Video Article

Analysis of Microglia and Monocyte-derived Macrophages from the Central Nervous System by Flow Cytometry

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Abstract

Numerous studies have demonstrated the role of immune cells, in particular macrophages, in central nervous system (CNS) pathologies. There are two main macrophage populations in the CNS: (i) the microglia, which are the resident macrophages of the CNS and are derived from yolk sac progenitors during embryogenesis, and (ii) the monocyte-derived macrophages (MDM), which can infiltrate the CNS during disease and are derived from bone marrow progenitors. The roles of each macrophage subpopulation differ depending on the pathology being studied. Furthermore, there is no consensus on the histological markers or the distinguishing criteria used for these macrophage subpopulations. However, the analysis of the expression profiles of the CD11b and CD45 markers by flow cytometry allows us to distinguish the microglia ($CD11b^{+}CD45^{med}$) from the MDM ($CD11b^{+}CD45^{high}$). In this protocol, we show that the density gradient centrifugation and the flow cytometry analysis can be used to characterize these CNS macrophage subpopulations, and to study several markers of interest expressed by these cells as we recently published. Thus, this technique can further our understanding of the role of macrophages in mouse models of neurological diseases and can also be used to evaluate drug effects on these cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/55781/

Introduction

The microglia are the parenchymal tissue-resident macrophages of the central nervous system (CNS). They play two key functional roles: immune defense and maintenance of the CNS homeostasis. In contrast to the MDM, which are renewed continually from the hematopoietic stem cells in the bone marrow, the microglial cells differentiate from primitive hematopoietic progenitor cells originating in the yolk sac (YS) that colonized the brain during embryonic development[1,2,3]. In rodents, the transcription factor Myb plays a crucial role in the development of all bone marrow derived monocytes and macrophages, but for YS derived microglia, this factor is dispensable and differentiation remains dependent on the transcription factor PU.1[4].

In the healthy CNS, microglia are dynamic cells that constantly sample their environment, scanning and surveying for invading pathogens or tissue damage[5]. The detection of such signals initiates a pathway to resolve the injury. The microglia rapidly switch from a ramified morphology to an amoeboid one, which is followed by phagocytosis and release of various mediators, such as pro- or anti-inflammatory cytokines. Thus, depending on their microenvironment, activated microglia can acquire a spectrum of distinct priming states[6].

The microglia profoundly impact the development and progression of many neurological disorders. In the rodent models of Alzheimer's disease (AD)[7], Amyotrophic Lateral Sclerosis (ALS)[8], Multiple Sclerosis (MS)[9] or Parkinson disease (PD)[10], the microglia are shown to play a dual role, either inducing detrimental neurotoxicity or acting in a neuroprotective manner, which is dependent on the specific disease, the disease stage, and whether the disease was influenced by the systemic immune compartment[7,8,9,10,11]. Most of the CNS lesions observed in the diseases cited above contain a heterogeneous population of myeloid cells, including not only parenchymal microglia, but also perivascular and meningeal macrophages, as well as CNS-infiltrating MDM. These cell types may differentially contribute to the pathophysiologic mechanisms related to injury and repair[7,12,13,14,15]. The current challenge for investigators who are studying these disease models is to establish whether the peripheral monocytes and macrophages infiltrate the CNS and if so, to distinguish the resident microglia from these cells. Indeed, the microglial cells are very plastic; when they are activated, the microglia re-express markers that are usually expressed by peripheral monocytes and macrophages. The issue, therefore, relies on identifying markers that can distinguish the resident microglia from the infiltrating monocytes and macrophages.

The discrimination of these populations on brain slices by immunohistological applications is limited due to the lack of specific antibodies. However, flow cytometry analysis is an efficient technique to assess the expression of several markers and to distinguish cell populations (for example, lymphocytes, macrophages/MDM $CD11b^{+}CD45^{high}$, and microglia $CD11b^{+}CD45^{med}$), as well as cell subpopulations[16,17,18]. This

protocol describes the procedures for isolating the mononuclear cells from the mouse CNS in neurologic disease models by using an optimized, enzymatic tissue dissociation and a density gradient centrifugation; as well as, a method for differentiating the microglia and MDM populations in the CNS by using flow cytometry.

Another approach is to eliminate the myelin and purify the cells by using magnetic beads conjugated to specific antibodies[19,20,21]. Myelin removal using anti-myelin magnetic beads is more expensive and affects the viability and the yield of isolated cells[22]. This step and the following immunomagnetic separation of the microglia, limit further studies of specific immune cell populations[21,22].

