrophages can be further divided into two distinct populations: embryo-derived self-renewing and bone marrow-derived non-self-renewing macrophages. Typical self-renewing macrophages derived from the embryonic yolk sac or fetal liver include microglia, Kupffer cells, alveolar macrophages, and Langerhans cells. Bone marrow-derived non-self-renewing resident macrophages, which must be replenished by circulating monocytes at tissue-specific levels, include tissue-resident macrophages in the intestine, pancreas, and dermis (Chakarov *et al.*, 2019). Non-resident macrophages are derived from bone marrow progenitor cells and infiltrate tissues following injury or infection (Kratofil *et al.*, 2016).

Macrophages are highly versatile cells that are capable of ingesting and digesting pathogens as well as necrotic and infected cells, activating T and B lymphocytes, and inducing or suppressing inflammation (Shapouri-Moghaddam *et al.*, 2018). The functional diversity of macrophages is well represented by

their dynamic polarization abilities. Depending on signals from the local environment, macrophages can be polarized toward functionally opposite roles: pro-inflammatory M1 or anti-inflammatory M2 subtypes. Cytokines produced by Th1 (T helper type 1) lymphocytes, including interferon γ (IFNγ or IFNG) and tumor necrosis factor (TNF), polarize macrophages to the M1 subtype, while cytokines produced by Th2 lymphocytes, such as interleukin (IL) 4 and IL13, promote macrophage M2 polarization (Mills *et al.*, 2000; Martinez *et al.,* 2008). Polarized M1 macrophages induce inflammation, destroy pathogens, and clean up cell debris, partly through upregulation of the nitric oxide synthase (NOS) pathway (Rath *et al.*, 2014). By contrast, M2 macrophages suppress inflammation and promote tissue repair, partially through upregulation of the arginase pathway (Rath *et al.*, 2014). While M1 and M2 are well-known macrophage subtypes, more recent single-cell studies have identified additional subtypes in several mouse tissues (Chakarov *et al.*, 2019; Jaitin *et al.*, 2019). These subtypes share similarities and differences with M1 and M2, which further reveal the heterogeneity and versatility of macrophages.

Macrophages adapt to individual tissues and largely act in a tissue-dependent manner. Macrophages from different tissues possess distinct gene expression profiles and transcriptional regulatory pathways (Gautier *et al.*, 2012). Recent studies suggest that local environmental factors in each tissue contribute to the tissue specificity of resident macrophages (Gosselin *et al.*, 2014; Lavin *et al.*, 2014). For instance, tumor growth factor β (TGFβ or TGFB) promotes the development of microglia by affecting the enhancer/promoter landscape of brain macrophages, while retinoic acid determines peritoneal macrophage specificity (Hoeksema and Glass, 2012). These studies have further uncovered the capacity of macrophages to adapt to local environments and acquire tissue-specific identities.

As the largest organ in mammals, skeletal muscle contains numerous and diverse resident macrophages. In skeletal muscle, macrophages are localized in the perimysium and endomysium (Cui *et al.*, 2019), where they resolve infections and repair injury (Arnold *et al.*, 2007; Tidball, 2011 and 2017). For example, when skeletal muscle is damaged, monocytes from the bloodstream differentiate and polarize into pro-inflammatory M1 macrophages, which eliminate pathogens and clean up tissue debris. Subsequently, M1 macrophages convert to M2 macrophages to suppress inflammation and repair tissues along with resident M2 macrophages (Yang and Hu, 2018; Cui and Ferrucci, 2020). Recently, skeletal muscle macrophages have been associated with physiological adaptations to exercise that differ between young and elderly individuals (Walton *et al.*, 2019; Jensen *et al.*, 2020), although the full spectrum of macrophage subtypes in skeletal muscle and their functions are only partially known. We have recently found that macrophages residing in human and mouse skeletal muscle are mostly of the M2 subtype (Cui *et al.*, 2019); however, recent single-cell analyses from skeletal muscle and other tissues suggest that the identities of skeletal muscle macrophages are likely more complex (Chakarov *et al.*, 2019; Jaitin *et al.*, 2019; Wang *et al.*, 2020). Furthermore, skeletal muscle macrophages have been shown to have mixed origins, including the embryonic yolk sac, fetal liver, and adult bone marrow (Wang *et al.*, 2020). It remains unclear whether macrophages from various origins behave differently, and the function of each macrophage subtype in skeletal muscle physiology and aging is yet to be elucidated. To answer these questions, it is critical to isolate macrophages from skeletal muscle. In a recent report, Liu and coworkers established a protocol for the isolation of muscle stem cells that was

also effective in isolating macrophages from human skeletal muscle (Liu *et al.*, 2015; Kosmac *et al.*, 2018). Here, we report modifications to this methodology for the isolation and purification of resident macrophages from young and old mouse skeletal muscle, allowing the characterization of resident macrophages by flow cytometry analysis and single-cell transcriptomics.

