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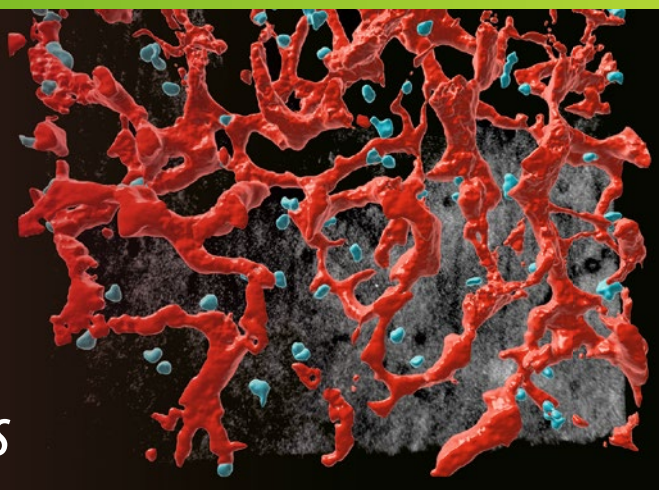

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Myeloid-Derived Suppressor Cells

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Myeloid-Derived Suppressor Cells

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Cover Caption: Exemplary picture of immune cells and blood vessels in the murine tibial bone marrow recorded by intravital 2-photon microscopy. Cytotoxic $CD8^+$ T cells (turquoise) are visualized by an endogenous fluorescent protein and the vessels (red) by an i.v. injection of Q dots. The bone surface (grey) is displayed by the second-harmonic generation signal.

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Preface

Research in myeloid-derived suppressor cells (MDSC) went through a dynamic evolution over the past decades. Initially, these cells were termed myeloid suppressor cells (MSC) and their immunosuppressive features were broadly recognized in the later 1990s. As of today, there are several thousand hits in NCBI-Pubmed and MDSC activity is recognized as a major immunoregulatory mechanism and a key immune resistance mechanism in modern cancer immunotherapy. Next to cancer, these cells have also been described in various inflammatory and infectious diseases, some of which are covered in the current volume. A debate on whether the term MDSC is appropriate, as it may suggest the existence of a distinct "cell type", continues nowadays. It has been argued that functionally specialized neutrophils, monocytes, macrophages and other myeloid cells carry out the immunosuppressive activity ascribed to MDSC. Without any doubt, both polymorphonuclear and mononuclear myeloid cells can obtain immunoregulatory and/or suppressive activity in the context of pathological expansion. Thus, the regulatory activity of myeloid cells truly represents an important immunoregulatory mechanism in many diseases. In the context of this volume, we use the term "MDSC" as it most clearly defines what the chapters describe.

Besides the semantic issues, in its initial phase, the research on immunoregulatory activity of myeloid cells has indeed suffered from some uncertainties as to the identity, phenotype and functional characterization of MDSC. The field has now matured and with this first volume of *MDSC—Methods and Protocols* we hope to further support this path and facilitate experiments addressing the complex biology of myeloid regulatory cells.

The volume details several experimental approaches that can be employed to investigate MDSC or may be useful for a more comprehensive characterization of these cells in the future. The chapters cover topics of relevance for investigators in various research fields, including biology of myeloid cells, cancer, infection and inflammation. Moreover, these protocols are relevant for experimentations in various research sectors, notably biology, medicine and veterinary medicine. Detailed protocols are provided for the study of MDSC in human patient samples and experimental models employing flow cytometry or magnetic enrichment, as well as for immunophenotyping using multi-parameter flow cytometry and mass cytometry. Methods for functional characterization of MDSC by biochemical, immunological and microscopy approaches along with high-resolution genomics are detailed. Moreover, protocols for in vitro generation of MDSC, as well as their characterization upon interactions with medically relevant microbes, are provided. Novel methods for high-resolution visualization in living tissue and for purification of granulocytes, which may encompass MDSC, in experimental animals and livestock are also included in this chapter.

It is our hope that this edition will guide novices in the field and help them to start their research on MDSC. We hope that it will also be useful for experienced investigators wishing to establish new methods in their labs and/or align their own studies to protocols employed

by other investigators. It is important to realize that regulatory myeloid cells are complex and the field is dynamic. This represents a challenge to our understanding of MDSC biology. We are optimistic that this collection of protocols will support these dynamic developments.

Finally, we thank all contributing authors for sharing their laboratory protocols and technical expertise. Without your generous input this volume would not have been possible!

Greifswald, Germany — *Anca Dorhoi*
Essen, Germany — *Sven Brandau*

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Chapter 1

Immunophenotyping of Circulating Myeloid-Derived Suppressor Cells (MDSC) in the Peripheral Blood of Cancer Patients

Kirsten Bruderek, Ronja Schirrmann, and Sven Brandau

Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of pathologically expanded myeloid cells with immunosuppressive activity. According to their phenotype, MDSC can be divided into three major subpopulations: early stage MDSC (e-MDSC), lacking myeloid lineage markers, monocytic MDSC (M-MDSC), and granulocytic MDSC (PMN-MDSC). Additionally, PMN-MDSC can be subdivided based on their activation and differentiation status, although it is not clear how this status contributes to immunosuppression and disease pathology. Here, we describe an immunophenotyping and gating strategy for the identification and isolation of MDSC subsets based on fluorescence-activated cell sorting. This method allows direct comparison of MDSC subsets in clinical settings.

Key words Circulating MDSC, Immunophenotyping, Flow cytometry, Cancer, Neutrophils, Immunosuppression

1 Introduction

Myeloid-derived suppressor cells (MDSC) are pathologically expanded myeloid cells that acquire immunosuppressive properties under the influence of host-derived factors [1]. Elevated frequencies of circulating MDSC have been reported in human cancer patients. The correlation of high frequencies of MDSC in the peripheral blood with poor survival of cancer patients suggests a clinical relevance for these cells [2]. However, no standardized and uniform isolation protocols, surface marker panels, and gating strategies for human MDSC exist. This still impedes human MDSC research. Consequently, several attempts to harmonize and standardize MDSC analysis are underway or were initiated [3–5].

In humans, several subsets of MDSC exist. Circulating granulocytic MDSC (PMN-MDSC) express the neutrophil markers CD15 and CD66b, the myeloid marker CD33, but lack the

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monocytic marker CD14. Furthermore, CD16 and CD11b distinguish immature and mature PMN-MDSC [6, 7]. The monocytic MDSC (M-MDSC) are CD66b and CD15 negative (or very low), express higher levels of CD33 compared to PMN-MDSC, and they are positive for CD14. Furthermore, M-MDSC can be distinguished from monocytes by their low or even absent expression of HLA-DR. Early stage MDSC (e-MDSC) express the myeloid markers CD33 and CD11b and have low or absent levels HLA-DR. They lack or have low expression of the myeloid lineage markers CD14 and CD66b/CD15 [1, 4]. Recent studies proposed the use of CD123 to exclude basophils from e-MDSC [8, 9].

Here we present an easy-to-follow protocol for the isolation and immunophenotyping of MDSC subsets with minimal requirements to facilitate the comparison of their clinical relevance in different kinds of cancer (*see* **Note 1**).

2 Materials

Perform all steps at room temperature unless indicated otherwise (*see* **Note 2**).

2.1 Density Centrifugation

1. 10 mL 9NC blood collection tubes (recommended, followed by K3EDTA) (*see* **Note 3**).
2. Conical centrifuge tubes 50 mL.
3. Isotonic separation medium for density centrifugation (density 1.077 g/mL).
4. DPBS—Dulbecco's phosphate-buffered saline without calcium and magnesium.
5. Heat-inactivated fetal calf serum: Thaw fetal calf serum over night at 4 °C and incubate for 30 min at 56 °C. Filter inactivated serum over 0.2 μm filter unit. Store in aliquots at −20 °C (*see* **Note 4**).
6. Culture medium: RPMI Medium 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin/100 μg/mL streptomycin. Add 50 mL heat-inactivated fetal calf serum and 5 mL 10,000 U/mL penicillin/10,000 μg/mL streptomycin to 445 mL RPMI Medium 1640 (1×) supplemented with L-Glutamine. Store at 4 °C.
7. Disposable Pasteur pipette.
8. Neubauer chamber or automatic cell counter CASY. If using a Neubauer chamber perform live/dead staining by mixing 100 μL cell suspension with 400 μL 0.4% trypan blue.

Table 1
Recommended antibodies for MDSC immunophenotyping

Antigen	Clone	Fluorochrome
CD66b	80H3	FITC
CD14	HCD14	PerCP-Cy5.5
CD33	WM53	PE
HLA-DR	G46-6	APC
CD11b	ICRF44	APC-Cy7
CD16	3G8	PE-Cy7
Lineage cocktail CD3/CD19/CD20/CD56	SK7/H1B19/2H7/HCD56	All Brilliant Violet 421
CD123	REA918	VioGreen

2.2 Staining Reagents and Antigens

1. Falcon® Round-Bottom Polystyrene Tubes, 5 mL (FACS tubes).
2. 96-well Round-Bottom Plate.
3. Human Serum (HS): Incubate pooled human AB serum for 30 min at 56 °C. Store in aliquots at −20 °C.
4. DPBS/HS: To prepare staining buffer DPBS/3% HS add 7.5 mL HS to 242.5 mL DPBS and filter sterile using 0.2 μm filter membranes. Store at 4 °C.
5. Fluorochrome conjugated anti-CD66b, anti-CD14, anti-CD33, anti-HLA-DR, anti-CD3, anti-CD19, anti-CD20, anti-CD56, anti-CD123, anti-CD11b, and anti-CD16 (*see* Table 1 for suggested clones and fluorochromes).
6. Lineage cocktail: Prepare Lineage Cocktails by mixing pre-titrated anti-CD3/CD19/CD20/CD56 antibodies conjugated with the same fluorochrome (*see* **Note 5**).

   Example preparation of master mix lineage cocktail

Antigen (same fluorochrome)	μL per sample	μL for 20 samples
CD3 Brilliant Violet 421 1:400	0.125	2.5
CD19 Brilliant Violet 421 1:200	0.25	5
CD20 Brilliant Violet 421 1:200	0.25	5
CD56 Brilliant Violet 421 1:50	1	20
Fill up to 2.5 μL with DPBS/HS	0.875	17.5

7. Flow cytometer: Please verify your flow cytometers capability for exciting and detecting fluorochromes. To perform analysis with the suggested clones and fluorochromes BD FACS Canto II with 488 nm, 635 nm, and 450 nm lasers can be used.

3 Methods

Perform all procedures at room temperature unless indicated otherwise (*see* **Note 6**).

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC) from Cancer Patients

1. Dilute blood 1:1 with DPBS (for example 10 mL of blood + 10 mL of DPBS).
2. Pipette 15 mL of separation medium for density centrifugation into one conical centrifuge 50 mL tube.
3. Overlay separation medium for density centrifugation with up to 33 mL pre-diluted blood (*see* **Note 3**).
4. Centrifuge at room temperature with 400 × *g* for 30 min without acceleration and break (to prevent mixing of resulting phases).
5. After centrifugation, aspirate plasma until approximately 5 cm above the PBMC fraction.
6. Use a disposable Pasteur pipette to collect PBMC into a new 50 mL tube. Do not collect any separation medium.
7. Wash PBMC by filling tube with DPBS up to 50 mL.
8. Centrifuge at 300 × *g* for 10 min.
9. Discard supernatant so that around 2 mL remain in the tube and resuspend the pellet.
10. Repeat the washing **steps 7–10** until the supernatant is clear.
11. Discard supernatant completely.
12. Resuspend pellet in 1 mL culture medium and add additional 9 mL medium.
13. Determine the cell concentration by using an automatic cell counter CASY or Neubauer chamber. If using a Neubauer chamber perform live/dead staining by mixing 100 µL cell suspension with 400 µL 0.4% trypan blue.
14. Use 2.5 × 10^6 PBMC for immunophenotyping.

3.2 Staining for Immunophenotyping (See Note 7)

1. Wash 2.5 × 10^6 cells with 1 mL DPBS.
2. Centrifuge tube at 460 × *g* for 5 min at 4 °C.
3. Resuspend cells in 70 µL DPBS/HS.
4. Pipette 30 µL of cell suspension in two different wells of a 96-well Round-Bottom Plate.
5. Add 20 µL of antibody mix for FMO control to one well and 20 µL of specific staining to the second well (*see* Table 2).
6. Incubate for 30 min at 4 °C.
7. Wash by adding 200 µL DPBS.

Table 2
Proposed FACS-panel for MDSC immunophenotyping

Antigen	Dilution	FMO control (μL per sample)	Specific staining (μL per sample)
CD66B FITC	1:20	2.5	2.5
CD14 PerCP-Cy5.5	1:100	0.5	0.5
CD33 PE	1:6.6	7.5	7.5
Lineage cocktail CD3/CD19/CD20/CD56 Brilliant Violet 421	See 2.2.6	2.5	2.5
CD11B APC-Cy7	1:20 of 1:10 pre dilution		2.5
CD16 PE-Cy7	1:100 of 1:10 pre dilution		0.5
CD123 VioGreen	1:100		0.5
Fill up to 20 μL with DPBS/HS		6.5	1

8. Centrifuge at 460 × *g* for 5 min at 4 °C.
9. Decant the supernatant.
10. Resuspend cells in 200 μL DPBS/HS.

3.3 Flow Cytometer Acquisition and Gating (See Note 8)

1. Gate on singlets (P1) in FSC-A vs. FSC-H.
2. Plot singlets in SSC-A vs. FSC-A and exclude debris (P2).
3. To analyze MDSC subsets (*see* Fig. 1a) show cells without debris in HLA-DR vs. CD33. HLA-DR^{neg}/CD33^{high} (P3) cells represent M-MDSC.
4. Set another gate for HLA-DR^{neg}/CD33^{dim} (P4).
5. Show P4 in LIN vs. CD66b. HLA-DR^{neg}/CD33^{dim}/LIN^{-}/CD66b^{+} (P5) cells are classified as PMN-MDSC (P5) and HLA-DR^{neg}/CD33^{dim}/LIN^{-}/CD66b^{-} (P6) as e-MDSC.
6. To exclude basophils from e-MDSC show (P6) in CD123 vs. CD66b. E-MDSC are CD123 negative.
7. To determine the differentiation and maturation status of PMN-MDSC plot (P5) for CD11b vs. CD16 (*see* Fig. 1b). CD11b^{-}/CD16^{-}, CD11b^{+}/CD16^{-} are immature PMN-MDSC whereas mature PMN-MDSC are CD11b^{+}/CD16^{+}. Refer to reference [2, 5] for biological features of these PMN-MDSC subsets.
8. Preferentially acquire the whole sample, but at least 10,000 events in P5 (PMN-MDSC gate).

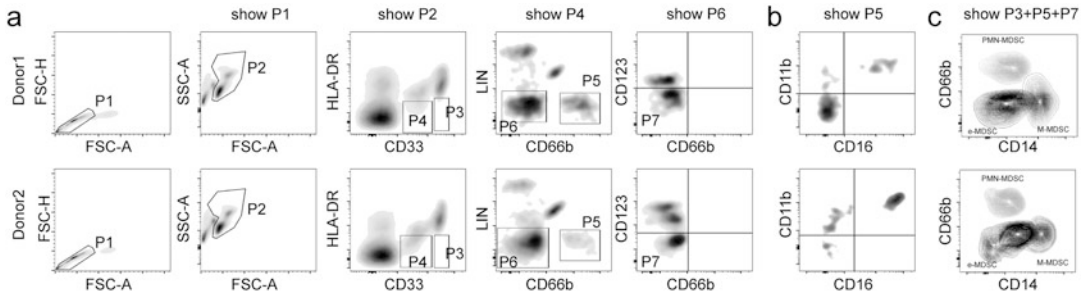

Fig. 1 Exemplary gating strategy for the immunophenotyping of circulating MDSC. PBMCs are isolated from the peripheral blood of cancer patients by density gradient centrifugation. (**a**) MDSC Subsets are classified as $CD33^{high}/HLA\text{-}DR^{low/-}/CD14^{+}$ for M-MDSC (P3), $CD33^{dim}/HLA\text{-}DR^{-}/LIN^{-}/CD66b^{+}$ for PMN-MDSC (P5), and $HLA\text{-}DR^{-}/CD33^{dim}/LIN^{-}/CD66b^{-}/CD123^{-}$ (P7) for e-MDSC. (**b**) CD11b and CD16 expression is used to determine the differentiation status of PMN-MDSC. Promyelocytes are identified as $CD66b^{+}/CD11b^{-}/CD16^{-}$, early myelo- and metamyelocytes as $CD66b^{+}/CD11b^{+}/CD16^{-}$, and $CD66b^{+}/CD11b^{+}/CD16^{+}$ as banded- and segmented cells. (**c**) Expression of CD14 and CD66b on M-MDSC (P3), PMN-MDSC (P5), and e-MDSC (P7). Note that PMN-MDSC are negative for CD14, M-MDSC are negative for CD66b, and e-MDSC are negative or low for both myeloid markers

4 Notes

1. This protocol is also suitable for circulating MDSC in nonmalignant diseases, inflammation, and infection. However, it must then be tested and validated for the patient cohort of interest.
2. To avoid activation of cells by endotoxin contaminations we highly recommend using buffers and reagents produced under good manufacturing practice conditions. Use reagents with endotoxin levels <0.5 EU/mL.
3. 10 mL blood are sufficient for phenotyping. For distinguishing PMN-MDSC subsets in most patients 50 mL of blood will be needed. Avoid the combination of Heparin-containing blood collection tubes and Ficoll-Paque™. This might lead to artificially high amounts of PMN-MDSC in cancer patients (and appearance of substantial amounts of otherwise absent PMN-MDSC in healthy donor controls).
4. Cool the heat inactivated serum to 4 °C prior to filtration to reduce clogging of pores during filtration.
5. Optimized lineage cocktails from companies can be used, but must not include CD16 and CD14.
6. After blood collection from cancer patients, start processing within 1 h. During this time, keep blood at room temperature. Make sure that all tubes are filled completely. Noncompliance may also lead to artificially increased amounts of PMN-MDSC.

7. The CD33 staining has to differentiate three subsets: CD33 negative, CD33 dim, and CD33 high. Make sure to use a sufficiently titrated antibody concentration to achieve this.
8. Before acquisition of the sample on the flow cytometer activate the logarithmic function for SSC display.

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Chapter 2

Phenotyping of Myeloid-Derived Suppressor Cells in Nonhuman Primates

Ang Lin and Karin Loré

Abstract

While myeloid-derived suppressor cells (MDSCs) in humans and mice have been intensively investigated, there is limited knowledge of these cells in nonhuman primates (NHPs). NHPs serve as critical models for late-stage testing of several biomedical inventions before proceeding with clinical trials and it is therefore important to fully understand their immune compartments and similarities with humans. Here, using antihuman cross-reactive antibodies, we provide flow cytometric analysis protocols for identification of MDSCs in the blood of rhesus macaques, one of the major NHP species as experimental models. Discrepancies and similarities between rhesus and human MDSCs are discussed.

Key words MDSC, Phenotyping, Nonhuman primate, Rhesus macaque, Human, Flow cytometry, CD33, CD66abce, CD14

1 Introduction

Myeloid-derived suppressor cells (MDSCs) appear under conditions such as inflammation, infection, and cancer [1]. As immune regulatory cells, their suppressive effects on T cell responses have been closely associated with disease progression. However, there is a challenge to specifically identify these cells since expression of surface antigens has a large degree of overlap between MDSCs with other myeloid cells. Functional assessment of MDSCs is therefore required and has been widely accepted to be the "gold standard" to identify them [2].

MDSCs have been intensively studied in humans and mice. In contrast, knowledge of these cells in NHPs is limited. A few studies on MDSCs in rhesus macaques have been published, but the phenotypic criteria of these cells vary and are even conflicting between studies [3–6]. Part of this problem stems from subtle differences in the phenotype of immune cell subsets between humans and rhesus and the use of antihuman antibodies with limited cross-reactivity to rhesus samples. As the field is in its infancy, it is therefore important

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to carefully explore and validate methods to correctly identify rhesus MDSCs to ensure an accurate monitoring of these cells.

We have recently found that two subsets of MDSCs including monocytic (M)-MDSCs and polymorphonuclear (PMN)-MDSCs were identified in rhesus blood from the low-density fraction after Ficoll separation [3]. While the phenotypes of MDSCs in rhesus blood were largely similar with that in humans, some differences exist in the expression of some surface antigens important for the identification. In this chapter, we describe a phenotyping procedure of MDSCs in rhesus macaques, including experimental methods, data analysis, and the potential factors causing artifacts.

2 Materials

2.1 Cell Isolation

1. 10 mL BD Vacutainer K2-EDTA tube for blood collection.
2. Ficoll-Paque PLUS density gradient solution (density = 1.077 g/mL).
3. Sterile phosphate-buffered saline (PBS).
4. Complete culture medium: RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 unit/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL L-glutamine.
5. 1 × Red blood cell (RBC) lysis buffer: 1 L of sterile deionized water (diH_2O) containing 8.02 g NH_4Cl, 0.84 g $NaHCO_3$, and 0.37 g EDTA. Autoclaving sterilized, stored at 4 °C.
6. 50 mL sterile polypropylene centrifuge tubes.
7. Centrifuge and cell counter.

2.2 Cell Staining

1. 5 mL sterile round-bottom polystyrene flow cytometry tubes.
2. Flow cytometry buffer: sterile PBS containing 2% heat-inactivated FBS (v/v).
3. Dye for determination of viable cells: Live/Dead fixable blue dead cell stain kit (ThermoFisher Scientific).
4. Reagent for blocking unspecific binding of antibodies to Fc receptor-expressing cells: Fc receptor blocking reagent.
5. Staining antibodies (Table 1) (*see* **Notes 1** and **2**).
6. Fixation buffer: 1% paraformaldehyde (PFA).

2.3 Data Acquisition and Analysis

1. Flow cytometer: For example, a BD LSRFortesssa™ cell analyzer with five lasers. Channels and configuration related to our staining panel is available in Table 1.
2. Software for data analysis: FlowJo (Version 10, Treestar).

Table 1
Antibody staining panel for phenotyping MDSCs in rhesus macaques

Laser	Filter	Fluorochrome	Antigen		Clone
Blue (488 nm)	530/30	FITC	CD66abce		TET2
YellowGreen (561 nm)	586/15 610/20	PE PE-Texas Red	CD33 HLA-DR		AC104.3E3 Tü36
Red (640 nm)	780/60	APC-Cy7 APC-Cy7 APC-Cy7	Lin	CD3 CD8 CD20	SP34-2 RPA-T8 L27
Violet (405 nm)	450/50	Pacific blue	CD11b		ICRF44
	525/50	BV510	CD14		M5E2
Ultraviolet (355 nm)	450/50	Live/dead blue			

3 Methods

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Rhesus Macaques

1. Collect 10 mL of fresh venous blood in a K2-EDTA tube followed by gentle mixing (*see* **Notes 3** and **4**).
2. Dilute the blood 1:1 with PBS. Invert the tube to mix well.
3. Add 15 mL of Ficoll-Paque PLUS density gradient solution into a 50 mL tube.
4. Layer 20 mL blood/PBS mixture onto the surface of Ficoll-Paque PLUS density gradient solution gently.
5. Centrifuge for 30 min at 400 rcf (relative centrifugal force) at room temperature (RT), without acceleration and brake.
6. After centrifugation, the sample is stratified into three phases. An interface between top phase (plasma) and middle phase (Ficoll-Paque solution). The interface of cells consists of low-density cells.
7. Aspirate the plasma in the top phase until around 2 centimeter (cm) is left above the interface. Transfer the interface (low-density cells) into a 50 mL tube.
8. Wash the cells twice with 40 mL of PBS (350 rcf, 5 min, RT).
9. Discard supernatant and resuspend cells in 5 mL of RBC lysis buffer to lyse erythrocytes (5 min, RT).
10. Wash the cells once with 40 mL of PBS (350 rcf, 5 min, RT).
11. Resuspend cells in an appropriate volume of complete RPMI-1640 medium and take an aliquot for cell counting.

3.2 Cell Staining

1. Prepare the antibody cocktail in flow cytometry buffer up to a volume of 50 μL according to the staining panel in Table 1.

2. Transfer 1 × 10^6 cells into a 5 mL flow cytometry tube. Wash the cells once with 1 mL of PBS (350 rcf, 5 min, RT).
3. Discard supernatant and resuspend cells in 1 mL of PBS.
4. Add 1 μL of the reconstituted Live/Dead fixable blue dead cell dye to the cell suspension and mix well.
5. Incubate at RT for 20 min in the dark.
6. Wash the cells once with 1 mL of PBS (350 rcf, 5 min, RT), and resuspend cells in 50 μL of FACS buffer.
7. Add 5 μL of Fc receptor blocking reagent and mix well.
8. Add 50 μL of antibody cocktail to the cell suspension and mix well by pipetting gently.
9. Incubate for 20 min at RT in the dark.
10. Wash the cells twice with flow cytometry buffer (350 rcf, 5 min, RT).
11. Discard supernatant and add 100 μL of 1% PFA fixation buffer (*see* **Note 5**).

3.3 Data Acquisition

1. Start up the flow cytometer, run cytometer setup and tracking (CS&T) performance check to ensure optimal conditions of machine.
2. Set an appropriate voltage for each fluorochrome channel and perform compensation. BD CompBeads or rhesus cells can be used to prepare compensation controls.
3. After data acquisition, single FCS file will be saved and exported under FCS 3.0 format. The raw data can be analyzed using FlowJo or other compatible software.

3.4 Data Analysis

1. Figure 1 shows the gating strategy for identification of M-MDSCs and low-density neutrophils (LDNs) in rhesus blood and the comparison with the human blood counterparts. In rhesus blood, LDNs express CD66abce and have a dim expression of CD14 [7, 8]. M-MDSCs are $CD14^{high}$ but are $CD66abce^{dim}$ (*see* **Note 6**). In contrast, human LDNs and M-MDSCs do not express CD14 and CD15, respectively.
2. Rhesus LDNs can be further divided into two subsets, $CD33^-$ LDNs and $CD33^+$ LDNs (Fig. 2). Among the two populations, $CD33^+$ LDNs have recently been shown to have suppressive effect on T cells, thus representing PMN-MDSCs [3]. In contrast, human LDNs comprise a uniform cell population with intermediate expression of CD33 (Fig. 2). Due to the so far limited knowledge of unique markers that can discriminate suppressive PMN-MDSCs from non-suppressive LDNs, PMN-MDSCs in human blood are usually defined as the LDNs as a whole [2, 9] (*see* **Note 7**).

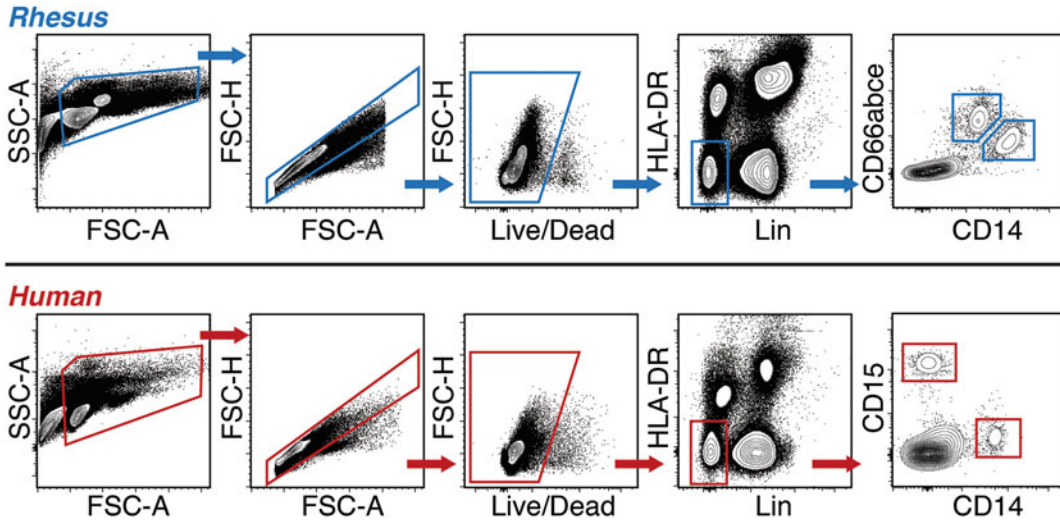

Fig. 1 The gating strategy for phenotypic identification of LDNs and M-MDSCs in rhesus and human PBMCs. Forward scatter (FSC) and side scatter (SSC) are used to gate out the whole white blood cell populations. FSC-A and FSC-H are used to exclude cell aggregates. Live/Dead is used to exclude dead cells. Lin is used to exclude lymphocytes (T cells, B cells, and natural killer cells). CD66abce and CD15 are used to identify rhesus and human neutrophils, respectively. CD14 is expressed on M-MDSCs

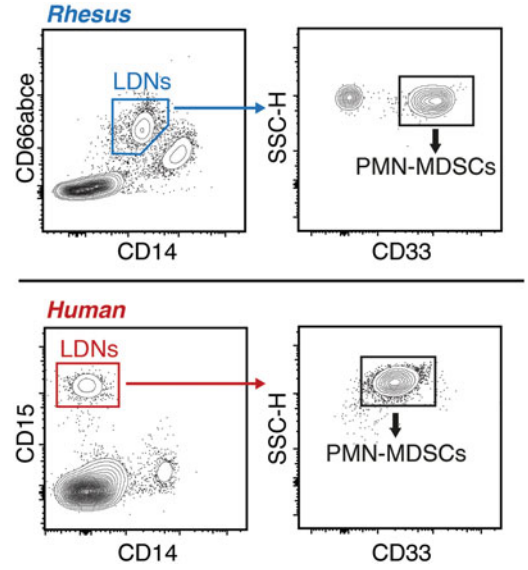

Fig. 2 Expression of CD33 on rhesus and human LDNs. Rhesus LDNs contain suppressive PMN-MDSCs ($CD33^+$ LDNs) and non-suppressive $CD33^-$ LDNs. Human LDNs are all $CD33^+$ cells

3. In human blood, M-MDSCs can be identified as HLA-DR$^{-/low}$Lin^{-}CD14^{+} cells [2]. CD11b and CD33 are also present, but not uniquely, on M-MDSCs since they are also expressed by classical monocytes, myeloid dendritic cells, and neutrophils [10–12]. In rhesus blood, M-MDSCs are positive of CD11b [3]. However, conflicting results exist with regard to CD33 expression [3, 6] (*see* **Note 8**).

4 Notes

1. Antibody selection and panel design. There are limited numbers of commercially available monoclonal antibodies that are specially designed to react with rhesus antigens. However, it is usually feasible to phenotype multiple rhesus immune cells by flow cytometry due to the cross-reactivity of many antihuman antibodies. Information on the cross-reactive antihuman antibody clones is available on the NHP Reagent Resource website (http://www.nhpreagents.org). In addition, we strongly recommend testing the cross-reactivity of all antibodies to be included in a staining panel for NHPs. The antibodies should also be titrated to obtain the optimal brightness in each channel.
2. In the staining panel, some antibodies need particular attention. As for the phenotyping of neutrophils, an anti-CD15 antibody (clone: SSEA-1) although reported to react with rhesus neutrophils [5], we found that this antibody showed a very weak cross-reactivity when compared with an anti-CD66abce antibody (clone: TET2) (Fig. 3). Therefore, we recommend to use anti-CD66abce antibody and not anti-CD15 antibody for phenotyping of rhesus neutrophils and PMN-MDSCs. To exclude lymphoid-lineage cells, anti-CD3,

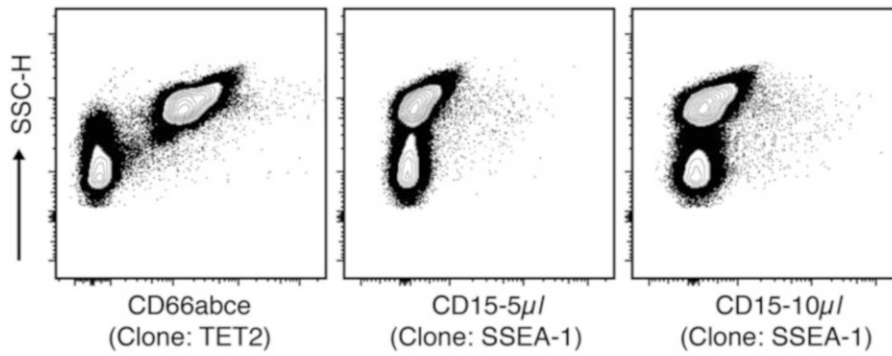

Fig. 3 Cross-reactivity of anti-CD66abce and anti-CD15 antibodies with rhesus neutrophils. Rhesus neutrophils are isolated using dextran sedimentation assay [3]. A mixture of cells containing neutrophils and PBMCs are stained with anti-CD66abce (clone: TET2) or anti-CD15 (clone: SSEA-1) antibody

anti-CD8, and anti-CD20 antibodies are used. Among them, anti-CD8 antibody is used to stain natural killer (NK) cells as CD8 is expressed on rhesus NK cells [13]. In this regard, anti-CD56 antibody cannot be used because CD56 is also expressed on monocytes in rhesus macaques [13].

3. Blood collection tubes are normally designed to draw a certain volume of blood to ensure a proper blood-to-anticoagulant ratio. It is recommended to fill the tube during blood collection. Once the blood collection is finished, invert the tube up and down gently and thoroughly to avoid blood clotting.
4. We strongly recommend using fresh blood and processing the blood within 1 h after collection. This is mainly due to the fragility and short life span of neutrophils. In addition, when functionally evaluating MDSCs, especially PMN-MDSCs, frozen samples cannot be used because the freeze/thaw procedure will induce cell death and compromise their suppressive functions [14].
5. NHP cells should always be considered potentially hazardous because they may carry pathogens that can infect humans, e.g., Herpes Simplex Virus and simian type D retrovirus [15, 16]. Therefore, fixative reagents should be used to inactivate the pathogens.
6. Using anti-CD66abce and anti-CD14 antibodies is usually enough to discriminate M-MDSCs from LDNs. In addition, CCR2 can be used as an additional marker since M-MDSCs have a much higher expression of CCR2 than LDNs (Fig. 4). However, alteration in expression of some markers could occur under specific conditions. To ensure the correct gating, we recommend checking the SSC value of these cells based on the difference in cell granularity. LDNs are higher in SSC than M-MDSCs (Fig. 4).

