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Title: Isolation and purification of tissue resident macrophages for the analysis of nuclear receptor activity.

Running head: Isolation of tissue resident macrophages.

Isolation and purification of tissue resident macrophages for the analysis of nuclear receptor activity.

Abstract:

Tissue resident macrophages (TRM) are multifunctional immune cells present in all tissues, contributing to the correct development, homeostasis, and protection against pathogens and injury. TRMs are morphologically and functionally heterogeneous, as a result of both the diversity of tissue environments in which they reside and their complex origin. Furthermore, some specific TRM populations are controlled by nuclear receptors. A widely used method for studying the role of nuclear receptors in immune cells is flow cytometry. Although flow cytometry is widely used in tissues such as peripheral blood, lymph nodes, peritoneal cavity, and bone marrow, there is a need for protocols for the study TRMs in solid tissues.

In this chapter, we describe a comprehensive protocol for obtaining single-cell suspensions of resident macrophages from pleural cavity, heart, lung, spleen, and kidney, and we present detailed gating strategies for the study of nuclear receptor activity in different TRM subsets within these tissues.

Keywords: tissue resident macrophages, heart, pleural cavity, kidney, spleen, lung, nuclear receptors, tissue digestion, flow cytometry.

1 Introduction

The term macrophage was first coined by Élie Metchnikoff in 1892 to identify cells involved in the phagocytosis process during inflammation (Metchnikoff, 1892). Macrophages are immune cells that play roles in many specific processes in order to maintain tissue homeostasis[1]. Tissue resident macrophages (TRM) are

multifunctional, specialized, and heterogeneous cells found in most mammalian tissues. They act as sentinel cells or as an immune barrier against pathogens, protecting against homeostatic imbalance and tissue damage. However, TRMs have many other functions required not only in adulthood but also during development; moreover, many of these functions vary from one tissue to another[2, 3]. Macrophages were for many years thought to always originate from adult bone marrow; however, lately it has become evident that most adult TRMs originate during embryonic development and not from circulating monocytes[2, 4-6]. The contribution of circulating monocytes to TRM populations is restricted to a few specific tissues, including the gut, the skin, and the heart or during inflammation and injury[4, 7, 8]. In adulthood, most TRMs are replaced by local self-renewal independently of bone-marrow-derived precursors[9-11].

Some TRMs subtypes are regulated by specific types of nuclear receptor (NR)[12-15]. NRs are ligand-dependent TFs that regulate diverse aspects of development and homeostasis[16-18]. Important NRs involved in TRM regulation include LXRα in marginal zone splenic macrophages[12], PPARγ in alveolar macrophages[14], and RAR in peritoneal macrophages[13]. The complex transcriptional control of TMR subsets creates a need for powerful tools allowing scientists to distinguish among the many different populations.

Flow cytometry is a powerful and established tool in the study of the immune system (e.g. immunophenotyping of peripheral blood cells, analysis of apoptosis and detection of cytokines)[19, 20]. Flow cytometry is based on the light scattering properties of the cells under investigation. Light scattering at different angles can distinguish differences in size and internal complexity; in addition, light emitted from fluorescently labeled antibodies can identify a great variety of cell surface and

cytoplasmic antigens[21]. These properties make flow cytometry a powerful tool for the rapid and detailed analysis of complex populations. State-of-the-art flow cytometers are able to analyze up to millions of cells, recognizing and differentiating, depending on the system, more than 10 fluorochromes, and thus allowing the precise identification of several populations in a single sample. Flow cytometry techniques are improving continually. Moreover, the combination of flow cytometry with time-of-flight detectors (CyTOF) and sophisticated algorithms (tSNE) is helping researchers to identify new subsets within already known TRM populations, ensuring an unbiased study of the cells.

In this chapter, we describe how to harvest, digest (if needed), and prepare single-cell suspension of purified TRM samples from pleural cavity, heart, lung, spleen, and kidney. These protocols generate high yields of leukocytes, and specifically macrophages, from the indicated tissues. Furthermore, the staining antibodies we propose here are optimized to give excellent fluorescence resolution, allowing the identification of diverse TRM subsets within each tissue.