These procedures provide an easy way to study the macrophage subpopulations in disease development, and to determine the drug effects or gene modifications on macrophage phenotypes and activation states.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee at the ICM Institute and by the Darwin French Ethic Animal Committee, and are covered under the protocol 01407.02.

1. Preparation

1. Prepare the digestion cocktail in a 1.5 mL tube by combining the following for each mouse: 1 mL Phosphate Buffered Saline (PBS); 123 µL digestion enzyme (see table of materials) at 13 wunsch/mL (stock solution), final concentration 1.6 wunsch/mL; and 5 µL DNase I (see table of materials) at 100 mg/mL (stock solution), final concentration 0.5 mg/mL.

2. Perfusion and Dissection

1. Euthanize the mice with a lethal dose of pentobarbital (100 µL at 400 mg/mL).
2. Place the animal in supine position and wet the body with 70% ethanol.
3. Cut the ventral skin with fine scissors just below the xyphoid process to expose the thoracic cavity.
4. Make an incision in the diaphragm and cut the lateral aspects of the rib cage with fine scissorsin a caudal to rostral direction to expose the heart (and avoid cutting other organs). Identify the left ventricle (LV) and the right atrium (RA).
5. Insert a butterfly catheter with a 25 G needle inside the LV. Make an incision on the RA with fine scissors and at the beginning of blood flow, start perfusion at 10 mL/min flow rate through the LV with 20 mL PBS to remove cells and blood from vessels; successful perfusion is noted by the blanching of the lungs and the liver as blood is displaced.
6. Decapitate the animal with medium scissors. Dissect the spinal column with fine scissors and separate it from the body by excising the abdominal wall musculature on the ventral side of the spinal column and by cutting the vertebral column at the base of the tail.
7. Insert a 10 mL syringe containing PBS and a mounted 200 µL pipette tip into the lumbar side of the spinal column and flush the spinal cord on the cervical side.
   NOTE: The 200 µL pipette tip is cut with scissors at its widest end (approximately 1 cm) and tightly inserted on a 10 mL syringe previously filled with PBS.
8. Remove the skin above the skull and use forceps to expose the brain. Insert a scissor blade at the base of the skull and cut the cranium following the sagittal suture. Insert small forceps into the cut and gently lift the skull. Dissect the brain from the mouse head and remove the olfactory bulb and cerebellum.
9. Collect the brain and spinal cord in a 1.5 mL tube containing 1.128 mL PBS with digestion cocktail (from step 1.1).

3. Cell Dissociation

1. Finely cut the brain and spinal cord in the 1.5 mL tube (step 2.9) into 1-2 mm^3 pieces with small scissors (**Figure 1**).
2. Incubate the 1.5 mL tube containing the minced tissues for 30 min in an incubator at 37 °C with 5% CO_2.
   NOTE: Prepare the density gradient at this step.
3. Add 20 µL 0.5 M EDTA (stock solution), final concentration 10 mM, to the 1.5 mL tube to stop the enzymatic reaction.
4. Gently make a cell suspension by pipetting up and down with a 5 mL pipette.
5. Pass the cell suspension through a nylon mesh (70 µm pore size) in a 50 mL tube using the plunger of a 10 mL syringe.
6. Wash the nylon mesh with 20 mL 5% Fetal Bovine Serum (FBS)-PBS.
7. Centrifuge for 7 min (300 x g) at room temperature (18 °C). Discard the supernatant by aspiration and proceed to the next step. Be extremely careful when removing the supernatant; do not disturb the pellet, which can easily be aspirated by the aspirating pipette.

4. Density Gradient

1. Prepare a stock solution of the density gradient medium by adding the appropriate amount of 10x PBS to the density gradient medium.
   NOTE: Add one part 10X PBS to nine parts density gradient medium.
2. Dilute the stock density gradient medium with 1x PBS for the different percentages as described in **Table 1**.
3. Resuspend the pellet (from step 3.7) with 4 mL 30% density gradient medium and transfer to a 15 mL tube.
4. Place a Pasteur pipette in the bottom of the 15 mL tube and slowly underlay 4 mL of 37% density gradient medium.
5. Add 4 mL of 70% density gradient medium, as described above.
6. Centrifuge the gradient for 40 min (800 x g) at room temperature (18 °C). Ensure that the centrifuge stops with minimal or no brake so that the interphase is not disturbed.
   NOTE: The tube will appear stratified. The cells layered at the 70% - 37% density gradient interface are the mononucleated immune cells (microglia and macrophage subpopulations); the upper levels are cell debris and myelin, respectively (**Figure 2**).