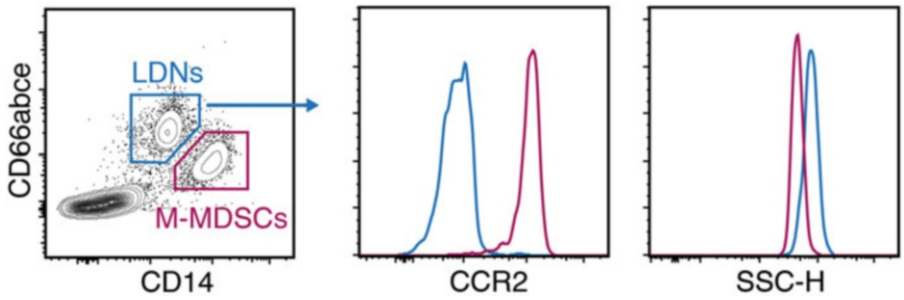

Fig. 4 Expression of CCR2 and SSC on rhesus LDNs and M-MDSCs. Anti-CCR2 (clone: 48607) is used for cell staining

7. Recently, lectin-type oxidized LDL receptor-1 (LOX-1) was shown to be a specific marker for PMN-MDSCs in cancer patients [17]. We found an anti-LOX-1 antibody (clone: 15C4) that is cross-reactive with rhesus cells. However, whether LOX-1 can be used as a specific marker to identify PMN-MDSCs in rhesus macaques requires further investigation.
8. According to studies including our own work [3, 18] and the NHP Reagent Resource website (http://www.nhpreagents.org), the only commercial anti-CD33 antibody (clone: AC104.3E3) that is cross-reactive with rhesus cells could only stain rhesus neutrophils, not M-MDSCs or monocytes. However, some studies showed that rhesus M-MDSCs express CD33 [6]. Therefore, to avoid potential artifacts, we propose not using CD33 as a marker to identify rhesus M-MDSCs.

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Chapter 3

Isolation and Phenotyping of Splenic Myeloid-Derived Suppressor Cells in Murine Cancer Models

Emilio Sanseviero, Rina Kim, and Dmitry I. Gabrilovich

Abstract

Myeloid-derived suppressor cells (MDSC) are immunosuppressive myeloid cells that accumulate in tumor sites and peripheral lymphoid organs such as the spleen. In murine cancer models, the spleen is a major reservoir for MDSC, representing an easily accessible tissue from which to isolate high numbers of these cell population for downstream applications. Here we describe an efficient method to phenotype as well as to isolate and assess the functionality of murine splenic MDSC.

Key words Splenic MDSC, Tumor immunology, Suppression assay, Murine cancer, MDSC characterization

1 Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of pathologically activated myeloid cells in tumor-bearing (TB) mice. These cells accumulate in the tumor site and in lymphoid peripheral organs such as lymph node and spleen [1, 2]. These cells are classified into two different subsets, monocytic (M)-MDSC and polymorphonuclear (PMN)-MDSC. M-MDSC are phenotypically and morphologically similar to monocytes as are PMN-MDSC to neutrophils. However, monocytes, neutrophils, and MDSC have unique biochemical, metabolic, and gene expression profiles that reflect their distinct functionalities [1, 3]. While monocytes and neutrophils activate T cell response, MDSC inhibit T cell activation and function [4, 5]. Thus, MDSC support cancer progression, and play a critical role in the efficacy of cancer treatments, including immune checkpoint inhibitors and CAR-T cells [6–9].

MDSC's ability to suppress T cell function is considered the golden standard in characterizing these cells [10]. Since MDSC cannot be easily generated in vitro, isolation of MDSC from murine TB spleen represents a convenient method to study these cells

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[2, 11]. Here we suggest a fast and efficient method to phenotypically characterize MDSC in the spleen of TB mice by flow cytometry. Moreover, we suggest a method to isolate these cells and to assess the ability of MDSC to inhibit T cell function.

2 Materials

Keep all the solutions, columns, and medium cold (4 °C), except for the complete medium that needs to be kept at room temperature (RT, 25 °C). Do not use complete medium that is older than 2 weeks. If the complete medium changes color, discard and prepare a fresh one. Different fetal bovine serums from multiple sources may need to be tested to optimize conditions for the T cell proliferation.

2.1 Spleen Isolation and Processing

1. MACS buffer: DPBS w/o Ca^{2+} and Mg^{2+} with 1% of fetal bovine serum (heat inactivated, FBS) and 2 mM EDTA. Remove 14 mL of DPBS from 1 L DPBS bottle. Then add 10 mL of FBS and 4 mL of 0.5 M Ultrapure EDTA. Keep it cold (4 °C).
2. RBC 1× (Red Blood Cell lysis buffer): Dilute 10 mL of eBioscience™ 10× RBC Lysis Buffer (Multi-species) with 90 mL of DI culture grade water.
3. Cell strainer 70 μm (Thermo Fisher).
4. 1 mL syringe plunger (BD).
5. 60 mm diameter cell culture treated dish (CELLTREAT).
6. Trypan Blue dye for counting cells (Corning).

2.2 Phenotyping of PMN-MDSC and M-MDSC from TB Spleen

1. Naïve mouse spleen (Control).
2. TB mouse spleen.
3. MACS buffer.
4. Flow Cytometry Panel.
   (a) Fc blocking antibody (clone 2.4g2) BD Bioscience Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™).
   (b) LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (Aqua LIVE/DEAD).
   (c) Anti-mouse CD11b clone M1/70 Brilliant Violet 421 (BV421).
   (d) Ly6C APC or PerCP-Cy5.5 clone HK1.4.
   (e) Ly6G FITC clone 1A8.

5. FACS tubes.
6. Flow cytometer equipped with appropriate lasers and detectors.

2.3 PMN-MDSC Isolation

1. TB mouse spleen.
2. MACS buffer.
3. Fc blocking antibody.
4. Ly6G Anti-Ly-6G MicroBeads UltraPure, mouse (Miltenyi Biotec).
5. LS columns (Miltenyi Biotec).
6. Miltenyi MidiMACS or QuadroMACS separators.

2.4 M-MDSC Isolation

1. TB mouse spleen.
2. MACS buffer.
3. Flow Cytometry Panel.
   (a) Aqua LIVE/DEAD.
   (b) Anti-mouse CD11b clone M1/70 BV421.
   (c) Ly6C APC or PerCP-Cy5.5 clone HK1.4.
   (d) Ly6G FITC clone 1A8.
4. FACS Sorter equipped with appropriate lasers and detectors.

2.5 Suppression Assay

1. PMEL or OT-1 transgenic mouse spleen.
2. Naïve mouse spleen.
3. PMN-MDSC and/or M-MDSC.
4. Complete medium: RPMI 1640 with L-glutamine and 25 mM HEPES, 10% FBS, 50 U/mL Penicillin-Streptomycin, 2-mercaptoethanol 33 μM.
5. Mouse gp100 peptide, EGSRNQDWL sequence, (ANASPEC) or mouse OVA peptide, SIINFEKL sequence.
6. 96 multi-well U-bottom plate.
7. Thymidine, [Methyl-^{3}H], in 10% ethanol, 1 mCi (Perkin Elmer).
8. Glass Fiber Filters (Perkin Elmer).
9. Omnifilter holders (Perkin Elmer).
10. Omnifilter-96 Cell Harvester (Perkin Elmer).
11. Plastic adhesive strip top seal (Perkin Elmer).
12. MicroScint, Scintillation Liquid (Perkin Elmer).
13. β-counter for plates (i.e., top count NXT Perkin Elmer).

3 Methods

3.1 Processing of the Spleen

1. Euthanize the mouse according to best animal management practice, harvest the spleen via incision on the left side of the mouse. Put the spleen in 6 mL of MACS buffer and transfer everything onto a 70 μm cell strainer in a 60 mm dish.
2. Using the plunger end of the 1 mL syringe gently mash the spleen through the cell strainer onto the dish.
3. Mix the solution in the dish and transfer the cell suspension to a 15 mL conical tube.
4. Centrifuge the conical tube at 400 × *g* at 4 °C for 5 min, discard the supernatant, and resuspend the pellet in 3 mL of 1× RBC. Let the red blood cells lyse for 3 min at RT.
5. After incubation, fill the 15 mL tube by adding 11 mL of MACS buffer and spin down again for 5 min at 400 × *g* at 4 °C and discard the supernatant.
6. Resuspend the pellet in 10 mL of MACS buffer and filter the suspension through a 70 μm cell strainer in a 50 mL conical tube.
7. Count the cells by diluting the cell suspension 1:25 with Trypan Blue dye.

3.2 Phenotype of PMN-MDSC and M-MDSC

1. Transfer 2×10^6 splenocytes to FACS tube.
2. Spin down the cells by centrifuging the tube at 400 × *g* for 4 min at 4 °C.
3. Discard the supernatant.
4. Resuspend the cell pellet in 50 μL of MACS buffer containing Fc blocking antibody (diluted 1:100).
5. Incubate the FACS tube at 4 °C for 10 min.
6. Prepare a master mix with MACS buffer with Aqua LIVE/DEAD (diluted 1:200), CD11b-BV421 (diluted 1:100), Ly6C-PerCP-Cy5.5 (diluted 1:100), and Ly6G-FITC (diluted 1:100).
7. Add 50 μL of the antibody master mix on the top of the cells incubated with the Fc blocking antibody, mix well, and incubate in the dark, at 4 °C for 20 min.
8. After incubation, wash the cells by adding 1 ml of MACS buffer to the tube, spinning down the tube for 4 min at 400 × *g* at 4 °C and removing the supernatant.
9. Acquire the cells in the FACS tube with a flow cytometer (*see* **Note 1**).

3.3 Isolation of PMN-MDSC from the Spleen

Below, we will consider protocol of MDSC isolation per 5×10^7 splenocytes. For higher starting splenocyte numbers, scale up all the reagents.

1. Spin down the splenocytes in a 50 mL conical tube for 4 min at $400 \times g$ at 4 °C and remove the supernatant.
2. Resuspend the cell pellet in 450 μL of MACS buffer.
3. Add to the suspension 5 μL of Fc blocking antibody and mix well.
4. Incubate for 10 min at 4 °C.
5. Add 50 μL of Anti-Ly-6G MicroBeads UltraPure, mouse to the suspension and mix well.
6. Incubate for 10 min at 4 °C.
7. Wash the cells by adding 50 mL of MACS buffer, spin down the cells, and remove the supernatant.
8. (*See* **Note 2**) Place a Miltenyi LS column onto a MidiMACS or QuadroMACS separator and wash the column with 3 mL of MACS buffer. Discard the flow-through.
9. Resuspend the cell pellets into 500 μL of MACS buffer and transfer the cell suspension in the washed LS column.
10. Wait for the reservoir to be empty and wash the column with 3 mL of MACS buffer three times. Be sure that the reservoir of the column is empty before adding the MACS buffer every time.
11. Collect the flow-through for subsequent M-MDSC isolation. Put the flow-through on ice.
12. Once the LS column has been washed three times, detach the column from the magnet, put the column in a 15 mL conical tube, add 5 mL of MACS buffer in the top of the LS column and with the special plunger push the MACS buffer through the column and collect the flow-through containing the enriched PMN-MDSC.
13. Start a second round of purification on a LS column by centrifuging the PMN-MDSC and pelleting down the cells.
14. In the meantime, on the centrifuge place a Miltenyi LS column onto a MidiMACS or QuadroMACS separator and wash the column with 3 mL of MACS buffer. Discard the flow-through.
15. Resuspend the cell pellets from **step 13** in 500 μL of MACS buffer and transfer the cell suspension in the washed LS column.
16. Wait for the reservoir to be empty and wash the column with 3 mL of MACS three times. Be sure the reservoir of the column is empty before adding the MACS buffer every time.

17. Once the LS column has been washed three times, detach the column from the magnet, put the column in a 15 mL conical tube, add 5 mL of MACS buffer in the top of the LS column and with the special plunger push the MACS buffer through the column and collect the flow-through containing the purified PMN-MDSC (*see* **Note 3**).

3.4 Isolation of M-MDSC

Here, we describe the isolation of M-MDSC per 5×10^7 splenocytes or from the flow-through of the previous purification. Scale up the quantity of every reagent if you need (*see* **Note 4**)

1. Count the cells and spin them down in a 50 mL conical tube.
2. Remove the supernatant and resuspend the pellet in 450 μL of MACS buffer.
3. Add 5 μL of Fc blocking antibody, mix well, and incubate for 10 min at 4 °C (*see* **Note 5**).
4. Add 2.5 μL of Aqua LIVE/DEAD, 5 μL of CD11b-BV421, 5 μL of Ly6C-PerCP-Cy5.5, and 5 μL of Ly6G-FITC, mix well, and incubate for 20 min at 4 °C.
5. Wash the cells with 50 mL of MACS buffer, spin the cells down.
6. Resuspend the cell pellet in 1 mL of MACS buffer.
7. Use a cell sorter equipped with the proper laser to isolate M-MDSC. Gate the population as single cells/Aqua LIVE/DEAD-/CD11b+/Ly6G-/Ly6C high (Fig. 1 for the gating strategy) (*see* **Note 6**).

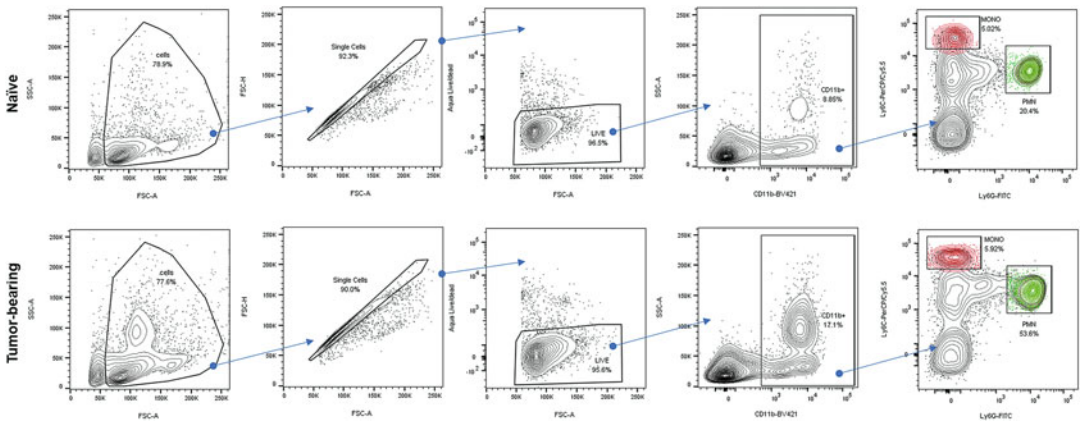

Fig. 1 Phenotype of splenic MDSC. Flow cytometry analysis and gating strategy used to identify and cell sort MDSC in spleen. Monocytes (naïve mouse) or M-MDSC (tumor-bearing mouse) are identified as single cells/Aqua LIVE/DEAD-CD11b+ Ly6ChighLy6G− (in red) and neutrophils (naïve mouse) or PMN-MDSC (tumor-bearing mouse) are identified as single cells/Aqua LIVE/DEAD-CD11b+ Ly6CintLy6G+ (in green). The upper panel shows the phenotype of myeloid cells in a spleen from naïve mouse, the lower panel shows the phenotype of myeloid cells from tumor-bearing mouse (EL4 subcutaneous tumor)

3.5 Suppression Assay

For the suppression assay TCR transgenic T cells (i.e., PMEL or OT-1) are cultured in the presence of splenic cells that provide antigen-presenting cells to present the TCR cognate antigen (i.e., gp100 for PMEL or OVA for OT-1). Suppression assay is performed by measuring proliferation of PMEL or OT-1T cells induced by gp100 or OVA in the presence of different ratio of MDSC. The starting ratio for PMN-MDSC and M-MDSC in the assay can be different given stronger M-MDSC suppressive abilities. Usually PMN-MDSC are used at 1:1 highest ratio with splenocytes and M-MDSC at 1:2 highest ratio. Each well will have a different number of MDSC and 1×10^5 splenocytes. Each condition should be assessed in triplicates.

1. Prepare splenocytes mix suspension containing 1 part of PMEL or OT-1 splenocytes with 4 part of naïve spleen at the final concentration of 2×10^6 cells/mL in complete medium. Prepare 15 mL of splenocytes mix suspension using 6×10^6 PMEL or OT-1 splenocytes plus 2.4×10^7 naïve splenocytes in a final volume of 15 mL of complete medium.
2. Prepare a 4× solution of the gp100 peptide diluted in complete medium at the concentration of 0.4 μg/mL or prepare a 4× solution of the OVA peptide diluted in complete medium at the concentration of 0.4 ng/mL.
3. Prepare 700 μL of PMN-MDSC suspension in complete medium at the concentration of 1×10^6 cells/mL (1:1 concentration). By serial dilution, add 350 μL of 1:1 PMN-MDSC suspension to 350 μL of complete medium (1:2 concentration) and repeat the procedure again to prepare 1:4 concentration.
4. Prepare 700 μL of M-MDSC suspension in complete medium at the concentration of 5×10^5 cells/mL (1:2 concentration). By serial dilution, add 350 μL of 1:1 M-MDSC suspension to 350 μL of complete medium (1:4 concentration) and repeat the procedure again to prepare 1:8 concentration.
5. In a 96 well U-bottom plate dispense 100 μL of each MDSC suspension to each well in triplicate. Add to the same well 50 μL of splenocyte mix suspension and 50 μL of 4× gp100 solution or 4× OVA solution.
6. In three wells plate with 100 μL of complete medium plus 50 μL of splenocyte mix suspension and 50 μL of 4× gp100 solution or 4× OVA solution (this is your positive control for the proliferation).
7. In three wells plate with 150 μL of complete medium plus 50 μL of splenocyte mix suspension (this is your negative control for the proliferation).
8. Incubate the plate in a CO_2 incubator (5% humidity) for 40 h.

9. After 40 h prepare a thymidine solution containing 29 μL of complete medium plus 1 μL of thymidine-H3 (1 μCi) per well, for 20 wells prepare 580 μL of complete medium plus 20 μL of Thymidine-H3.
10. Add 30 μL of the thymidine solution to each well of your plate and incubate in the CO_2 incubator for additional 8 h.
11. After total 48 h of incubation freeze the plate for at least 2 h at −80° (*see* **Note 7**).
12. Thaw the plate.
13. Transfer the liquid of each well to a glass fiber filter using the filter mate harvester.
14. Remove the filter from the harvester and let it completely dry (*see* **Note 8**).
15. Assemble the filter into the omnifilter holder.
16. Add 25 μL of Scintillation Liquid to each well.
17. Seal the plate with the adhesive strip.
18. Acquire the plate in a β-counter to have the CPM of your well (*see* **Note 9**).

4 Notes

1. You expect to identify monocytes and PMN as in Fig. 1. MDSC are usually expanded in tumor-bearing mice as compared to naïve mice. There is no specific marker to identify MDSC. The optimal classification of PMN and M-MDSC involve the assessment of their suppressive abilities.
2. Start this step during the centrifugation in the previous step.
3. PMN-MDSC are ready to use. We suggest assessing cell purity after the isolation step and to work very fast with these cells because of their sensitivity to rapid activation. *See* Fig. 2 for a typical assessment of the purity after PMN-MDSC isolation.
4. M-MDSC can be isolated either from the first flow-through after PMN-MDSC isolation or directly from the spleen by cell sorting. M-MDSC are less abundant compared to PMN-MDSC in the spleen of TB mice, but have a more pronounced ability to suppress T cell function.
5. If you are isolating M-MDSC from the previous flow-through you can skip this step.
6. After sorting we advise to assess cell purity. *See* Fig. 2 for a typical sorting experiment check purity.
7. The plate can be stored in the −80 for 2–3 days before proceeding with further steps.

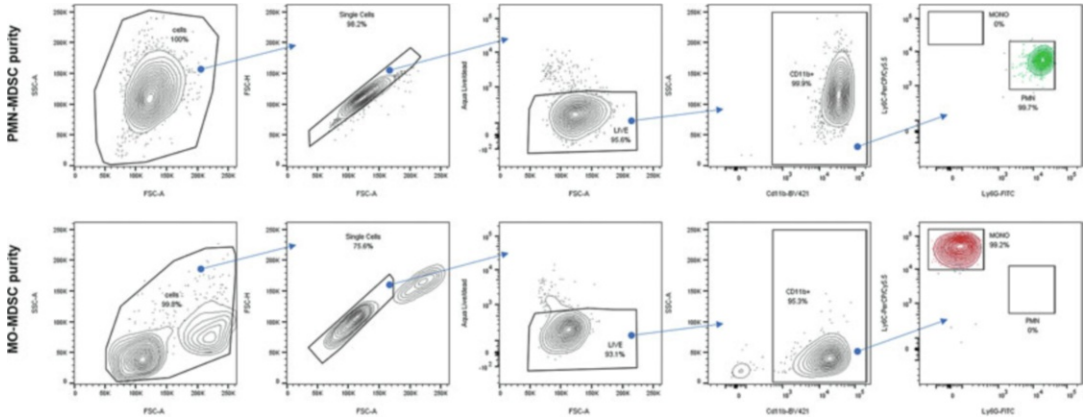

Fig. 2 Purity check of M-MDSC and PMN-MDSC after isolation. Flow cytometry analysis of PMN-MDSC isolated from spleen of tumor-bearing mouse (EL4 subcutaneous tumor) using Ly6G-UltraPure MicroBeads (upper panels) and flow cytometry analysis of M-MDSC isolated from the spleen of tumor-bearing mouse (EL4 subcutaneous tumor) through cell sorting (lower panels)

8. You can use a dry incubator to speed up the process.
9. T cell proliferation is proportional to the CPM that you obtain. Using this condition, we normally obtain a value of CPM for the positive control that is in the range of 5×10^4–6×10^4 CPM.

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Chapter 4

Phenotypical Characterization and Isolation of Tumor-Derived Mouse Myeloid-Derived Suppressor Cells

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Abstract

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population composed of mature and immature cells of myeloid origin that play a major role in tumor progression by inhibiting the antitumor immune responses mediated by T cells. In this chapter, we describe protocols for isolation, phenotypical and functional evaluation of MDSCs isolated from mouse tumors, with the aim at unifying and standardizing protocols set up by different laboratories.

Key words Myeloid-derived suppressor cells (MDSCs), Tumor digestion, Immunosuppression, ELISPOT, Cell sorting

1 Introduction

Cancer cells exploit a series of mechanisms in order to develop, like corrupting cells of the tumor microenvironment and sustaining emergency myelopoiesis in the bone marrow, which generates myeloid cells with an immature phenotype known as myeloid-derived suppressor cells (MDSCs) [1, 2]. As suggested by the name, MDSCs are highly immunosuppressive, favoring several steps of both tumor development and progression [3]. While MDSCs have been identified in several organisms, for the purpose of this work we will focus on mouse MDSCs, highlighting the phenotypical and functional activity of MDSCs specifically isolated from tumor tissues.

1.1 Phenotypical Characterization of Mouse MDSCs

In mice, MDSCs are phenotypically characterized by the expression of two distinct markers, Ly6C and Ly6G among the $CD11b^+$ myeloid cells [4]. While Ly6C identifies the monocytic lineage

Roza Maria Barouni and Chiara Musiu contributed equally to this work.

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(M-MDSCs), Ly6G recognizes the granulocytic lineage (PMN-MDSCs) [5, 6]. Although these markers can be used in order to easily detect and purify MDSCs from tumor tissues, restraining the analysis to the phenotypical cue is not enough. Therefore, it is mandatory to evaluate their functional activity, as it has been stated in several publications [5]. The advance of the high-throughput technologies has not only facilitated the genetic, epigenetic, and metabolic understanding of the two main MDSC subsets but also underscored atypical cells, like the segregated-nucleus-containing atypical monocytes (SatM) and neutrophil-like monocytes [7, 8]. Interestingly, while the formers are missing the expression of monocytes classical marker Ly6C, since they are phenotypically defined as Ceacam1^{+}Msr1^{+}Ly6C^{-}F4/80^{-}Mac1^{+} cells, the second do not express Ly6G marker and they are identified as proliferating CD11b^{+}Ly6G^{-}CD115^{+}Ly6C^{hi} cells even though they are generated by granulocyte-monocyte progenitor cells (GMPs). Despite the fact that these alternative cell populations have been discovered in diverse pathological settings other than tumors, it has been speculated that they might contribute to tumor onset by suppressing antitumor immune responses. However, to our knowledge, data describing their suppressive activity are still lacking.

1.2 Suppressive Mechanisms of Mouse MDSCs

A plethora of studies on the MDSCs have also revealed the mechanisms through which they suppress the antitumor immune response. Mainly, MDSCs exert suppression on T lymphocytes by upregulating immune-regulatory enzymes, like arginase-1 (ARG1), nitric oxide synthase 2 (NOS2), indoleamine-pyrrole 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2). Additionally, MDSCs secrete cytokines and generate reactive oxygen and nitrogen species (ROS and RNS, respectively), which subsequently inhibit T cell proliferation [9, 10].

1.3 Aim of the Chapter

Taking into consideration the background information stated above, in this chapter we describe the methods to isolate and characterize tumor-derived mouse MDSCs. Specifically, Subheading 3.1 will present methods on how to digest tumor tissues according to the tumor-specific characteristics; Subheading 3.2 describes protocols to isolate MDSCs by either fluorescence-based or immunomagnetic cell sorting from tumors. As previously mentioned, to define MDSCs, the suppressive activity must be evaluated, consequently in Subheading 3.3 we are proposing different assays to assess the T cell proliferation and cytotoxic activity, as we previously published [11], together with the newly inserted ELISPOT assay to determine IFN-γ release. Finally, in Subheading 3.4 we refer to techniques, kits, and reagents employed to evaluate the immunoregulatory molecules used by MDSCs to suppress T cell responses.

2 Materials

2.1 Digestion of Mouse Tumor Tissues

1. RPMI containing 10% FBS: RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 100 U/mL penicillin/streptomycin, 50 μM β-mercaptoethanol, 88 μM folic acid, 1 mM sodium pyruvate (NaPyr), 2 mM L-Glutamine.
2. FBS heat inactivated.
3. Ammonium–chloride–potassium lysis buffer (ACK).
4. Enzymatic digestion mix: Collagenase IV [1 mg/mL], Hylurodinase [0.1 mg/mL], DNase [0.003 μ/mL] or [4.5 mg/mL] (*see* **Notes 1–4**).
5. 6-Well plates.
6. 70 μm and 40 μm nylon cell strainer.
7. 2 mL Syringes without needle.

2.2 Isolation of MDSC Subsets from Mouse Tumor Tissues

1. Tumors from BALB/c or C57BL/6 mice.
2. RPMI containing 10% of FBS (*see* recipe).
3. Dulbecco's phosphate-buffered saline dPBS.

2.2.1 Fluorescence-Based Cell Sorting

4. Sorting buffer (autoMACS Running Buffer or PBE Buffer (PBS 1×, BSA 0.5%, EDTA 2 mM)).
5. Antibodies: anti-mouse Fc-receptor (FcR) blocking reagent, anti-mouse CD45.1 or CD45.2, anti-mouse CD11b, anti-mouse Ly6G, anti-mouse Ly6C, and a fixable viability dye.
6. FBS heat inactivated.

2.2.2 Immunomagnetic Cell Sorting

1. Sorting buffer (autoMACS Running Buffer).
2. Mouse FcR blocking reagent.
3. Microbeads: mouse anti-Ly6G biotin, anti-biotin microbeads, mouse anti-Gr-1 biotin, μMAC-Streptavidin microbeads, mouse/human CD11b Microbeads.
4. SuperMACS II separator.
5. Adapter for MS and LS columns to use with SuperMACS II magnetic separator.
6. MS or LS columns.

2.3 Evaluation of MDSC Suppressive Activity

2.3.1 Ex Vivo Analysis of IFN-γ Production: Evaluation of Intracellular IFN-γ Levels by FACS

1. Phorbol 12-myristate 13-acetate (PMA).
2. Ionomycin.
3. Golgi plug.
4. BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit.
5. IFN-γ (clone XMG1.2).
6. 96-Well plate.
7. RPMI containing 10% of FBS.

2.3.2 Ex Vivo Analysis of IFN-γ Production: Determination of T Cell-Dependent IFN-γ Secretion (ELISPOT)

1. 0.45 μm Surfactant-Free Mixed Cellulose Ester Membrane 96-well filtration plate.
2. Coating antibody: ELISPOT purified mouse anti-IFN-γ.
3. Detection antibody: Biotinylated mouse anti-IFN-γ.
4. AKP streptavidin.
5. Substrate 1-Step NBT/BCIP.
6. Tween-20 100% Nonionic Detergent.
7. BSA.

3 Methods

3.1 Digestion of Mouse Tumor Tissues

1. Isolate tumors by removing irrelevant tissues and put them in RPMI containing 10% FBS under sterile conditions.
2. Place the tumor in 6-well plate dish and depending on the size of the tumor add the appropriate type and volume of enzymatic digestion mix. Here below we list a series of digestive mixes which are used for the most common mouse tumor models. According to the tumor type, we recommend the enzymes, their concentrations, and the optimal incubation time (*see* **Note 4**—Table 1).
3. Add an amount of digestive mix and mince the tumor in small pieces.
4. Incubate the tumor at 37 °C for the time period indicated in Table 1.
5. Every 10/15 min disaggregate mechanically the tumor pieces using a 2 mL syringe without needle.
6. At the end of the incubation collect everything and filter the suspension by passing through a cell strainer with a 70 μm pore size.
7. Rinse the strainer with RPMI containing 10% FBS.
8. Centrifuge for 5 min at 300 × *g*, 4 °C.
9. Discard the supernatant.

Table 1
Proposed tumor digestions mixes and incubation times for different tumor models (*see* Note 21)

Digestion mix	Tumor model	Incubation time	References
Mouse Tumor Dissociation Kit (Miltenyi Biotec)	EL4, LLC, CT26, KPC[a] MOC1, MOC2, LLC LLC, RENCA	40 min 30 min	[24] [25, 26] [27]
DNase I [0.1 mg/mL] Collagenase D [0.2 mg/mL]	B16-F10	45 min	[14]
DNase I [150 IU/mL] Liberase [25 μg/mL]	LLC, 3LL, MCA-38, EL-4, B16 (F10), ID8, B16-GM-GSF, MCA-205, MC38, AT3	1 h	[28] [29] [30]
Collagenase type I [1 mg/mL] Collagenase type IV [0.4 mg/mL] DNase I [10 μg/mL]	Hepa 1–6	30 min	[31]
Collagenase type I [0.025 mg/mL] Collagenase type IV [0.05 mg/mL] Hyaluronidase [0.025 mg/mL] DNase I [0.01 mg/mL] Soybean trypsin inhibitor [0.2 unit/mL]	MCA 38, GL261	15 min	[32]
Collagenase IV [1 mg/mL] Hylurodinase [0.1 mg/mL] DNase [0.003 μ/mL] or [4.5 mg/mL]	EL4, EG7, MN-MCA1, MMTV-PyMT[a] E0771, KPC syngeneic cell lines	1 h 45 min	[18], Lab experience Lab experience
Collagenase IV [0.5 mg/mL] DNase I [0.5 mg/mL] Dispase II [3 mg/mL]	CAC	20 min	[33]

[a]Sample isolated from mice that develop spontaneous tumors

10. If tumor samples contain a consistent amount of red blood cells (RBC), proceed to lyse RBC by resuspending the sample in 5 mL ACK lysis buffer and incubate for 10 min at 25 °C.
11. Block the reaction by adding an equal volume of RPMI-containing 10% FBS and mix gently.
12. Centrifuge the samples for 5 min at 300 × *g*, 4 °C.
13. Resuspend the tumor mix and filter it through a cell strainer, 40 μm pore size.
14. Estimate the living cells by trypan blue assay (*see* **Notes 5** and **6**).

3.2 Isolation of MDSC Subsets from Mouse Tumor Tissues

Here below we describe the basic protocol 1 from Solito et al. adapted to specifically isolate MDSC subsets from different tumor tissues [11].

3.2.1 Fluorescence-Based Cell Sorting

1. Digest the tumor mass as previously mentioned (*see* "Digestion of Mouse Tumor Tissues" section).
2. Centrifuge the suspension for 5 min at 300 × *g* at 4 °C and discard the supernatant.
3. Resuspend cell pellet in 10 mL/tumor of DPBS, filter through a 70 μm cell strainer placed on the top of a 50-mL tube, and collect the suspension.
4. Take an aliquot of the single-cell suspension and estimate the number of viable cells by trypan blue assay [12].
5. Transfer 3×10^7 viable cells to a new 15-mL conical tube, and then add 8–10 mL of sorting buffer to wash the cells.
6. Centrifuge the single-cell suspension for 5 min at 300 × *g* at 4 °C and discard the supernatant.
7. Add 10 mL of sorting buffer and wash cells by gently pipetting.
8. Centrifuge the suspension for 5 min at 300 × *g* at 4 °C and discard the supernatant (*see* **Note 7**).
9. Block nonspecific binding with 50 μL of FcR blocking reagent mix for 10 min at 4 °C.
10. Stain with 50 μL of a mix composed of anti-mouse CD45.1 or CD45.2, anti-mouse CD11b, anti-mouse Ly6G, anti-mouse Ly6C antibodies, and a fixable viability dye in staining buffer, for 30 min at 4 °C. For concentration of antibodies, please refer to Table 2 (*see* **Note 8**).
11. Wash twice with sorting buffer. Centrifuge the suspension for 5 min at 300 × *g* at 4 °C and discard the supernatant.
12. After the labeling, resuspend samples at the concentration of 3×10^7 cells/mL of sorting buffer.
13. Filter the sample with a 40 μm pore size cell strainer and proceed to sort the cells by FACS ARIA.
14. Proceed with the FACS separation, using the gating strategy reported in Fig. 1 (*see* **Notes 9–12**).