Materials and Reagents

1. 100-mm Falcon™ bacteriological Petri dishes with lid (Fisher Scientific, catalog number: 08-757-100D)
2. GentleMACS C-tubes (Miltenyi Biotec, catalog number: 130-093-237 or 130-096-334)
3. 5-ml Falcon™ polypropylene round-bottomed tubes (Corning, catalog number: 352063)
4. 15-ml Falcon™ tubes
5. 50-ml Falcon™ tubes
6. 1.5-ml Eppendorf™ tubes
7. Polystyrene containers; “sticky” for cells but can be used after BSA coating
8. PluriStrainer, 50 µm (pluriSelect, catalog number: 43-50050-01)
9. BD 10-ml syringe, Luer-Lok tip (BD, catalog number: 309604)
10. 20G × 1.5" blunt tip dispensing fill needles (CML Supply, catalog number: 901-20-150)
11. Countess™ cell counting chamber slides (Invitrogen, catalog number: C10228)
12. Skeletal muscles from the hind limbs of 3-month-old and 18-month-old C57BL/6J mice (see below)
13. 70% ethanol
14. DMEM (ThermoFisher, catalog number: 11965-092)
15. Fetal bovine serum, heat inactivated (ThermoFisher, catalog number: 10438-026)
16. Penicillin and streptomycin solution (10,000 U/ml) (100×) (ThermoFisher Scientific, catalog number: 15140122)
17. PBS, pH 7.2 (ThermoFisher, catalog number: 20012-027), free of Ca^{2+} and Mg^{2+}
18. RNAseZAP™ zaps (Ambion, catalog number: 9786-9788)
19. Skeletal Muscle Dissociation Kit (Miltenyi Biotec, catalog number: 130-098-305)
20. Debris removal solution (Miltenyi Biotec, catalog number: 130-109-398)
21. Red blood cell lysis solution (Miltenyi Biotec, catalog number: 130-094-183)
22. Auto MACS™ rinsing solution (Miltenyi Biotec, catalog number: 130-091-222)
23. MACS™ BSA stock solution (Miltenyi Biotec, catalog number: 130-091-376)
24. 0.5 M EDTA (ThermoFisher, catalog number: 15575020)
25. Trypan Blue solution (Invitrogen, catalog number: T10282)
26. Antibodies:
    a. PE anti-mouse/human CD11b antibody (Biolegend, catalog number: 101208, Clone M1/70)
    b. PE Rat IgG2b, κ Isotype Ctrl antibody (Biolegend, catalog number: 400607, Clone RTK4530)
    c. APC anti-mouse CD45 antibody (Biolegend, catalog number: 103111, Clone 30-F11)

d. APC Rat IgG2b, κ Isotype Ctrl antibody (Biolegend, catalog number: 400611, RTK4530)
e. FITC anti-mouse CD206 (MMR) antibody (Biolegend, catalog number: 141703, Clone C068C2)
f. FITC Rat IgG2a, κ Isotype Ctrl antibody (Biolegend, catalog number: 400505, Clone RTK2758)

27. TruStain FcX™ (anti-mouse CD16/32) antibody (Fc blocker) (Biolegend, catalog number: 101319)
28. eFluor780 (Invitrogen, catalog number: 65-0865-14)
29. Medium (see Recipes)
    DMEM-I
    DMEM-II
30. Digestive enzymes (see Recipes)
31. Buffers for cell isolation and flow cytometry (see Recipes)
    PEB buffer
32. Reagent combination to label macrophages for flow cytometry (see Recipes)

Equipment

1. Dissection tools: forceps, scalpels, and scissors
2. Pipettes
3. -80°C freezer, -20°C freezer, 4°C refrigerator
4. GentleMACS™ Octo Dissociator with Heaters (Miltenyi Biotec, catalog number: 130-096-427)
5. Countess™ II FL Automated Cell Counter (Invitrogen, catalog number: AMQAF1000)
6. BD FACSCanto™ II Cell Analyzer (BD, catalog number: REF338960)
7. Centrifuge 5702R (Eppendorf, catalog number: 022626205)
8. Centrifuge 5415R (Eppendorf, catalog number: 22-62-140-8)

Procedure

Note: The following protocol was designed for isolating mononuclear cells from skeletal muscle from 2 adult C57BL/6J mice.