2 Materials

- Adult (8-12 weeks old) C57BL/6 mice.
- Collagenase type IV from *Clostridium histolyticum.*
- Hybridization oven with rocker.
- 70% Ethanol in distilled water.
- Sterile surgical tools: tweezers and scissors.
- Cold (around 4ºC) 1X PBS.
- 1, 2, and 10 mL syringes.
- 18, 21, and 25 G needles.

- 1.5, 15, and 50 mL polipropylene conical test tubes.
- CO_2 chamber.
- 1.5 mL tubes.
- Red blood cell lysis buffer: 4.13 g NH_4Cl, 0.5 g $KHCO_3$, 100 µL EDTA, 500 mL distilled H_20.
- Portable electric pipette controller.
- Sterile 10, 200, and 1000 plastic tips.
- Compensation beads.
- Pipettes.
- 10 mL glass pipettes.
- Vortex.
- Precision balance.
- Neubauer chamber.
- Trypan blue.
- Centrifuge.
- Bright field microscope.
- Aluminum foil.
- Tape.
- FACS buffer: 1X PBS, 1% inactive fetal bovine serum (FBS) and 5mM EDTA.
- Heart and kidney digestion buffer: 1X PBS with 100 µg/mL collagenase IV.
- Lung digestion buffer: 1X PBS with 10 µg/mL collagenase IV.
- Cytometer tubes.
- Antibodies for flow cytometry (see **Table 1**).
- 70 and 100 µm nylon cell strainers.

- 70 µm conical filters.
- Flow cytometer/cell sorter.

3 Methods

3.1. Setting up tools and reagents in the lab

3.1.1. Set up the hybridization oven at 37ºC.

3.1.2. Set up the centrifuge at 4ºC.

3.1.3. Thaw collagenase type IV from -20ºC to 4ºC.

3.1.4. Remove the red blood cell lysis buffer from the fridge and place it at room temperature.

3.2. Mouse sacrifice and tissue harvesting

3.2.1. Euthanize the mouse in the CO_2 chamber or by cervical dislocation.

3.2.2. Place the mouse on its back. Clean the chest and abdomen by soaking all the fur with 70 % ethanol.

3.2.3. Using tweezers and scissors, open the animal (see **Note 1**). First, make a small incision in the skin with the scissors. With the scissors near-closed, introduce them under the skin and then open them up. Remove the scissors and repeat the processed until you have completely separated the skin from the peritoneum and ribs. After this, gently pull the fur back to expose the inner skin lining the peritoneal cavity and chest (see **Note 2**).

3.2.4. Cap a 2 mL syringe with a 25 G needle and insert the needle between the 5^{th} and 6^{th} rib of the mouse (see **Note 3**). Introduce 2 mL of cold 1X PBS into the pleural cavity.

3.2.5. Using a 2 mL syringe capped with a 20 G needle, recover as much as possible of the pleural lavage. We suggest injecting the needle in the

inflated part close to the liver. It is extremely important not to damage the lungs.

3.2.6. Transfer the pleural lavage into a 15 mL test tube and fill it to 10 mL with cold 1X PBS.

3.2.7. Use the scissors to open the chest under the sternum and cut the ribs to expose the heart. Cut the right atrium and, using a 10 mL syringe capped with a 25 G needle, gently perfuse the animal through the apex with 20 mL of cold 1X PBS (see **Note 4**).

