Chapter 16

Methods of Isolation and Analysis of TREG Immune Infiltrates from Injured and Dystrophic Skeletal Muscle

Dalia Burzyn, Amy Wagers, Diane Mathis, and Massimiliano Cerletti

Abstract

The immune infiltrate present in acutely injured or dystrophic skeletal muscle has been shown to play an important role in the process of muscle regeneration. Our work has described, for the first time, muscle regulatory T cells (Tregs), a unique population in phenotype and function capable of promoting skeletal muscle repair. Herein, we describe the methods we have optimized to study muscle Tregs, including their isolation from injured muscle, immuno-labeling for analysis/separation by flow cytometry, and measurement of their proliferation status.

Key words Regulatory T cell, Skeletal muscle infiltrate, Tissue-resident leukocytes, Treg proliferation, Skeletal muscle injury, Muscular dystrophy

1 Introduction

In addition to primary and secondary lymphoid tissues, numerous immune cell populations can be found residing in nonlymphoid tissues. Growing evidence indicates that these tissue-resident leukocytes are important players in the maintenance of tissue homeostasis and immune tolerance. Resident innate and adaptive immune cells must keep a fine balance that allows efficient immune responses without causing detrimental effects on the tissue. Interestingly, CD4+ regulatory T cells expressing the transcription factor Foxp3 (Tregs) have been shown to be part of the nonlymphoid tissue-resident immune system in several organs. Because of their strong immunosuppressive capacity, Tregs have a key role in controlling immune over-reactivity in autoimmunity, allergies, infectious diseases, and cancer. Tregs residing in peripheral nonlymphoid tissues have a distinctive phenotype, and are essential for keeping tissue homeostasis and immune tolerance in place [1–3].

The repair of skeletal muscle injuries resulting from trauma, neuromuscular diseases, or other insults, depends essentially on the activity of satellite cells, a pool of skeletal muscle precursors with

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regenerative potential [4–11]. However, successful regeneration can only be achieved with the support of other cellular and molecular systems present at the site of injury. The immune infiltrate that accompanies the repair process has been shown to play an important role in the resolution of the injury, through complex interactions with muscle precursors, dead and nascent fibers, and stroma cells [12, 13]. In particular, our work has shown that a phenotypically and functionally distinct population Foxp3$^+$ regulatory T cells (Tregs) accumulates at the injury site in high frequency and number, and that these cells are required for proper recovery of an acute injury and stabilization of dystrophic disease. Muscle Tregs are important to control the inflammatory response and myofiber regeneration. Muscle Tregs express Amphiregulin, a growth factor that acts directly on satellite cells in vitro and improves muscle repair in vivo [14].

In this chapter we describe the methods for isolation and characterization of immune cells from mouse skeletal muscle, in particular muscle Tregs. The isolation protocol combines enzymatic digestion and density separation, allowing an excellent recovery of infiltrating leukocytes in terms of quality and cell numbers. To analyze the isolated infiltrates, flow cytometry is the main method of choice, given its versatility to study several surface and intracellular markers simultaneously. Success in using this technique will depend on the careful selection of antibody panels and an appropriate gating strategy.

2 Materials

2.1 Immune Cell Isolation Reagents

1. *Processing buffer*: DMEM without phenol red complemented with 2% fetal bovine serum (FBS) and HEPES 10 mM.
2. *2× Digestion buffer*: 0.4% collagenase II (Invitrogen) and 300 μg/ml DNAse I (Sigma) in processing buffer.
3. *Percoll*: Before preparing the different density solutions, osmolality needs to be adjusted by mixing 9 parts of Percoll with 1 part of 10× PBS. Then, 40 and 80% Percoll solutions are prepared by diluting adjusted Percoll with 1× PBS to the desired concentration.