A. Muscle preparation

1. Decontaminate the tools for muscle dissection, including forceps, scalpels, and scissors, with RNaseZAP™ wipes and rinse thoroughly with double-distilled water (ddH_2O). Decontaminate the procedure area by spraying with 70% ethanol.
2. Prepare three 100-mm Petri dishes each containing 10 ml DMEM-I (see Recipes below), place on ice for washing and trimming of isolated muscles. To collect dissected muscles from two mice, prepare two 50-ml Falcon tubes each containing 5 ml DMEM-I and place on ice.

3. Sacrifice a mouse by cervical dislocation or CO_2 asphyxiation and spray 70% ethanol over the entire mouse. Incise the skin at the ankle and peel off the skin from the hind limb. Dissect out all muscles surrounding the femur, tibia, and fibula (Figure 1A), and place into the 100-mm dishes containing DMEM-I. Cut out the fat, blood vessels, tendons, and damaged tissues under a dissection microscope. Collect the trimmed muscles in the 50-ml Falcon tube containing DMEM-I; weigh the tube before and after sampling and record the muscle weight. Two hind limbs from an adult mouse provide approximately 1 g muscle. Collect the muscles from the second mouse following the same procedure.
4. Cut and slice the harvested muscles with scissors on ice, leaving 1–2 ml DMEM-I on the Petri dish and removing the rest. Holding one end of a piece of muscle with forceps, use the scissors to cut the muscle into small pieces (approximately 1-mm^3 fragments). After cutting, gather all the muscle pieces in the center of the dish and mince with scissors for an additional 2–3 min [Figure 1B (a)].

B. Mononuclear cell isolation
1. Transfer the minced muscle tissues from one mouse into two C-tubes containing the enzyme mix (5 ml each) and close the tubes tightly.
2. Mount the C-tubes onto the gentleMAC Octo Dissociator with Heaters [Figure 1B (b)]. Choose the 37C-mr-SMDK-2 program. Start the digestion, which takes approximately 1.5 h.
3. After digestion, briefly centrifuge the C-tubes [Figure 1B (c)]. At this step, combine the two C-tubes containing muscle suspensions that were dissected from the same mouse (5 + 5 = 10 ml) into one tube. Use a 10- or 15-ml syringe and a 20 G blunt needle (pre-washed with 70% ethanol and distilled water or with DMEM-I) to slowly aspirate and eject the muscle suspension 10 times. Eject the suspension toward the wall of the tube to avoid foaming. This step further breaks down the tissue debris and increases cell yield.
4. Add 10 ml DMEM-II to each suspension to dilute and deactivate the enzymes. Pipette the suspension onto a cell strainer (50-μm mesh size) and collect the flowthrough into a 50-ml Falcon tube. Wash each strainer with 10 ml DMEM-II twice. Use a 200-μl pipette to collect any remaining liquid from the underside of the strainer. Each tube will contain 40 ml cell suspension.
5. Discard the strainer and centrifuge the cell suspension at 600 × *g* for 15 min at room temperature. A clear pellet can be seen at the bottom of the tube [Figure 1B (d)]. Discard the supernatant completely. Dissolve the pellet in 1 ml DMEM-II and transfer to a 1.5-ml Eppendorf tube.
6. Centrifuge at 500 × *g* for 10 min at 4°C in a bench-top centrifuge. Discard the supernatant and resuspend the pellet in 0.5 ml cold PBS. Place on ice.

C. Debris removal

Note: This step efficiently removes fiber debris from the digested skeletal muscles and significantly improves the purity of mononuclear cells (Figure 1C).