3.2.8. Repeat step 3.2.7., but this time perfuse through the right ventricle.

3.2.9. Dissect the atria from the ventricles and discard.

3.2.10. Transfer the ventricles to 1.5 mL tubes containing 1 mL cold 1X PBS.

3.2.11. Collect the lungs. Introduce the multilobular part in cold 1X PBS in 1.5 mL tubes.

3.2.12. Cut the splenic vessels and remove the spleen with the help of tweezers.

3.2.13. Next, cut the renal vessels and harvest the kidneys in cold 1X PBS in 1.5 mL tubes.

3.2.14. Weigh the solid tissues on a precision balance (see **Note 5**).

3.3. **Preparation of single-cell suspension from pleural resident macrophages.**

3.3.1. Transfer the pleural lavage to a 15 mL test tube and fill until 10 mL with cold 1X PBS.

3.3.2. Centrifuge for 5 minutes at 1500 rpm and discard the supernatant.

3.3.3. If blood is visible: resuspend the pellet with a vortex in 300 μl of red blood cell lysis buffer for 3 minutes. Then wash with 30 mL of 1X PBS (see **Notes 6** and **7**).

3.3.4. Centrifuge for 5 minutes at 1500 rpm and discard the supernatant.

3.4. **Preparation of single-cell suspension from cardiac resident macrophages.**

3.4.1. Place each heart in one 1.5 mL tube with 1mL of digestion buffer (see **Note 8**).

3.4.2. Finely chop the heart into small pieces with small sterile scissors (see **Notes 9, 10** and **11**).

3.4.3. To digest the heart, place the sample in the hybridization oven and incubate at 37ºC for 45 minutes with gentle shaking (30 rpm). Hold the samples in place by sticking them to the oven surface with tape (see **Note 11**).

3.4.4. Mechanically homogenize the digested tissue with up and down motions in a 1 mL syringe capped with a 18 G needle. Work on ice from this point on (see **Note 12)**.

3.4.5. To remove the tissue stroma, transfer the digested sample to a 50 mL tube through a 100 µm nylon cell strainer. Gently press the digested tissue against the cell strainer and wash it with around 30 mL of 1X PBS (see **Note 13**).

3.4.6. Centrifuge for 10 minutes at 1500 rpm at 4ºC and discard the supernatant.

3.4.7. To eliminate erythrocytes, resuspend the pellet with vortexing in 600 µL of red blood cell lysis buffer. Incubate for 3 minutes at room temperature (see **Note 14**).

3.4.8. Wash with 10 mL FACS buffer, centrifuge for 5 minutes at 1500 rpm at 4ºC, and discard the supernatant.

3.5. **Preparation of single-cell suspension from alveolar macrophages.**

3.5.1. Place each lung sample in one 1.5 mL tube with 1 mL of lung digestion buffer (see **Note 8**).

3.5.2. Finely chop the lung sample into small pieces with small sterile scissors (see **Notes 9, 10** and **11**).

3.5.3. To digest the lung tissue, place the sample in the hybridization oven and incubate at 37ºC for 30 minutes with gentle shaking (30 rpm). Hold the samples in place by sticking them to the oven surface with tape (see **Note 11**).

3.5.4. Mechanically homogenize the digested tissue with up and down motions in a 1 mL syringe capped with a 18 G needle Work on ice from this point on (see **Note 12)**.

3.5.5. To remove the tissue stroma, transfer the digested sample to a 50 mL tube through a 70 µm nylon cell strainer. Gently press the digested tissue against the cell strainer and wash it with around 30 mL of 1X PBS (see **Note 13**).

3.5.6. Centrifuge for 5 minutes at 1.500 rpm at 4ºC and discard the supernatant.

3.5.7. To eliminate erythrocytes, resuspend the pellet with vortexing in 500 µL of red blood cell lysis buffer. Incubate for 3 minutes at room temperature (see **Note 14**).

3.5.8. Wash with 10 mL FAC buffer, centrifuge for 5 minutes at 1500 rpm at 4ºC, and discard the supernatant.

3.6. **Preparation of single-cell suspension from spleen resident macrophages.**

3.6.1. Place the spleen on a pre-wetted (with 1X PBS) 100 µm nylon filter over an open 50 mL tube. Squeeze the spleen against the filter with the plunger of a 2 mL syringe.

3.6.2. Pass 50 mL of 1X PBS through the filter.

3.6.3. Centrifuge for 5 minutes at 1500 rpm at 4ºC and discard the supernatant.

3.6.4. Resuspend the pellet by vortexing in 500 μL of red blood cell lysis buffer for 5 minutes at room temperature. Then wash with 30 mL of 1X PBS.