7. Using a transfer pipette, gently remove the layer of myelin.
8. Collect 3-4 mL of the 70-37% density gradient interphase containing the macrophage subpopulations cells into a clean 15 mL tube.
9. Wash the cells 3x with PBS, total volume of 15 mL. Make sure that the density gradient medium containing the cells is diluted at least three times with PBS, and to centrifuge for 7 min (500 x g, 4 °C) with the brake "on".
10. Resuspend the cell pellet with 1 mL PBS for cell labeling or for other functional assays.

5. Cell Labeling for Flow Cytometry

1. Transfer the cells (step 4.10) into a flow cytometry tube.
2. Add 1 µL of fixable dead cell stain reagent to each cell sample obtained in step 4.10. Incubate for 30 min on ice, protected from light.
3. Wash the cells once with 2 mL PBS and centrifuge for 5 min (300 x g, 4 °C).
4. Remove the supernatant, and resuspend the cell pellet with 400 µL PBS 1% BSA, 0.1% azide.
5. Add 1 µg CD16/CD32 antibodies to 100 µL of the cells to block the Fc receptors. Incubate 10-15 min on ice, protected from light.
6. Prepare the primary antibody mix in PBS 1% BSA, 0.1% azide or PBS 1% BSA, 0.1% azide, 0.25% saponin, for cell permeabilization for intracellular staining.
7. Add 100 µL of primary antibodies mix (2x) and incubate for 30 min on ice, protected from light.
   NOTE: All antibodies should be titrated prior to conducting the experiment to ensure optimal results.
8. Wash the cells with 2 mL PBS and centrifuge for 5 min (300 x g, 4 °C). Repeat this step three times.
9. Add 100 µL of a secondary antibody if the primary antibodies are not directly conjugated to a fluorophore, and incubate for 30 min on ice, protected from light.
10. Wash the cells with 2 mL PBS and centrifuge for 5 min at 300 x g at 4 °C). Repeat this step three times.
11. Fix the cells with 100 µL 1% PFA for 15 min at room temperature, protected from light.
    NOTE: Caution: PFA is toxic. Use in a fume hood and wear personal protective equipment.
12. Wash the cells with 2 mL PBS and centrifuge for 5 min at 300 x g at 4 °C. Repeat this step three times.
13. Resuspend the cell pellet with 200 µL PBS and proceed to flow cytometry acquisition and analysis. Follow the gating strategy in **Figure 3**.

Representative Results

After the density gradient centrifugation and antibody staining, the cells were acquired on a flow cytometer and analyzed using a morphological gating strategy as follows. A first gate was defined in the dot plot Forward-Scattered-Area (FSC-A) *versus* Forward-Scattered-Height (FSC-H) to discriminate single cells from doublets (**Figure 3A**). The single cells were then gated on FSC-A *versus*Side-Scattered-Area (SSC-A) dot plots to exclude cell debris and pyknotic cells, based on the relative cell size and cell granularity (**Figure 3B**).Then, the microglia and the MDM were identified based on their expression levels of the CD11b and CD45 markers (**Figure 3C, 3D**). Finally, other expression markers were analyzed for each macrophage subpopulation (**Figure 3E-3G**). The microglia were reported to express the CX3CR1 but not the CCR2 marker in contrast to MDM[23]. Recently, it was demonstrated that Tmem119 is a specific marker for microglia[19]. The following markers (Tmem119, CX3CR1, and CCR2) were assessed by flow cytometry using detector antibodies specific for each marker; all $CD11b^{+}CD45^{med}$ cells were shown to be positive for the Tmem119 and CX3CR1 markers but not for the CCR2 marker, which validates this gating strategy (**Figure 3E-3G**).

In the absence of the density gradient isolation, the macrophage subpopulations could not be defined in the dot plot FSC-A *versus* SSC-A due to the presence of many cells and myelin debris (**Figure 3H**). Of note, the permeabilization treatment induces a cell shrinkage and thus the macrophage subpopulations appeared smaller in the FSC *versus* SSC dot plot (**Figure 3I, J**) as previously described[24]. The viability of the cells was determined using the fixable dead cell stain kit. After the density gradient, 85-95% of cells are alive (**Figure 3K**). The microglia and MDM populations can be defined by the CD45 and CD11b markers on a dot plot after isolation of the brain and spinal cord cells in a single sample (**Figure 3L**), and also from the brain or spinal cord alone (**Figure 3M, 3N**).