2.2 Immunostaining Reagents

1. *Staining buffer*: DMEM without phenol red (or PBS) complemented with 2% FBS and HEPES 10 mM.
2. *Antibodies*. Unless noted, all antibodies are from Biolegend, *Treg staining panel*: Pacific Blue anti-mouse CD45 (clone 30-F11), FITC anti-mouse TCRβ (clone H5-7597), PerCP-Cy5.5 anti-mouse CD4 (clone GK1.5 or RM4-5), PE anti-mouse CD25 (clone PC61), APC anti-mouse Foxp3 (clone FJK-16s), biotin anti-mouse amphiregulin (polyclonal,

R&D), streptavidin-PE, anti-KI-67 (clone B56, BD Biosciences). *Myeloid cell staining panel*: Pacific Blue anti-mouse CD45 (clone 30-F11), APC-Cy7 anti-mouse Ly6G (clone 1A8), PerCP-Cy5.5 anti-mouse CD11b (clone M1/70), PE anti-mouse I-A/I-E (clone M5/114.15.2), APC anti-mouse F4/80 (clone BM8), PE-efluor610 anti-mouse CD11c (clone N418), FITC anti-mouse Ly6c (clone HK1.4). For proliferation analysis: PE anti-mouse Ki67 (clone B56, BD Biosciences).

3. *FcBlock*: Commercial (such as anti-CD16/CD32) or supernatant from 2.4G2 hybridoma at a concentration of 1:5–1:10 of final staining volume.
4. *PFA Fixation buffer*: 2% paraformaldehyde in PBS. The solution can be stored for long time at 4 °C, protected from light.
5. *Fixation/permeabilization buffers for intracellular staining*: For nuclear antigens such as Foxp3, we use eBioscience Foxp3/Transcription Factor Staining Buffer Set. To prepare Fix/perm buffer: mix 1 volume of Fix/perm with 3 volumes of Fix/Perm diluent. Prepare only the amount needed each time, typically, 200 μl/well. To prepare permeabilization (Perm) buffer: mix 1 volume of 10× Perm buffer with 9 volumes of filtered water. Prepare only the amount needed each time.
6. *Click-iT EdU kit (Molecular Probes)*: EdU reconstitution, 5 mg/ml in PBS. Aliquot and store at −20 °C.

3 Methods

3.1 Muscle Dissection

1. Euthanize the mouse with CO_2.
2. Open skin and peritoneum to expose peritoneal cavity.
3. Cut the diaphragm to expose the heart, cut the cava vein, and immediately perfuse the mouse by injecting 30 ml of PBS into the left ventricle (slowly).
4. Dissect muscles. We usually use three hind limb muscles: tibialis anterior, gastrocnemius, and quadriceps (*see* **Note 1**). Avoid taking popliteal lymph nodes, tendons, and fat.
5. Place the muscles in an Eppendorf tube with 0.8–1 ml of buffer on ice.
6. Weigh tissue to allow calculation of cell numbers per gram of muscle.

3.2 Digesting the Tissue

1. With straight scissors, chop muscles into 0.1 in. pieces.
2. Using a plastic transfer pipette (with the tip cut) transfer tissue and buffer to a 50 ml tube containing 4 ml of processing buffer and add 5 ml of 2× digestion buffer (prepared the same day).

Final digestion volume will be 10 ml. If digesting muscles from two hind limbs (six muscles total), increase the digestion volume to 15 ml.

3. Place the tube horizontally in a 37 °C water bath with gentle shaking and digest for 30 min.
4. Filter through a 70 μm cell strainer, mashing the rest of the tissue with a syringe plunge. Add 20–30 ml of fresh buffer during the process to neutralize collagenase and clean the mesh. Centrifuge 400 × *g*, for 10 min at 4 °C. Carefully discard the supernatant, vortex the pellet, and add 15 ml of processing buffer. Centrifuge 5 min at 400 × *g* and carefully discard the supernatant.

3.3 Leukocyte Isolation by Density Gradient

1. Resuspend pellet in 6 ml of 40% Percoll and transfer to a 15 ml tube. Carefully underlay 6 ml of 80% Percoll. Spin for 25 min at 400 × *g*, at room temperature, with the brake off.
2. After centrifugation, leukocytes will band at the 40–80% interface. With a plastic transfer pipette, take the top layer of fat and dead cells and discard. With a clean pipette then carefully take all the white interface and transfer to a clean tube. Fill the tube with processing buffer, mix by inverting the tube 2–3 times, and centrifuge 400 × *g*, for 10 min at 4 °C. Repeat the washing step. Cells are now ready for use (*see* **Note 2**).