3.6.5. Centrifuge for 5 minutes at 1500 rpm and discard the supernatant.

3.7. **Preparation of single-cell suspension from kidney resident macrophages.**

3.7.1. Cut the kidney into two halves with sterile scissors.

3.7.2. Select the half that to be processed and weigh it in the precision balance (see **Note 15**).

3.7.3. Finely mince the tissue in 500 μL digestion buffer in a 1.5 mL tube. Ensure that no visible tissue pieces remain (see **Notes 9, 10** and **11**).

3.7.4. To digest the half kidney, place it in the hybridization oven and incubate at 37ºC for 45 minutes with gentle shaking (30 rpm). Hold the samples in place by sticking them to the oven surface with tape (see **Note 16**).

3.7.5. Transfer the digested tissue to a 50 mL test tube through a 100 μm nylon cell strainer. Ensure you recover all the sample by washing the digestion tube with up to 30 ml of 1X PBS.

3.7.6. Centrifuge for 5 minutes at 1500 rpm and discard the supernatant.

3.7.7. Resuspend the pellet by vortexing in 500 μl of red blood cell lysis buffer for 3 minutes at room temperature. Wash with 30 mL of 1X PBS.

3.7.8. Centrifuge for 5 minutes at 1500 rpm and discard the supernatant.

3.8. **Staining of TRMs for flow cytometry**

The following steps are the same for all five tissues.

3.8.1. **Cell counting.**

3.8.1.1. Mix 10 μL of the cell suspension with 10 μL Trypan blue.

3.8.1.2. Count the viable cells in a Neubauer chamber under a bright field microscope fitted with a 10 X objective.

3.8.1.3. Centrifuge the cell suspension for 5 minutes at 1500 rpm and discard the supernatant.

3.8.2. **Staining of pleural, cardiac, alveolar, spleen, and kidney resident macrophages for flow cytometry**

You will need to use the full cell suspension for the pleural cavity and heart. For spleen resident macrophages, use and $5x10^6$ cells, for alveolar macrophages use 1/7 of the sample, and for kidney resident macrophages use 2/5 of the sample.

3.8.2.1. Block the cells by adding 100 µL of anti-CD16/CD32 Ab (1:100) in FACS buffer per sample.

3.8.2.2. Incubate for 10 to 15 minutes at 4ºC.

3.8.2.3. For staining, transfer the samples to a round-bottom p96-well plate or cytometer tubes (see **Note 17** and **18)**.

3.8.2.4. Wash with FACS buffer: 200 µL for p96-well plate or 1 mL for cytometer tubes.

3.8.2.5. Centrifuge for 5 minutes at 1500 rpm at 4ºC and discard the supernatant.

3.8.2.6. Use compensation beads to establish the cytometer settings according to your staining panel. Put one drop of compensation beads in empty wells of the p96-well plate or in fresh cytometer tubes (one for each fluorochrome used). Add one tested antibody to one well or tube containing compensation beads; the antibody should have the same concentration you will use in the staining panel. From this point, the compensation beads and experimental samples must follow the same steps of incubation, washing, and centrifugation.

3.8.2.7. Add 50 µL (p96-well plate) or 100 µL (cytometer tube) of antibody mix per sample. Incubate for 30 min at 4ºC with gentle shacking (30 rpm). Protect samples from light with aluminum foil (see **Notes 19** and **20**).

3.8.2.8. Proposed antibody panels (see **Table 1**).

	Antibody	**Clone**	**Concentration (µg/ml)**
Pleural macrophages	CD45-APC-Cy7	30-F11	2
	B220-PerCPCy5.5	RA3-6B2	2
	CD11b-AF647	M1/70	2
	CD115-PE	AFS98	2
	F4/80-PECy7	BM8	2
	MHC-II-BV605	M5/114.15.2	0.67
Cardiac macrophages	CD45-PerCPCy5.5	30-F11	2
	CD11b-PECy7	M1/70	2
	F4/80-PE	BM8	6
	Ly6C-APC	AL-21	4
	MHC-II-BV605	M5/114.15.2	0.67
	CCR2-APC	475301	5
Alveolar macrophages	CD45-PerCPCy5.5	30-F11	2
	CD11b-PECy7	M1/70	2
	CD11c-FITC	N418	5
	SiglecF-PE	E50-2440	4
Splenic macrophages	CD45-APC-Cy7	30-F11	2
	CD11b-AF647	M1/70	2