1. Transfer the cell suspension carefully to a 15-ml Falcon tube containing 5.7 ml cold (4°C) PBS (0.5 ml cell suspension + 5.7 ml PBS = 6.2 ml).
2. Add 1.8 ml cold Debris Removal Solution; mix well by slowly pipetting up and down 10 times using a 10-ml pipette.
3. Tilt the tube slightly and overlay the cell suspension with 4 ml cold PBS very slowly and gently. Ensure that the PBS and the cell suspension phases do not mix.
4. Centrifuge at 3,000 × *g* for 10 min at 4°C. Discard the supernatant completely; this contains tissue debris [Figure 1C (a)]. The cell pellet at the bottom contains the mononuclear cells [Figure 1C (b)].
5. Add cold PBS to a final volume of 15 ml. Gently invert the tube three times. Do not vortex.
6. Centrifuge at 1,000 × *g* for 10 min at 4°C. Discard the supernatant completely.
7. Resuspend cells in 0.5 ml PEB (see Recipes) by pipetting gently and slowly. Place on ice.

D. Red blood cell lysis
1. Make 10 ml of 1× Red Blood Cell Lysis Solution by diluting 10× lysis solution with double-distilled water (ddH_2O). Do not use deionized water (including deionized DEPC water). Store at room temperature.
2. Mix one volume cell suspension with 10 volumes 1× Red Blood Cell Lysis Solution (*e.g.*, 0.5 ml cell suspension + 5 ml 1× lysis solution) in a 15-ml tube. Vortex for 5 s and incubate for 2 min at room temperature. Longer incubation may damage the cells.
3. Centrifuge at 500 × *g* for 10 min at room temperature [Figure 1C (c)]. Discard the supernatant completely and resuspend the pellet in 0.5 ml PEB. Place cells on ice.
4. Mix 5 µl cells with 5 µl Trypan blue and count the number of cells using the Countess™ machine. Approximately 2 million live mononuclear cells can be obtained from a mouse using the above procedure.

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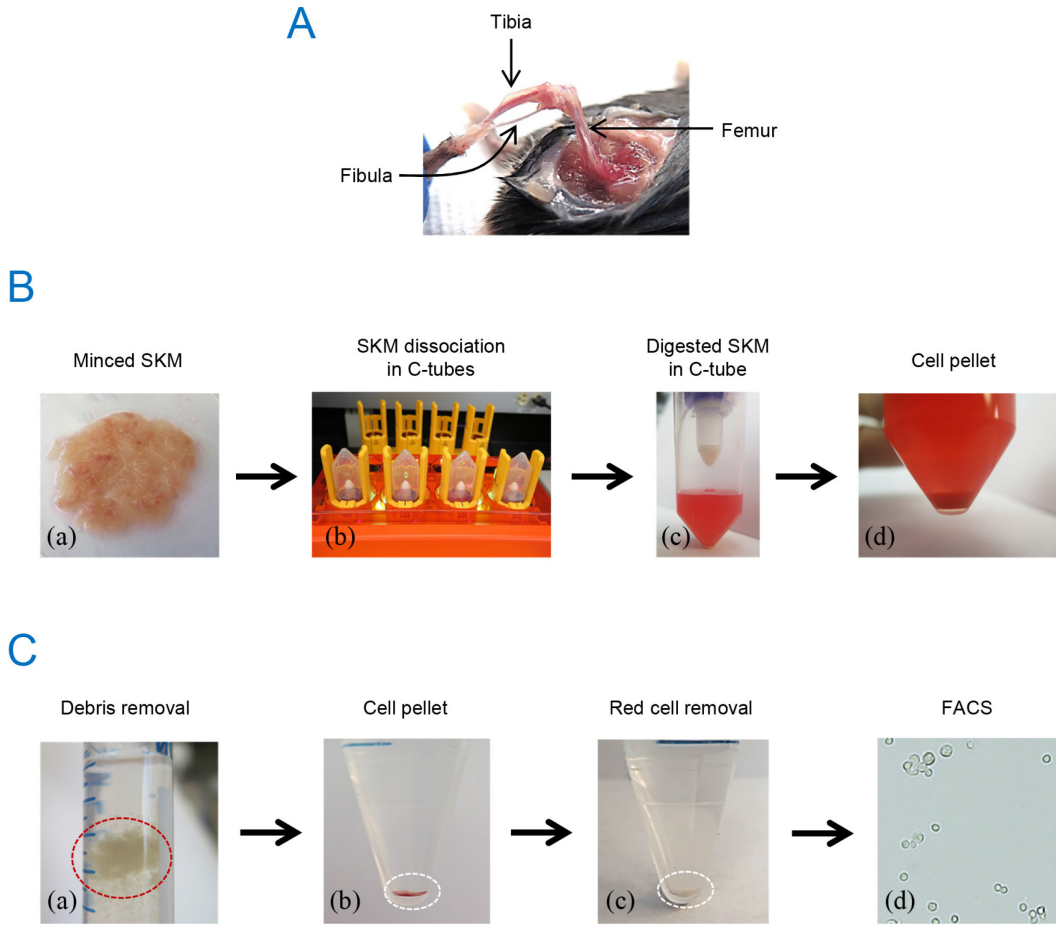