For flow cytometry labeling, it is important to know whether the protein and/or the epitope recognized by the antibody is intra- or extracellular. In the case of intracellular antigens, a permeabilization buffer is required. Tmem119 is a transmembrane protein but the epitope recognized by the antibody stains as intracellular[19]. In the absence of cell permeabilization, there was no labeling (**Figure 3O**). The absence of this permeabilization step could lead to false negative results. Since the Tmem119 antibody is not directly conjugated to a fluorophore, the cells were labeled with a secondary antibody; the negative control was secondary antibody alone (black line) (**Figure 3E, 3O-3R**). When directly conjugated antibodies are used, there are two main strategies to assess specific labeling: isotype antibodies as a negative control or Fluorescence Minus One (FMO) controls. FMO controls contain every labeling antibody in the mixture except the control antibody for that sample. Thus, an FMO control is necessary for each marker, while only one labeling mixture is needed for the isotype antibodies control, per sample. For the labeling of the brain immune cells, we chose the isotype antibodies control since a limited number of cells (400,000 cells) is obtained after the density gradient from one mouse brain.

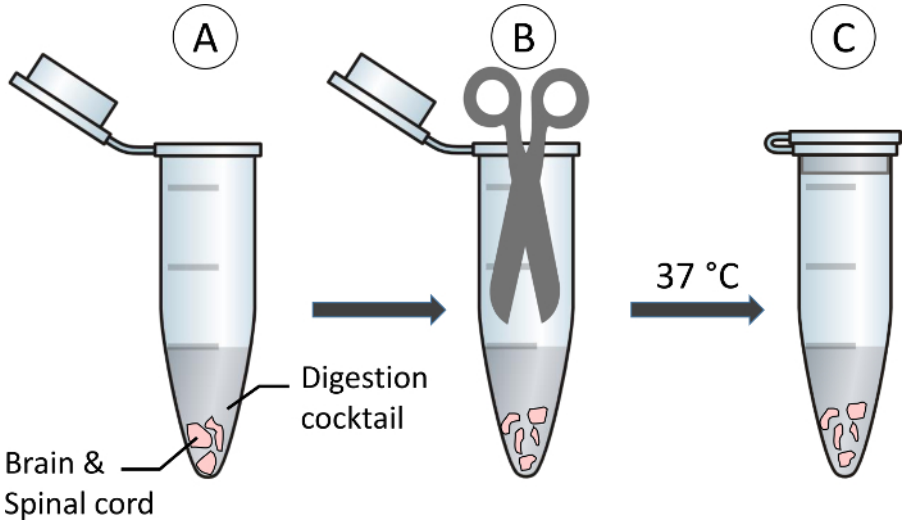

Figure 1: Steps of the Cell Dissociation Procedure. (**A**) The tissue was placed in a tube with digestion cocktail enzymes. (**B**) The tissue was finely cut into small pieces with scissors. (**C**) The small pieces of tissue were incubated for 30 min (37 °C) for efficient tissue digestion. Please click here to view a larger version of this figure.