	F4/80-PECy7	BM8	2
	MHC-II-BV605	M5/114.15.2	0.67
Kidney macrophages	CD45-APC-Cy7	30-F12	2
	CD11b-AF647	M1/71	2
	F4/80-PECy7	BM9	2
	Tim 4-PE	RMT4-54	2

Table 1. Proposed panel of antibodies for the gating strategies described in Fig (X-Y). For each antibody, we indicate the clone we use and the concentration. Note that the concentration needs to be adjusted according to the fluorescence intensity of the fluorochrome selected for each clone.

3.8.2.9. Transfer samples stained in p96 well plates to cytometer tubes. Wash with 1 mL FACS buffer and centrifuge for 5 minutes at 1500 rpm at 4ºC and discard the supernatant.

3.8.2.10. Resuspend in 200-300 µL FACS buffer.

3.8.2.11. Filter the samples through 70 µm conical filters and transfer them to fresh cytometer tubes.

3.8.2.12. Place the samples on ice and go to the cytometer.

3.8.3. **In the cytometer/cell sorter**

3.8.3.1. Use a small fraction of one stained sample to adjust the voltage settings. Remember to do this for each tissue analyzed.

3.8.3.2. Run the compensation beads to create the compensation matrix. Remember to do this for every antibody panel.

3.8.3.3. Run the negative and FMO controls to create the layout of the gating strategy for every tissue.

3.8.3.4. Run your stained samples and acquire the data.

3.8.3.5. Export the data for later analysis with the software of your choice.

3.8.4. **Flow cytometry analysis of pleural resident macrophages**

Within the single viable cell population, we gate leukocytes ($CD45^+$ cells). We then exclude B cells by gating only $B220^-$ cells (see **Fig. 1**) (see **Note 21**). Macrophages are gated within the $CD11b^+$ population. We can then differentiate 2 macrophage populations based on their levels of F4/80 and MHCII expression: large pleural macrophages (LPMs) are $F4/80^{high}MHCII^{-/low}$ cells, and small pleural macrophages (SPMs) are defined as $F4/80^{-/low}MHCII^{high}$ cells (see **Fig. 1**).

3.8.5. **Flow cytometry analysis of cardiac resident macrophages**

Within the single viable cell population, we gate leukocytes ($CD45^+$ cells). We then select macrophages ($CD11b^+F4/80^+$ cells) and differentiate two cardiac macrophage populations based on their level of MHCII expression: $MHCII^{high}Ly6C^{low}$ macrophages and $MHCII^{low}Ly6C^{low}$ macrophages (see **Fig. 2**). We can also identify monocytes as $Ly6C^{hi}$ cells.

3.1.1. **Flow cytometry analysis of alveolar macrophages**

Within the single viable cell population, we gate leukocytes ($CD45^+$ cells). We then select $Ly6G^-/CD11b^+$ cells to exclude neutrophils. Alveolar macrophages are distinguished as $CD11c^{high}SiglecF^{high}$ cells (see **Fig. 3**).

3.1.2. **Flow cytometry analysis of spleen resident macrophages**

Within the single viable cell population, we gate leukocytes ($CD45^+$ cells). We then select myeloid cells as $CD11b^+$ cells. Two macrophage subsets can be differentiated according to the expression F4/80 and Tim-4: red pulp macrophages are $F4/80^+$, whereas marginal zone macrophages are $Tim\text{-}4^+$ (see **Fig. 4**).