Figure 1. Outline of macrophage isolation from mouse skeletal muscle. A. Hind limb after muscle harvest. B. Images of minced muscle from mouse hind limbs (a), cell dissociator (b), digested muscle in a C-tube (c), and cell pellet after centrifugation (d). C. Images of debris removed by the debris removal solution (a), cell pellet after debris removal (b), cell pellet after red cell removal (c), and cells after sorting (d, 20× brightfield image).

E. Flow cytometry analysis of isolated mononuclear cells

For analytical studies, evenly divide approximately 2 million cells into nine 1.5-ml Eppendorf tubes (50 µl in each), including a tube with unstained live cells, a tube with heat-induced dead cells, and tubes with isotype controls, single antibody controls, or combined antibodies. Add eFluor 780, Fc blocker, isotype control, or antibodies to each tube as explained below.

F. Procedures for staining

1. Add 50 µl PEB to tube 1 (unstained) and place on ice.
2. Incubate tube 2 at 65°C for 10 min to generate the dead cell control. Subsequently, add 950 µl PEB to bring the volume to 1 ml. Place on ice.

3. Add 950 µl PEB to the remaining tubes to bring the volume to 1 ml. Add 1 µl Viability Dye (eFluor 780) to tubes 2, 3, 5, 7, and 9 (tubes 3, 5, and 7 are IgG isotype controls). Mix immediately by inverting the tubes 5–10 times. Incubate for 10 min at 4°C.
4. Centrifuge tubes 3, 5, 7, and 9 at 500 × *g* for 5 min, discard the supernatants, and resuspend in 1 ml PEB. Centrifuge tube 2 at 12,000 × *g* for 5 min at 4°C, discard the supernatant, and resuspend the pellet in 100 µl PEB. Place tube 2 on ice.
5. Add 1 µl Fc blocker to tubes 3–9 and gently vortex for 1 s; repeat 3 times. Incubate for 5 min at room temperature (~25°C).
6. Centrifuge tubes 3–9 at 500 × *g* for 5 min at 4°C. Discard supernatants and resuspend the pellet in 100 µl PEB.
7. Add 1 µl IgG-PE, 1 µl IgG-APC, or 1 µl IgG-FITC to tubes 3, 5, and 7, respectively.
8. Add 1 µl CD11b-PE antibody, 1 µl CD45-APC antibody, or 1 µl CD206-FITC antibody to tubes 4, 6, and 8, respectively (tubes 4, 6, and 8 are for compensation). Add 1 µl CD11b-PE, 1 µl CD45-APC, and 1 µl CD206-FITC to tube 9. Gently vortex for 1 s and repeat 3 times. Incubate for 40 min at 4°C.
9. Centrifuge tubes 3–9 at 500 × *g* for 5 min at 4°C, discard the supernatant and resuspend the pellet in 500 µl PEB. Gently vortex for 1 s and repeat 3 times.
10. Spin down tubes 3–9 at 500 × *g* for 5 min at 4°C, aspirate the supernatant and resuspend the cells in 100 µl PEB. Cells are ready for flow cytometry analysis. Figure 2 shows the results of flow cytometry analysis of isolated mononuclear cells.