3.2.2 Immunomagnetic Cell Sorting

1. Prepare sample according to tumor digestion protocol described above or other strategy of digestion.
2. Determine cell number and centrifuge the single-cell suspension for 10 min at 300 × *g* at 4 °C and discard the supernatant.
3. Follow the anti-Ly6G microbeads ultrapure kit, positive selection, manufacturer's instructions.

Table 2
Proposed antibodies for the isolation of MDSCs with fluorescent activated cell sorting

Antibody	Clone	Conjugate	Stock concentration (mg/mL)	Amount per 10^6 cells (μg)
Purified anti-mouse CD16/32	93		0.5	0.2
Anti-mouse CD45.1	A20	PE	0.2	0.06
Anti-mouse CD45.2	104	PE	0.2	0.06
Anti-mouse CD11b	M1/70	PerCP/Cy5.5	0.2	0.06
Anti-mouse Ly6G	1A8	APC/Cy7	0.2	0.06
Anti-mouse Ly6C	HK1.4	eFluor450	0.2	0.06
Fixable viability dye				

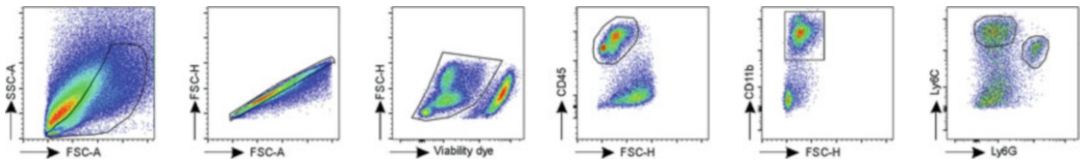

Fig. 1 Gating strategy to isolate Ly6C$^+$ and Ly6G$^+$ cells from tumor tissues

4. We recommend to maintain the temperature of incubation at 4 °C to avoid nonspecific binding.
5. Proceed to magnetic separation choosing an appropriate MACS column and MACS Separator according to the number of total cells (*see* **Note 13**).
6. To isolate tumor-infiltrating M-MDSCs (CD11b$^+$Ly6C$^+$Ly6G$^-$ cells) we will proceed as follows: count the cells present in the Ly6G$^-$ fraction, then incubate them with an anti-Gr-1 antibody, following the manufacturer's instructions. Add the corresponding amount of μMAC-Streptavidin microbeads and incubate the sample as indicated by the datasheet. Proceed to magnetic separation. The eluted fraction (enriched in Ly6C$^+$ cells) will be further incubated with CD11b microbeads, as recommended by the manufacturer's instructions. We think that with this approach we substantially avoid the contamination of Ly6C-expressing memory CD8$^+$ T cells [13] as well as limit the amount of tumor-infiltrating macrophages (TAM) expressing CD11b but negative for Ly6C. In conclusion, with this approach we can obtain CD11b$^+$Ly6C$^+$ cells with a good purity (80–90%) (*see* **Note 14**).

3.3 Evaluation of MDSC Suppressive Activity

3.3.1 Ex Vivo Analysis of IFN-γ Production: Evaluation of Intracellular IFN-γ Levels by FACS

1. Resuspend $CD45^+$ cells isolated from tumors in RPMI complete (*see* recipe) containing 50 ng/mL PMA, 2 μg/mL ionomycin, and Golgi plug (dilution 1/1000) in 96-well plate.
2. Incubate for 6 h at 37 °C.
3. Collect the cells and stain for IFN-γ using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit and following the manufacturer's instructions and Alissafi et al. paper [14].

3.3.2 Ex Vivo Analysis of IFN-γ Production: Determination of T Cell-Dependent IFN-γ Secretion (ELISPOT)

1. Add 100 μL per well of the coating antibody anti-IFN-γ diluted in PBS in final concentration 5 μg/mL in the ELISPOT plate.
2. Incubate overnight at 4 °C.
3. Wash three times the plate with PBS.
4. Add 200 μL per well of 5% BSA-PBS and incubate for 2 h at room temperature.
5. Wash three times the plate with PBS.
6. Add 100 μL/well of RPMI complete (*see* recipe) and incubate for 10 min at room temperature.
7. Add the antigen-presenting cells (APCs) and effector cells in a final volume of 100 μL/well and incubate the plate for 24 h at 37 ° C (*see* **Note 15**).
8. Wash ten times the plate with 0.05% Tween-20—PBS.
9. Add 200 μL/well of distilled water and incubate for 10 min at room temperature.
10. Add 100 μL/well of the biotinylated anti-IFN-γ antibody diluted in 2% BSA-PBS in final concentration 1 μg/mL.
11. Incubate for 5 h at room temperature.
12. Wash three times the plate with 0.05% Tween-20—PBS.
13. Wash three times the plate with PBS.
14. Add 100 μL/well of AKP streptavidin diluted 1:6000 in 2% BSA-PBS.
15. Incubate for 1 h at room temperature.
16. Wash three times the plate with 0.05% Tween-20—PBS.
17. Wash three times the plate with PBS.
18. Add 100 μL/well of the substrate 1-Step NBT/BCIP and incubate in the dark for 30 min at room temperature.
19. Wash three times the plate with distilled water.
20. Let the plate dry for 24 h, after removing the bottom of the plate.
21. Count the spots in the appropriate machine (*see* **Notes 16–19**).

3.4 Evaluation of the Suppressive Mechanisms

1. Colorimetric evaluation of urea production (please refer to [15]).
2. Proteomic analysis (HPLC; LC/MS-MS).

3.4.1 ARG1 Activity

3.4.2 NOS2 Activity

1. Colorimetric evaluation of NO production, using the Griess reagent (please refer to [16, 17]).
2. Colorimetric evaluation of NO production, based on DAF-FM Diacetate (4-Amino-5 Methylamino-2′, 7′ Difluorofluorescein Diacetate) detection (please refer to [18]).

3.4.3 DO Activity

1. Kynunerine assay (please refer to [15]).
2. HPLC analysis of kynurenine production (please refer to [19]).

3.4.4 PGE2

Please refer to [19].

3.4.5 ROS Production

1. Detection of H_2O_2 (please refer to [20] and the Thermo Fisher Scientific manufacturer's instructions, Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, cat. No. A22188).
2. ROS production (please refer to [21] and to the products CM-H2DCFDA or DAR-4M).
3. Proteomic analysis (LC/MS-MS) of different metabolites, like L-arginine, L-ornithine, proline, and kynurenine.

3.5 Summary

Despite the increasing interest and the increasing amount on scientific papers, it has been observed a great variability and incoherence in several experimental approaches used to identify MDSCs among the different laboratories. For this reason, in this chapter, we propose protocols for the isolation, phenotypic and functional characterization of MDSCs isolated from mouse tumors with the view to standardize the current available protocols.

As mentioned in this manuscript and in several other publications, the phenotypic characterization of MDSCs is not sufficient. It is imperative to test their suppressive capacity and the mechanisms involved, in order to safely name these cells as MDSCs [22]. Considering the heterogeneity of the tumor microenvironment present in each tumor model and its ability to shape differently the immunosuppressive activity of MDSCs [23], we think that understanding the mechanisms set to dampen the antitumor immune response is a fundamental step for a therapeutic intervention (*see* **Note 20**). We believe that unifying the experimental procedures will help not only to have great insights on the molecular mechanisms used by "traditional" MDSCs but also to disclose new MDSC subsets.

4 Notes

1. All protocols using animals must be approved by an institutional ethics committee and must be executed in accordance with governing laws, directives, and guidelines.
2. Solutions and equipment used to isolate and purify cells must be sterile, and aseptic technique must be used accordingly.
3. Cells and tissues are generally maintained in a humidified incubator 37 °C, 5% CO_2 incubator unless otherwise stated.
4. Noteworthy, for each tumor model the composition of the enzymatic digestion mix and the time of digestion must be evaluated taking into account the tissue composition of the model (*see* Table 1).
5. To avoid counting red blood cells use acridine orange/ethidium bromide solution. Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity, consequently live cells will appear uniformly green while dead cells will stain orange and red cells will not appear.
6. Obtained cells should be processed immediately for downstream applications.
7. Staining procedure requires specific antibody concentration; thus, the supernatant must be carefully removed.
8. All antibodies must be titrated before use. The fluorochrome choice must take into account the FACS configuration setting. For suggested concentration of antibodies, please refer to Table 2.
9. We recommend to sort the MDSC subsets by purity- and not by yield-based approach, specifically when a clear information regarding the source of suppression is needed. Moreover, to obtain both cell subsets without altering their functions, we suggest to use a cell rate of 3000–5000 events/s. On BD instruments, the parameter flow-rate operates in a range between 1 and 10, with a better performance at three flow-rate, in order to avoid the coefficient of variation of fluorescence increment. Consequently, to increase the flow-rate we suggest to increase the concentration of the single-cell suspension. Noteworthy, monocytes ($Ly6C^+$ cells) sorting should be performed at cold temperature (4–8 °C), while for neutrophils ($Ly6G^+$ cells) the temperature of sorting should be maintained around 23–24 °C.
10. We recommend to use 100 μm-nozzle to purify MDSCs out of the tumors.

11. Collect sorted M-MDSCs and PMN-MDSCs in different 15-mL polypropylene tubes previously coated for at least 1 h with heat-inactivated fetal bovine serum. After the separation, wash and resuspend the sorted MDSCs in 10 mL of 10% FBS—RPMI medium. Take an aliquot of single-cell suspension, and check by trypan blue assay the number of viable cells. We recommend to check the purity of both MDSC subsets after sorting.
12. FACS sorting could stress MDSCs, resulting in altered functions. Depending on the aim of the experiment and when feasible, we suggest to leave sorted cells in culture, allowing overnight recovery.
13. To increase the purity of the magnetically labeled Ly6G cells, we recommend to perform a second round of purification over MS or LS columns.
14. We recommend to evaluate the cell purity of isolated M-MDSCs, in particular the contamination of F4/80-expressing cells, which normally refer to TAMs.
15. Incubation time has to be previously tested. The incubation time range is between 16 and 40 h.
16. The quantification of IFN-γ is expressed as number of spots per number of plated cells.
17. T cells stimulated with anti-CD3 and anti-CD28 can be used as positive control for this assay.
18. Different types of antigen-presenting cells could be assayed with ELISPOT. In case of tumor cells, we suggest to γ-irradiate tumor cells at 7000–1000 rad.
19. IFN-γ intracellular staining is quantitative and suitable for testing the intracellular accumulation of the cytokine, but in order to safely draw conclusions about the activation status of T cells, it is necessary to test the IFN-γ secretion by ELISPOT. In addition, ELISPOT has higher sensitivity as compared to the intracellular staining by FACS.
20. As we stated earlier, it is mandatory to correlate T cell activity with two or more MDSC-associated immunosuppressive mechanisms [5]. Therefore, the functional activity of tumor-infiltrating T cells must be linked to both the tumor-associated MDSC frequency and MDSC functional properties. Here we highlight, by a Pearson correlation plot, a possible interpolation between T cell activity and MDSC-associated immunosuppressive functions (Fig. 2).
21. To standardize the difference among various companies' reagents, enzyme concentrations have to be calculated in unit/mL.

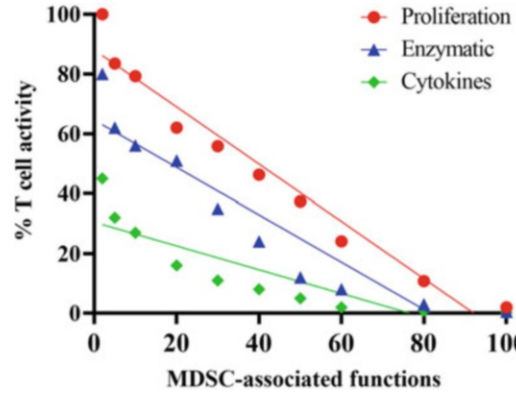

Fig. 2 Possible Pearson correlation plot of T cell activity and MDSC-associated functions

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Chapter 5

Isolation of Human Circulating Myeloid-Derived Suppressor Cells and Analysis of Their Immunosuppressive Activity

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Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of myeloid cells with potent immunosuppressive activity and characterized by a pathological state of activation. The T cell suppression assay is the most common method to evaluate the suppressive capacity of MDSC. Identifying the suppressive potential of different MDSC subsets within individual donors is key for understanding the biology of MDSC and their clinical relevance. Here we describe assays to ascertain and quantify the suppression of autologous T cells by human MDSC. These include the dye dilution proliferation assay for flow cytometry and the detection of IFNγ production by T cells using flow cytometry and sandwich ELISA.

Key words MDSC, T cell suppression assay, IFNγ secretion assay

1 Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of myeloid cells with potent immunosuppressive activity and characterized by a pathological state of activation [1, 2]. Two MDSC subsets, $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ (M-MDSC) and $CD11b^{+}Ly6C^{lo}Ly6G^{+}$ (PMN-MDSC) [1, 3], were described in tumor-bearing mice. Analysis of blood circulating MDSC in cancer patients revealed an additional subset, the early-stage MDSC (eMDSC) [1, 4]. The phenotyping of these three subsets is described elsewhere (*see* Chapter on "Immunophenotyping of circulating MDSC in the peripheral blood of cancer patients" in this volume). The ability to suppress T cell functions is a key feature of MDSC and commonly investigated using polyclonal activators or antigen-specific stimulation of T cells [1, 5]. Recently, we compared the suppressive capacity and clinical relevance of circulating M-MDSC, PMN-MDSC, and e-MDSC in patients with cancer [6].

Different modalities to trigger T cell activation (e.g., polyclonal, lectin-based, cytokine-based, antigen-specific, or mixed lymphocyte reaction) may be employed in suppression assays.

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Here we describe a robust MDSC-mediated suppression assay that is based on polyclonal T cell stimulation of autologous T cells. We describe different T cell readouts to comparatively evaluate the suppressive capacity of the three human MDSC subsets in microwell assays ex vivo.

2 Materials

Perform all steps at room temperature unless indicated otherwise (*see* **Note 1**).

2.1 Density Centrifugation of Peripheral Blood

1. 5× 10 mL 9NC blood collection tubes (recommended, followed by K3EDTA).
2. Isotonic separation medium for density centrifugation (density 1.077 g/mL).
3. Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS).
4. Conical centrifuge tubes 50 mL.
5. Heat-inactivated fetal calf serum: Thaw fetal calf serum over night at 4 °C and incubate for 30 min at 56 °C. Filter inactivated serum over 0.2 μm filter membrane. Store in aliquots at −20 °C (*see* **Note 2**).
6. Culture medium: RPMI Medium 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin/100 μg/mL streptomycin. Add 50 mL heat-inactivated fetal calf serum and 5 mL 10,000 U/mL penicillin/10,000 μg/mL streptomycin to 445 mL RPMI Medium 1640 (1×) supplemented with L-glutamine. Store at 4 °C.
7. Disposable Pasteur pipette.
8. Neubauer chamber or automatic cell counter CASY. If using a Neubauer chamber perform live/dead staining by mixing 100 μL cell suspension with 400 μL 0.4% trypan blue.

2.2 MACS Separation

1. Anti-human CD3 MicroBeads (Miltenyi BioTec, Bergisch-Gladbach, Germany).
2. MACS buffer: Add one bottle of Miltenyi MACS BSA Stock solution in one bottle of Miltenyi autoMACS Rinsing Solution to obtain a solution containing DPBS (pH 7.2), 0.5% BSA and 2 mM EDTA.
3. Miltenyi MACS LS-Columns and Miltenyi QuadroMACS Separators.

2.3 Fluorescence-Activated Cell Sorting

1. Falcon® Round-Bottom Polystyrene Tubes, 5 mL (FACS tubes).
2. Human Serum (HS): Incubate pooled human AB serum for 30 min at 56 °C. Store in aliquots at −20 °C.
3. DPBS/HS: To prepare staining buffer DPBS/3% HS add 7.5 mL HS to 242.5 mL DPBS and filter sterile using 0.2 μm filter membranes. Store at 4 °C.
4. Fluorochrome conjugated anti-CD66b, anti-CD33, anti-HLA-DR, anti-CD3, anti-CD19, anti-CD20, anti-CD56, anti-CD11b, and anti-CD16 (*see* Table 1 for suggested clones and fluorochromes). It is recommended to titrate the antibodies on the own system.
5. Lineage cocktail: Prepare Lineage Cocktails by mixing pre-titrated anti-CD3/CD19/CD20/CD56 antibodies conjugated with the same fluorochrome (*see* **Note 3**).
6. 100× L-Lysine: Dissolve 200 mg L-Lysine hydrochloride in 50 mL aqua dest in a 50 mL centrifugation tube. Use a 0.2 μm syringe filter and a 50 mL perfusion syringe for sterile filtration into a new 50 mL tube. Store at 4–8 °C.
7. 15 mM L-Arginine: Dissolve 26 mg L-Arginine in 10 mL DPBS. Use a 0.2 μm syringe filter and a 10 mL syringe for sterile filtration into a new tube.
8. Arginine-free medium: RPMI Medium 1640 (Arginine and Lysine free) supplemented with 10% fetal calf serum, 100 U/mL penicillin/100 μg/mL streptomycin, 0.04 mg/mL L-Lysine. Supplement 88 mL of RPMI 1640 Medium (Arginine and Lysine free) with 10 mL heat-inactivated fetal calf serum, 1 mL 10,000 U/mL penicillin/10,000 μg/mL streptomycin, and 1 mL of 100× L-Lysine. Store the medium at 4 °C.

Table 1
Antibodies used for MDSC FACS sorting

Antigen	Clone	Fluorochrome
CD66b	80H3	FITC
CD33	WM53	PE
HLA-DR	G46-6	APC
CD11b	ICRF44	APC-Cy7
CD16	3G8	PE-Cy7
Lineage cocktail CD3/CD19/CD20/CD56	SK7/H1B19/2H7/HCD56	All Brilliant Violet 421

9. Arginine low medium: Add 100 μL of freshly prepared 15 mM L-Arginine solution (150 μM final concertation) per 10 mL of Arginine-free medium (*see* **Note 4**).
10. 50 μm Sterile cell strainers.

2.4 T Cell Assays

1. Arginine low medium or culture medium as described above.
2. 10 mM Cell Proliferation Dye eFluor 450 (Thermofischer scientific) or comparable.
3. 96-well Round-Bottom Plate.
4. Anti-CD3 clone OKT3 (1 mg/mL) and anti-CD28 clone 28.2 (0.1 mg/mL) (*see* **Note 5**).
5. ModFit LT™ Software (or similar analysis software).
6. IFNγ ELISA Kit.
7. 1.5 mL Reaction tubes.
8. BD Bioscience Fixation/Permeabilization Solution Kit: Dilute 5 mL 10× PermWash buffer with 45 mL aqua dest to obtain 1× PermWash buffer. Store at 4 °C.
9. Monensin (protein transport inhibitor; BD GolgiStop™).
10. Fluorochrome conjugated anti-human IFNγ (clone 45-15) and anti-CD3 (clone BW264/56). It is recommended to titrate the antibodies on the own system.

3 Methods

Perform all steps at room temperature unless indicated otherwise (*see* **Note 6**).

3.1 Isolation of Peripheral Blood Mononuclear Cell (PBMC) from Cancer Patients

1. Dilute 50 mL of blood 1:1 with 50 mL of DPBS (for three 50 mL separation tubes).
2. Pipette 15 mL of separation medium for density centrifugation into three conical centrifuge 50 mL tubes.
3. Overlay separation medium with up to 33 mL of pre-diluted blood.
4. Centrifuge at room temperature and at 400 × *g* for 30 min, without acceleration and break (to prevent mixing of resulting phases).
5. After centrifugation, aspirate plasma until approximately 5 cm above the PBMC fraction.
6. Use a disposable Pasteur pipette to collect PBMC into two to three 50 mL tubes. Do not collect any separation medium.
7. Wash PBMC by filling tubes with DPBS up to 50 mL.
8. Centrifuge at 300 × *g* for 10 min room at temperature.

9. Discard supernatant allowing about 2 mL to remain in the tube and resuspend the pellet in this rest volume.
10. Combine pellets in one 50 mL tube and fill up with DPBS.
11. Repeat **steps 7–9** until the supernatant is clear.
12. Resuspend pellet in 1 mL of culture medium and add additional 19 mL medium for determination of cell concentration using an automatic cell counter or Neubauer chamber.
13. If needed, keep 2.5×10^6 PBMC for immunophenotyping.

3.2 Depletion/ Isolation of $CD3^+$ Responder Cells from PBMC of Cancer Patients

1. Centrifuge isolated PBMC at $300 \times g$ for 10 min at 4 °C.
2. Discard the supernatant and resuspend the cell pellet in 80 μL MACS buffer and 20 μL of CD3 MicroBeads per 10^7 total cells.
3. Mix well and incubate for 15 min at 4 °C.
4. Wash cells by adding 2 mL MACS buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 min.
5. Discard the supernatant completely and resuspend the cell pellet, adjusting the volume to 500 μL MACS buffer per 10^8 cells.
6. Place the LS column in magnetic field (separator) and put a 15 mL collection tube below.
7. Prepare the column by rinsing with 3 mL MACS buffer and change the collection tube.
8. Apply the cell suspension on the column.
9. Wash the column with 3×3 mL of MACS buffer. Perform washing steps by adding buffer three times, each time once the column reservoir is empty.
10. The collected cells represent $CD3^-$ cells. This fraction contains the enriched MDSC (*see* **Note 7**).
11. Remove the column from the separator and place it on a new collection tube.
12. Pipette 5 mL of buffer onto the column. Immediately flush out fraction with the magnetically labeled $CD3^+$ cells by firmly applying the plunger supplied with the column.
13. Centrifuge the $CD3^+$ and $CD3^-$ cells at $300 \times g$ for 10 min at 4 °C.
14. Resuspend the cells in 1 mL of culture medium.
15. Determine the cell concentration by using an automatic cell counter CASY or Neubauer chamber. If using a Neubauer chamber perform live/dead staining by mixing 100 μL cell suspension with 400 μL 0.4% trypan blue.

16. Proceed with the $CD3^-$ cells to Subheading 3.3, namely labeling for FACS sorting of MDSC Subsets.
17. Keep the $CD3^+$ cells as responder cells for the T cell proliferation assay. Store until usage at 37 °C and 5% CO_2 (*see* **Note 8**).

3.3 Labeling the $CD3^-$ Cell Fraction for FACS Sorting of MDSC Subsets

1. Wash cells by filling up the tube with DPBS and centrifuge at 300 × *g* for 10 min, 4 °C.
2. Resuspend up to 70 × 10^6 cells in 350 µL DPBS/HS and stain for 30 min at 4 °C with either CD66b/CD33/HLA-DR/Lineage cocktail isolating the total PMN-, M-MDSC and e-MDSC subsets **or** CD66b/CD11b/CD16 to isolate and separate the mature and immature PMN-MDSC subsets.
3. Wash the cells with 1 mL DPBS.
4. Centrifuge at 300 × *g* for 10 min, 4 °C.
5. Discard supernatant and resuspend the cell pellet in 500 µL Arginine low medium (*see* **Note 4**).
6. Keep the cells on ice until sorting.

3.4 FACS Sorting of MDSC Subsets Using BD FACS Aria III

1. Filter stained cells through a sterile cell strainer into one FACS tube.
2. Rinse strainer with 250 µL Arginine low medium to collect as many cells as possible.
3. Set following sort instructions in the sort layout window: Device on 4 Tubes, Precision on 4-Way, and target events on continuous.
4. Use a 70 µm nozzle for sorting.
5. Adjust the stream on lowest flow rate and sort no more than 25,000 cells/s. If necessary, dilute the sample with Arginine low medium.
6. Add 1 mL of Arginine low medium into three sorting collection tubes (fourth position remains empty).
7. For the isolation of MDSC subsets sort the M-MDSC into the left, PMN-MDSC in right, and e-MDSC in far-right tube (*see* **Note 9**).
8. Plot singlets in SSC-A vs. FSC-A to exclude debris (P2 in Fig. 1a).
9. To differentiate MDSC subsets (*see* Fig. 1a) show cells without debris in HLA-DR vs. CD33. HLA-DR^-/$CD33^{high}$ (P3) cells are M-MDSC.
10. Set gate for HLA-DR^-/$CD33^{dim}$ (P4).
11. Show P4 in LIN vs. CD66b. HLA-DR^-/$CD33^{dim}$/LIN^-/$CD66b^+$ (P5) cells are classified as PMN-MDSC and HLA-DR^-/$CD33^{dim}$/LIN^-/$CD66b^-$ (P6) as e-MDSC (*see* **Note 10**).

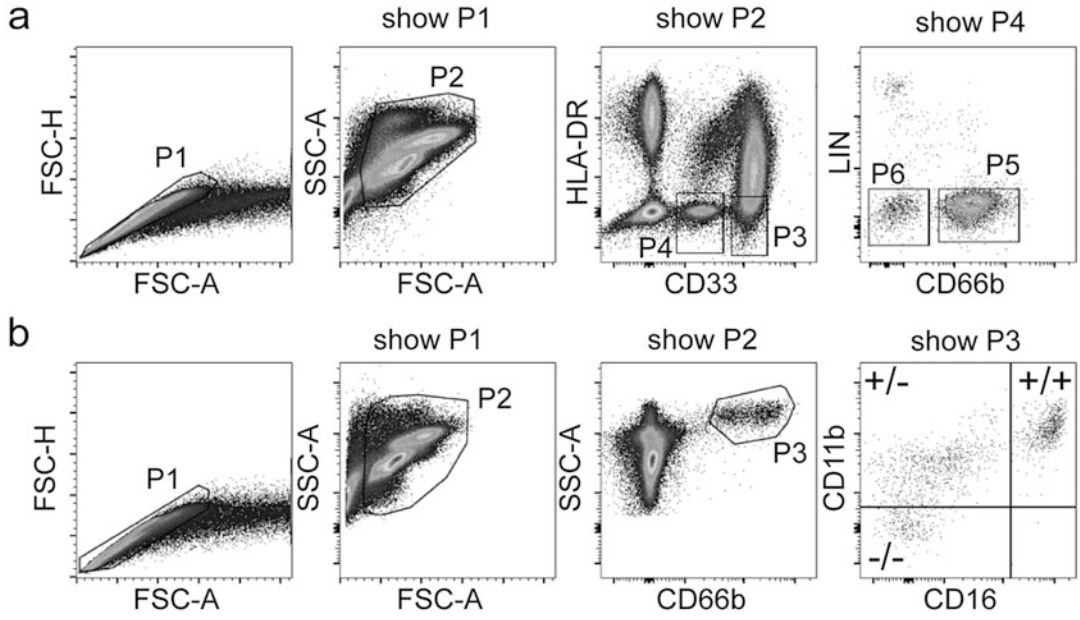

Fig. 1 Gating strategy for the isolation of MDSC and PMN-MDSC subsets. PBMC that are depleted for CD3 cells were stained with CD66b/CD33/HLA-DR/Lineage cocktail to isolate MDSC subsets or CD66b/CD11b/CD16 to isolate PMN-MDSC subsets. (**a**) Shows the gating for HLA-DR$^-$/CD33^{high} (P3) M-MDSC, HLA-DR$^-$/CD33^{dim}/LIN$^-$/CD66b$^+$ (P5) PMN-MDSC and HLA-DR$^-$/CD33^{dim}/LIN$^-$/CD66b$^-$ (P6) e-MDSC subsets and (**b**) the gating for immature (CD11b$^-$/CD16$^-$, CD11b$^+$/CD16$^-$) and mature (CD11b$^+$/CD16$^+$) PMN-MDSC

12. To avoid more centrifugation steps after sorting to determine the cell number activate the sort counters and write down the sort rate (events) of the locations at the end of the sort.
13. Add 1 mL of Arginine low medium to the cells after sorting.
14. Keep cells on ice.

3.5 FACS Sorting of PMN-MDSC Subsets Using BD FACS Aria III

1. See primary adjustments of FACS Aria III in Subheading 3.4 (**steps 1–6**).
2. Sort CD11b$^-$/CD16$^-$ into the left, CD11b$^+$/CD16$^+$ into the right, and CD11b$^+$/CD16$^-$ into the far-right tube (*see* **Note 9**).
3. Set the gate on singlets (P1) by plotting FSC-A vs. FSC-H.
4. Plot singlets in SSC-A vs. FSC-A to exclude debris (P2 in Fig. 1b).
5. To sort mature and immature PMN-MDSC subsets (*see* Fig. 1b) gate on SSC-A vs. CD66b and set the gate on CD66b$^+$ cells (P3).
6. Prepare plots for CD11b vs. CD16 to distinguish between CD11b$^-$/CD16$^-$ (Promyelocytes), CD11b$^+$/CD16$^-$ (early myelo- and metamyelocytes), and CD11b$^+$/CD16$^+$ (banded and segmented cells) subsets (*see* **Note 10**).

7. To avoid more centrifugation steps after sorting to determine the cell number activate the sort counters and write down the sort rate (events) of the locations at the end of the sort.
8. Add 1 mL of Arginine low medium to the cells after sorting.
9. Keep cells on ice.

3.6 Labeling of CD3+ Responder Cells for the T Cell Suppression Assay

1. Prior to labeling the $CD3^+$ responder cells, wash cells twice by filling up the tube with DPBS to remove serum.
2. Centrifuge at 300 × *g* for 10 min, 4 °C.
3. Resuspend cells up to a concentration of 20×10^6/mL. If labeling less than 5×10^6 cells use at least 0.5 mL DPBS, do not use lower volumes.
4. Prepare a 20 μM solution of 10 mM Cell Proliferation Dye eFlour405 (CPDye) in DPBS. Mix 1:1 with the cell suspension. For example, resuspend 10×10^6 $CD3^+$ responder cells in 250 μL DPBS and add 250 μL of the 20 μM dye solution and mix well. Final labeling concertation is 10 μM.
5. Incubate for 10 min at room temperature in the dark.
6. Stop labeling by filling up the tube with medium (*see* **Note 11**).
7. Centrifuge for 10 min, 300 × *g*, at 4 °C.
8. Discard supernatant and repeat washing steps twice (repeat **steps 7** and **8**).
9. Resuspend labeled cells in 1 mL medium.
10. Determine cell concentration and adjust to 0.5×10^6 cells/mL in medium for T cell proliferation assay.
11. Keep cells at 37 °C/5% CO_2 until usage (*see* **Note 8**).

3.7 T Cell Proliferation (Suppression) Assay and Collection of Supernatants for IFNγ Secretion

1. If possible, perform assay in intra-experimental duplicates. This depends on the amount of isolated MDSC.
2. For polyclonal stimulation coat a sufficient amount of wells (at least four wells are needed: only T cells and T cells with the three different MDSC subsets) with 100 μL of 1 μg/mL CD3 clone OKT3 and 2 μg/mL CD28.2 in DPBS for at least 2 h at 37 °C/5% CO_2 (*see* **Note 12**).
3. After the isolation of MDSC subsets, centrifuge them at 460 × *g* for 5 min at 4 °C and resuspend according to event rate from sorting counter to a final concentration of 0.25×10^6 cells/mL in Arginine low medium (*see* **Note 13**).
4. Discard the CD3/28 antibody solution from the well. Do not wash the wells.
5. Pipette 100 μL of Arginine low medium into an uncoated well. This is for the unstimulated T cells only control (*see* diagram in Fig. 2).

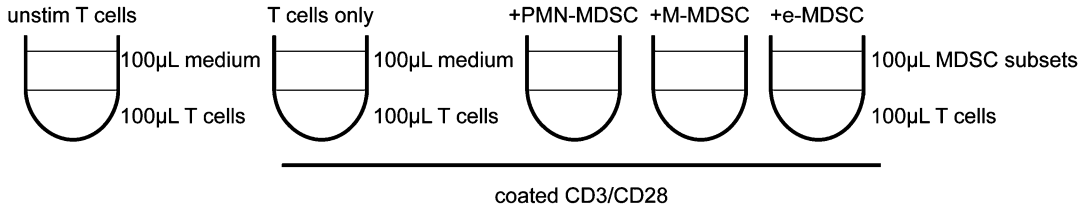

Fig. 2 Schematic representation of the experimental design for setup of the T cell proliferation assay and intracellular IFNγ assay

6. Pipette 100 µL of Arginine low medium into an antibody-coated well. This is for the stimulated T cells only control.
7. Pipette 100 µL of MDSC cell suspension to antibody-coated wells.
8. Add 100 µL of CPDye labeled $CD3^+$ responder cells (T cells) into all wells.
9. Incubate the plate for 4 days at 37 °C/5% CO_2.
10. Centrifuge the plate at 460 × *g*, 5 min, 4 °C.
11. Carefully transfer 150 µL of supernatant into 1.5 mL reaction tubes. Store samples at −20 °C for the determination of secreted IFNγ by ELISA according to manufacturer protocol (any human IFNγ ELISA kit can be used).
12. Wash cells in wells by adding 200 µL DPBS.
13. Centrifuge the plate at 460 × *g* for 5 min, at 4 °C.
14. Resuspend cells in 200 µL of DPBS/HS and subsequently measure T cell proliferation (*see* **Note 14**).
15. To determine the intensity of CPDye set first gate in SSC-A vs. FSC-A (P1) to exclude debris (*see* **Note 15**).
16. Plot SSC-A vs. CPDye and gate on $CPDye^+$ cells (P2 in Fig. 3a). Set Voltage of PMT according to unstimulated/non-proliferated cells (prepared as detailed at **step 5**). These cells are bright and show a high fluorescence, 10^4–10^5 log fluorescence intensity.
17. Depict the fluorescence intensity in a histogram.
18. The ModFit LT software calculates the proliferation index based on an algorithm provided by the software. The proliferation index is the sum of the cells in all generations divided by the computed number of original parent T cells theoretically present at the start of the experiment. Thus, the proliferation index reflects the increase in cell number in the culture over the course of the experiment. To compare the samples (and quantify the level of suppression), subtract the index of the non-proliferated fraction and set the index of the T cells without MDSC as 100%.