3.1.3. **Flow cytometry analysis of kidney resident macrophages**

Within the single viable cellpopulation, we gate leukocytes ($CD45^+$ cells). We can then differentiate two macrophage subsets according to the expression levels of CD11b and F4/80: $CD11b^{low}F480^{high}$ and $CD11b^{high}F480^{low}$ (see **Fig. 5**).

4. **Applications:**

The protocol described here is intended for the study of the contribution of NRs to TRM development, identity, and function. The protocol can be used to characterize TRMs from wild type and nuclear-receptor knockout mice in order to analyze the role of the nuclear receptor under study.

This protocol can also be used to obtain pure TRM populations by cell sorting for further analysis, such as genome-wide studies (RNA-Seq, ATAC-Seq, ChIP-Seq, GRO-seq), qPCR, and ligand-based activation of NRs in cell culture.

5. **Notes**

1. We recommend use blunt-ended scissors.

2. If you also intend to do a peritoneal lavage, be careful not to break the peritoneum.

3. It is extremely important to introduce only the needle tip; otherwise, lungs can be damaged and the pleural lavage will be contaminated with red blood cells

and circulating monocytes. A sign that the pleural inflation is proceding well is movement of the liver toward the intestine.

4. It is important to perfuse the mouse well in order to eliminate erythrocytes and circulating cells as much as possible. This avoids having a mix of TRMs and circulating myeloid cells during the flow cytometry analysis.

5. Weighing the tissues allows calculation of TRM abundance (TRMs/mg of tissue).

6. Sometimes, blood may be visible due to the injection or inflation. We recommend lysing red blood cells to ensure correct labeling in subsequent steps.

7. If the lungs are damaged during lavage, we strongly recommend discarding the sample because of the risk of contamination by circulating leukocytes.

8. We strongly recommend that you fine tune the conditions for your own lab, varying digestion times by 10 minutes among tests, trying different collagenase concentrations, and even using digestion buffers different from those proposed here, for example, Hanks' balanced salt solution with Ca^{2+}. Be careful with collagenase incubation times; exposures longer than 1 hour will dramatically decrease the yield of viable cells.

9. Tissue pieces must be small enough to pass through a 18 G needle.

10. If you have more than one sample, keep the already minced tissues on ice while mincing the rest.

11. Thorough mincing with shaking is very important to ensure proper cell extraction.

12. The combination of enzyme digestion and mechanical tissue disruption improves TRM yield during digestion and isolation.

13. We recommend use of the soft end of a 2 mL syringe plunger to squeeze the sample residues.

14. Although the mouse is perfused through the heart, erythrocyte lysis is required afterwards to eliminate residual red blood cells. Note that before the lysis, the pellet is still red, and after the lysis it is completely white.

15. Maintain the other half in 1X PBS on ice; you many need it in case something goes wrong.

16. We strongly recommend shaking the tissue by hand every 5 minutes to increase the cell yield.

17. When several stainings are required, we recommend dividing the sample among different wells or tubes. Nevertheless, avoid this practice with small cell populations.

18. When testing a new antibody, it is advisable to use fractions of the cell sample for a negative control (50 000 cells) and a fluorescence minus one (FMO) control (50 000 cells). We recommend that these fractions are taken from the sample with the highest cell number. In the negative control, cells are not labeled with antibody, which allows the negative and the positive populations to be discerned. In the FMO control, the sample is stained with all the antibodies except the one being measured, revealing false-positive signals due to the effect of the other fluorochromes. In both controls, the cells must be processed with the same protocol as the experimental samples.

19. You can adjust the incubation time to your needs (minimum 15 minutes, maximum 2 hours).

20. It is very important to protect antibodies from light in order to prevent bleaching of conjugated fluorochromes.

21. Macrophages are highly autofluorescent; as a result, the population of $F4/80^+$ cells in the $B220^-$ gate seems to bepartly $B220^+$.

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Figure 1. Example gating strategy for pleural resident macrophages.

Figure 2. Example gating strategy for cardiac resident macrophages.

Figure 3. Example gating strategy for alveolar macrophages.

Figure 4. Example gating strategy e for splenic resident macrophages.

Figure 5. Example gating strategy for kidney resident macrophages.

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