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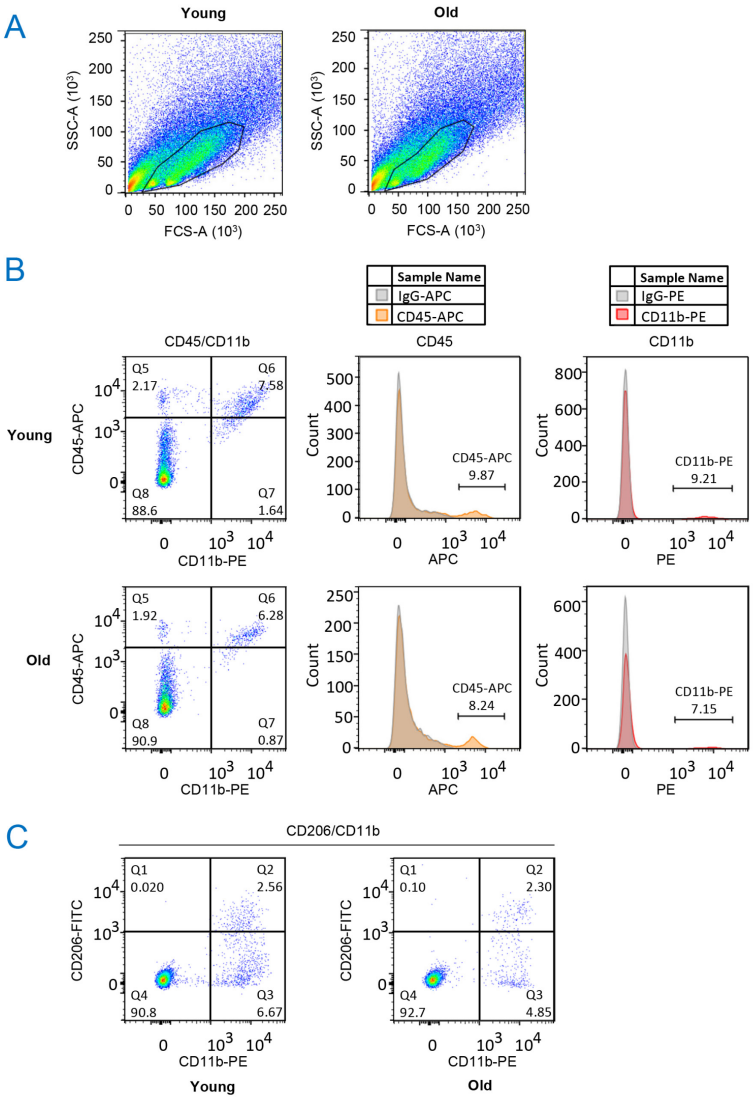

Figure 2. Flow cytometry analysis of isolated mononuclear cells. A. Isolated mononuclear cells from young and old skeletal muscle were gated in a Forward/Side scatter plot. B. Gated cells (from A) were further analyzed for CD45 and CD11b expression. $CD45^+/CD11b^+$ cells were separated from the main cell population (left, gate Q6). The double-positive cells accounted for 7.58% of the total cells in the young preparations and 6.28% in the old preparations (left). $CD45^+$ cells accounted for 9.87% of the total cells in the young preparations and 8.24% in the old preparations (center). $CD11b^+$ cells accounted for 9.21% of the total cells in the young preparations and 7.15% in the old preparations (right). $CD11b^+$ cells were clearly separated from the main population, and almost all $CD11b^+$ cells were $CD45^+$. C. Putative M2 macrophage marker CD206 was highly expressed (gate Q2) in 27.7% of $CD11b^+$ cells (gates Q2+Q3) in the young preparations (left) and in 32.1% of $CD11b^+$ cells in the old preparations (right). BD FACSCanto™ II Cell Analyzer and FlowJo 10 software were used for analysis.