3.4 Immunolabeling of Muscle Infiltrate for Cytofluorometric Analysis of Surface and Intracellular Markers

We routinely stain using two antibody panels, one for analysis of Tregs and other T cells, and one for analysis of myeloid populations. Both panels require staining of surface markers, and the Treg panel requires a second step of permeabilization and intracellular staining for the transcription factor Foxp3. Inclusion of an anti-CD45 antibody in both panels is helpful to set the gating strategy. Although the Percoll separation step produces a clean leukocyte population, some debris is still present which might interfere with the analysis (Fig. 1). The basic panel for Tregs includes, in addition to Foxp3, CD25 (IL-2 receptor alpha), expressed by the majority of Tregs, which will facilitate gating during analysis (Fig. 1). Other markers can be added to both panels if the available flow cytometer allows for detection of more colors. Alternatively, some of the basic markers can be replaced by new markers of interest.

3.4.1 Surface Staining

(a) Aliquot cells for staining. In general, 0.1–2 × 10^6 cells are acceptable numbers for a good staining. If looking for rare populations (e.g., a subset of muscle Tregs), it is recommendable to use the whole cell suspension obtained from one mouse for one staining, as many events will need to be recorded in order to get significant results (*see* **Note 3**). For myeloid cell staining, set apart one fifth to one tenth of the whole cell suspension.

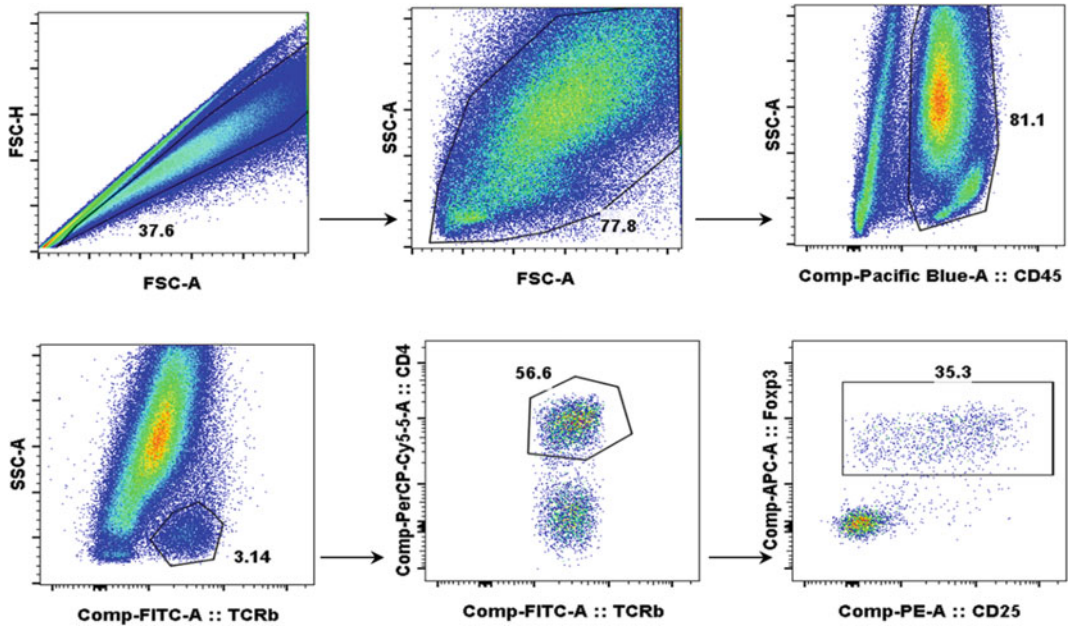

Fig. 1 Gating strategy for analysis of muscle Tregs. Careful exclusion of debris and cell doublets, gating on CD45 positive cells and gating on the TCR beta^{+}/side-scatter^{low} population are key steps to obtain a clean separation of Foxp3 vs. CD25 staining

(b) Set apart some cells for staining controls and isotype controls (*see* **Notes 4** and **5**). All the compensation controls and isotype controls should be treated as the samples of interest (fixation, permeabilization, etc.).

(c) Centrifuge cells 400 × *g*, for 3 min at 4 °C and resuspend the pellet in 50 μl of staining buffer containing FcBlock and the antibodies of interest (*see* **Notes 6–8**) to stain surface markers at the concentrations indicated by the manufacturer or by titration of the reagents. Skip APC-Cy7 conjugated antibodies in this step if doing a fix/perm step later (*see* **Note 9**). Incubate for 20–25 min on ice or at 4 °C, protected from light.