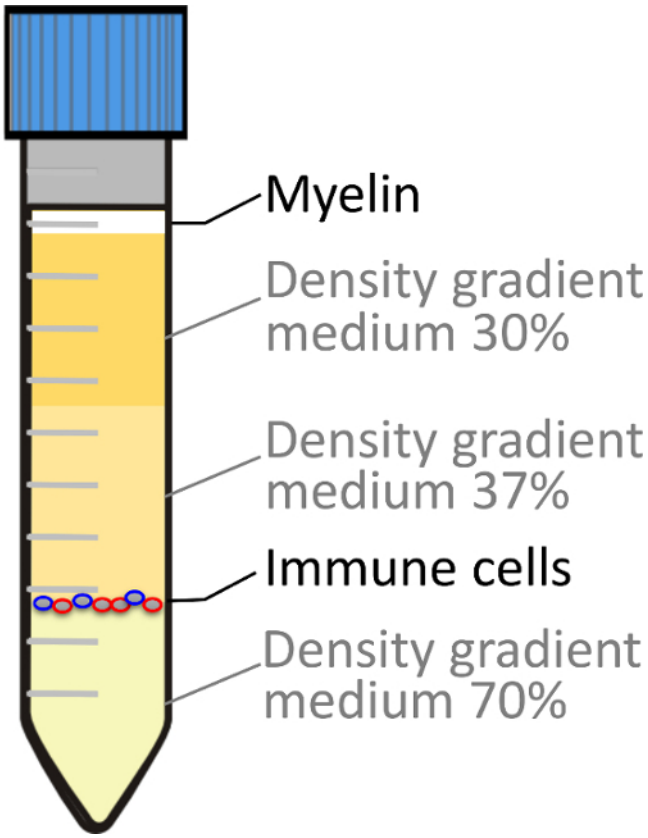

Figure 2: Schema of the Density Gradient After Centrifugation Step. After centrifugation, myelin is present at the top of the density gradient and immune cells containing macrophage populations are found at the interphase between the 37% and 70% density gradient layers. Please click here to view a larger version of this figure.