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Notes

1. A practical protocol for stem cell isolation from mouse skeletal muscle was reported by Liu *et al.* (2015). The same protocol was effective for macrophage isolation from skeletal muscle (Kosmac *et al.*, 2018). Here, we tested a modified approach; we used a commercial skeletal muscle dissociation kit combined with a programmable tissue dissociator and added a debris removal step. This protocol allowed the isolation of high-purity skeletal muscle macrophages. We generally obtain approximately 2 million live mononuclear cells from two hind limbs of a mouse, among which ~5–9% of cells are $CD11b^+/CD45^+$. This number is sufficient for FACS and single-cell transcriptomics analyses.
2. Several steps in our protocol were included to improve the purity and yield of macrophages. Firstly, the combination of tissue weight and enzyme mix affects cell yield. In our experience, 0.5 g muscle per 5 ml enzyme mix provided superior muscle digestion and cell yield as compared with 1 g muscle per 5 ml enzyme mix. Hence, we use two C-tubes, each containing approximately 0.5 g muscle and 5 ml enzyme mix, for one mouse. Secondly, we compared two digestion programs – 37C-mr-SMDK-1 (1 h) and 37C-mr-SMDK-2 (1.5 h) – in a gentleMAC octo dissociator with heaters. The 1.5-h program provided more thorough digestion and better cell yield. Thirdly, the debris removal solution effectively removed most of fiber debris, which allowed for much cleaner cell preparations. We highly recommend the debris removal steps.
3. Collectively, our protocol provides an alternative, improved option for primary macrophage isolation from young and old mouse skeletal muscle.

Recipes

1. Medium
   a. DMEM-I
      DMEM containing 1× penicillin and streptomycin solution
   b. DMEM-II
      DMEM-I supplemented with 5% heat-inactivated fetal bovine serum
2. Digestive enzymes
   A skeletal muscle dissociation kit (see Reagents above) was used for mononuclear cell isolation. Upon arrival of the kit, reconstitute and aliquot enzymes D, P, and A, and store at -20°C.
   a. Prepare an enzyme mix for cell dissociation. Remove the enzymes from the -20°C freezer and place at room temperature (~25°C) for 5–10 min. An enzyme master mix will be prepared first.
   b. Each gentleMACS C-tube will include 4.7 ml DMEM supplemented with:
      1× Antibiotics
      200 µl Enzyme D
      50 µl Enzyme P

36 µl Enzyme A

Total 5 ml

c. Make 20 ml enzyme master mix for 4 C-tubes to digest muscles from 2 mice, as follows:

20.21 ml DMEM

860 µl Enzyme D

215 µl Enzyme P

154.8 µl Enzyme A (1 tube × 4.3)

Total 21.65 ml

d. Resuspend the enzyme master mix.

e. Add 5 ml enzyme mix to each of the 4 gentleMACS C-tubes and place on ice. Use two C-tubes to digest skeletal muscles from one mouse. Given that approximately 1 g skeletal muscle can be obtained from two hind legs of a mouse, approximately 0.5 g muscle tissues will be digested in 5 ml enzyme mix in each C-tube.

3. Buffers for flow cytometry analysis

PEB buffer:

1,450 ml Auto MACS Rinsing Solution

150 ml MACS BSA Stock Solution

24 ml 0.5 M EDTA

The PEB buffer contains 1% BSA and 10 mM EDTA

Store at 4°C

4. Reagent combination to label macrophages for flow cytometry

Evenly divide approximately 2 million cells into nine 1.5-ml Eppendorf tubes (50 µl in each). Tube 1 contains unstained live cells and tube 2 contains heat-induced dead cells. Tubes 3, 5, and 7 contain isotype controls, and tubes 4, 6, and 8 contain single antibody controls. Tube 9 contains the combined antibodies. Add eFluor 780, Fc blocker, isotype control, or antibodies to each tube as described below. See Procedures for Staining for more details.

Tube 1: Unstained live cells

Tube 2: 1 µl Viability Dye (eFluor 780)

Tube 3: 1 µl Viability Dye (eFluor 780), 1 µl Fc blocker, and 1 µl IgG-PE

Tube 4: 1 µl Fc blocker and 1 µl CD11b-PE antibody

Tube 5: 1 µl Viability Dye (eFluor 780), 1 µl Fc blocker, and 1 µl IgG-APC

Tube 6: 1 µl Fc blocker and 1 µl CD45-APC antibody

Tube 7: 1 µl Viability Dye (eFluor 780), 1 µl Fc blocker, and 1 µl IgG-FITC

Tube 8: 1 µl Fc blocker and 1 µl CD206-FITC

Tube 9: 1 µl Viability Dye (eFluor 780), 1 µl Fc blocker, 1 µl CD11b-PE, 1 µl CD45-APC, and 1 µl CD206-FITC

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Competing interests

The authors declare no competing interests.

Ethics

Animal use was approved by the Animal Care and Use Committee of the National Institute on Aging, NIH. Approved ID is 476-LGG-2024, which is effective until 2024.

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