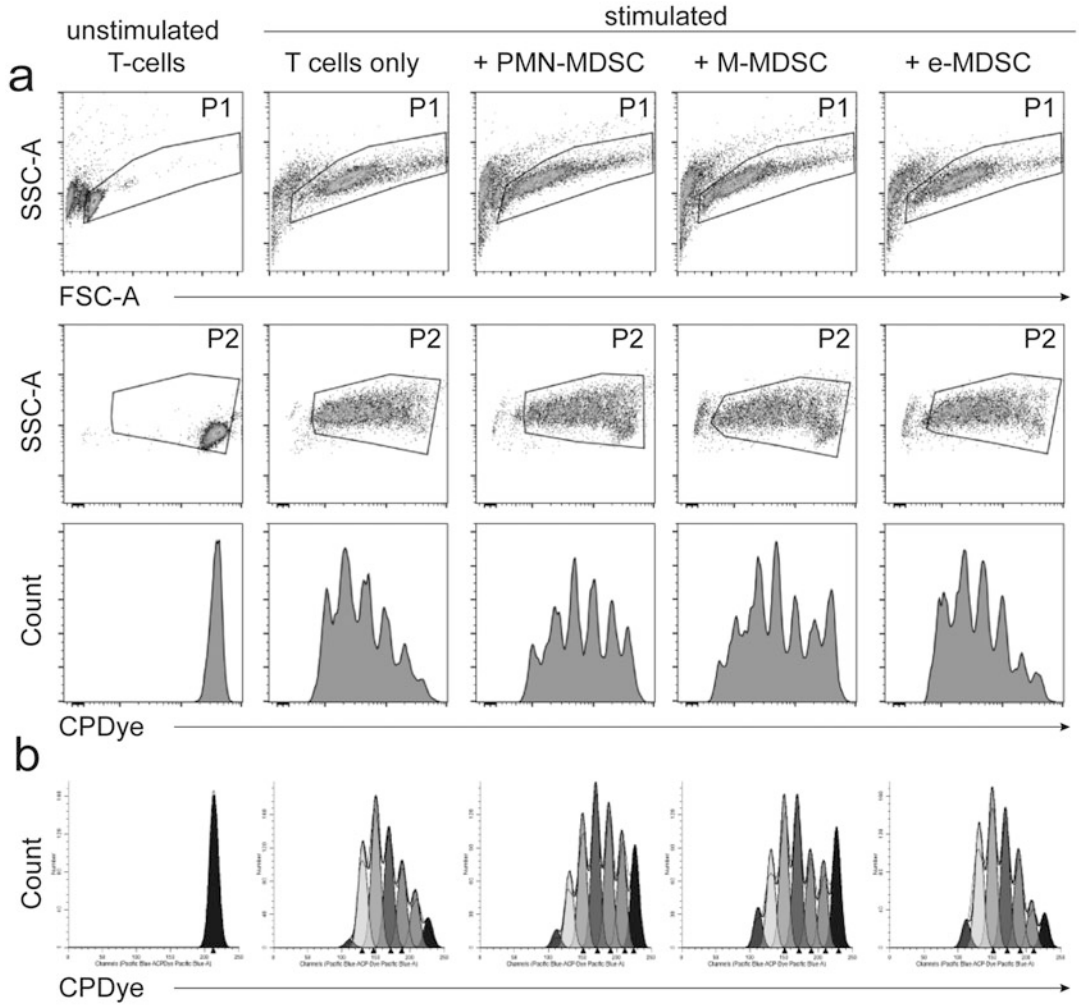

Fig. 3 Gating strategy for evaluation of T cell proliferation. $CD3^+$ responder T cells were labeled with cell proliferation dye and subsequently activated for 4 days by polyclonal stimulation with 1 μg/mL CD3 and 2 μg/mL CD28 in the presence of sorted MDSC. (**a**) Cell proliferation dye (CPDye) dilution of T cells under different experimental conditions is shown. Please note the dilution of CPDye after activation of T cells. (**b**) Data analysis with ModFIT software. Please note the reduced CPDye dilution after addition of PMN-MDSC and M-MDSC

3.8 Intracellular IFNγ Secretion Assay

1. If possible, perform assay in intra-experimental duplicates. This depends on the amounts of isolated MDSC.
2. For polyclonal stimulation coat wells (at least four wells are needed: only T cells and T cells with three different MDSC subsets) with 100 μL of 2μg/mL CD3 clone OKT3 and 2 μg/mL CD28.2 in DPBS for at least 2 h at 37 °C/5% CO_2 (*see* **Note 11**).

3. Adjust numbers of $CD3^+$ responder cells to 1×10^6 cells/mL in culture medium. Allow cells to rest after the isolation for at least 1 h at 37 °C/5% CO_2.
4. To inhibit protein transport, add 1:750 Monensin to T cells.
5. After isolation of MDSC subsets, centrifuge MDSC at 460 × *g* for 5 min at 4 °C and resuspend according to event rate from sorting counter to a final concentration of 0.5×10^6 cells/mL in culture medium.
6. Discard the CD3/28 antibody solution from the wells and do not wash the wells.
7. Pipette 100 μL of Arginine low medium into an uncoated well. This is for the unstimulated T cells only control.
8. Pipette 100 μL of Arginine low medium into an antibody-coated well. This is for the stimulated T cells only control.
9. Pipette 100 μL of MDSC cell suspension to antibody-coated wells.
10. Add 100 μL suspension of responder T cells to all wells.
11. Incubate for 10–12 h at 37 °C/5% CO_2. It is not recommended to keep Monensin longer than 12 h in cell culture.
12. At the end of the incubation, centrifuge the plate at 460 × *g* for 5 min, at 4 °C.
13. Decant the supernatant carefully and wash cells by adding 200 μL DPBS per well.
14. Centrifuge plate at 460 × *g* for 5 min, at 4 °C.
15. Decant the supernatant carefully and resuspend cells in 50 μL anti-human CD3 diluted in DPBS/HS.
16. Incubate for 30 min at 4 °C.
17. Wash cells by adding 200 μL DPBS per well.
18. Centrifuge plate at 460 × *g* for 5 min at 4 °C and decant the supernatant carefully.
19. Add 200 μL Cytofix/Cytoperm per well to fix and permeabilize the sample, mix cells by pipetting up and down, and incubate for 30 min at 4 °C.
20. Divide each sample by pipetting 100 μL of cell suspension into another well, thereby duplicating each sample.
21. Wash all wells with 150 μL 1× PermWash, centrifuge the plate at 500 × *g* for 5 min at 4 °C, and decant the supernatant carefully.
22. Resuspend one well per sample in 50 μL 1× PermWash; this represents the unstained control. Resuspend the second well of the same sample in 50 μL anti-human IFNγ.
23. Incubate for 30 min at 4 °C.

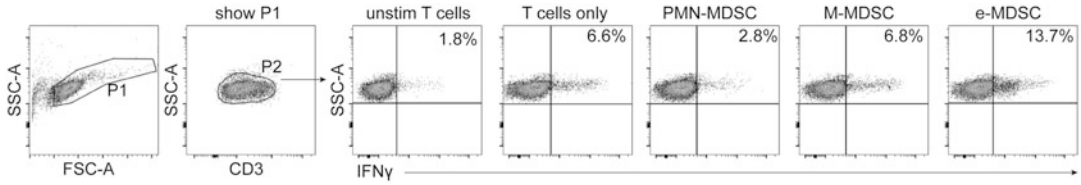

Fig. 4 Gating strategy for detection of intracellular IFNγ in T cells. $CD3^+$ responder T cells were activated for 10–12 h by polyclonal stimulation with 2 μg/mL CD3 and 2 μg/mL CD28 in the presence of sorted MDSC

24. Wash cells by adding 200 μL 1× PermWash per well, centrifuge plate at 500 × *g* for 5 min at 4 °C, and decant the supernatant carefully.
25. Resuspend cells in 200 μL DPBS/HS per well.
26. To determine the frequency of IFNγ positive T cells set first gate for SSC-A vs. FSC-A (P1 in Fig. 4) to exclude cell debris (*see* **Note 14**).
27. Plot SSC-A vs. CD3 and set gate on $CD3^+$ cells (P2) followed by analysis for IFNγ positive cells in SSC-A vs. IFNγ.

4 Notes

1. To avoid activation of cells by endotoxin contaminations we highly recommend using buffers, reagents produced under good manufacturing practice conditions. Use reagents with endotoxin levels <0.5 EU/mL.
2. Cool the heat inactivated serum to 4 °C prior to filtration to reduce clogging of pores during filtration.

   We could observe different induction levels of T cell proliferation and IFNγ secretion with different batches of fetal calf serum. Therefore, we would recommend testing the batch in advance (*see* Fig. 5).
3. Optimized Lineage cocktails from companies can be used. However, make sure that these commercial reagents do not include CD16 and CD14.
4. Always prepare L-Arginine solution and Arginine low medium freshly.
5. We noticed that CD28 clone 28.2 from different suppliers have different adsorption on plastic. We recommend IM1376 from Beckmann coulter. Reconstitute one vial with 2 mL distilled water to obtain 0.1 mg/mL. Store in aliquots at −20 °C.
6. Start processing the sample within 1 h after blood collection from cancer patients. During this time, keep blood at room temperature. Make sure that all blood collection tubes are filled completely. Noncompliance may lead to artificial increases in PMN-MDSC frequency.

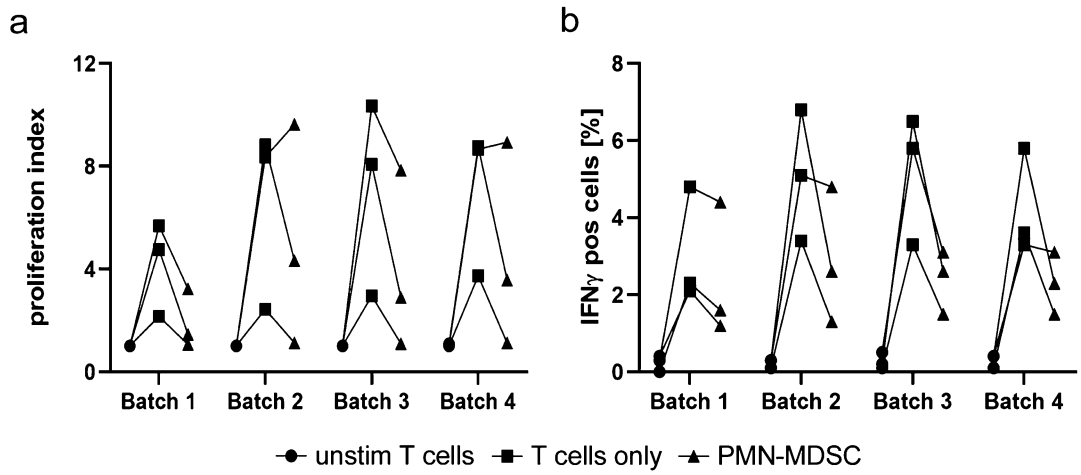

Fig. 5 Responses of T cells to polyclonal stimulation using different batches of fetal calf serum. $CD3^+$ responder T cells of three donors were activated by polyclonal stimulation. (**a**) T cell proliferation assay was analyzed after 4 days and (**b**) intracellular IFNγ release was measured after 10–12 h. Both assays were performed in four different batches of FCS

7. Subheadings 3.1 and 3.2 can be performed T cells independently. However, in many cancer patients, the sample size and the cell number are limited. Therefore, this protocol was designed for maximal recovery of MDSC from the patient blood sample and no separate tube is reserved for T cell isolation only.
8. Avoid using thawed responder T cells due to their reduced response to polyclonal stimulation.
9. To obtain purest separation of populations, the population with the highest frequency needs to be sorted into the right tube. This is mostly PMN-MDSC.
10. Please note that Subheadings 3.4 and 3.5 use simplified, minimal markers combinations for flow cytometry sorting workflows. Further validation of the respective MDSC immunophenotypes can be found in published references [6].
11. Always use Arginine low Medium for T cell proliferation suppression assay since the proliferation is Arginine-dependent and minor effects of MDSC suppression might not be detectable in regular culture medium. Use culture medium for intracellular IFNγ assay.
12. Start antibody coating of 96-well Round-Bottom plate during preparation of responder cells.
13. It is possible to reduce the number of seeded cells up to four times without altering sensitivity of the assay. The ratio of responder cells to suppressor cells should remain 2:1. Therefore, the lowest ratio is 12,500 T cells: 6250 MDSC per well.

14. In this step, a staining for CD4 and/or CD8 can be included to determine the suppressive capacity on different T cell populations.
15. Do not eliminate doublets using FSC-A vs. FSC-H gating. Activation of T cells may increase values for FSC and SSC.

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Chapter 6

High-Dimensional Phenotyping of Human Myeloid-Derived Suppressor Cells/Tumor-Associated Macrophages in Tissue by Mass Cytometry

Juliette Ferrant, Simon Le Gallou, Guillaume Manson, Steve Genebrier, Frederic Mourcin, Karin Tarte, and Mikael Roussel

Abstract

Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) are heterogeneous cells that share myeloid markers and are not easily distinguishable in human tumors due to their lack of specific markers. These cells are a major player in the tumor microenvironment and are involved in the prognosis and physiopathology of various tumors. Here is presented a scheme to decipher these cells by mass cytometry.

Key words Mass cytometry, Tumor-associated macrophages, Myeloid-derived suppressor cells, Tissue

1 Introduction

In most solid cancers—including melanoma, renal, lung, liver, or prostate cancer—the abundance of circulating myeloid-derived suppressor cells (MDSCs) is correlated with tumor stage and volume, and disease prognosis [1–3]. In B cell lymphomas, an increase in circulating MDSCs is also correlated with a poor prognosis [4–7].

In mice tumors, MDSCs and tumor-associated macrophages (TAMs) coexist and MDSCs have been shown to differentiate into TAMs [8–10]. In human tissue, because of the lack of specific markers, the delineation between MDSCs and TAMs is inconsistent [11]. TAM phenotype and function have not been fully defined yet within the human B cell lymphoma microenvironment [12], and their heterogeneity has been explored with low-resolution approaches, using few markers (mostly CD68 and/or CD163 expression evaluated by immunohistochemistry) [12, 13].

High-resolution approaches such as mass cytometry (also known as cytometry by time-of-flight, or CyTOF) enhance the understanding of cellular diversity and function. CyTOF allows

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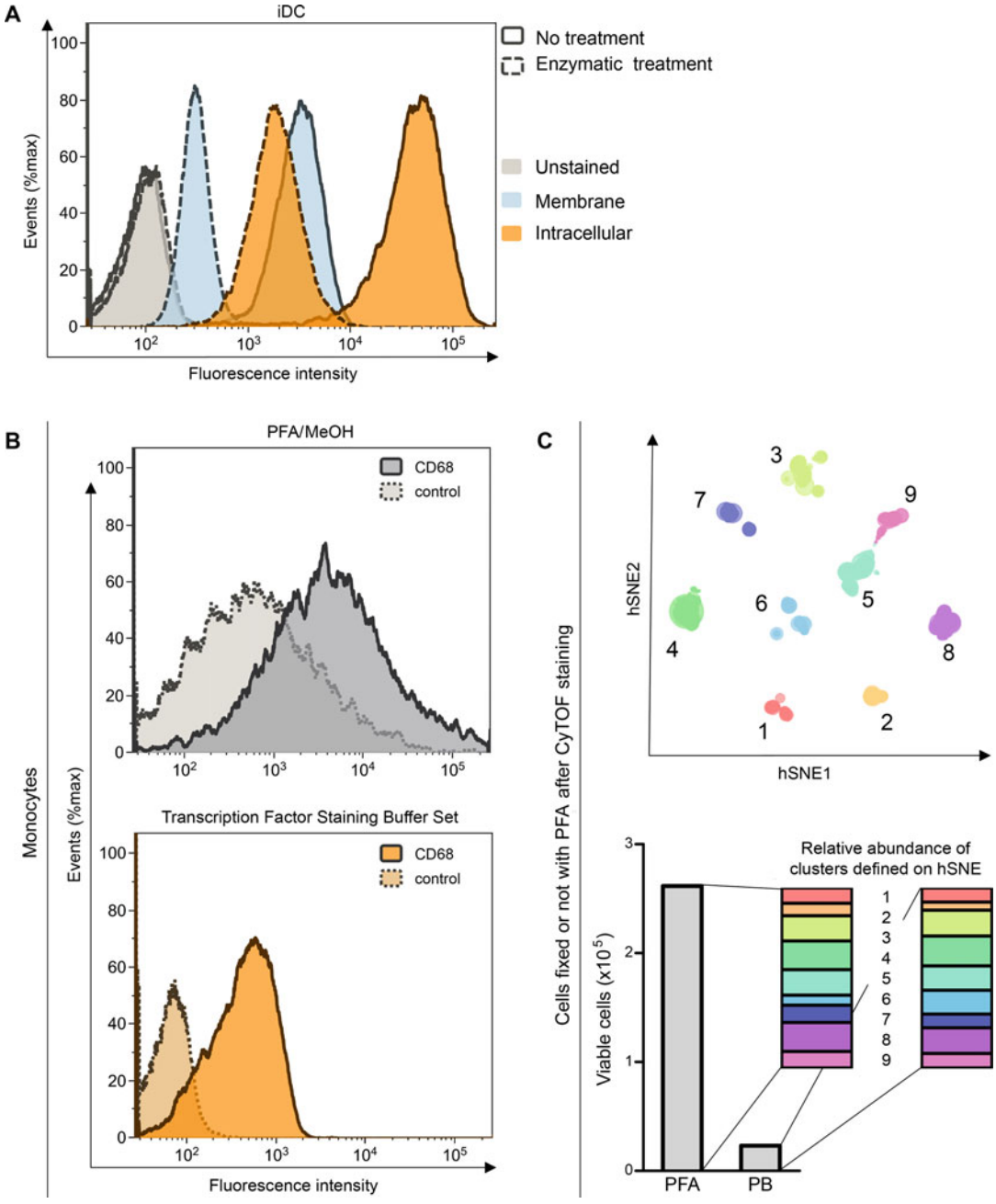

Fig. 1 Optimization steps are necessary to set up a tissular CyTOF panel. (**a**) Enzymatic dissociation is required to analyze myeloid cells from tissues; potential modulation of antigen expression can be prevented by cytoplasmic staining. To illustrate the relevance of intracellular staining for markers expressed on cell membrane, immature dendritic cells (iDCs) were treated (dashed line) or not (plain line) for 40 min in enzymatic dissociation buffer. Then cells were stained with a primary anti-CD209 mass antibody followed by a secondary fluorescent antibody, acquisition was performed on LSR X20 (Becton Dickinson). Membrane staining (light blue, plain line) overlaps with unstained cells after enzymatic dissociation (light blue, dashed line). The expression can be restored by intracellular staining (orange, dashed line). (**b**) Signal-to-noise ratio

the analysis of around 40 protein expression at the single cell level, thus enabling the exploration of the highly heterogeneous myeloid compartment. Recently, myeloid cell populations (including dendritic cells, monocytes, and TAMs) were deciphered by mass cytometry in human healthy and tumor tissues from renal and lung carcinomas [14–18]. Additionally, a monocyte subset ($CD14^{pos}CD16^{neg}HLA\text{-}DR^{high}$) identified by mass cytometry was predictive of the response to anti-PD-1 immunotherapy in melanoma [19]. Using mass cytometry to characterize B cell lymphoma tumors and nonmalignant human tissues, we recently identified phenotypically distinct intra-tumor macrophage subsets based on abnormal marker expression profiles. Interestingly, these discrete myeloid cell subsets were associated with lymphoma tumor types [20].

Here we provide a protocol to characterize myeloid cells, including MDSCs and TAMs, from lymph nodes and tonsils. We particularly focus on (1) the specificity of the tissue dissociation technique for an optimal recovery of viable myeloid cells (Fig. 1a) and (2) the optimization of the fixation and permeabilization protocol (Fig. 1b, c). Finally, reactive secondary lymphoid organs and lymphoma tissues were analyzed by mass cytometry to characterize the myeloid compartment phenotype (Fig. 2).

2 Material

2.1 Tissue Preparation

1. Depletion buffer: 1% Human serum albumin (HSA), 5 mM $MgCl_2$, 6.7 mg/mL sodium citrate in phosphate-buffered saline (PBS).
2. FcR blocking solution: 12.5 μg/mL Tegeline© in depletion buffer (*see* **Note 1**).
3. Dissociation buffer: 10 U/mL DNase, 200 U/mL collagenase IV (Worthington Biochemical), 1.6 U/mL neutral protease (Worthington Biochemical), 5 mM $MgCl_2$, 1% Penicillin/Streptomycin in RPMI-1640.

Fig. 1 (continued) can be increased by modifying the fixation/permeabilization protocol. Monocytes were stained intracellularly with an anti-CD68 mass antibody followed by a secondary fluorescent antibody using two different staining protocols: PFA/methanol or Transcription Factor Staining Buffer Set (Miltenyi Biotec), acquisition was performed on LSR X20 (Becton Dickinson). We observed less background and better signal-to-noise ratio with the Transcription Factor Staining Buffer Set. (**c**) Recovery of viable cells can be increased by modifying the fixation/permeabilization protocol. A mix of different myeloid control cell types was stained with a CyTOF panel using the Transcription Factor Staining Buffer Set, then split into two equal fractions before nucleic acid staining and final fixation with 2% PFA or with Permeabilization buffer 1× (PB) (Miltenyi Biotec). A hSNE algorithm (top) was performed with Cytosplore on 23,100 viable cells ($cisplatin^{neg}$) for each condition, nine clusters were identified. More viable cells were recovered with PFA whereas cluster abundance was not modified (bottom)

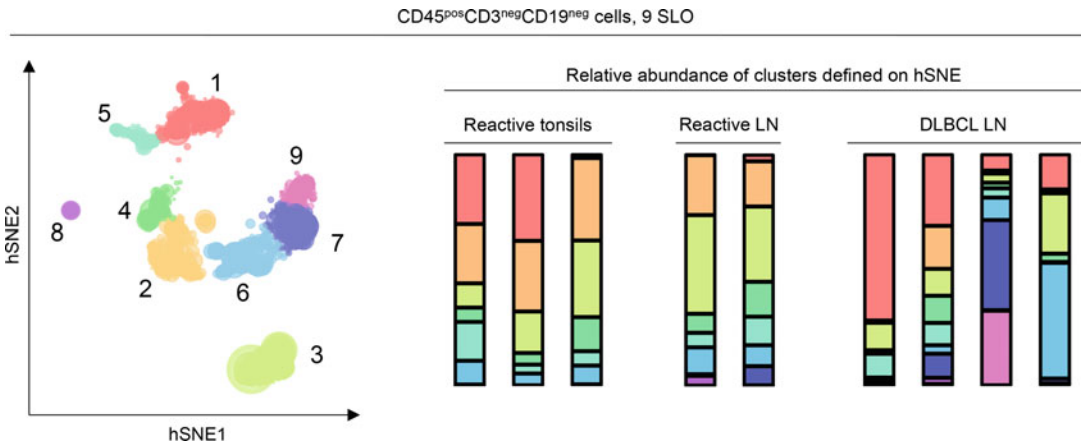

Fig. 2 High level of myeloid cell heterogeneity is detected by mass cytometry. Nine secondary lymphoid organs (SLO) from nine different donors (three reactive tonsils, two reactive lymph nodes [reactive LN], four diffuse large B cell lymphomas [DLBCL LN]) were dissociated and stained with a dedicated TAM/MDSC CyTOF panel of 37 parameters. Nine clusters were identified after hSNE (Cytosplore) performed on viable cells, $CD45^{pos}$, $CD3^{neg}$, and $CD19^{neg}$ cells (left). The abundance of the nine clusters was assessed for each sample (right). CyTOF analysis allows the exploration of the heterogeneity of the myeloid compartment and the identification of different TAM/MDSC subpopulations, whose distribution varies depending on the organs and pathological contexts

4. GentleMACS™ dissociator (Miltenyi Biotec).
5. GentleMACS™ C tubes (Miltenyi Biotec).
6. Dry bath incubator Eppendorf ThermoMixer®.
7. Dynabeads™ anti-CD3 (Invitrogen).
8. Dynabeads™ anti-CD19 (Invitrogen).
9. DynaMag™ Magnet for depletion with magnetic beads (Invitrogen).
10. Nutating platform mixer.

2.2 Antibody Staining and Mass Cytometry

1. Metal isotope-conjugated antibodies (*see* **Note 2**).
2. PBS.
3. RPMI-1640 Medium.
4. Room temperature medium: RPMI-1640, 10% fetal calf serum (FCS).
5. Staining buffer (SB): 0.5% Bovine serum albumin (BSA) in PBS, filter-sterilized before use.
6. 1 mM Cisplatin Cell-ID™ (Fluidigm).
7. 500 μM Cell-ID™ Intercalator-Ir (Fluidigm)
8. DNA intercalator solution: 2.5% Paraformaldehyde (PFA), 1:3200 Cell-ID™ Intercalator-Ir in PBS.

9. Transcription Factor Staining Buffer Set (Miltenyi Biotec).
10. Ultrapure water.
11. CoolCell® cooling device (Biocision).
12. CyTOF Calibration Beads (Fluidigm).
13. Mass cytometer (Helios™, Fluidigm).
14. 100 μm Cell strainer.
15. Polypropylene FACS tube.

3 Methods

3.1 Tissue Preparation

3.1.1 Tissue Dissociation and FcR Blocking

1. Mince tissue samples into small fragments of about 2–3 mm^3 (*see* **Note 3**).
2. Incubate tissue fragments in dissociation buffer (15 mL for 1 g of tissue) at 37 °C for 5 min.
3. Transfer to GentleMACS™ C tube and mechanically disperse with GentleMACS™ Dissociator using pre-installed program B.01 (31 s).
4. Transfer to an appropriate tube and incubate at 37 °C in an Eppendorf ThermoMixer® at 350 rpm for 40 min.
5. Strain with a 100 μm cell strainer.
6. Wash with depletion buffer and centrifuge for 5 min at 600 × *g* (*see* **Note 4**).
7. Discard supernatant.
8. Resuspend cells in 500 μL of FcR blocking solution.
9. Keep on ice for 5 min before proceeding to myeloid enrichment.

3.1.2 Myeloid Enrichment

1. Resuspend the beads in the vial by vortexing >30s.
2. According to manufacturer's recommendations, transfer the desired volume of beads (25 μL of Dynabeads™ anti-CD3 for 1×10^7 cells and 25 μL of Dynabeads™ anti-CD19 for 2.5×10^7 cells, *see* **Note 5**) to a new 50-mL tube.
3. Add the same amount of depletion buffer on beads and place the tube on the magnet for 1 min.
4. Discard supernatant.
5. Remove the tube from the magnet and resuspend the washed beads with the same volume of depletion buffer as the initial volume of beads.
6. Transfer washed beads with the cells previously obtained from tissue dissociation and adjust concentration to 1×10^7 cells/mL by adding depletion buffer (*see* **Note 6**).

7. Incubate for 30 min at 4 °C with constant rocking on a nutating platform mixer.
8. Place the tube on the magnet for 2 min and then transfer supernatant to a new 50 mL Falcon tube.
9. Place this tube on the magnet for 2 additional minutes and then transfer supernatant to a new 50 mL Falcon tube.
10. Centrifuge at 600 × *g* for 5 min and discard supernatant (*see* **Note 5**).
11. Resuspend in RPMI at 2×10^6 cells/mL and proceed to mass cytometry staining.

3.2 Antibody Staining

3.2.1 Prepare Staining Cocktails and Staining Reagents

1. Prepare Fixation/permeabilization Solution and Permeabilization Buffer 1× from the Transcription Factor Staining Buffer Set according to manufacturer's recommendations.
2. Prepare the appropriate quantity of surface staining cocktail in SB for the number of samples to be stained, plus 10% overage for pipetting errors. Each antibody should be previously titrated and diluted to a concentration of 100×. Samples will be stained in a final volume of 100 μL, for up to approximately 5×10^6 cells. Considering a residual volume of 40 μL per sample tube, the antibody cocktail should be completed with SB to a final volume of 60 μL.
3. Prepare the appropriate quantity of intracellular/intranuclear staining cocktail (*see* **Note 7**) in Permeabilization Buffer 1× for the number of samples to be stained, plus 10% overage for pipetting errors. Each antibody should be previously titrated and diluted to a concentration of 100×. Samples will be stained in a final volume of 100 μL, for up to approximately 5×10^6 cells. Considering a residual volume of 40 μL per sample tube, the antibody cocktail should be completed with Permeabilization Buffer 1× to a final volume of 60 μL.

3.2.2 Dead Cell Identification

1. Transfer the cell suspension (2×10^6 cells/mL in RPMI) to a new polypropylene FACS tube.
2. Add Cisplatin Cell-ID™ to the cell suspension for a final concentration of 0.5 μM.
3. Vortex to mix.
4. Incubate for 5 min at room temperature.
5. Wash cells with room temperature medium.
6. Centrifuge for 5 min at 600 × *g*.
7. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.

3.2.3 Surface Staining

1. Add 60 μL of surface staining cocktail per tube (final volume of 100 μL).
2. Vortex to mix.
3. Stain for 30 min at room temperature.
4. Wash cells with 4 mL SB.
5. Centrifuge for 5 min at 600 × *g*.
6. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.

3.2.4 Cell Fixation

1. Resuspend and fix the cells by adding the Fixation/permeabilization Solution to a final concentration of 1×10^6 cells/mL, with a maximum of 4 mL per FACS tube for up to 1×10^7 cells.
2. Incubate for 30 min at 4 °C.
3. Wash cells with 4 mL SB.
4. Centrifuge for 5 min at 900 × *g*.
5. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.

3.2.5 Cell Permeabilization

1. Permeabilize by adding 4 mL of Permeabilization Buffer 1×.
2. Centrifuge for 5 min at 900 × *g*.
3. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.

3.2.6 Intracellular and Intranuclear Staining (If Required)

1. Add 60 μL of intracellular/intranuclear staining cocktail per tube (final volume of 100 μL).
2. Vortex to mix.
3. Incubate for 30 min at 4 °C.
4. Wash cells with 4 mL of Permeabilization Buffer 1×.
5. Centrifuge for 5 min at 900 × *g*.
6. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.
7. Wash with 4 mL of SB.
8. Centrifuge for 5 min at 900 × *g*.
9. Invert the tube to discard supernatant, place the inverted tube on absorbent paper to remove excess liquid, and resuspend the cells in the residual volume.

3.2.7 Staining of the Cells in DNA Intercalator Solution

1. Add 160 µL of DNA intercalator solution (*see* **Note 8**).
2. Vortex to mix.
3. Transfer cell suspension to cryovials (*see* **Note 9**).
4. Incubate overnight at 4 °C.
5. Transfer cryovials at −80 °C into a CoolCell® device (Biocision).
6. The next day, samples can be removed from the CoolCell® device and kept for weeks at −80 °C.

3.3 Running Samples on CyTOF and Data Analysis

1. Thaw samples.
2. Run samples on a CyTOF cytometer according to the manufacturer's protocol.
3. Analyze data (*see* **Note 10**).

4 Notes

1. Pre-conjugated antibodies can be bought from Fluidigm or antibodies from any supplier can be self-conjugated using the Fluidigm Maxpar conjugation kit.
2. Tegeline© is a pool of human intravenous polyclonal immuno globulin isolated by fractionation.
3. Tissue fragments can be processed directly or cryopreserved before dissociation. If cryopreserved tissue samples are used, small tissue fragments can be transferred into cryovials containing cell cryopreservation media such as Cryostor CS10 (Sigma) and then stored in liquid nitrogen.
4. If fresh samples are processed, an erythrocyte lysis step can be added.
5. The volume of anti-CD19 beads should be doubled (i.e., 50 µL for 2.5×10^7 cells) for secondary lymphoid organs (SLO), which contain more than 10% B cells. Depending on the number of cells obtained after one step of anti-CD3 anti-CD19 depletion, a second step of depletion may be necessary.
6. If the total volume of the cell suspension is greater than 12.5 mL, the suspension should be split into several 50-mL falcon tubes for optimal agitation.
7. An enzymatic dissociation can lead to the modulation of some antigen expression; using intracellular staining can prevent this issue (*see* Fig. 1a). Using the Transcription Factor Buffer Set allows better intracellular staining for intracellular target epitopes (*see* Fig. 1b), as well as intranuclear staining in the same step.

8. With a residual volume of 40 μL, the final volume for the nucleic acid staining will be 200 μL. The cells will thus be fixed in a final concentration of 2% PFA, 1:4000 Cell-ID™ Intercalator-Ir (0.125 μM). This second fixation with PFA allows a better viable cells recovery (*see* Fig. 1c).
9. After staining, cells can be run on CyTOF or cryopreserved for further analysis.
10. High-dimensional analysis include various tools for data normalization and cleaning, dimension reduction, visualization, and cluster identification. These algorithms, scripts, or software are embedded in workflows reviewed in the following references [21–25]. As an example, we used hSNE (Hierarchical Stochastic Neighbor Embedding) from Cytosplore in Figs. 1 and 2 [26].

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Chapter 7

Depletion and Maturation of Myeloid-Derived Suppressor Cells in Murine Cancer Models

Christopher Groth, Rebekka Weber, Jochen Utikal, and Viktor Umansky

Abstract

Myeloid-derived suppressor cells (MDSC) are known to inhibit functions of T and NK cells. MDSC have been shown to be generated and to accumulate under chronic inflammatory conditions that are typical for cancer. Therefore, it would be highly beneficial to find ways to diminish the number and immunosuppressive functions of these cells in tumor-bearing hosts. Here we describe current protocols to deplete MDSC or induce their maturation in preclinical tumor models that could lead to the attenuation of their immunosuppressive functions.

Key words Myeloid-derived suppressor cells, Immunosuppression, Cancer immunotherapy, Chronic inflammation, MDSC depletion, MDSC maturation, Paclitaxel, ATRA

1 Introduction

1.1 Features of Myeloid-Derived Suppressor Cells (MDSC)

MDSC represent a highly heterogeneous population of both immature and mature myeloid cells. Characterized by a strong immunosuppressive effect on effector cells of the immune system (such as T and NK cells), MDSC are enriched and activated during chronic inflammation and tumor progression, representing one of the major mechanisms of immunosuppression developing in the tumor microenvironment [1]. Therefore, these cells are considered as one of the main obstacles for successful cancer therapies including novel approaches using immune checkpoint inhibitors [1]. In addition, MDSC have been shown to contribute to pathogen persistence and immunosuppression during chronic infections [2].