(d) Wash twice by adding staining buffer (0.2 ml if staining in plates, 0.5–1 ml if using tubes) and centrifuging at 4 °C.

(e) If a secondary step is needed, resuspend the cells in 50 μl of staining buffer containing the appropriate secondary reagent (fluorochrome-conjugated streptavidin or secondary antibody). Incubate for 15 min on ice or at 4 °C, protected from light.

(f) Wash twice by adding staining buffer and centrifuging at 4 °C.

(g) If intracellular staining will follow, proceed to Subheading 3.4.2.

(h) If no intracellular staining is required, cells are ready to run in the flow cytometer. If running samples immediately, resuspend the cells in 200 μl of staining buffer and keep at 4 °C until the moment of running. If samples are analyzed the following day/s, fix in 200 μl of PFA fixation buffer and store at 4 °C until the moment of running. Before running, spin down the cells and resuspend in 200 μl of staining buffer or PBS.

3.4.2 Intracellular Staining

(a) *Fixation*: After surface staining and washing, fix cells with 200 μl 1× Fix/perm buffer (eBioscience). Vortex or resuspend very well immediately. Incubate at 4 °C for at least 1.5 h and up to 16 h.

(b) *Wash step*: Centrifuge at 500 × *g*, 4 °C, discard the supernatant, and resuspend in 200 μl of staining buffer or PBS. Centrifuge again. Discard the supernatant.

The following permeabilization and staining steps should be done on the same day the samples will be run in the flow cytometer. If acquisition will not happen on the same day than the fixation step, the protocol can be put on hold by resuspending the cells in 200 μl of staining buffer or PBS and storing them at 4 °C (up to a couple of days). The day of acquisition, centrifuge at 500 × *g*, 4 °C, discard the supernatant and proceed with **step c**.

(c) *Permeabilization*: Resuspend cell pellets in 200 μl of 1× Perm buffer. Centrifuge at 500 × *g*, 4 °C. Discard the supernatant.

(d) *Staining*: The staining should be done in 1× Perm Buffer. Block Fc receptors and unspecific binding by resuspending the cells in 50 μl of 1× Perm Buffer with FcBlock in the appropriate concentration. Incubate 5–10′ at room temperature. Then add the antibodies for intracellular staining and APC-Cy7 conjugated antibody if applicable (*see* Subheading 3.4.1, **step c** and **Note 9**). Incubate for 45 minutes at room temperature, protected from light.

(e) *Wash step*: Add 100 ml of 1× Perm Buffer. Centrifuge at 500 × *g*, 4 °C, discard the supernatant, and resuspend in 200 μl of 1× Perm Buffer. Centrifuge again. Discard the supernatant.

(f) Resuspend the cells in 200 μl of staining buffer and keep at 4 °C until the moment of running.

3.5 Analysis of Treg Cell Proliferation in Skeletal Muscle Infiltrates

In this section we describe two methods to study proliferation in skeletal muscle infiltrates by flow cytometry. The first one is detection of the Ki67 antigen, which is expressed intracellularly by cycling cells (only undetectable during G_0 phase). The second protocol, measurement of EdU (5-ethynyl-2′-deoxyuridine)

incorporation, allows the detection of cells that were in S phase at the time of EdU exposure. EdU is a nucleoside analog that can be used in replacement of the classical BrdU (bromo-deoxyuridine). As with BrdU, EdU is administered to mice to allow in vivo labeling of cycling cells, followed by staining of isolated cells to detect the presence of EdU-containing DNA. With the EdU detection method, mild fixation and detergent permeabilization are sufficient for the small molecule-based detection reagent to gain access to the DNA, as opposed to the BrdU detection method, which uses anti-BrdU antibodies following DNA denaturation by harsh methods (HCl, heat, or enzymes) to expose the BrdU molecules, affecting sample integrity and quality. We successfully combined Foxp3 intracellular staining with EdU detection protocol to study Treg proliferation.

3.5.1 Staining Protocol for Analysis of Ki-67 Expression

(a) Stain surface markers following instructions in Subheading 3.4.1.