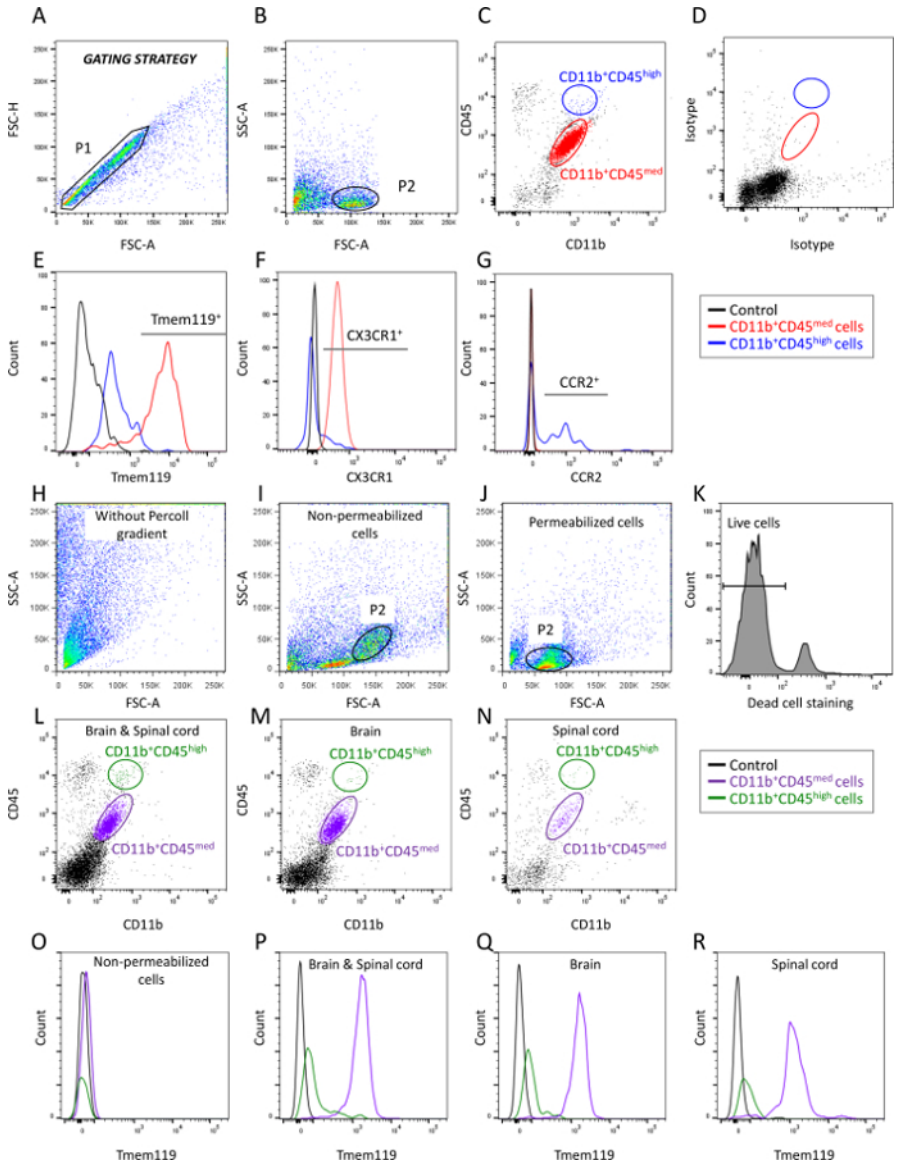

Figure 3: Representative Flow Cytometry Analysis of Macrophages. A logical gating strategy was applied: (**A**) The doublet discrimination refers to dot plot FSC-A *versus* FSC-H. (**B**) The morphological gating strategy refers to dot plot FSC-A *versus* SSC-A. (**C**) The macrophage subpopulations refers to dot plot CD45 *versus* CD11b. (**D**) The dot plot of cells stained with isotype antibodies as a control for CD45 and CD11b staining. The analysis of the markers expressed by these two macrophage subpopulations: Tmem119 (**E**), CX3CR1 (**F**), and CCR2 (**G**) (10,000 events in gate P2 were acquired). Dot plots showing the cell size (FSC-A) and granularity (SSC-A) of the brain and spinal cord cells before the density gradient (**H**) or after the density gradient isolation and with permeabilizing treatment (**I**) or without (**J**). (**K**) Flow cytometry histogram illustrates the percentage of dead cells after the density gradient. Gates shown in the dot plots illustrate the $CD11b^{+}CD45^{med}$ microglia population and the $CD11b^{+}CD45^{high}$ MDM population in the brain and spinal cord (**L**) or in the brain (**M**) or the spinal cord (**N**) separately. Analysis of Tmem119 expressed by the macrophage subpopulations in the brain and spinal cord cells permeabilized (**O**) or non-permeabilized (**P**), and in the brain (**Q**) or the spinal cord (**R**) permeabilized cells. Please click here to view a larger version of this figure.

Volume for 1 tube (mL)	Density gradient medium 1x	PBS 10x	PBS 1x
Density gradient medium 30%	1.5	0.15	3.35
Density gradient medium 37%	1.85	0.185	2.965
Density gradient medium 70%	3.5	0.35	1.15

Table 1: Composition of the Different Percentages of the Density Gradient Medium.

Discussion

It has been demonstrated that the microglia and MDM have different functions and phenotypes in the CNS, and thus the identification and the analysis of these macrophage subpopulations are essential in order to better understand neurological diseases[9,18,25]. Flow cytometry analysis

using two markers (CD11b and CD45) allows for the distinction between each subpopulation (**Figure 3C**). This strategy was previously validated by using other specific markers, such as CCR2 for the MDM and CX3CR1 for the microglia, and a recently developed approach with an antibody to Tmem119 (**Figure 3E-3G**). We performed experiments with CCR2 and CX3CR1 antibodies (**Figure 3F, 3G**) as we previously published[18]. The $CD11b^{+}CD45^{med}$ population highly expressed Tmem119 and CX3CR1 but not CCR2 in contrast to the $CD11b^{+}CD45^{high}$ population. The $CD11b^{+}CD45^{high}$ population is composed of various subpopulations with or without CCR2 expression. The identification and characterization of these macrophage subpopulations (perivascular macrophages, infiltrating macrophages, *etc.*) are an extensive area of research. Furthermore, the markers for these populations can change depending on the pathological processes. Indeed, CCR2 is down-regulated when the monocytes cross the blood brain barrier[26].

The CD45 and CD11b antibodies recognized the cell surface antigens, so the permeabilization treatment is unnecessary. Therefore, this technique could also be used to purify each macrophage subpopulation by cell sorting for mRNA analysis or other functional assays. It is of note that the use of the enzyme and density gradient can induce, to a certain extent, microglia activation. We have previously observed that after isolation, the microglia and macrophages express the inducible nitric oxide synthase (iNOS). Nevertheless, increased iNOS expression level observed in the pathologic *versus* the normal condition can be highlighted using this method[18]. Furthermore, this technique achieves a well-separated cell population (singlets) with a high proportion of live cells.

For the successful replication of this protocol, there are several critical parameters. The setup of the gradient is most critical. Our experience indicates that preparing the gradients in 15 mL tubes versus 50 mL ones achieves better separations. If no cells are seen in the 70-37% interface, the amount of CNS tissue used for cell isolation should be considered. We usually collect ~400,000 cells from one mouse brain. Although this yield fluctuates from various experimental conditions, one for a better visualization of the 70-37% interface is to make the 37% density gradient layer with PBS and phenol red.

With the help of different lasers and filters in the flow cytometer, this protocol can quantify several markers expressed by each of the macrophage subpopulations[18]. This technique is also useful for assessing the involvement of other immune cells such as T cells and B cellsin the same sample. Indeed, techniques using immunomagnetic enrichment only allow for the study of the populations previously selected[21].

Overall, this technique constitutes a useful tool to characterize the different macrophage subpopulations in mouse models of neurological diseases. This unique alternative can assess the effects of treatment on inflammatory processes in pathological conditions.

Disclosures

The authors have nothing to disclose

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