In mice, MDSC can be identified through the co-expression of the pan-myeloid marker CD11b and the myeloid differentiation antigen Gr1 [3, 4]. Gr1 consists of two subunits, Ly6C and Ly6G, which allows to distinguish $Ly6C^{high}Ly6G^{-}$ M-MDSC from $Ly6C^{low}Ly6G^{+}$ PMN-MDSC. Since it is not possible to distinguish murine M-MDSC from $Ly6C^{+}$ monocytes and PMN-MDSC from

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Ly6G$^+$ neutrophils by morphology and marker expression, MDSC can only be identified through their immunosuppressive capacity [5].

1.2 Depletion of MDSC

Depletion of MDSC could be achieved by the application of classical chemotherapeutics including 5-fluorouracil (5-FU), gemcitabine, and paclitaxel in therapeutic doses [6]. Mice bearing tumors of the size of approximately 100 mm^2 were treated with a single intraperitoneal (i.p.) injection of gemcitabine (120 mg/kg) or 5-FU (50 mg/kg) [6]. In addition, the tyrosine kinase inhibitor sunitinib and the VEGFR antagonist bevacizumab could significantly reduce the numbers of MDSC in murine tumor models [7]. Depletion of MDSC using sunitinib was described in murine model of subcutaneously (s.c.) injected TC1 lung cancer cells. Sunitinib solubilized in PBS was given i.p. starting from day 15 after tumor cell inoculation and using three different doses (20, 40, 60 mg/kg) for 9 consecutive days. This resulted in MDSC depletion in a dose-dependent manner. Application of 60 mg/kg led to a considerable weight loss of the treated mice. Since 40 mg/kg sunitinib caused a significant decrease in tumor-infiltrating and splenic MDSC, this dose was considered to be enough for this depletion.

We applied ultralow dose paclitaxel in the *RET* transgenic spontaneous skin melanoma mouse model that closely resemble human melanoma [8]. We found a significant reduction of MDSC frequencies in melanoma lesions correlated with a partial recovery of tumor-specific T cell responses, leading to profound anti-melanoma effects [8].

A significant depletion of MDSC could be also achieved in mice by a single i.p. injection of anti-Gr1 monoclonal antibodies (mAbs; Clone RB6-8C5) or of anti-Ly6G mAbs (Clone 1A8) [9].

1.3 Induction of MDSC Maturation

A promising approach is dealing with an induction of MDSC maturation into mature myeloid cells. Vitamin A was shown to drive this differentiation, and vitamin A deficiency caused an expansion of immature myeloid cells (IMC) in mice [10]. Closely related to vitamin A, all-trans-retinoic acid (ATRA) was demonstrated to eliminate IMC from tumor-bearing mice, which resulted in an improved antitumor T cell response [11]. Further studies indicated that this differentiation process was attributed to the stimulation of glutathione synthesis [12]. Glutathione is a major intracellular antioxidant molecule, which highlights the importance of free radicals in inhibiting IMC differentiation. In line with these findings, MDSC from tumor-bearing mice displayed higher level of reactive oxygen species (ROS), than IMC from tumor-free mice. The differentiation process of normal IMC was blocked upon their transfer into tumor-bearing mice. In addition, scavenging of free radicals by catalase could induce differentiation of MDSC from tumor-bearing animals in vitro [13].

Another factor responsible for impaired IMC maturation is S100A9, which recruits leukocytes to the site of inflammation. Mice missing S100A9 could reject implanted tumors and showed an increased differentiation of IMC into macrophages and dendritic cells [14]. Therefore, pharmacological inhibition of S100A9 might also be a useful way to induce MDSC maturation in mice.

Here we provide protocols for the depletion of MSDC or for their differentiation into mature myeloid cells.

2 Materials

2.1 Tumor-Bearing Mice

MDSC maturation and depletion can be studied in mice with transplanted or spontaneously developed tumors (*see* **Note 1**). Mice should be kept under specific pathogen-free conditions and tumor growth should be monitored.

1. C57BL/6 mice.
2. *RET* transgenic tumor-bearing mice line 304/B6.

2.2 Maintenance of Tumor Cells

E.G7 lymphoma cells (used for 5-FU depletion), EL-4 lymphoma cells (used for gemcitabine depletion), 3LL lung carcinoma cells (used for anti-Gr1 and anti-Ly6G depletion), and C3 fibrosarcoma cells (used for ATRA-mediated maturation) are cultivated in cell culture media. All cells are incubated at 37 ° C at 5% CO_2 and regularly tested for mycoplasma contamination.

1. RPMI 1640 medium for E.G7 cells: 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 0.4 mg/mL G418, 10% fetal calf serum (FCS).
2. RPMI 1640 medium for EL-4 cells: 0.4 mmol/L sodium pyruvate, 4 mmol/L HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, 10% FCS.
3. RPMI 1640 medium for 3LL cells: 2 mmol/L sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, 10% FCS.
4. DMEM medium for C3 fibrosarcoma cells: 100 units/mL penicillin, 100 μg/mL streptomycin, 25 mm HEPES, 2 mm glutamine, 10% FCS.

2.3 Reagents for MDSC Depletion, Maturation, and for the Processing of the Murine Tissue

Prepare freshly all solutions to be administered to tumor-bearing mice (*see* **Note 2**). Store reagents at 4 °C (unless indicated otherwise).

1. Sterile phosphate buffer saline (PBS).
2. Paclitaxel, delivered at 1 mg/kg (0.1 mg/mL in sterile PBS, for 20 g mice).

Table 1
The list of directly conjugated mAbs for flow cytometry

Maker	Fluorochrome	Clone	Supplier	Recommended dilution
FcR Blocking Reagent		2.4G2	BD Biosciences	1:200
CD45	V500	30-F11	BD Biosciences	1:100
Gr1	PE-Cy7	RB6-8C5	BD Biosciences	1:400
CD11b	APC-Cy7	M1/70	BD Biosciences	1:200

3. 5-FU, delivered at 50 mg/kg (5 mg/mL in sterile PBS, for 20 g mice).
4. Gemcitabine, delivered at 120 mg/kg (12 mg/mL in sterile PBS, for 20 g mice).
5. Anti-Gr1 mAbs (clone RB6-8C5; 2 mg/mL in sterile PBS).
6. Anti-Ly6G mAbs (clone 1A8; 2 mg/mL in sterile PBS).
7. *All-trans* retinoic acid (ATRA) pellets, 5 mg.
8. 1 mL sterile syringe and sterile 30G ½, 0.3 × 13 mm needle for injections.
9. 14G mouse trocar.
10. Erythrocyte lysis buffer to remove contaminating erythrocytes.
11. 40-μM cell strainer for the filtration of cell suspension.
12. Vacuum pump to remove supernatants upon the centrifugation.
13. Pasteur pipettes to remove supernatants upon the centrifugation.
14. FACS buffer: 2% FCS, 0.05% NaN_3 in PBS.
15. FcR Blocking Reagent, use at the concentration indicated by vendor.
16. Anti-mouse directly conjugated mAbs against CD11b, Gr1, and CD45 (*see* Table 1 for details on clones and concentrations).
17. Chamber for euthanasia of tumor-bearing mice with CO_2. The amount of CO_2 should be increased slowly in the chamber to avoid distress of the animals.
18. Appropriate scissors and tweezers for tissue dissection and tubes for organ collection and processing.
19. Flow cytometer with laser configuration allowing measurements of the fluorochromes as indicated in Table 1.
20. FACS tubes or 96-well plates.
21. Laboratory centrifuge.
22. Cell counting devices or Neubauer chamber.

3 Methods

3.1 MDSC Depletion with Chemotherapeutics

1. Inject *RET* transgenic tumor-bearing mice after appearance of macroscopic skin tumors with paclitaxel weekly i.p. three times (*see* **Note 3**).
2. Inject 5-FU once i.p. 7 days after s.c. inoculation of 10^6 E.G7 cells (*see* **Note 4**).
3. Inject gemcitabine once i.p. into mice transplanted with 2×10^5 EL4 cells when tumor surface reaches 100 mm^2.
4. Collect skin primary tumor, metastatic lymph nodes, tibia, femur, and spleen at day 10 after the end of paclitaxel treatment (*see* **Note 5**).
5. Isolate tumor and lymphatic organs from mice treated with 5-FU when tumors reach a diameter of 15 mm.
6. Remove tumor and lymphatic organs from mice treated with gemcitabine 5 days after start of treatment (*see* **Note 6**).
7. Prepare single cell suspensions of tumors, lymph nodes, and spleen through mechanical dissociation with scissors followed by pushing the tissue through a 40-μM cell strainer.
8. Flush bone marrow cells using sterile PBS followed by filtration with a 40-μM cell strainer.
9. Centrifuge cell suspension at 300 × *g* for 7 min at 4 °C.
10. Remove supernatants using a vacuum pump and Pasteur pipettes.
11. Resuspend the pellets in 1 mL PBS (for lymph nodes) or 1 mL erythrocyte lysis buffer (for tumors, spleens, and BM).
12. Add 10 mL sterile PBS to restore normal isotonic solution.
13. Centrifuge cell suspension at 300 × *g* for 7 min at 4 °C.
14. Resuspend the pellet in 1 mL FACS buffer, count the cells, and proceed with staining. Use 0.5–1 × 10^7 cells/mL and distribute 100 μL cell suspension either in FACS tube or 96-well plates for staining.
15. Incubate the cells with FcR Blocking Reagent to prevent an unspecific binding of mAbs.
16. Stain the cells with mAbs against CD11b, Gr1, and CD45 (Table 1).
17. Acquire cells on a flow cytometry device. Gating for $CD45^+$ leukocytes significantly enhances the efficiency of the detection of tumor-infiltrating MDSC (Fig. 1).

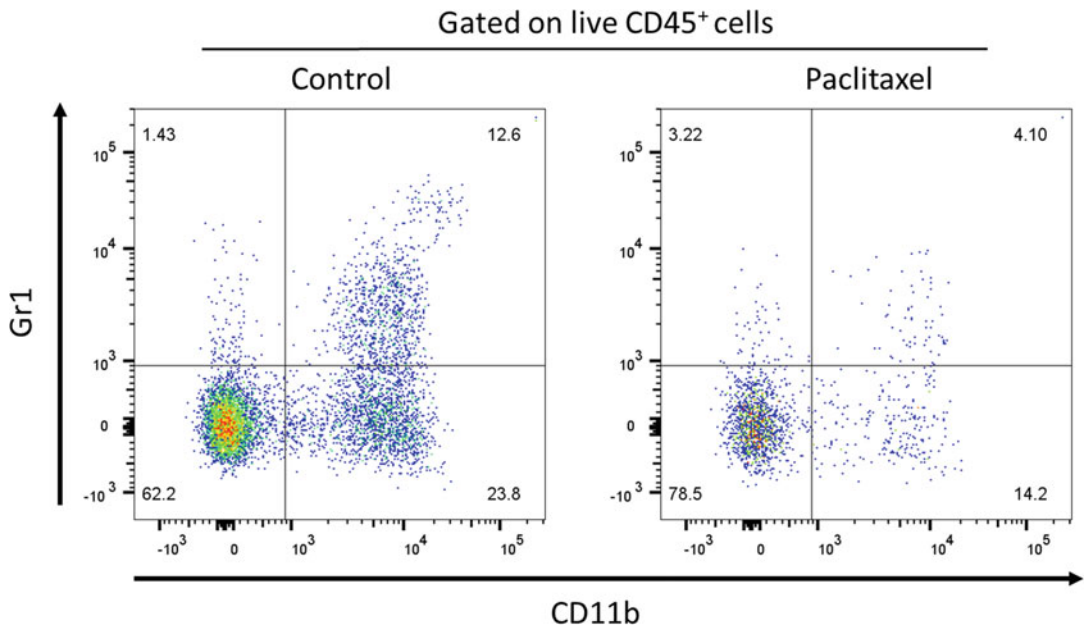

Fig. 1 Paclitaxel depletes tumor-infiltrating MDSC in *RET* transgenic mice. The single cell suspension is treated with FcR Blocking Reagent and stained with antibodies against CD45, CD11b, and Gr1. MDSC are identified as $CD45^+CD11b^+$ $Gr1^+$ cells

3.2 MDSC Depletion with Anti-Gr1 mAbs

1. Inject 2×10^5 3LL cells s.c. in the right supra scapular region of C57BL/6 mice.
2. When tumors become palpable, inject 100 μL anti-Gr1 or 100 μL anti-Ly6G mAbs for 2 weeks, every 48 h (*see* **Note 7**).
3. Determine tumor-infiltrating MDSC 21 days after tumor inoculation by flow cytometry (*see* Subheading 3.1, **steps 14–17**) (*see* **Note 8**).

3.3 MDSC Maturation with ATRA

1. Inoculate 5×10^5 C3 fibrosarcoma cells in 100 μL PBS s.c. into the shaved right flank of female C57BL/6 mice.
2. Implant ATRA s.c. contralateral when tumors reached a size of 4–5 mm in diameter via a trocar injection approach. The release of ATRA continues during 21 days, leading to a constant level of circulating ATRA of <0.5 μM (*see* **Note 9**).
3. Study tumor-infiltrating MDSC at day 21 after tumor inoculation using flow cytometry (*see* Subheading 3.1, **steps 14–17**).

4 Notes

1. Tumor development in individual mice could vary in both transplantation and spontaneous tumor models. Therefore, assignment of individual mice to treatment or control groups should be performed immediately before the treatment initiation to reduce differences attributed to different tumor sizes.
2. Depending on the reagent used for depletion or maturation of MDSC and the desired final concentration of the reagent, the solubility in PBS might not be sufficient. Alternatively, compounds can be solubilized in a buffer containing DMSO or ethanol. The injections for the control group have to be chosen accordingly. Paclitaxel dilution in PBS was prepared directly before each experiment but can be kept at 2–8 °C for up to 16 days [15].
3. The depletion of MDSC with chemotherapeutic agents is highly dependent on their dosage. While 1 mg/kg paclitaxel was able to significantly reduce the number of MDSC, no effect was observed at a dose 36 mg/kg [8]. Although the tumor models used in these studies differ, the efficacy of MDSC depletion may depend on the dose and duration of the treatment with MDSC depleting agents.
4. The dose of 40 mg/kg 5-FU was considered ideal since it did not induce severe side effects as weight loss or ruffled fur of animals. In addition, the equivalent dose for patients has been shown to be only minimal symptomatic [16].
5. To get representative results of the depletion success, mice should be carefully skinned after euthanasia to detect tumor nodules that are not visible during the general inspection of mice.
6. The frequency of tumor-infiltrating MDSC in tumor-bearing mice is in the range of 10–40% among total $CD45^+$ leukocytes. Since the frequency of MDSC within leukocytes in lymph nodes is significantly lower (<0.5%), a contamination of the isolated tumor tissue by lymph nodes could give misleading results. Therefore, a careful evaluation of prepared tissue for a potential lymph node contamination is recommended.
7. Depletion of MDSC using anti-Gr1 mAbs resulted in a decreased tumor volume and weight [17, 18]. This effect was observed in old (17–19 months old) but not in young (4–8 weeks old) C57BL/6 mice bearing subcutaneous B16F10 melanoma [18]. In addition, the treatment with anti-Gr1 mAbs led to a stronger effect on PMN-MDSC than on M-MDSC [19]. Since Ly6G and Gr1 are expressed also on normal neutrophils and monocytes, respectively, the

application of anti-Gr1 or anti-Ly6G mAbs may also affect these cells, impairing thereby antitumor immune responses. Both mAbs have been used for systemic MDSC depletion systemically, but also via intra-tumoral injection to deplete these cells in the tumor microenvironment.

8. Since the MDSC frequency increases with tumor progression, the time point of initiating the depletion of MDSC may have an impact on the outcome of the treatment. In addition, PMN-MDSC have a relatively short half-life span (6–8 h) that could affect the efficacy of the depleting approach.
9. Since ATRA has a very short half-life, the use of pellets is superior over i.p. injections of ATRA. In addition, the low but continuous release of ATRA has no cytotoxic effect.

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Chapter 8

In Vitro Generation of Human Neutrophilic Myeloid-Derived Suppressor Cells

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Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells of myeloid origin. MDSC are functionally defined by their capacity to suppress T, NK, and B cell responses and henceforth altering the disease outcome in various pathological conditions. MDSC are further subdivided into three distinct subsets: monocytic (M-) MDSC, neutrophilic or polymorphonuclear (PMN-) MDSC, and early-stage (e-) MDSC. However, since surface markers utilized to define MDSC are expressed on other myeloid cells too, it is mandatory to functionally assess the suppressive activity for characterizing these cells. Here, we provide a protocol for generation of PMN-MDSC in vitro from freshly isolated human peripheral blood mononuclear cells. These MDSC can be used further to perform functional assays to determine their immunosuppressive potential or test their activities in various biological conditions, for instance in infection and cancer.

Key words Myeloid-derived suppressor cells, MDSC, T cells, Immunosuppression, Immunomodulation, In vitro cell culture

1 Introduction

Due to their immunosuppressive properties, there is continuously growing interest in the role of myeloid-derived suppressor cells (MDSC) in disease conditions including cancers. MDSC accumulation in the patients has been described as a prognostic factor in many cancer types. Increased frequencies of MDSC were found to be associated with deterioration of disease state and decreased survival rate in cancer patients with different tumor conditions [1–3]. Apart from cancer, MDSC are also known to play a crucial role in several other pathological conditions like inflammation [4], transplant [5], sepsis, and infections [6, 7]. MDSC are difficult to define due to the lack of lineage specific markers. The heterogeneity as an inherent characteristic of MDSC contributes to this issue. Different combinations of markers are used to define human MDSC. Phenotypically, human PMN-MDSC have been described

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as $CD66b^+CD33^+CD11b^+CD14^-CD15^+$ and M-MDSC as $CD33^+CD14^+HLA\text{-}DR^{low}$ [8]. Due to their crucial role in a diverse range of disease conditions, MDSC emerge as one of the particularly interesting therapeutic targets. However, a better understanding of the processes and mechanisms by which they exert their immunosuppressive activity, particularly toward T cells, is required. Therefore, a robust method for generation of MDSC in vitro is essential to study immune suppression mechanism and exploit the therapeutic potential of the expanded cells. By using a broad array of in vitro and in vivo assays, we have previously reported that MDSC play a crucial role during fungal infections and cystic fibrosis [9–11].

In this chapter, we describe a culture method for in vitro generation of PMN-MDSC from freshly isolated human peripheral blood mononuclear cells (PBMCs), modified and adapted from a previously published study [12]. This protocol is robust and enables expanding PMN-MDSC using human granulocyte macrophage colony stimulating factor (hGM-CSF) for studying their functionality (Fig. 1). PMN-MDSC generated by this method showed a significant ability to suppress T cell responses in polyclonal proliferation assays [9–11] and this method can be easily tweaked with other stimulants in cell culture, and hGM-CSF can be used as an effective positive control for PMN-MDSC generation. The protocol described here indicates usage of 12-well or 24-well culture plates. However, this method can be adapted to other well plates as well as to cell culture flasks, provided the concentration of cells and stimulants are kept constant (*see* **Note 1**). In vitro MDSC generation in cell culture flasks is especially beneficial when large numbers of cells are needed at the end of the experiment for separation and purification of cells by magnetic-activated cell sorting (MACS) to conduct functional assays.

2 Materials

This protocol includes the experimental procedure that our laboratory established and modified to generate PMN-MDSC from freshly isolated PBMCs, in a 6-day-long cell culture with stimulants, in order to immunophenotype and characterize the PMN-MDSC.

2.1 PMN-MDSC Generation

1. General cell culture equipment and sterile disposables such as pipettes, tips, tubes, culture plates, and FACS tubes.
2. Laminar airflow bench.
3. Cell culture incubator set at 37 °C, 5% CO.
4. Centrifuge.

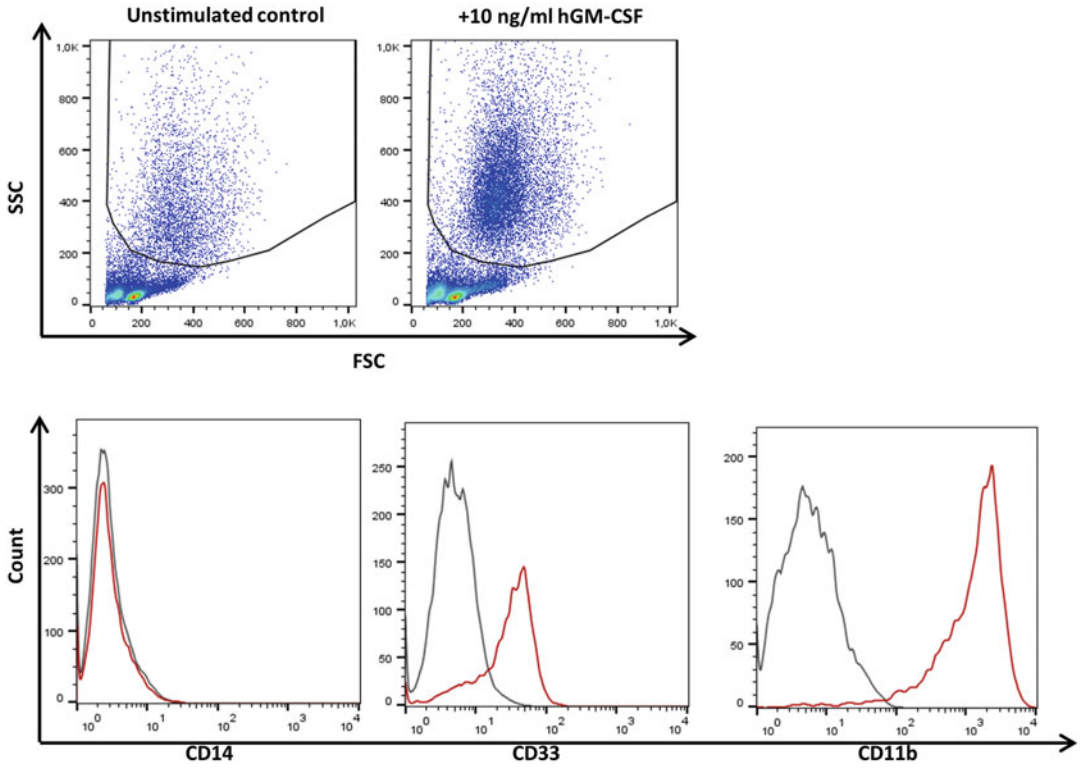

Fig. 1 Characterization of in vitro cultured human MDSC. Human MDSC are generated by incubating freshly isolated PBMCs (5×10^5/mL in 24-well plates) from healthy donors with 10 ng/mL hGM-CSF for 6 days. On day 6, MDSC are analyzed for phenotypic markers using flow cytometry. PMN-MDSC are identified as $CD33^+CD14^-CD11b^+$ cells. This population is distinct from lymphocytes or debris. Gray histograms represent respective unstained controls

5. Dulbecco's phosphate-buffered saline (DPBS).
6. Ficoll-Hypaque solution (density 1.077 g/L).
7. Cell counter or counting chamber for manual counting.
8. Trypan blue solution 0.4% (w/v) in DPBS.
9. Peripheral blood mononuclear cells (PBMC) from healthy donors.
10. RPMI 1640 Medium: 2.0 g/L $NaHCO_3$, without glutamine, low endotoxin.
11. MDSC culture medium (complete medium): RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin.
12. hGM-CSF (human granulocyte macrophage colony stimulating factor, Genzyme). Stored in a stock solution of 250 μg/mL.

Table 1
Antibody mix for FACS staining

Antibody/reagents	Clone	Amount per staining (μL)
CD14-FITC	Clone MφP9	2.5
CD33-PE	Clone AC104.3E3	1
CD11b-APC	Clone M1/70.15.11.5	1
Rabbit serum		0.2

2.2 Flow Cytometric Measurements

1. Detachin cell detachment solution.
2. DPBS.
3. Rabbit serum.
4. Fluorescence conjugated antibodies against CD14, CD11b, and CD33 for flow cytometry (*see* Table 1).
5. Flow cytometer BD FACSCalibur or other flow cytometry analyzers.

3 Methods

All steps should be performed under sterile conditions. All equipment should be sterilized by spraying with 70% ethanol (v/v in water).

3.1 PMN-MDSC Generation

1. Isolate PBMC from the blood of healthy volunteer donors, by using standard density gradient separation protocol (using Ficoll-Hypaque) under sterile conditions (*see* **Notes 2–4**).
2. Adjust the PBMC cell count to a concentration of 5×10^5/mL with the complete medium. For this experimental approach, the cells are seeded in 12-well plates (2 mL per well) resulting in a concentration of 1×10^6cells per well. Alternatively, 24-well plates can also be used (1 mL per well, 5×10^5 cells per well).
3. Add the stimulants (10 ng/mL hGM-CSF). Do not forget to set up a negative and positive control. For PMN-MDSC, hGM-CSF is also used a positive control. Cells in media only are used as a negative control.
4. Pipette the appropriate amounts in the wells and then resuspend thoroughly and gently.
5. Incubate at 37 °C, 5% CO_2 for 4 days. On day 4 proceed with cell feeding/addition of supplements (**steps 6–12**).
6. Warm complete medium in water bath at 37 °C.
7. Take the 12-well plate out of the incubator carefully.

8. Centrifuge at 400 × *g*, for 10 min, and 20 °C. A smeared pellet will be visible in the middle of the plate.
9. Carefully remove 1400 μL, almost the entire liquid within the well—a small amount is left over so as not to accidently remove the cells as well. In case of using 24-well plates, 600 μL media is removed. At this stage, culture supernatant can be frozen for further cytokine studies by ELISA.
10. Add 2 mL of pre-warmed fresh complete medium. In case of 24-well plates, 1 mL media is added.
11. Add the appropriate stimulants (according to day 0) and resuspend cells carefully (*see* **Note 5**).
12. Put the plates back in the incubator and incubate at 37 °C, 5% CO_2 until day 6.

3.2 FACS Staining and MDSC Characterization

After co-incubation with stimulants, on day 6 cultured MDSC are analyzed and characterized using flow cytometry. Also, on this day MDSC can be sorted, separated, and purified using respective magnetic beads and MACS® separation columns.

1. Take the plate out of incubator carefully.
2. Collect the content of the wells by repeated pipetting and transfer into 15 mL falcon tubes.
3. Rinse the bottom of the wells with 200 μL of DPBS and transfer afterward into respective falcon tubes.
4. Add 100 μL of sterile Detachin solution into the wells, and distribute it equally on the bottom of the well by gently swiveling the plate so that it covers the whole well.
5. Put the well plates back into the incubator (10 min, 37 °C, 5% CO_2). After this step, tap the plates gently to detach all the cells from the bottom of the well.
6. Add 200 μL of DPBS, mix well and transfer the content within the wells into the appropriate falcon tube.
7. The falcon tubes are centrifuged at 400 × *g* for 10 min, 20 °C.
8. Discard the supernatant using a pipette. Resuspend the pellet in 1 mL of DPBS.
9. Determine the cell count with a Neubauer counting chamber.
10. Approximately 4×10^5 cells are required for the FACS analysis.
11. If cell number is less than above, use whole volume (1 mL) of cell suspension for FACS.
12. Transfer the cells to the "unstained" FACS tube. Fill this tube up with 3 mL of DPBS and mix by inverting carefully. Centrifuge at 10 min, 20 °C, 300 × *g*.
13. Discard supernatant. Measure remaining volume (=solution which has run back down to the bottom of the tube) with a

pipette. If the ascertained volume is less than 100 μL fill it up to 100 μL with DPBS. Transfer half of solution to the "stained" FACS tube.

14. The "stained" tube is prepared with antibody mix as indicated in Table 1 (*see* **Note 6**).
15. Add the antibody mix (*see* Table 1) and include unstained, FMO and isotype controls.
16. Incubate the FACS tubes in the dark for 20 min at room temperature.
17. Fill up the FACS tubes with 3 mL of DPBS and mix by inverting carefully. Centrifuge at 300 × *g*, 10 min, 20 °C.
18. Discard the supernatant in a way that little amount is left in the tube to resuspend cells and proceed with sample acquisition on a flow cytometer.

4 Notes

There are some important caveats and pitfalls to consider before the experimental setup.

1. Before starting the assay, all steps should be standardized in individual laboratory conditions to minimize pre-analytical factors and get reproducible data.
2. Care should be taken before selecting the donors for experiments to minimize donor-dependent variability (consider smoking, allergies, infections, medications). MDSC numbers may vary according to donors' physiological state. For example, donors with a preexisting medical condition, an inflammatory disorder, or an infection might have a larger number of MDSC or MDSC-like cells which can also be functionally impaired. Therefore, while selecting a donor for a 6-day cell culture, a healthy volunteer should be chosen for blood donation.
3. Always use freshly drawn blood to isolate PBMCs for cell culture. According to our experience, PBMC isolated from fresh blood work better in a 6-day cell culture than cells isolated from buffy coats.
4. According to our experience for in vitro culture, tubes containing Na-Heparin as anticoagulant work the best. Always use same tubes and avoid EDTA.
5. On day 4 while feeding the cells, only soluble and fine stimulants need to be re-added. Stimulants which are particulate in nature (for example, heat-killed cells, cell fragments) need not be added. Nevertheless, this step should be standardized for individual stimulants.

6. FACS read-out quality depends on cell numbers/blood volume. MDSC numbers vary according to samples, and since MDSC population is largely donor dependent, it is important to perform experiments with multiple donors to get a robust data set amenable to statistical analysis. Use FACS antibodies that have been titrated properly and work well in experimental settings.

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Chapter 9

Measuring Suppressive Activity and Autophagy in Myeloid-Derived Suppressor Cells

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Abstract

Myeloid-derived suppressor cells (MDSC) are potent suppressor cells that accumulate in tumor microenvironment and inhibit anti-tumor responses. Assessment of cell-autonomous MDSC responses allows the precise characterization of MDSCs in various disease settings and elucidates the underlying mechanisms of MDSC-mediated immune suppression. Here we describe a protocol for the isolation of tumor infiltrating or splenic MDSC, as well as their subpopulations, from melanoma-inoculated mice using Fluorescent Activated Cell Sorting (FACS). We further provide protocols for investigation of the autophagy pathway and ex vivo assessment of MDSC suppressive function using lymph node responder cells. These assays allow a comprehensive characterization of MDSC in murine experimental models.

Key words MDSC, Fluorescent activated cell sorting, Co-culture, Autophagy, Lymph node cells (LNC), MDSC subsets

1 Introduction

Despite major advances in cancer immunotherapy and our understanding of tumor tolerance mechanisms, cancer remains one of the leading causes of death globally. Fundamental discoveries made over the last decade have unequivocally shown that the immune system plays an essential role in tumor development with tumors exploiting sophisticated immune tolerance networks to avoid immune recognition and elimination. Myeloid-derived suppressor cells (MDSC) comprise a heterogeneous population of cells with potent suppressive activity that accumulate into tumors where they impede the anti-tumor immunity and hamper the effectiveness of immunotherapy [1–4]. Moreover, the immunosuppressive function of MDSC is well established in almost all pathological situations including autoimmunity, infectious diseases, and transplantation [5–8].

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Major effort has been undertaken to reach a consensus regarding the phenotypic characterization of human and murine MDSC. Mouse MDSC are characterized as $CD11c^{-}CD11b^{+}Gr1^{+}$ cells comprising two distinct cell subpopulations: monocytic MDSC (M-MDSC ($CD11c^{-}CD11b^{+}Ly6C^{+}Ly6G^{-}$ cells)) and polymorphonuclear MDSC (PMN-MDSC ($CD11c^{-}CD11b^{+}Ly6C^{dim}Ly6G^{hi}$ cells)) [9–11]. In humans, M-MDSC are currently characterized in peripheral blood as $CD33^{+}CD11b^{+}CD14^{+}HLA\text{-}DR^{-/lo}CD15^{-}CD66b^{-}$ and have a monocytic morphology and PMN-MDSC are characterized as $Lin^{-}CD33^{+}CD11b^{+}CD14^{-}CD15^{+}HLA\text{-}DR^{-}$ or $Lin^{-}CD33^{+}CD11b^{+}CD14^{-}CD66b^{+}$ [4, 9] having granulocyte-like morphology. Recently, additional markers have been reported, for instance CD84 for tumor residing MDSC [12], or for the characterization of the human PMN-MDSC compartment, Lectin-type oxidized LDL receptor 1 (LOX1) which can differentiate these cells from mature neutrophils [13, 14].

Various mechanisms have been identified via which MDSC exert their suppressive activity. Among these are production of reactive oxygen and nitrogen species, depletion of essential amino acids (i.e., arginine, cysteine), induction of regulatory T cells (Tregs), and production of immunosuppressive cytokines, such as IL-10 and TGF-β [1, 13]. Recently, autophagy was shown to dictate the MDSC suppressive program in the tumor microenvironment [15]. Considering the central role of MDSC in the suppression of anti-tumor immunity and their prominent role in the unresponsiveness to immunotherapy, it is important to utilize standardized methods to assess the phenotype, frequencies, and function of these cells in mouse models of disease. We describe protocols for characterization and isolation of MDSC, specifically their cell-autonomous responses, notably autophagy. We provide a detailed method for measuring suppressive activity of MDSC using lymph node responder lymphocytes.

2 Materials

2.1 Induction of Melanoma

1. Cryopreserved B16-F10 cells.
2. Complete medium: RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, heat inactivation is done by incubation at 56 °C for 1 h), 100 U/mL penicillin/streptomycin, and 50 μM β-mercaptoethanol.
3. Sterile phosphate buffered saline (PBS).
4. Trypsin solution: 0.25% in PBS, prepared from stock solution of 0.5%Trypsin-EDTA.
5. T75 cell culture flasks.

6. 15 mL falcon tubes.
7. 50 mL falcon tubes.
8. Trypan blue ((0.5 w/v) stock solution which is diluted 1/5 before use).
9. Hematocytometer Neubauer.
10. Centrifuge.
11. Cell incubator.
12. C57BL/6 mice, 6–12 weeks old.