(b) Follow instructions for intracellular staining in Subheading 3.4.2, including the anti-Ki67 clone 56. Titration and use of an isotype control are highly recommendable (*see* **Note 10**).

3.5.2 EdU Incorporation

(a) Inject intra vein 1 mg EdU per adult mouse, 4 or 24 h before collecting tissues (*see* **Note 11**).

(b) Isolate muscle infiltrate as described above and stain for cell surface and intracellular markers as usual. Important: avoid using PE, PE-TXRD, APC-Cy7, or other tandem colors, which are incompatible with EdU detection reagents.

(c) After the last wash with Perm buffer of the intracellular staining protocol, add 100 μl EdU mix (prepared following the manufacturer's protocol) and incubate for 30 min at room temperature.

(d) Wash twice with Foxp3 Perm buffer by centrifuging at 500 × *g*, 4 °C.

(e) If necessary, stain with APC-Cy7-conjugated antibody in staining buffer (include FcBlock).

4 Notes

1. Diaphragm from dystrophin-deficient mice (mdx) can also be dissected and processed with this protocol. For perfusion, make an incision between ribs instead of cutting the diaphragm. Reduce digestion volume to 5 ml, the wash volumes by half, and the Percoll band volume to 4 ml.

2. Cell counting is advisable at this point, since the Percoll procedure yields a very clean population of leukocytes with little fiber debris and dead cells that facilitates the counting.
3. The number of cells to stain depends on the abundance of the cell population of interest. In an acute injury model, total leukocyte numbers infiltrating the muscle vary with time, peaking between days 2 and 4 after injury [14]. If working at these time points, injuring three muscles of one hind limb will yield enough cells for running $0.5–1.5 \times 10^6$ events in the flow cytometer. Staining can be done in round-bottom 96-well plates or FACS tubes.
4. Single color staining controls are needed for compensation to correct for spectral overlap during multicolor flow cytometry. Although it is always recommended to use an aliquot of the sample cells to make these controls, we routinely use cells from mouse lymph node or spleen without any complication.
5. Isotype controls (i.e., samples in which a particular antibody is replaced by a nonspecific antibody of the same immunoglobulin isotype as the antibody of interest) are particularly important for antigens that do not stain a clear cell population or have low expression, in order to be able to set the gates during sample acquisition in the flow cytometer, and later for analysis. Unlike single color controls (*see* **Note 4**), isotype controls must be prepared from the same cells that are being analyzed. The use of isotype controls is highly recommended when a new antibody is being used for the first time, and every time if a particular antigen gives a dim, low, or unclear staining pattern.
6. When staining immune cells isolated from non-immune tissues, it is important to include an anti-CD45 antibody to facilitate the analysis (Fig. 1).
7. Fluorochrome conjugates suggested in this protocol can be modified according to user preference. However, it is advisable to test new color combinations since occasionally different conjugates of the same clone can give variable levels of staining and/or trouble with compensation.
8. Percoll separation allows enrichment of live cells. However, a viability dye, actively excluded from live cells, can be included in the protocol. For unfixed cells, propidium iodide (2 μg/ml final) or DAPI (0.1 μg/ml final) can be added right before running the sample in the flow cytometer. For fixed cells (with or without permeabilization step) alternative methods exist that are compatible with fixation, in which the dye (such as LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Molecular Probes) is added and washed before fixation and it remains stable after the fixative is added.

9. APC-Cy7 conjugates can be affected by the fix/perm step. To overcome this issue, we add the APC-Cy7-conjugated antibodies at the intracellular staining step. We have successfully tested this with several markers (TCRβ, CD8, CD4, CD25, CD45). If using an APC-Cy7 conjugate to a different antigen, take into account that some epitopes can be modified after fixation, affecting antibody binding.
10. Clone B56 is sold as anti-human Ki-67 but cross-reactivity with mouse Ki67 has been shown, and this clone has been widely used in mouse assays. Please note that the antibody is sold in a low concentration solution, so a higher volume needs to be used (typically 10–20 μl/sample). Titration and use of isotype control are highly recommendable.
11. For analysis of proliferation in muscle infiltrates, we found that intra vein injection delivers higher levels of EdU to the muscle than intraperitoneal injection.

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