2.2 Preparation of MDSC from Tumor Spleen and Tumor

1. Surgical scissors.
2. Petri dishes.
3. PBS containing 5% FBS (PBS-5%FBS).
4. Digestion mix: 0.1 mg/mL DNaseI and 0.2 mg/mL collagenase D diluted in RPMI.
5. 16 mL u-bottom tube.
6. 40 μm strainer.
7. 2.5 mL syringe plunger.
8. FACS tubes.
9. Eppendorf tubes.
10. PBS containing 20% FBS (PBS-20%FBS).
11. Extracellular staining panel (Table 1).

2.3 Measurement of Autophagy in MDSC Using Confocal Microscopy

1. 24-well plate.
2. Poly-L-lysine treated coverslips.
3. Fixation buffer (4% paraformaldehyde in PBS).
4. Methanol (should be kept at −20 °C).
5. Permeabilization/Blocking Buffer (0.1% saponin, 2% BSA in PBS).
6. Primary antibody panel (Table 2).
7. Secondary antibody panel (Table 3).

Table 1
Antibody staining panel for phenotyping/sorting of MDSC

Antibody name	Clone
Anti-CD45	30-F11
Anti-CD11c	N418
Anti-CD11b	M1/70
Anti-GR1	RB8-8C5

Table 2
Primary antibody panel for autophagy assessment

Antibody name	Host species	Clone	Dilution (in permeabilization buffer)
Anti-LC3	Mouse	5F10	1:20
Anti-LAMP-1	Rat	1D4B	1:400
Anti-p62	Rabbit	SQSTM1	1:500

Table 3
Secondary antibody panel for autophagy assessment

Antibody name	Fluorochrome	Cat number	Dilution (in permeabilization buffer)
Anti-mouse IgG	Alexa Fluor 555	A28180	1:500
Anti-rat IgG	Alexa Fluor 488	A11006	1:250
Anti-rabbit IgG	Alexa Fluor 647	A21245	1:200

2.4 Evaluation of PI3K/AKT/mTOR Pathway Using Flow Cytometry

1. Surgical scissors.
2. Petri dishes.
3. PBS containing 5% FBS (PBS-5%FBS).
4. Digestion mix: 0.1 mg/mL DNaseI and 0.2 mg/mL collagenase D diluted in RPMI.
5. 16 mL u-bottom tube.
6. 40 μm strainer.
7. 2.5 mL syringe plunger.
8. FACS tubes.
9. Eppendorf tubes.
10. eBioscience™ Intracellular Fixation and Permeabilization Buffer Set (Cat 88-8824-00).
11. Extracellular staining panel (Table 1).
12. Intracellular staining panel (Table 4).

2.5 Preparation of Lymph Node Cells (LNC) (T Cells)

1. Surgical scissors.
2. Petri dishes.
3. PBS containing 5% FBS (PBS-5%FBS).
4. 40 μm strainer.

Table 4
Antibody staining panel for the phospho-proteins

Antibody name	Clone
Anti-phosphor-mTOR	MRRBY
Anti-phosphor-AKT	S473
Anti-phosphor-S6	S236

Table 5
Antibody staining panel for the suppression assays

Antibody name	Clone
Anti-CD4	GK1.5
Anti-CD44	IM7

5. 2.5 mL syringe plunger.
6. 15 mL falcon tubes.
7. Hematocytometer Neubauer.
8. Centrifuge.
9. CellTrace Violet.
10. Cell incubator.

2.6 Suppression Assay Plate Setup

1. Dynabeads™ Mouse T-Activator CD3/CD28.
2. Dynabeads wash buffer: PBS supplemented with 0.1% bovine serum albumin and 2 mM EDTA, pH 7.4.
3. Dynabeads magnet.
4. 96 well U bottom.
5. Antibody panel (Table 5).

3 Methods

3.1 Maintenance of B16-F10 Cells (See Note 1)

1. Thaw cryopreserved B16-F10 cells (*see* **Note 2**) and immediately dilute them in 9 mL complete medium (*see* **Note 3**).
2. Centrifuge cells at 520 × *g* for 5 min at Room Temperature (RT) and discard the supernatant.
3. Resuspend pelleted cells in 1 mL of complete medium, culture them in a T75 flask containing 20 mL complete medium, and incubate at 37 °C, 5% CO_2, and absolute humidity.

4. Maintain B16-F10 cells by splitting 1/10 when cells reach 90% confluency (*see* **Note 4**). Remove the medium and rinse the flask with 5 mL PBS.
5. Add 2 mL of Trypsin solution and incubate at 37 °C for 1–2 min (*see* **Note 5**).
6. Upon cell detachment immediately inactivate trypsin by adding 10 mL of complete medium (*see* **Note 6**) and transfer the cells to a 15 mL falcon tube.
7. Centrifuge at 520 × *g* for 5 min, discard supernatant, resuspend cells in 1 mL of complete medium, and transfer 100 μL to a new T75 flask containing 20 mL complete medium (*see* **Note 7**).

3.2 Induction of Melanoma in C57BL/6 Mice

1. Prepare cells for injections when they reach 90% confluency.
2. Remove the medium, rinse the flask(s) with 5 mL PBS and discard PBS.
3. Add 2 mL of Trypsin solution per flask and incubate at 37 °C for 1–2 min.
4. When cells are detached immediately inactivate trypsin by adding 10 mL of complete medium per flask and transfer all the cells from different flasks into a common 50 mL falcon tube.
5. Centrifuge at 520 × *g* for 5 min, discard supernatant, resuspend cells in 1 mL PBS per flask.
6. Count cells with a hemocytometer using trypan blue to exclude dead cells (*see* **Note 8**).
7. Dilute cells with PBS as to reach a concentration of 3×10^6 cells/mL.
8. Inject C57BL/6 female (*see* **Note 9**) mice subcutaneously at the base of the tail, with 100 μL of cell solution (300,000 cells/mouse) [16].

3.3 Preparation of MDSC from Tumor (See Note 10)

1. Sacrifice mice 15 days after tumor inoculation. Tumor volume, which is calculated with the formula (length × width^2)/2, should not excide 1100 mm^3.
2. Excise the tumor, remove the skin using surgical scissors, and place it in a Petri dish with 5 mL PBS–5%FBS on ice (*see* **Note 11**).
3. Weigh the tumor, transfer it to an empty Petri dish, and finely mince it with surgical scissors until it looks like a paste.
4. Transfer the minced tumor to a u-bottom 16 mL tube containing 2 mL of digestion mix and incubate for 45 min at 37 °C (*see* **Note 12**).
5. Pass digested tumor cells through a 40 μm cell strainer and dissociate any remaining tumor pieces by smashing them with a 2.5 mL syringe plunger.

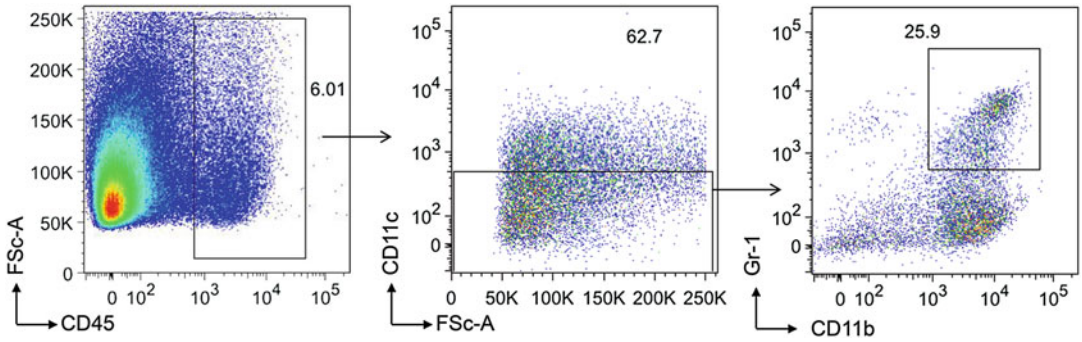

Fig. 1 Gating Strategy for sorting of total MDSC. Cells are isolated from excised melanoma tumors of B16-F10–inoculated mice at 15 days post inoculation. Highly purified total MDSC (purity >95%) are sorted on a FACS ARIA III as $CD45^{+}CD11c^{-}CD11b^{+}GR\text{-}1^{+}$ cells

6. Wash the strainer with 10 mL PBS and collect the cell suspension in a 15 mL falcon tube.
7. Centrifuge the single cell tumor suspension at 520 × *g* for 10 min at 4 °C. Discard supernatant.
8. Prepare the staining master mix by adding the antibodies detailed in Table 1 (anti-CD45, anti-CD11c, anti-CD11b, anti-GR1) in a 1/200 dilution in PBS-5%FBS (*see* **Note 13**).
9. Each tumor cell sample should be resuspended in an equal to the tumor weight volume of staining master mix. For example, the single cell pellet of the tumor that weighed 300 mg should be resuspended in 300 μL master mix (*see* **Note 14**).
10. Incubate for 20 min in dark at 4 °C (*see* **Note 15**).
11. Wash the cells by filling the falcon with PBS and centrifuge at 520 × *g* for 10 min at 4 °C.
12. Discard supernatant and resuspend pelleted cells in PBS-5% FBS at a concentration of 5×10^{7} cells/mL.
13. Pass the cell suspension through a 40 μm strainer in a FACS tube.
14. Using a FACS sorter, sort MDSC according to the gating strategy presented in Fig. 1 in an Eppendorf tube containing 500 μL of sterile PBS-20%FBS (*see* **Note 16**).
15. Acquire desired number of cells in the Eppendorf tube and bring the Eppendorf tube afterward in a laminar flow hood and add till 1.5 mL sterile PBS-5%FBS.
16. Centrifuge the Eppendorf contained sorted cells at 520 × *g* for 20 min at 4 °C.
17. Discard supernatant and resuspend cells in complete medium at a concentration of 1×10^{6} cells/mL.

3.4 Measurement of Autophagy in MDSC Using Confocal Microscopy

1. Seed sorted MDSC on coverslips pretreated with poly-L-Lysine in a 24 well plate.
2. Fix the cells by adding 500 µL 4% paraformaldehyde per well and incubate for 15 min at RT.
3. Discard supernatant and wash the cells with 1 mL PBS per well for 5–10 min at RT (*see* **Note 17**).
4. Continue the fixation by adding 500 µL of ice-cold methanol per well and incubate for 10 min at RT.
5. Discard supernatant and wash the cells with 1 mL PBS per well for 5–10 min at RT.
6. Remove the coverslips from the 24 well plate and put them on parafilm in a Petri dish (*see* **Note 18**).
7. Add 100 µL Permeabilization/Blocking Buffer to each coverslip and incubate for 15 min at RT (*see* **Note 19**).
8. Discard supernatant. Prepare the master mix for the primary antibodies in Permeabilization/Blocking Buffer detailed in Table 2.
9. Add 100 µL of Primary antibody mix per coverslip and incubate for 1 h at RT.
10. Discard supernatant and wash three times with Permeabilization/Blocking Buffer for 5–10 min at RT.
11. Prepare the master mix for the Secondary antibodies in Permeabilization/Blocking Buffer as detailed in Table 3.
12. Add 100 µL of Secondary antibody mix per coverslip and incubate for 1 h at RT in the dark (*see* **Note 20**).
13. Discard supernatant and wash three times with Permeabilization/Blocking Buffer for 5–10 min at RT.
14. For visualization of the nuclei, stain with DAPI diluted 1:100 in Permeabilization/Blocking buffer for 3 min at RT.
15. Wash twice with Permeabilization/Blocking Buffer for 5–10 min at RT.
16. Wash with PBS for 5–10 min at RT.
17. Mount the samples with mowiol. Add 5 µL for each coverslip on a super frost slide and place the coverslips with the surface carrying the seeded cells placed on the drop of mowiol (*see* **Note 21**).
18. Let the slides dry for at least 2 h at RT in the dark.
19. Store slides at 4 °C protected from light (*see* **Note 22**).
20. Visualization with 63× magnifying oil lens on confocal microscope, e.g., Leica SP5 inverted confocal live cell imaging system (Fig. 2).

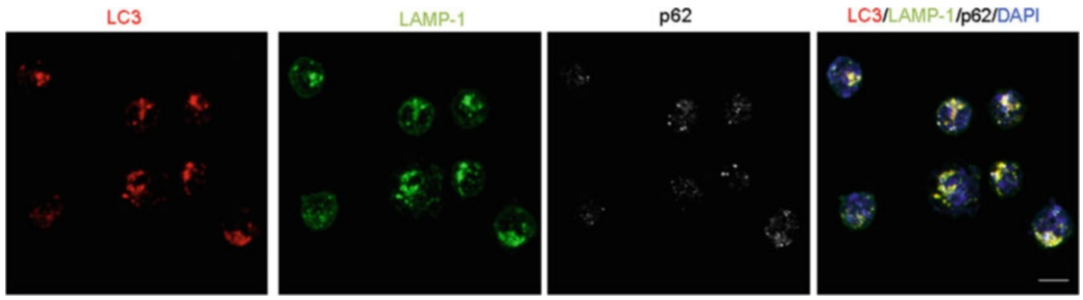

Fig. 2 Monitoring of autophagolysosomal formation through confocal microscopy. Representative immunofluorescence confocal images for LC3 (red), LAMP-1 (green), p62 (silver white), and DAPI (blue), in sorted M-MDSC from tumors of B16-F10–inoculated mice. Scale bar: 10 μm

21. Calculate the numbers of LC3 puncta/cell, p62 puncta/cell, and LAMP-1 puncta/cell with Fiji software using a macro script developed by our group [17].

3.5 Evaluation of PI3K/AKT/mTOR Pathway Using Flow Cytometry

1. Prepare a single tumor cell suspension and stain for MDSC as describe on Subheading 3.3 (**until step 11**).
2. Discard supernatant and continue the procedure for the intracellular phosphoprotein staining. Use intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to the manufacturer's instructions.
3. Resuspend the pellet in 100 μL intracellular fixation buffer. Incubate for 45 min in the dark at RT.
4. Wash with 500 μL Permeabilization Buffer and centrifuge at 520 × *g* for 10 min at 4 °C.
5. Prepare phosphoprotein staining master mix by adding the antibodies detailed in Table 4 in a dilution 1:50 in Permeabilization Buffer.
6. Discard supernatant and resuspend pelleted cells in 100 μL of staining master mix.
7. Incubate for 45 min in the dark at RT.
8. Wash with 500 μL Permeabilization Buffer and centrifuge at 520 × *g* for 10 min at 4 °C.
9. Discard supernatant and resuspend pelleted cells in 200 μL PBS-5%FBS.
10. Pass the cell suspension through a 40 μm strainer in a FACS tube.
11. Use a flow cytometer for acquisition of the samples.
12. Evaluate abundance of the phosphoproteins by plotting their Mean Fluorescence Intensity (MFI).

3.6 Preparation of Lymphocytes from Lymph Nodes (See Note 23)

1. Sacrifice one naïve C57/BL/6 mouse of the same gender as the mouse used for MDSC isolation, collect inguinal, brachial, cervical lymph nodes in 1 mL PBS-5%FBS and store organs on ice.
2. Prepare a single cell suspension by passing the lymph nodes and 1 mL PBS-5%FBS solution through a 40 μm mesh using the plastic plunger of 2.5 mL syringe in a Petri dish.
3. Wash the plunger and mesh with a 3 mL PBS-5%FBS and collect the cell suspension with a pipette in a 15 mL tube and fill up the tube with PBS-5%FBS.
4. Centrifuge at 881 × *g* for 10 min at 4 °C. Discard the supernatant.
5. Resuspend the pellet in 1 mL pre-warmed PBS and count cells with Neubauer Chamber.
6. Dilute the cell suspension with PBS in order to obtain a concentration of 1×10^6 cells/mL.
7. Stain cells with CellTrace violet according to manufacturer guidelines. In detail, add 1 μL of CellTrace violet per 1×10^6 cells/mL and incubate for 20 min in the cell incubator.
8. Fill up the 15 mL falcon with complete medium and incubate for 15 min in the cell incubator.
9. Centrifuge at 881 × *g* for 10 min at 4 °C. Discard supernatant and resuspend the pellet in complete medium in order to obtain a concentration of 300,000 cells/mL.

3.7 Suppression Assay Plate Setup

1. Prepare Dynabeads™ Mouse T-Activator CD3/CD28 according to manufacturer's instructions. In detail, vortex beads thoroughly and transfer the volume needed into an Eppendorf tube. Add 1 mL of Dynabeads wash buffer and vortex 3 × 10 s. Place the Eppendorf tube on the magnet for 1 min and discard the washing buffer with a pipette. Remove the vial from the magnet and resuspend them in complete medium in order to achieve a concentration of 2.5×10^6 beads/mL.
2. In a 96-well U-bottom plate add 50 μL of CellTrace-labeled lymph node cells per well (150,000 cells).
3. Add 20 μL of aCD3/aCD28 (50,000 beads, Beads/LNC ratio = 1/5) in all wells.
4. Add different volume of MDSC cell suspension in wells in order to achieve different ratios of MDSC/LNC (for example, for a ratio of 1:2 add 75 μL of MDSC cell suspension per well—75,000 cells—or for a ratio of 1:6 add 25 μL per well—25,000 cells). As a control use one well of LNC with beads without adding MDSC.
5. Fill the wells up to 200 μL with complete medium and mix well by pipetting five times up and down.

6. Incubate cells at 37 °C, 5% CO_2, and absolute humidity for 4 days.
7. Collect cells from the wells in Eppendorf tubes, wash each well with 200 μL PBS and collect in the same tube as the cells.
8. Centrifuge at 520 × *g* for 10 min. Discard supernatant.
9. Prepare a staining master mix using antibody panel in Table 5 in a dilution of 1/200. The volume of the master mix is calculated by multiplying the number of wells with 100 μL.
10. Resuspend the pellets in 100 μL master mix each and incubate for 20 min at 4 °C in the dark.
11. Wash cells with 1 mL PBS and centrifuge at 520 × *g* for 10 min. Discard supernatant. Resuspend the cell pellet in 200 μL of PBS-5%FBS.
12. Acquire samples on flow cytometer using the gating strategy displayed in Fig. 3.

4 Notes

1. All buffers should be sterile and all workflow must be done in laminar cabinet.
2. It is important to use B16-F10 cells of early passages.
3. When working with cell cultures all reagents should be at RT unless stated otherwise.
4. Cells will reach 90% confluency every 3 days.
5. Cells detached from the bottom of the flask will float and have a round shape under the light microscope. In case this phenotype does not appear after 2 min continue the incubation until cells are detached.
6. Long-time incubation with trypsin diminishes cell viability and this is minimized by addition of the FBS, which inactivates trypsin.
7. Consider preparing a number of T75 flasks according to the number of mice to be injected. Each flask at 90% confluency may contain 4–9 × 10^6 cells (1 flask/20 mice).
8. A dilution of 1/10 with trypan blue should be adequate to correctly count the cells.
9. Male mice can be used as well, but with caution that T cell should be isolated from an age/sex-matched mouse.
10. Splenic MDSC can also be isolated and used for this assay.
11. In order to remove the skin efficiently use forceps to grab the skin from the periphery of the tumor, carefully cut the skin with surgical scissors, and keep them at an angle to avoid cutting any part of the tumor.

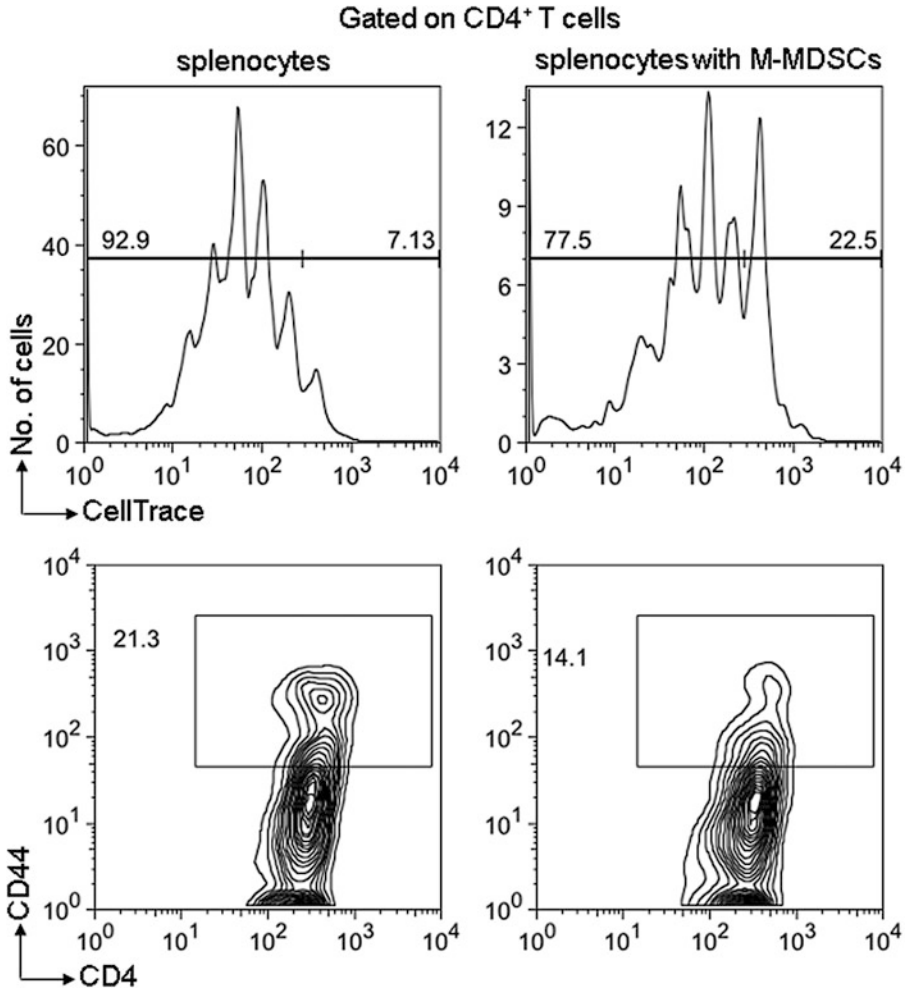

Fig. 3 Suppressive activity of MDSC. Representative histograms of $CD4^+$ T cell proliferation and flow cytometric analysis of CD44 expression on CellTrace-labeled lymph node cells (LNC) cultured with sorted M-MDSC from tumors of B16-F10–inoculated mice at 15 days post inoculation

12. For more efficient digestion pipette the solution up and down with a glass Pasteur pipette every 15 min.
13. Suppression assays can be also performed with isolated M-MDSC or G-MDSC. MDSC subsets can be isolated from tumor or spleen of tumor-bearing mice if samples are stained with CD45, CD11c, CD11b, Ly6C, and Ly6G, detailed in Table 6, and follow the gating strategy presented in Fig. 4.
14. Prepare master mix with one sample excess in order to bypass pipetting errors.
15. Vortex thoroughly the samples 10 min after initiation of the incubation because tumor cell suspensions tend to sediment fast.

Table 6
Antibody staining panel for phenotyping/sorting of MDSC subsets

Antibody name	Clone
Anti-CD45	30-F11
Anti-CD11c	N418
Anti-CD11b	M1/70
Anti-Ly6C	HK1.4
Anti-Ly6G	1A8

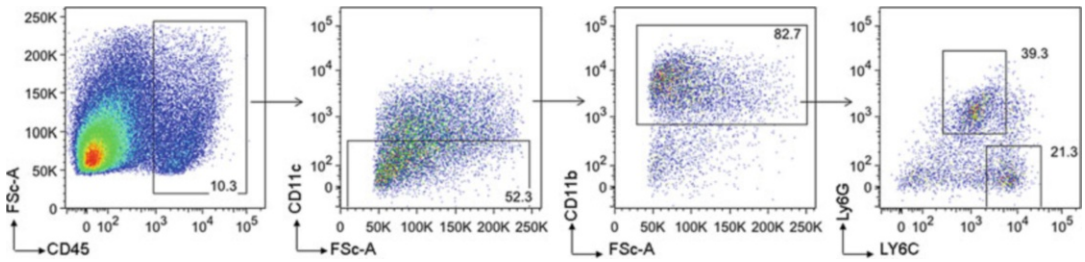

Fig. 4 Gating Strategy for sorting of MDSC subsets. Cells are isolated from excised melanoma tumors of B16-F10–inoculated mice at 15 days post inoculation. Highly purified (purity >95%) M-MDSC ($CD45^+CD11c^-CD11b^+Ly6C^+$ cells) or PMN-MDSC ($CD45^+CD11c^-$ $CD11b^+Ly6G^+$ cells) are sorted on a FACS ARIA III

16. Acquire on a flow cytometer a post-sort sample. The purity of the sorted population should be >95%.
17. Longer incubation or more washes reduce the background staining. Recommended washes at least 3 × 7 min.
18. Be careful with the transfer of coverslips. Use a 22 g needle and forceps with round end.
19. To reduce the background staining filter the Permeabilization Buffer before the blocking. Use a 0.45 μm filter membrane.
20. From this step and on the samples should be protected from light in order to avoid the loss of fluorescence.
21. Be careful when you place the coverslips on the slides. Foaming should be avoided because the air could dry the cells and reduce the fluorescence. Use a 22 g needle and a forceps with round end.
22. Visualization should be done as soon as possible, preferably once staining is completed in order to avoid loss of fluorescence. The staining is stable for at least 1 week if samples are kept as indicated.
23. Sorted $CD4^+$ T cells can be used instead of total LNC in the suppression assays. In this case, the lymph node cell suspension is stained with anti-CD4 and $CD4^+$ T cells are sorted.

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Chapter 10

In Vitro Generation of Murine Myeloid-Derived Suppressor Cells, Analysis of Markers, Developmental Commitment, and Function

Ina Eckert, Eliana Ribechini, and Manfred B. Lutz

Abstract

Myeloid-derived suppressor cells (MDSC) appear at relatively low frequencies in diseased organs such as tumors or infection sites, but accumulate systemically in the spleen. So far MDSC have been reported in humans and experimental animals such as mice, rats, and nonhuman primates. Therefore, methods to generate MDSC in large amounts in vitro can serve as an additional tool to study their biology. Here, we describe in detail the generation of murine MDSC with GM-CSF from bone marrow (BM). Both subsets of granulocytic (G-MDSC) and monocytic MDSC (M-MDSC) are generated by this cytokine. We provide panels of phenotypic markers to distinguish them from non-suppressive cells and define developmental stages of monocytes developing into M-MDSC by two subsequent steps in vitro.

Key words Myeloid-derived suppressor cells, In vitro generation, Differentiation, Activation, GM-CSF

1 Introduction

1.1 Basic MDSC Subsets, Biology, and Generation

Myeloid-derived suppressor cells (MDSC) are not detectable under healthy conditions, but appear during pregnancy, in newborns, and pathologic situations such as tumors or infections. The two major subsets that have been described consist of granulocytic (G-MDSC) and monocytic MDSC (M-MDSC) [1–3]. Their generation is dependent on tumor- or pathogen-induced growth factors such as GM-CSF, M-CSF, or G-CSF and their activation for suppression by pro-inflammatory cytokines such as IL-1β, TNF, IL-6, and IFN-γ. Therefore, these cytokines can be used for MDSC generation and activation in vitro [4, 5]. Although their origin in different pathological in vivo situations is often unclear, there is accumulating evidence that MDSC can be derived from epigenetically primed hematopoietic stem cells and myeloid progenitors [6]. Transcriptional and translational modifications of differentiated monocytes by GM-CSF or other factors may induce genesis

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of M-MDSC [7] and specific activation modes of immature neutrophils generate G-MDSC [8]. We have shown that in vitro conversion of classical murine Ly6C^{hi} and human CD14^{+} monocytes into M-MDSC requires two subsequent signaling steps and occurs in the absence of proliferation. The first step required a 3-day culture in GM-CSF, and since it was a strict prerequisite for subsequent M-MDSC generation, it was termed "monocyte licensing" (Fig. 1). The second step converts licensed monocytes (L-Mono) into M-MDSC and can be mediated by a cocktail of pro-inflammatory cytokines or pathogen signals or both (Fig. 1) [7].

Previously, we described a method to generate both subsets of murine MDSC from bone marrow (BM) and functionally test them for T cell suppression [9]. At that time MDSC were still termed "myeloid suppressor cells" (MSC) before renaming them as MDSC to avoid confusion with the MSC abbreviation for mesenchymal

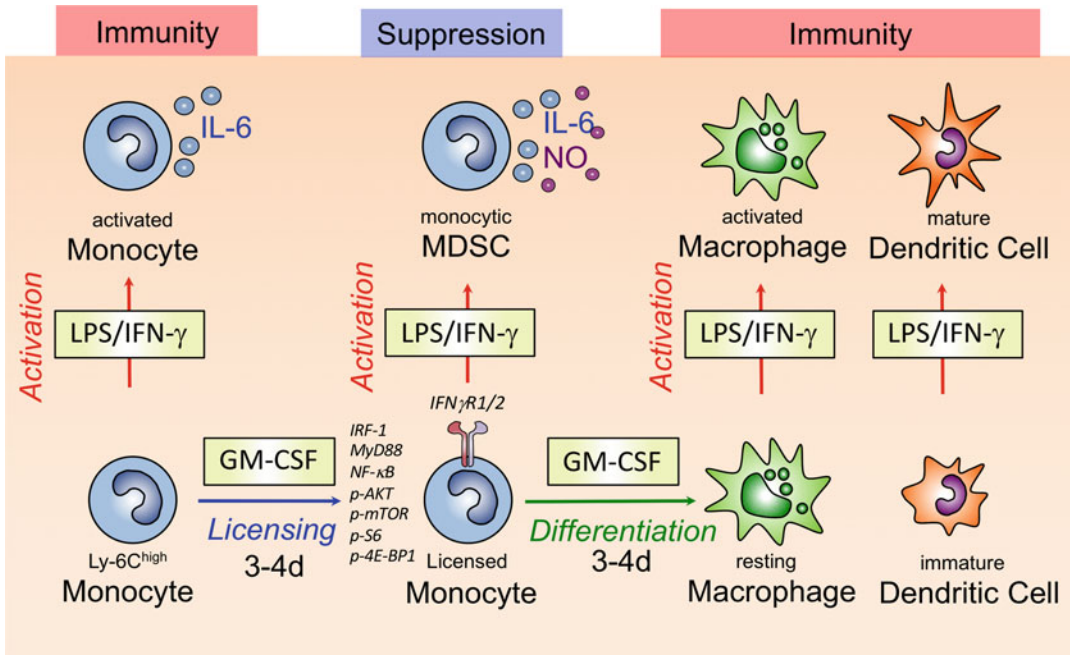

Fig. 1 Differentiation and activation stages of monocytes and monocyte-derived cells. Monocytes can differentiate or can be activated depending on the environmental cytokine or pathogen signals. Activation of monocytes by LPS/IFN-γ will result in an activated or inflammatory monocyte. Exposure of monocytes to GM-CSF over 3 days in culture induces several transcriptional and translational changes, that have been termed "licensing." Only GM-CSF-licensed but not normal fresh monocytes can be activated by LPS/IFN-γ into suppressive MDSCs that in addition to IL-6 secretion by activated monocytes now also release NO as a suppressive mediator. If activation of licensed monocytes does not occur at day 3 or 4 of culture but the cells are further maintained in GM-CSF for another 3–4 days, they will differentiate into monocyte-derived macrophages or DCs. The latter cell types will become immunogenic, activated macrophages or mature DCs upon stimulation with LPS/IFN-γ. Adapted from published reports [4, 7]

stem cells [10]. Surprisingly, the protocol to generate MDSC from BM required just a shorter culture period as compared to the generation of BM-DCs (Fig. 1) [11], representing murine monocyte-derived DCs (MoDCs) [12]. Although in later protocols the additional need of IL-6 in such BM cultures was proposed to generate murine MDSC from BM [13], IL-6 is not required following our protocol.

We analyzed the signaling pathways induced by the GM-CSF culture and their activation mechanisms that lead to the iNOS/NOS2-dependent release of suppressive NO, which was produced at much higher levels by M-MDSC compared to G-MDSC [3, 7]. Injection of in vitro-generated NO-producing M-MDSC have shown inhibition of transplant rejection after islet transplantation in diabetic mice [14] and bulk M/G-MDSC showed effects on allograft rejection [15] and experimental autoimmune encephalomyelitis (EAE) [7]. Although our cultures contain both MDSC subsets, G-MDSC appear the dominant suppressor population when injected into graft-versus-host disease models [16], while the in vitro suppression assay described here is dominated by iNOS- and NO-mediated suppression by the M-MDSC subset (Fig. 2).

1.2 MDSC Markers

The identification of MDSC among non-suppressive cells is difficult considering that monocytes can turn into M-MDSC just by transcriptional and translational changes, which may result in minimal alterations of their surface receptors. To distinguish L-Mono or M-MDSC from non-suppressive cells we suggest the staining of transcription factors, signaling and effector molecules of suppression (Fig. 1, Table 1). For transcription factor staining to identify L-Mono, please see published work [7]. Staining of the effector molecules iNOS and Arg1 to identify M-MDSC is described below and shown in Fig. 3.

Both non-suppressive monocytes and M-MDSC are characterized as $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$ cells. Therefore, additional markers are required to distinguish M-MDSC from resting and activated monocytes, licensed monocytes, and MoDCs (Table 1). CD103 has been proposed to be a specific marker for activated monocytes since it is not staining the surface of resting monocytes or M-MDSC [17]. The Gr-1 marker should not be used for MDSC or other myeloid cell detection since it recognizes two different molecules, Ly6G and Ly6C, and specific monoclonal antibodies against both individual molecules are available. Antibody co-staining of Gr-1 (clone RB6-8C5) with Ly6G (clone 1A8) is also not recommended since these antibody clones recognize the same Ly6G epitope and therefore compete for staining [18].

Immature murine neutrophils with a ring-shaped nucleus can be detected as $CD11b^{+}$ $Ly6G^{+}$ $CD101^{+}$ $Mcl1^{+}$ cells, while differentiated and polymorphonuclear mature neutrophils appear as

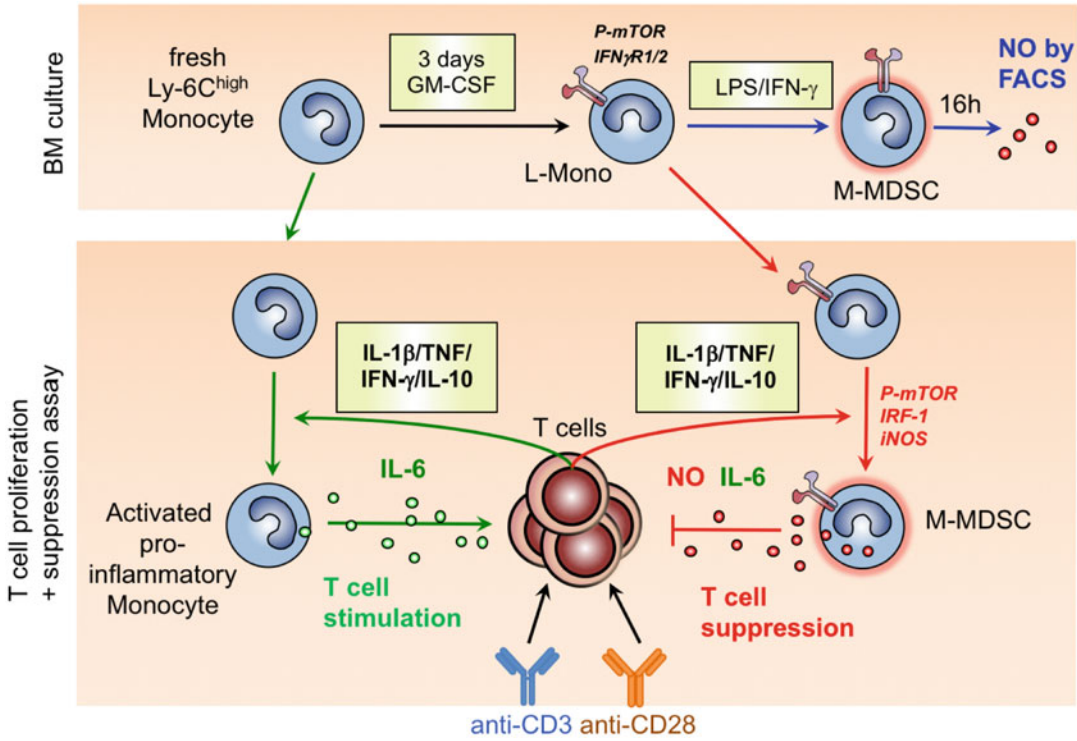

Fig. 2 Principle of T cell suppressor assay with in vitro generated M-MDSCs. Culture of mouse BM for 3 days in GM-CSF converts monocytes into licensed monocytes (L-Mono). These L-Mono can be further activated by LPS/IFN-γ to release NO after 16 h in culture (blue arrows). L-Mono or fresh monocytes can be cocultured with T cells that are stimulated to proliferate by αCD3 and αCD28 antibodies to test their suppressive activities. The activated T cells release a cocktail of cytokines (IL-1β, TNF, IFN-γ, IL-10). When monocytes are added to the T cells, the cytokine cocktail activates fresh monocytes into pro-inflammatory monocytes without suppressor capacity (green arrows). Alternatively, when L-Mono are added to the T cells, the same cocktail activates L-Mono to become NO-producing M-MDSC (red arrows). Activated monocytes do not impair T cell proliferation; however, addition of M-MDSC suppresses T cell responses and eventually kills these T cells

$CD11b^+$ $Ly6G^+$ $CD101^-$ $Mcl1^-$ cells, which have downregulated CD101 [19], the anti-apoptotic marker Mcl1 [18, 20, 21] and have no suppressive potential [3]. Of note, the Ly6G marker is downregulated in proliferating immature neutrophils [7], and therefore may not always report the presence of G-MDSC correctly.

1.3 M-MDSC Commitment or Monocyte Licensing

The generation of human or murine M-MDSC in vitro can be achieved by following a two-step protocol. First classical monocytes are converted by 3–4 days of culture with GM-CSF into "licensed" monocytes (L-Mono, $IFN\gamma R2^{pos}$, $pAKT^{hi}$, $pS6^{hi}$, IRF-1^{hi}) that are predisposed for suppression (Table 1, Fig. 1). Since these in vitro findings largely recapitulate the current understanding of MDSC generation in vivo, this concept may be of general relevance [5]. L-Mono can be considered as resting M-MDSC, and after a second step of activation L-Mono will convert into M-MDSC

Table 1
Markers used for MDSC characterization

Cell type	resting classical monocytes	activated classical monocytes	GM-CSF licensed monocytes	M-MDSC	MoDCs	mature neutrophils	G-MDSC
Suppressive	no	no	need activation	yes	no	no	yes
FACS Marker	$CD11b^{pos}$ $Ly\text{-}6G^{neg}$ $Ly\text{-}6C^{hi}$ $IFN\gamma R2^{neg}$ $pAKT^{lo}$ $pS6^{lo}$ $IRF\text{-}1^{lo}$ $CD103^{neg}$ $IL\text{-}6^{neg}$ $iNOS^{neg}$ $Arg1^{neg}$	$CD11b^{pos}$ $Ly\text{-}6G^{neg}$ $Ly\text{-}6C^{hi}$ $IFN\gamma R2^{neg}$ $pAKT^{lo}$ $pS6^{lo}$ $IRF\text{-}1^{lo}$ $CD103^{pos}$ $IL\text{-}6^{pos}$ $iNOS^{neg}$ $Arg1^{neg}$	$CD11b^{pos}$ $Ly\text{-}6G^{neg}$ $Ly\text{-}6C^{hi}$ $IFN\gamma R2^{pos}$ $pAKT^{hi}$ $pS6^{hi}$ $IRF\text{-}1^{hi}$ $CD103^{neg}$ $IL\text{-}6^{neg}$ $iNOS^{neg}$ $Arg1^{neg}$	$CD11b^{pos}$ $Ly\text{-}6G^{neg}$ $Ly\text{-}6C^{hi}$ $IFN\gamma R2^{pos}$ $pAKT^{hi}$ $pS6^{hi}$ $IRF\text{-}1^{hi}$ $CD103^{neg}$ $IL\text{-}6^{pos}$ $iNOS^{pos}$ $Arg1^{pos}$	$CD11b^{pos}$ $Ly\text{-}6G^{neg}$ $Ly\text{-}6C^{neg}$ $CD64^{low/pos}$ $Mertk^{pos}$ $MHC\,II^{pos}$ $CD11c^{pos}$	$CD11b^{pos}$ $Ly\text{-}6G^{pos}$ $Ly\text{-}6C^{lo}$ $CD101^{pos}$ $Mcl1^{neg}$	$CD11b^{pos}$ $Ly\text{-}6G^{pos}$ $Ly\text{-}6C^{lo}$ $CD101^{neg}$ $Mcl1^{pos}$ $CD84^{pos}$

Markers in red have been reported in [7], markers in blue in [18–21], markers in violet in [27], and markers in green in [17]

(Fig. 1) that release NO for suppression [7]. Combination of LPS + IFN-γ for the second step of L-Mono to M-MDSC activation appeared as one of the strongest signals [3]; however cocktails of pro-inflammatory cytokines such as IL-1β + TNF + IFN-γ + IL-10, as they may accumulate in tumors, were as well effective [7]. If activation does not occur at days 3 and 4 and the GM-CSF culture is continued, licensed monocytes lose their potential to become M-MDSC and instead develop into monocyte-derived macrophages or MoDCs that can be further activated, e.g., by LPS/IFN-γ (Fig. 1).

Although we did not analyze whether also a licensing process is required as an intermediate step for the generation of G-MDSC, measuring activation can serve to identify G-MDSC, since positive intracellular staining of iNOS or Arg1 occurs only after LPS or LPS/IFN-γ activation of 3-day GM-CSF cultures (Fig. 2).

In the following we provide a protocol for generation of murine MDSC from BM cells, staining with markers to distinguish suppressive MDSC from non-suppressive cells as well as testing MDSC for functional suppression of T cell proliferation.

2 Materials

2.1 BM Preparation

1. Ice-cold phosphate buffer saline (PBS).
2. R10 cell culture medium: 500 mL RPMI 1640 + 5 mL L-glutamine from a stock solution (stock: 10^4 U/mL) + 5 mL stock solution of penicillin-streptomycin (stock: 10 mg/mL) + 50 μM

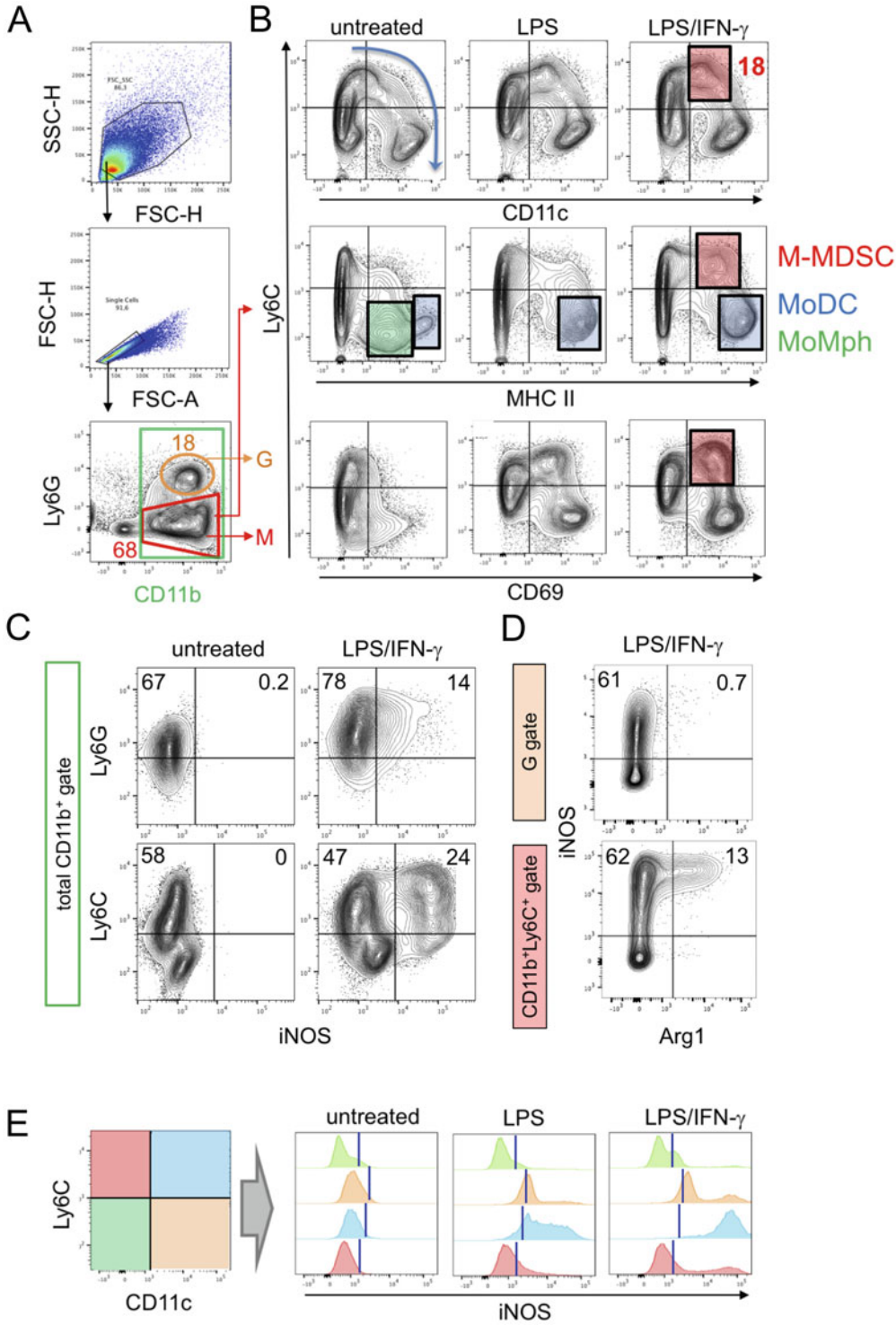

Fig. 3 Flow cytometric analysis of MDSC. Murine BM cells are cultured in GM-CSF for 3 days, then stimulated or not with LPS or LPS/IFN-γ overnight. Cultures are stained for the indicated markers. (**a**) Gating strategy to identify $CD11b^+$ cells (green gate) distinguish granulocytic (G, yellow gate) from all other myelomonocytic cells

final concentration of β-mercaptoethanol (use 34.8 μL of β-the 37 M mercaptoethanol in 10 mL PBS) + 50 mL fetal calf serum (FCS), heat inactivated for 30 min at 57 °C to inactivate complement (*see* **Note 1**).

3. 70% Ethanol (v/v in water) for disinfection.
4. Sterile injection needles (20 gauge, 0.4 × 19 mm).
5. Sterile syringe 10 mL.
6. Mouse strain: C57BL/6 female, 6–12 weeks old, same for BALB/c and most other inbred strains, NOD mice are different.

2.2 Culture of BM Cells

1. Petri dishes, 10 cm (*see* **Note 2**).
2. Recombinant mouse GM-CSF (rmGM-CSF): When specific doses are needed use murine rGM-CSF. Since the biological activity from different providers varies, we prefer its application by units, where ≥200 U/mL should be used, corresponding to 40 ng/mL by many providers (not 20 ng/mL as wrongly indicated earlier [11]) (*see* **Note 3**).
3. Alternative to no. 2: GM-CSF-supernatant. Transfectants producing murine GM-CSF can be used to obtain sufficiently high doses of GM-CSF instead of rGM-CSF [22]. This supernatant needs to be filtered (to avoid cell transfer) and used at 10% as a source for GM-CSF. The supernatant should contain 400–800 U/mL GM-CSF, this can be tested by ELISA and it is stable at 4 °C for more than 3 months.

Fig. 3 (continued) (M, red gate) such as monocytic MDSCs (M-MDSC), monocyte-derived DCs (MoDC), and monocyte-derived macrophages (MoMph). (**b**) Myelomonocytic cells (red gate M) are further stained for Ly6C and additional markers. Activated M-MDSC express low levels of CD11c, MHC II, and CD69 (red gates). Monocyte-derived macrophages (MoMph, green gate) and monocyte-derived DCs (MoDC, blue gate) at day 3 of culture as indicated above. The blue arrow indicates the "waterfall" of monocyte differentiation into MoDC under GM-CSF conditions without activation. LPS or LPS/IFN-γ treatment induces adherence of the majority of MoMph to the culture dish (not shown) and increases the frequencies of MHC II^{high} mature MoDCs. (**c**) Analysis by FACS within a total $CD11b^+$ gate for granulocytic $Ly6G^+$ or monocytic $Ly6C^+$ cells for their intracellular iNOS expression. $Ly6C^{high}$ M-MDSCs are the major population expressing iNOS after LPS/IFN-γ stimulation. (**d**) Granulocytic cells within gate G or $CD11b^+$ $Ly6C^+$ gated monocytes were further double-stained for intracellular iNOS and Arg-1. Granulocytes and monocytes show similar frequencies of cell expression iNOS, but monocytes contain higher amounts of iNOS. Only $Ly6C^+$ $iNOS^{high}$ monocytes also stain for Arg-1 after stimulation with LPS/IFN-γ. (**e**) Identification of cell types contributing to NO production. Cells generated as above with GM-CSF for day 3 and stimulated with LPS or LPS/IFN-γ or remained unstimulated. Cells are gated as $Ly6G^-$ and $CD11b^+$. Further staining for CD11c and Ly6C is indicated and intracellular iNOS staining is displayed as histograms of each color-coded quadrant. $Ly6C^{high}$ $CD11c^{low}$, MHC II^{low}, $CD69^+$ M-MDSC represent the most potent NO-producing cell type as indicated by the blue markers

2.3 MDSC Activation

1. LPS (0.1 μg/mL) + IFN-γ (100 U/mL). Final concentrations are indicated.

2.4 FACS Analysis

1. FACS buffer: PBS supplemented with 5% BSA and 0.5% sodium azide.
2. FcγR2/3 receptor blocking solution: 20 μg/mL 2.4G2 recombinant antibody (or 10% supernatant from 2.4G2 hybridoma cell line, ATCC) in FACS buffer.
3. Fixatives for intracellular staining: either 2% formaldehyde in PBS or 90–100% cold methanol (HPLC grade) depending on the applied antibody (Table 2).
4. "Perm buffer".
5. "Cytofix/Cytoperm solution".
6. "IC fixation buffer".
7. Intracellular cytokine staining: Phorbol 12-myristate 13-acetate (PMA; 0.01 μg/mL), ionomycin calcium salt (1 μg/mL), Brefeldin A (5 mg/mL in PBS).
8. For details of the suggested markers and required protocol, *see* Table 2.

Table 2
Antibodies used for MDSC characterization

Antibodies	Clone	Staining method	Staining Ref.
CD11b	M1/70	Surface	[7]
Ly6G	1A8	Surface	[7]
Ly6C	HK1.4	Surface	[7]
IFNγR2	MOB-47	Surface	[7]
Mcl1	Y37	Formaldehyde fixation + permeabilization on cytospin (not for FACS)	[18, 20, 21]
CD101	Moushi101	Surface	[19]
CD103	2E7	Surface	[17]
IRF-1	d5e4	On cytospin (not for FACS)	[7]
p-AKT	SDRNR	Methanol fixation	[7]
p-S6	D57.2.2E	Methanol fixation	[7]
IL-6	MP5-20F3	Befeldin A + formaldehyde fixation + permeabilization	[7]
iNOS	CXNFT	Formaldehyde fixation + permeabilization	[7] Fig. 3
Arg1	A1exF5	Formaldehyde fixation + permeabilization	Fig. 3
Ki67	16A8	Intranuclear staining	Fig. 4

2.5 T Cell Suppressor Assay

1. Sterile PBS.
2. 70 μm cell strainer.
3. Petri dish, 6 cm.
4. Sterile syringe, 1 mL.
5. R10 cell culture medium (*see* Subheading 2.1, **item 2**).
6. Erythrocyte lysis buffer: 15 mM ammonium chloride, 1 mM sodium bicarbonate, 0.01 mM EDTA in distilled or filtered water.
7. Sterile 96-well plate, U bottom.
8. CellTrace™ Violet proliferation dye (Invitrogen) or Ki67 antibody (clone 16A8).
9. Sterile αCD3 (2.5 μg/mL; clone 145-2C11) and αCD28 (2.5 μg/mL; clone E18) antibodies for stimulation.
10. αCD4 (clone GK1.5) and αCD8 (clone 53.6-7) antibodies for flow cytometry.

3 Methods

This method to generate G-MDSC and M-MDSC from murine BM has been described before [9] and is based on a modified protocol to generate BM-DCs [11]. The only difference between the protocols is the time point of harvest from the cultures. While optimal suppressor capacity is achieved from GM-CSF BM cultures at days 3 and 4, the highest yield and functionality of differentiated BM-DCs is at days 8 and 9. Of note, such bulk BM cultures never reveal pure populations of the indicated cell types which can cause confusion [23], due to simultaneous responses of hematopoietic stem cells, myeloid progenitors, and late stages of BM-monocytes to GM-CSF. Consequently, after any time point of culture different developmental stages of GM-CSF-responsive myeloid progenitor cells, but also differentiated neutrophils and macrophages will be generated and are simultaneously present [24]. Nonresponsive cells to GM-CSF die out between days 1 and 2 of culture and are cleared by phagocytic cells in the culture. Thus, these protocols only allow an enrichment of the desired cell population(s), MDSC or DCs at a given time point, but do not substitute for subsequent cell sorting to obtain a specific cell subset at high purity.

3.1 BM Cell Preparation

1. Carefully remove femur and tibia of both hind limbs without breaking the bones.
2. Remove muscles and other tissue surrounding the bones with scissors.

3. Remove remaining tissue surrounding the bones by rubbing with cleansing tissue (unsterile) to avoid fibroblast contaminations in the BM cultures.
4. Disinfect intact bones in 70% ethanol for 3–4 min.
5. Remove them from the disinfection tube with sterile forceps and rinse with sterile PBS to wash off the ethanol. From now on work sterile with sterile tools.
6. Cut off ends of femur and tibia and flush out the marrow with sterile PBS using a 10 mL syringe with 20-gauge needle into a fresh Petri dish until the bones appear white.
7. Collect cells and transfer them in a 50 mL tube.
8. Pipet up and down about ten times with a serological 10 mL pipet to disintegrate the big BM clumps.
9. Centrifuge for 5 min with 300 × *g* at room temperature.
10. Resuspend cell pellet in 10 mL R10 culture medium at room temperature.
11. Count cells. About 4–9 × 10^7 BM cells are obtained from four bones of a mouse, depending on age and sex.

3.2 BM Cell Culture with GM-CSF

1. Seed 3 × 10^6 cells per 10 cm dish in 10 mL R10 cell culture medium containing 10% GM-CSF-supernatant or ≥200 U/mL rmGM-CSF.
2. Culture at 37 °C, 5–7% CO_2 in a humidified incubator.
3. At day 3 of culture granulocytic and monocytic cells appear as major cell populations as shown by the FACS analysis in Fig. 3. These cells represent "resting" MDSC, or as outlined above for the monocytic fraction, "licensed monocytes", that are not yet expressing iNOS or Arg1.

3.3 Activation of Suppressor Molecules and Function

The expression of iNOS or Arg1 can be induced in GM-CSF-licensed monocytes and partially in granulocytic cells by treatment with pro-inflammatory cytokines or pathogen products [3, 7]. Only a fraction of monocytes and hardly the other cell types in the culture upregulate iNOS for suppression (Fig. 3).

1. Add 0.1 μg/mL LPS + 100 U/mL IFN-γ to the cultures for 16 h.
2. Induction of surface markers and iNOS or Arg1 can be measured by FACS (*see* Subheading 3.4 and Fig. 3).
3. Functional suppression can be measured in an in vitro suppressor assay (*see* Subheading 3.5 and Fig. 4).

3.4 FACS Analysis

1. Wash cells with FACS buffer and incubate with FcγR2/3 receptor blocking solution for 15 min at 4 °C.
2. Surface markers (Table 2) are stained for 30 min in the dark at 4 °C.
3. Intracellular antigens are stained after surface staining using either formaldehyde fixation or methanol fixation. Intracellular cytokine staining and intranuclear staining follow other protocols.
   (a) Formaldehyde fixation (e.g., iNOS, Arg1):
      - Cells are fixed with 2% formaldehyde for 20 min at room temperature.
      - After washing the cells with FACS buffer, the antibodies diluted in Perm buffer are incubated for 45–60 min at room temperature (Table 2).

   (b) Methanol fixation (phospho markers):
      - Stain surface markers using methanol resistant fluorochromes.
      - Incubate cells with IC fixation buffer for 30 min at room temperature.
      - After washing, fix cells with 90–100% cold methanol for 30 min at 4 °C.
      - Wash cells twice and then incubate with phospho antibodies in FACS buffer for 60 min at room temperature (Table 2).

   (c) Intranuclear staining (e.g., Ki67):
      - After surface staining, incubate cells in Cytofix/Cytoperm solution for 30–60 min at room temperature.
      - Stain intranuclear markers diluted in Perm buffer for 45–60 min at room temperature and wash cells with Perm buffer.

   (d) Intracellular cytokine staining (e.g., IL-6):
      - Restimulate cells with 0.01 μg/mL PMA and 1 μg/mL ionomycin in the presence of 5 mg/mL Brefeldin A for 16 h.
      - Stain surface markers as described above.
      - After 20 min formaldehyde fixation, perform the intracellular staining with antibodies diluted in Perm buffer for 45–60 min.
4. Resuspended cells in FACS buffer and proceed with the flow cytometry measurements.

3.5 In Vitro Suppressor Assay

Previously we used [^{3}H]-Thymidine incorporation to measure cell proliferation and suppression [3], but flow cytometry-based methods using CellTrace™ proliferation dyes or staining with the cell cycle marker Ki67 have several advantages, such as separate evaluation of $CD4^+$ and $CD8^+$ T cell suppression from bulk cultures. Since only a fraction of monocytes upregulates iNOS to produce NO, but all other cell types do not, the ratio between $iNOS^+$ and $iNOS^-$ cells in the culture can be decisive for the success of suppression. Are too many MoMph and MoDC generated as compared with $iNOS^+$ M-MDSC, suppression may not be the dominant function anymore by the bulk culture, but may turn into immune stimulation by matured MoDCs (Fig. 3b). In this case, cell sorting of $CD11b^+$ $Ly6C^{high}$ cells is recommended (*see* **Note 4**).

1. Remove syngeneic spleen and lymph nodes as a source of T cells under sterile conditions.
2. Transfer spleen and lymph nodes into separate cell strainers placed in Petri dishes filled with PBS and mash them with a sterile syringe plunger into the dish.
3. Centrifuge and resuspend spleen cells in 4 mL erythrocyte lysis buffer.
4. Incubate for 1 min.
5. Stop the lysis by adding 4 mL R10 cell culture medium.
6. Pool spleen and lymph node cells as a source of T cells. In case of CellTrace™ Violet proliferation dye usage, stain cells according to the manufacturer's instructions.
7. Harvest the MDSC and dispense in three wells each of 200,000 cells per well into a 96-well plate in R10 cell culture medium. Then pipet a serial dilution (*see* Fig. 4) of the triplicates (*see* **Note 5**).
8. Add 200,000 pooled spleen and lymph node cells per well to the MDSC. Prepare additional three wells with spleen and lymph node cells without MDSC as negative control.
9. Stimulate the cells with 2.5 μg/mL αCD3 and 2.5 μg/mL αCD28 antibodies.
10. Harvest the cells after 3 days in case of Ki67 staining or after 4–5 days when using CellTrace™ Violet proliferation dye and stain the cells for CD4 and CD8.
11. If Ki67 instead of CellTrace™ proliferation dye is used, stain now for intranuclear Ki67.

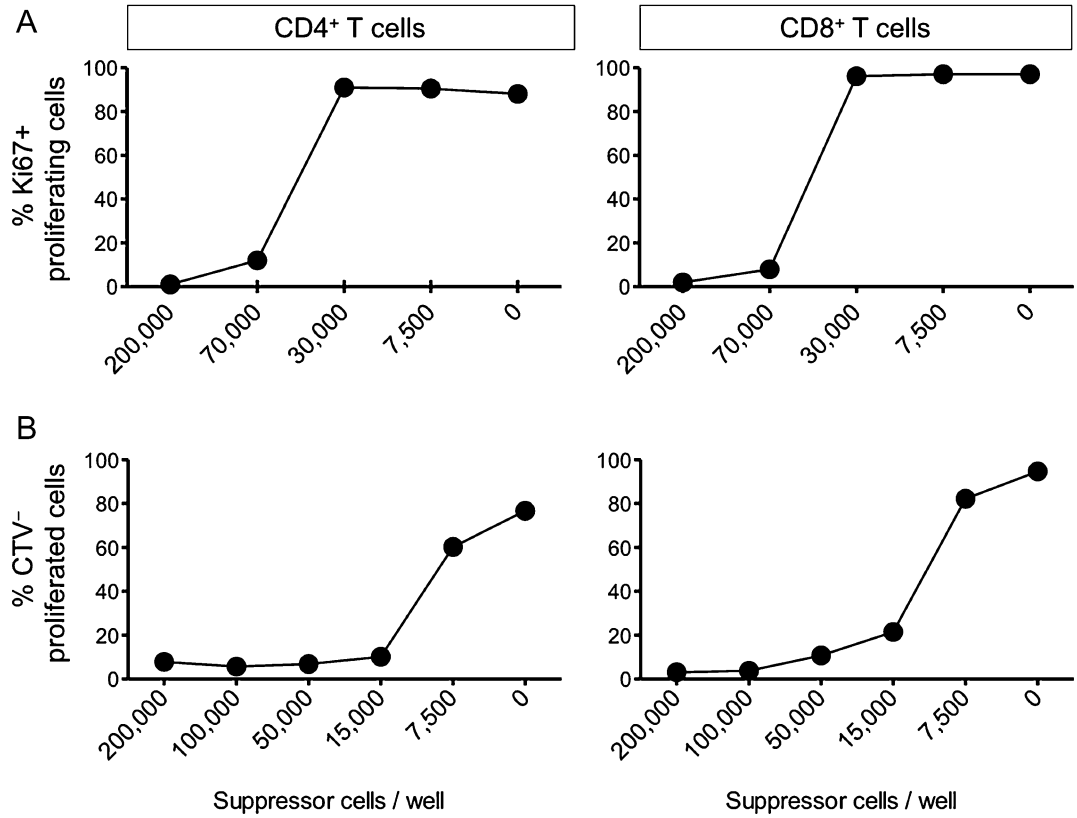

Fig. 4 T cell suppressor assays with GM-CSF-generated MDSC. Due to the high phenotypic similarity of suppressive and non-suppressive myeloid cells, the suppressive capacity of in vitro generated or isolated MDSC should be validated via a T cell suppression assay. Here, triplicate cultures of a 96-well plate are seeded with titrated amounts of in vitro generated MDSC. Antibodies against CD3 and CD28 are added in soluble form. (**a**) Pooled spleen and lymph node cells are used as a source for syngeneic T cells and added to the wells. After 3 days triplicate wells are pooled and T cell proliferation is assessed by staining for CD4 and CD8 and intracellular Ki67 and analyzed by flow cytometry. Percentages of $Ki67^+$ cells within the $CD4^+$ and $CD8^+$ T cell subsets are shown. (**b**) Pooled spleen and lymph node cells are used as a source for syngeneic T cells, labeled with the proliferation dye CellTrace™ Violet (CTV) and added to the wells containing MDSC. After 4 days triplicate wells are pooled and T cell proliferation is assessed by staining for CD4 and CD8 and analyzed by flow cytometry. Frequencies of CTV^- cells within the $CD4^+$ and $CD8^+$ T cell subsets are shown

4 Notes

1. The FCS source and quality can be critical for the yield of particular cell types and for the activation state of the cells, as we have observed for BM-DCs [25]. This applies also to MDSC cultures.
2. Bacterial quality dishes (e.g., *FALCON*, 1029) result in higher yields (~10%), with less macrophages and more DCs as compared to tissue culture quality dishes.

3. Higher doses than 200 U/mL do not yield more cells. If so, this may relate to differences in the definition of the specific activity in units by the provider. Lower doses (5–20 U/mL) lead to reduced cell yields [26] and the delayed generation of MDSC which in this case appear only at day 10 in these cultures [9].
4. The isolation of pure G-MDSC or M-MDSC from bulk BM cultures with GM-CSF at day 3 may require cell sorting or bead-based isolation of the desired subset, similar as from MDSC isolated from organs or tumors of mice. Since surface markers are likely to have also a function, they may not always suite cell sorting. The Gr-1 antibody has been found to trigger (a) up-regulation of macrophage markers and thus development via Ly6C signals, and (b) apoptosis of $Mcl1^{pos}$ neutrophils via Ly-6G signals [18]. Thus, any positive cell enrichment with the Gr-1 antibody may impair MDSC functions. This was not observed when using CD11b antibodies, which, however, does not distinguish between the MDSC subsets.
5. MDSC, like other fresh myeloid cells, but unlike T and B cells, respond with loss of functions and viability when placed on ice or in the cold. Thus, after harvesting MDSC from the culture plates cells should be kept at room temperature before their use for functional experiments in suppressor assays in vitro or before injection. FACS analyses on ice are not influenced when cells are kept continuously on ice.

Acknowledgments

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Chapter 11

Analysis of Antimicrobial Activity of Monocytic Myeloid-Derived Suppressor Cells in Infection with *Mycobacterium tuberculosis* and Human Immunodeficiency Virus

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Abstract

Myeloid-derived suppressor cells (MDSC) encompass a subset of myeloid cells, which suppress both innate and adaptive immune functions. Since *Mycobacterium tuberculosis* (*M. tuberculosis*) can infect these cells, interest has emerged to study the antimicrobial response of MDSC to mycobacteria causing tuberculosis. Reactive oxygen species (ROS) are critical mediators to control intracellular replication of *M. tuberculosis* and MDSC express high levels of these effector molecules. Here we describe the flow cytometric assessment of total cellular ROS produced by MDSC in response to infection with *M. tuberculosis* and compare it with the ROS activity of non-MDSC myeloid cells. To further understand the dynamics of host–pathogen interactions, we provide details on methods for measurement of the intracellular replication of *M. tuberculosis* within MDSC. Of note, these procedures were adopted for primary MDSC and non-MDSC subsets isolated from human immunodeficiency virus (HIV)-uninfected or HIV-infected individuals, in vitro infected with *M. tuberculosis* to mimic *M. tuberculosis* mono- or HIV-*M. tuberculosis* coinfection, respectively.

Key words Monocytic MDSC, HIV-*M. tuberculosis* coinfection, Cell ROS, Mitochondrial ROS, Antimicrobial activity, Flow cytometry, MDSC sorting

1 Introduction

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of myeloid origin that contributes to the negative regulation of immune function. Human MDSC are considered as lineage negative (Lin^-) cells that express common myeloid markers (CD11b, CD33) and low to zero levels of HLA-DR (HLA-$DR^{-/lo}$), and thus have $Lin^-CD11b^+CD33^+$HLA-$DR^{-/lo}$ phenotype [1, 2]. Depending on the presence of CD15/CD66b or CD14, MDSC have been classified as granulocytic ($CD15^+$ or/and $CD66b^+$) or monocytic ($CD14^+$) subsets, respectively [1, 3–5]. These cells suppress innate and adaptive immunity either by arginine depletion, reactive oxygen (ROS) and nitrogen (NOS)

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species generation, VEGF expression, and/or mediation of $CD4^+CD25^+FoxP3^+$ Treg cell expansion [3, 6–8]. Considerable research, predominantly performed in animal models, has demonstrated the inhibition of antitumor and antimicrobial activity by MDSC [9–12]. Additionally, recent evidence suggests that this inhibitory activity is present in patients with certain malignancies. Genesis of MDSC is regulated by various pro-inflammatory cytokines including IL-6, IL-10, prostaglandins, stem-cell factor, GM-CSF, TGF-β, VEGF, and TNFα [7, 8].

Infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) and human immunodeficiency virus type-1 (HIV) is associated with production of cytokines, which includes factors such as, IL-6, IL-10, prostaglandins, TGF-β, and TNF-α that could expand MDSC. Of note, both infections constitute the main burden of infectious disease worldwide [13, 14], potentiate one another, and accelerate disease development through mechanisms, which still remain undetermined [15–17]. Importantly, immunological suppression in individuals infected with *M. tuberculosis* and/or HIV persist post-treatment with respective drug regimens, preserving the risk of reactivation of *M. tuberculosis* both in mono- and HIV coinfection settings. We and others have demonstrated that $CD14^+HLA\text{-}DR^{-/lo}$ monocytic MDSC numbers decline in HIV patients on anti-retroviral therapy, but remain high as compared to healthy individuals [10, 11, 18–22]. We found that compared to $CD14^+HLA\text{-}DR^{hi}$ non-MDSC, the expression of ROS with anti-antimicrobial effect is higher in MDSC isolated from peripheral blood. Its expression declines in response to *M. tuberculosis* infection.

We provide a protocol for comparative analysis of cellular ROS in MDSC and non-MDSC subsets infected with *M. tuberculosis* using flow cytometry. We also provide a detailed methodology to determine the intracellular replication of *M. tuberculosis* in these cells isolated from peripheral blood, which has also been employed for in vitro-generated MDSC [23].

2 Materials

All the procedures must be carried out following the appropriate Biosafety Level (BSL) practices. *M. tuberculosis* should be handled in BSL-3 laboratory and flow cytometric analysis performed either in BSL-3 or outside BSL-3 laboratories after sample fixation and following the Institutional Biosafety Guidelines.

2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

1. Ficoll-Paque Premium (GE Healthcare).
2. Dulbecco's phosphate-buffered saline (D-PBS) (Life Technologies).
3. Hemocytometer.
4. 0.4% Trypan Blue in D-PBS (v/v) (*see* **Note 1**).

2.2 Flow Cytometry and Isolation of M-MDSC from PBMC

1. Anti-human-CD3-PerCP-Cy5.5 (clone SK7) antibody (dilution 1:100).
2. Anti-human-CD19-PerCP-Cy5.5 (clone SJ25C1) antibody (dilution 1:100).
3. Anti-human-CD66b-PerCP-Cy5.5 (clone G10F5) antibody (dilution 1:100).
4. Anti-human-CD11b-APC-eFlour780 (clone ICRF44) antibody (dilution 1:100).
5. Anti-human-CD33-Alexa Fluor 700 (clone WM55) antibody (dilution 1:100).
6. Anti-human-CD14-PE/Cy7 (clone ME5) antibody (dilution 1:100).
7. Anti-human-HLA DR- PE/Dazzel-594 (clone L243) antibody (dilution 1:100).
8. Aqua fluorescent LIVE/DEAD® Fixable Dead Cell Stain (Life Technologies) Excitation/Emission 367/526 nm (dilution 1:1000) (*see* **Notes 2** and **3**).
9. 2.5 mM CellROX® Deep Red Oxidative Stress Reagent (C10422; Life Technologies) Excitation/Emission 640/665 nm.
10. Human CD3 microbeads (Miltenyi Biotec).
11. LS magnetic columns.
12. MACS Magnetic Separators.
13. RBC lysis buffer (BD Biosciences).
14. Staining buffer: 1% bovine serum albumin in D-PBS.
15. Cell sorting buffer (Miltenyi Biotec).
16. Cell collection media: fetal bovine serum with 1% Penicillin-Streptomycin.

2.3 Cell Culture

2.3.1 M-MDSC Culture

1. RPMI1640 media.
2. 2.5 M HEPES.
3. 200 mM Glutamine.
4. 100 mM Sodium pyruvate.
5. 10,000 units/mL Penicillin-Streptomycin.
6. 10 mg/mL Gentamicin.
7. 10% Heat-inactivated autologous serum (*see* **Note 4**).
8. Culture medium: RPMI Medium 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/mL Penicillin-Streptomycin. Store at 4 °C.

9. Culture medium for infection with *M. tuberculosis*: RPMI Medium 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, and 1 mM sodium pyruvate. Store at 4 °C.
10. Culture medium to kill extracellular *M. tuberculosis*: RPMI Medium 1640 supplemented with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μg/mL gentamicin, prepare fresh.

2.3.2 M. tuberculosis Culture

1. Green Fluorescent Protein (GFP) expressing *M. tuberculosis* Erdman (gift from Dr. Larry Schlesinger, Texas Biomedical Research Institute, United States).
2. *M. tuberculosis* Erdman (BEI Resources, United States).
3. Middlebrook 7H9 broth.
4. Middlebrook 7H10 agar.
5. 10% Oleic Acid, Albumin, Dextrose, Catalase (OADC) enrichment.
6. 20% Tween 20 in sterile water.
7. 50% Glycerol in sterile water (*see* **Note 5**).
8. 10% Sodium dodecyl sulfate (SDS) in sterile water.
9. 100 mm Petri dishes.
10. 250-mL polypropylene non-vented screw-capped conical flasks.
11. Middlebrook 7H9 liquid medium: Dissolve 4.7 g Middlebrook 7H9 powder in 900 mL deionized water then add 10 mL 50% glycerol and 0.25 mL 20% Tween 20. Sterilize by autoclaving at 121 °C for 20 min and cool to room temperature. Supplement the media with 100 mL OADC and store at 4 °C.
12. Middlebrook 7H10 agar plates: Dissolve 19 g Middlebrook 7H10 powder in 900 mL deionized water, then add 10 mL glycerol. Sterilize by autoclaving at 121 °C for 20 min and cool for 20 min. Supplement the media with 100 mL OADC and pour approximately 20 mL to each Petri plate. Once solidified, store the plates at 4 °C.

3 Methods

3.1 Flow Cytometric Sorting of M-MDSC

3.1.1 Peripheral Blood Mononuclear Cell Isolation

1. Mix heparinized blood with 2–4 times volume of D-PBS.
2. Carefully layer 30 mL of diluted blood over 20 mL of Ficoll-Paque in a 50 mL conical tube and centrifuge at 400 × *g* for 30 min at 25 °C in a swinging bucket rotor without brakes. The acceleration and deceleration rates need to be optimized depending on the centrifuge being used. With Thermo

Scientific Sorvall ST 16R, acceleration and deceleration set at six gives an intact buffy coat layer.

3. Aspirate the top layer consisting of diluted plasma gently, without disturbing the white mononuclear blood cell layer.
4. Transfer the mononuclear cell layer to a new 50 mL conical tube. Fill the tube with D-PBS and centrifuge at 300 × *g* for 10 min at 25 °C. Remove the supernatant completely.
5. Resuspend the pellet in 50 mL D-PBS and centrifuge at 200 × *g* for 10 min at 25 °C. Remove the supernatant completely and proceed for CD3 depletion.

3.1.2 Depletion of $CD3^+$ Cells

1. Resuspend cells in D-PBS and determine cell number of PBMC using hemocytometer (*see* **Note 1**).
2. Centrifuge cell suspension at 300 × *g* for 10 min. Remove the supernatants completely.
3. Resuspend the cell pellet in 80 μL of MACS sorting buffer per 10^7 cells.
4. Add 20 μL of CD3 microbeads to 10^7 cells, mix, and incubate for 15 min at 4 °C.
5. Wash the cells with 5 mL buffer and centrifuge at 300 × *g* for 10 min. Remove the supernatant completely.
6. Resuspend cells in 600 μL of MACS buffer and proceed to magnetic separation using LS column.
7. Place the column in a MACS Separator and prepare by rinsing with 3 mL of MACS buffer.
8. Load the cell suspension onto the column. Collect the unlabeled cells which pass through the magnetic column. Wash the column by adding 3 mL buffer twice, each time once the column reservoir is empty.
9. The CD3+ cells are trapped in column and unlabeled CD3 depleted cell fraction enriched for myeloid cells is collected in the tube.

3.1.3 Sample Preparation for Cell Sorting

1. Centrifuge CD3+ cells depleted cell fraction at 300 × *g* for 10 min and resuspend in FACS staining buffer.
2. Count the cells and add pre-titrated CD11b, CD33, CD14, HLA-DR, and CD3/19/66b antibody cocktail (*see* **Note 6**).
3. Incubate cells for 30 min at 4 °C in dark.
4. Wash the cells twice with 3 mL FACS staining buffer by centrifuging at 300 × *g* for 10 min.
5. Resuspend the cells in cell sorting buffer and pass through 40 μM filter to get rid of cellular debris, which may clog the cell sorter.

6. Using the appropriate Cell Sorter capable of Biohazard cell sorting, gate on CD3/CD19/CD66b-cell population. Collect $CD11b^{+}CD33^{+}CD14^{+}HLA\text{-}DR^{hi}$ (non-MDSC) and $CD11b^{+}CD33^{+}CD14^{+}HLA\text{-}DR^{lo}$ (MDSC) cell fractions in separate collection tubes in appropriate collection media (*see* **Notes 7** and **8**).

3.2 Culture of Sorted Cells and Infection with M. tuberculosis

1. Centrifuge sorted cell fraction, resuspend in antibiotic-free complete RPMI1640 media, and count cells by Trypan Blue exclusion method (*see* **Note 1**).
2. Plate the cells at a minimum cell density of 0.5×10^6 cell/mL and minimum 8×10^4 cells/well in 24-well plate.
3. Keep the cells for resting for 2 h by incubating at 37 °C and 5% CO_2. We have not found any change in ROS expression if cells are rested overnight (ON). Subsequent methodology is shown for ON rested cells.
4. Gently aspirate the cell culture media and replace with fresh antibiotic-free complete RPMI1640 medium.
5. Infect cells with GFP-Erdman at a multiplicity of infection (MOI) 1:5 (1 cell: 5 bacteria). The total volume per well should not exceed 300 μL (*see* **Note 9**).
6. Incubate the cells for 1–2 h at 37 °C and 5% CO_2 for infection to happen.

3.3 Flow Cytometric Determination of ROS Production Following M. tuberculosis *Infection*

1. During the last 30 min of infection, add 1 μM of CellROX diluted in prewarmed complete RPMI1640 (*see* **Note 10**).
2. Wrap the plate in aluminum foil and transfer to incubator, 37 °C and 5% CO_2.
3. Gently scrape the cells and transfer to labeled FACS staining tubes.
4. Wash wells once with 2 mL D-PBS and add to respective staining tubes.
5. Centrifuge at $300 \times g$ for 10 min at 25 °C. Carefully aspirate the buffer.
6. Resuspend cells in 1 mL D-PBS and add 0.5 μL Aqua Live/Dead reagent. Incubate for 15 min at room temperature in dark.
7. Wash the cells with 2 mL FACS staining buffer by centrifuging at $300 \times g$ for 10 min.
8. Proceed with Fixation protocol approved by the Institutional Biosafety Committee to move samples out of BSL-3 for flow cytometry.
9. Samples are acquired on a flow cytometer analyzer with compatible lasers/filters combination and data analyzed using

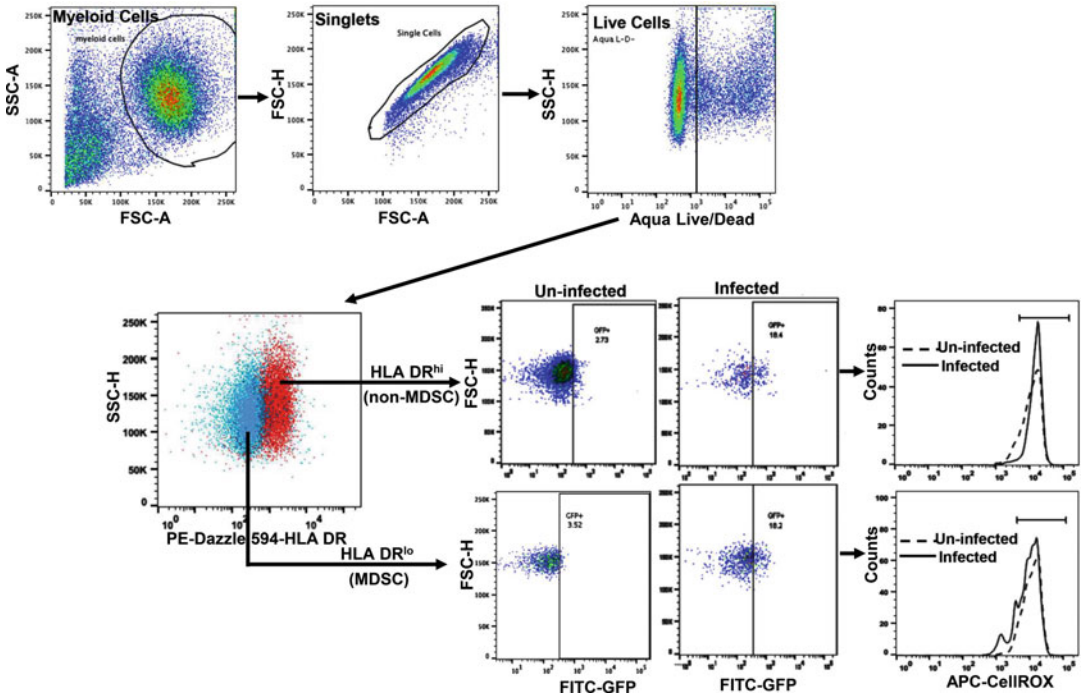

Fig. 1 Flow cytometric determination of reactive oxygen species by MDSC in response to *M. tuberculosis*. Sorted MDSC and non-MDSC (HLA-DR^{hi}) are infected with GFP-*M. tuberculosis* Erdman and incubated with CellROX for 30 min. Cells are stained with Live/Dead stain, formaldehyde fixed, and analyzed using flow cytometer. The gating strategy is shown

appropriate software or FlowJo. Dead cells are excluded and the expression of CellROX is analyzed in *M. tuberculosis* GFP+ cell gate (Fig. 1); and calculate Net ROS expression = [Mean fluorescence intensity of ROS by *M. tuberculosis* GFP+ non-MDSC—Mean fluorescence intensity of ROS by uninfected non-MDSC] and [Mean fluorescence intensity of ROS by *M. tuberculosis* GFP+ MDSC—Mean fluorescence intensity of ROS by uninfected MDSC]. The flow cytometry-based method can also be utilized to measure the rate of infection by measuring the percentage of GFP+ cells.

3.4 Measurement of Intracellular M. tuberculosis

1. Remove media after 2 h of infection in **step 6** of Subheading 3.2 and wash wells three times with 1 mL D-PBS to remove non-phagocytosed bacteria and loosely adherent cells.
2. Add 0.5 mL/well fresh media containing gentamicin (50 μg/mL) (*see* **Note 11**).
3. Incubate the cells for 1–2 h at 37 °C and 5% CO_2 to kill extracellular bacteria.
4. Remove the medium from wells and add to separate 15 mL centrifuge tubes containing 21 μL of 10% SDS. Add 3 mL

additional sterile water to have the final 0.07% SDS concentration (*see* **Note 12**).

5. Add 1 mL of 0.07% SDS to each of the wells to lyse adherent MDSC.
6. Remove the lysed MDSC to separate 15 mL centrifuge tubes.
7. Wash the wells twice with 0.07% SDS and add to previous tubes (total volume of 3 mL).
8. Centrifuge the tubes (*see* **steps 3** and 7) at 2000 × *g* for 20 min to pellet the mycobacteria.
9. Discard supernatant and resuspend the pellet in 1 mL Middlebrook 7H9 broth by gently tapping the sealed tube.
10. Prepare the serial dilutions in microtiter plate by adding infected cell lysate (100 μL) to the first well of a row and 90 μL of MiddleBrook 7H9 broth or D-PBS to each subsequent well. Transfer 10 μL from first well to next well and mix well by pipetting up and down to prepare 1:10 dilution. Further dilutions are made in the same manner. In triplicate plate, 10 μL of the dilution on to MiddleBrook 7H10 agar supplemented with 10% OADC and wait till dry. Once the spots are air-dried, incubate the plates at 37 °C following the Institutional Biosafety Guidelines for 3-weeks and count the colonies (Fig. 2).
11. Calculate colony forming units (CFUs) as: Number of colonies × Dilution factors/volume plated in mL (*see* **Note 13**).
12. Remaining infected MDSC are placed back to the incubator.
13. The above steps may be repeated at day-3 and -5 postinfection.

4 Notes

1. Trypan blue is used to count live cells using hemocytometer. For this, place the coverslip on the hemocytometer and apply 10 μL of cells (PBMC or sorted cells) diluted with 10 μL Trypan blue (Dilution factor 2). Grids of the hemocytometer are seen under the microscope, set of 4 × 4 squares are at each corner of the hemocytometer, count the number of cells in the 16-squares at each corner. Determine the average and calculate the cell number/mL as: Average cell count × Dilution Factor × 10^4/mL.
2. The antibody panel can be designed based on the lasers-filters combination of the flow cytometer accessible to the user. We have found this panel works best with a range of flow cytometers tested: MoFlo (Beckman Coulter), Canto II, Aria, and LSR II (all from Becton Dickinson) with very minimal to no spillover of fluorescence; this is particularly important to

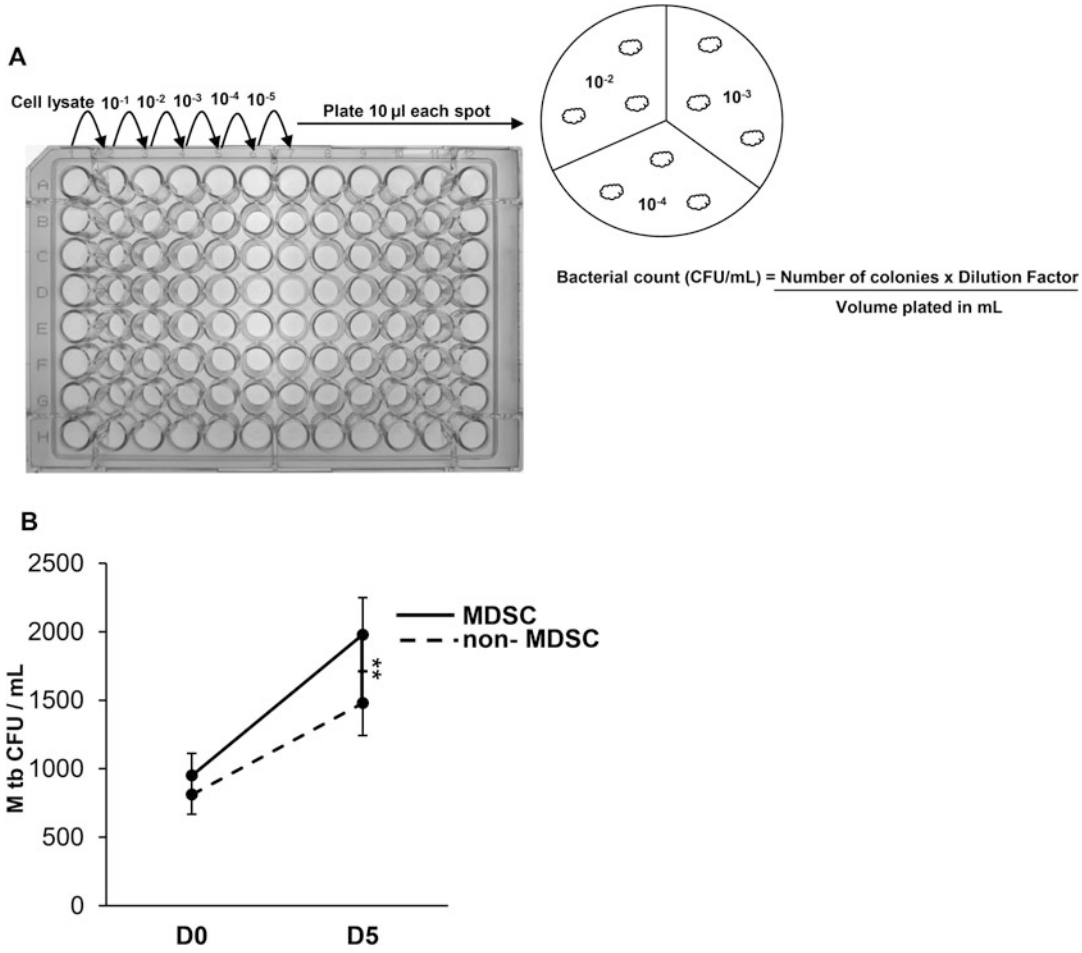

Fig. 2 Intracellular replication of *M. tuberculosis* in MDSC isolated from peripheral blood mononuclear cells. (**a**) Sorted MDSC and non-MDSC are infected with *M. tuberculosis* Erdman at multiplicity of infection of 5 for 3 h, washed with D-PBS to remove non-phagocytosed bacteria and loosely adherent cells. Cells are treated with gentamicin (50 μg/mL) for 1–2 h at 37 °C and 5% CO_2 to kill extracellular bacteria. Cells are lysed with 0.07% SDS and cellular lysates are serially diluted and plated in triplicate on Middlebrook 7H10 agar supplemented with OADC enrichment. The number of colonies are counted after 3 weeks and colony forming units (CFU)/ml determined. (**b**) Intracellular growth of *M. tuberculosis* shown at days-0 and -5 postinfection of MDSC and non-MDSC isolated from HIV-infected individuals. Data show mean values ±SEM; $N = 4$ donors. $^{**}p < 0.005$

avoid spillover of $CD14^{+}HLA\text{-}DR^{hi}$ into $CD14^{+}HLA\text{-}DR^{-/lo}$ population. The controls include unstained cells and fluorescence minus one (FMO). Due to the donor-to-donor variation in the expression of various markers, we recommend to use stained compensation beads (Comp Control) to calculate compensation.

3. Since monocytic MDSC are $CD3^{-}CD19^{-}CD66b^{-}$, using antibodies conjugated to same fluorophore place the cells expressing them in dump channel. This is helpful in removing contaminating cells and clean up for downstream analysis.

4. To avoid high level of variability between commercial serum lots and determine individual donor response, we recommend using autologous serum. We have found commercial human serum also gives similar readout. It is important to heat inactivate serum at 56 °C for 30 min, to inactivate complement and prevent cell lysis.
5. *M. tuberculosis* Erdman was cultured as previously described [24], with shaking at 70 rpm to avoid bacterial clumping. Infection stocks are prepared in Middlebrook 7H9 broth with OADC and 10% glycerol, CFU are determined after one freeze-thaw cycle, and MOI calculated for infection based on these values.
6. In order to avoid high background fluorescence, it is recommended to titrate antibodies and fluorescent probes for flow cytometry. For titration experiments use the cell number and experimental conditions identical to the actual assay and incorporate viability dye. A defined number of cells are stained in a total of 100 μL volume with antibodies diluted in staining buffer at 1:25, 1:50, 1:100, 1:200, and 1:400 dilution. To determine the optimal concentration, stain index is calculated: Stain index = Mean Fluorescence Intensity of (MFI) +ve population – Mean Fluorescence Intensity (MFI) of –ve population/2 Standard Deviation of –ve population. The dilution that gives the highest Stain index is the dilution to use (Fig. 3).
7. The selection of collection media depends on each individual laboratory, we have found collecting myeloid cells in fetal bovine serum (FBS) with 1% Penicillin-Streptomycin maintains good cell viability. It is important to wash FBS completely before proceeding for infection assays.
8. Since the cells are cultured post-sort, staining cells with viability dye before sorting can be avoided. Most of the currently available viability stains are dissolved in DMSO which can cause cell death of primary cells. If the cells are to be cultured post-sort, we do not prefer to include viability dye for sorting. However, it is highly recommended to include Live/Dead stain if collecting cells for gene expression or other molecular studies.
9. We used *M. tuberculosis* stocks frozen at –80 °C and actively growing cultures for infection; we found that actively growing culture gives a better readout of immediate effector molecules such as ROS.
10. CellROX kill the cells if used in excess. It is highly recommended that the optimal concentration to use is determined. The stained cells should be analyzed as soon as possible, latest within 24 h of fixation.

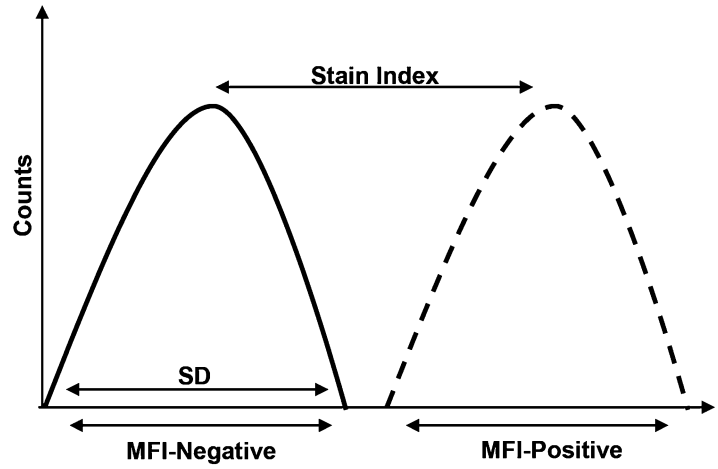

Fig. 3 Stain index. The stain index is the ratio of mean fluorescence intensity (MFI) positive population and the MFI of negative population, divided by two times the standard deviation of the negative population

11. This is to ensure that only intracellular bacteria are remaining in the wells. Amikacin at 100 μg/mL can also be used in place of gentamicin, as this antibiotic is impermeable to cells.
12. This step is very important at day 0 postinfection to determine the optimal antibiotic concentration that kills the extracellular bacteria.
13. With 0.1×10^5 cell infection at MOI 1:5, we observe 10^{-2} or 10^{-3} as the highest dilution that gives countable bacterial colonies.

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Chapter 12

Isolation and Functional Characterization of Myeloid-Derived Suppressor Cells in Infections Under High Containment

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Abstract

The current absence of markers unique to MDSC, particularly those expanded during human infection, necessitate concurrent demonstration of their suppressive capacity to ensure unequivocal identification. This is further complicated by the array of heterogeneous markers used to characterize MDSC in various conditions and models. Standardization of phenotypic and functional characterization, as well as isolation, from infectious biological samples of patients, are critical for accurately reporting MDSC dynamics, function, organ abundance, and establishment of their therapeutic value in infectious diseases. To illustrate, we report on our established method for MDSC isolation from bronchoalveolar lavage fluid and peripheral blood of pulmonary TB patients, as well as functional impact on T cells by measuring T cell activation, proliferation, and cytokine production.

Key words MDSC, Infectious disease, Bronchoalveolar lavage, Tuberculosis

1 Introduction

Two major MDSC subsets have been characterized in infectious disease conditions, namely monocytic-MDSC (M-MDSC) and polymorphonuclear-MDSC (PMN-MDSC) [1]. Identification of eosinophilic-MDSC (Eo-MDSC) in mice infected with *S. aureus*, expanded the repertoire of suppressive myeloid cells [2]. Phenotypic classification of MDSC differ considerably between laboratory animal models and humans. Even among various human conditions and organ compartments, the markers used to classify MDSC, the dominant subset, MDSC functions, and mechanisms of suppression, differ. In humans, MDSC are HLA-DR$^{-/low}$ whereas immature MDSC progenitor populations (e-MDSC) are Lin- and CD33$^{+/high}$ cells. Human M-MDSC and PMN-MDSC subtypes

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are $CD11b^+CD14^+CD15^-$ and $CD11b^+CD14^-CD15^+$, respectively. Additional markers have been added to the panel, notably S100A9 (M-MDSC) [3], LOX1 [4, 5], CD47 [6], and FATP2 [7]; however, these await validation in many infectious diseases. Differences in subtype reporting highlight the importance of standardizing MDSC phenotyping and isolation and a consensus for assessing MDSC suppressive potential.

The gold standard for MDSC isolation is undeniably by flow cytometric sorting, making use of fluorescently labeled monoclonal antibodies to the panel of markers described above. Unfortunately, this method is often unfeasible when working with infectious biological samples. A case in point is the isolation of MDSC from *Mycobacterium tuberculosis* (Mtb)-infected biological human samples. These samples are typically processed under biosafety-level-3 laboratory conditions, which often do not house sorting flow cytometers. In this illustration, another challenge is the incompatibility of bronchoalveolar lavage-derived cells, as proxy of site-of-disease immunity, with fluorescence-based assays. The extreme autofluorescence imparted by alveolar macrophages (AM), in particular those obtained from populations in regions with a high prevalence of smoking or biomass fuel burning such as everyday exposure to open/in-house wood fires. The particulate matter is ingested by AM and the intracellular hydrocarbon buildup and subsequent lysozyme activity within AM is likely at fault for the fluorescent spillover observed in most fluorescent channels.

Here, we provide a standardized protocol for the enrichment of total-MDSC from infectious BAL and pleural samples from pulmonary and pleural tuberculosis (TB) patients, along with corresponding peripheral blood samples using magnetic bead sorting to circumvent the need for fluorescence-based sorting within a BSL3 unit. We also offer the method used to verify MDSC suppressive function on T cell subsets by measuring T cell activation/proliferation by flow cytometry (cellular component) and secreted proteins by multiplex array (culture supernatant), following in vitro coculture.

2 Materials

All reagents and equipment are stored at room temperature (RT) unless otherwise stated.5

2.1 Cell Isolations

1. Ficoll-Paque (stored at RT) or Histopaque (stored at 4 °C).
2. EDTA or NaHep or LitHep vacutainers (*see* **Note 1**).
3. 1× Phosphate Buffered Saline (PBS): Dilute 100 mL of 10× PBS with 900 mL of autoclaved, distilled water (dH_2O). If the dH_2O was not autoclaved prior to the dilution, autoclave the prepared 1× PBS prior to use.

4. 1× ACK Lysis Buffer.
5. MACS Buffer: Prepare MACS buffer by adding 985 mL of 1× PBS, 10 mL 0.2 mM EDTA, and 5 mL Bovine serum albumin (BSA) (0.5%) in a sterile 1 L schott bottle (*see* **Note 2**). Do not sterilize by autoclave, filter sterilize prior to use. Always store MACS Buffer at 4 °C.
6. Counting solution: Add 10 μL of Trypan Blue to 80 μL of 1× PBS in a 1 mL Eppendorf tube.
7. 4% Paraformaldehyde (PFA): Dilute one 16% PFA ampule (10 mL) with 30 mL of 1× PBS in a sterile 50 mL Falcon tube. Use immediately or keep refrigerated for up to 1 week. Users may make appropriate 1:4 dilutions to the volume required for the experiment.
8. 70 μM Cell Strainer.
9. 2% FACS Buffer: Add 49 mL of 1× PBS with 1 mL fetal bovine serum (FBS). Store FACS Buffer at 4 °C.

2.2 Cell Adherence

1. Complete Roswell Park Memorial Institute Medium (cRPMI): RPMI-1640 supplemented with L-glutamine and 10% FBS.
2. L-Glutamine: Prepare 400 μL aliquots of L-glutamine in 2 mL Screw cap tubes, from 100 mL stock. Freeze away at –20 °C. Prepare working solution by taking out one aliquot of L-glutamine, thaw and transfer into 50 mL polypropylene tube containing 40 mL RPMI. Store at 4 °C until finished or until the solution turns pink.

2.3 MACS Microbead Isolations

1. CD3 Microbeads (positive selection).
2. HLA-DR Microbeads (positive selection).
3. CD33 Microbeads (positive selection).

2.4 Coculture

1. RPMI: RPMI-1640 (not supplemented).
2. Mtb Purified Protein Derivative (PPD) (1 mg/mL): Prepare a working solution of 200 μg/mL by creating a 1:5 dilution with 1× PBS. Prepare 20 μL aliquots of the working solution and keep at 4 °C. Upon use, thaw each aliquot required and add 80 μL RPMI and mix by pipetting up and down. Add 10 μL per 200 μL total well volume for a final concentration of 10 μg/mL per stimulation. These calculations have been designed for use in a 96-well culture plate.
3. Dimethyl sulfoxide (DMSO).
4. Brefeldin A (BFA): Reconstitute a 5 mg vial with 1 mL DMSO (5 mg/mL). Aliquot 20 μL into 2 mL Screw cap tubes and freeze away at –20 °C. Prepare a working solution of 500 μg/mL (1:10 dilution of stock solution). Add 180 μL 1× PBS into one aliquot of Brefeldin A. To reach a final concentration of 10 μg/mL, add 8 μL of working solution into each experimental tube.

5. Anti-CD3/CD28/CD2 MACSiBead Particles: Pipette 100 μL of CD2-Biotin, 100 μL of CD3-Biotin, and 100 μL of CD28-Biotin into a sealable 2 mL tube and mix well. Resuspend the Anti-Biotin MACSiBead Particles thoroughly by vortexing. Remove 500 μL of the Anti-Biotin MACSiBead Particles (1×10^8 Anti-Biotin MACSiBead Particles) and add to antibody mix. Add 200 μL of MACS buffer to adjust to a total volume of 1 mL. Incubate for 2 h at 2–8 °C under constant, gentle rotation by using MACSmix Tube Rotator at 4 rpm. The loaded Anti-Biotin MACSiBead Particles can be stored at 2–8 °C for up to 4 months.

2.5 Flow Cytometry

1. Conjugated Antibodies:
   (a) CD3-FITC (BD, Material number 555916, Clone UCHT1, Isotype Mouse IgG1).
   (b) HLA-DR-APC (BD, Material number 559866, Clone G46-6, Isotype Mouse IgG2a).
   (c) CD33-PE (BD, Material number 555450, Clone WM53, Isotype Mouse IgG1).
   (d) CD11b-PerCP-Cy5.5 (BD, Material number 550993, Clone M1/70, Isotype Rat IgG2b).
2. 10× Permeabilization Solution: BD Cytofix/Cytoperm (cat# 554722).
3. Fixation Buffer: BD Cytofix (cat# 554655).
4. Flow tubes: 12 × 75 mm round-bottom test tubes or 96-well round-bottom microtiter plates.

2.6 Luminex Immunoassays

1. Bio-Plex 200 suspension array system.
2. Bio-Plex Pro plate washer.
3. Bio Rad Acute Phase Assay kit: Contains antibody-conjugated beads (25×), detection antibodies (10×), acute phase standards (2 vials; lyophilized), acute phase controls (2 vials; lyophilized), serum-free diluent, serum-based diluent, and 96-well assay plates (×2).
4. Standard tissue culture grade 96-well plates for sample dilutions (may be non-sterile).
5. Bio Rad assay buffer.

3 Methods

Conduct the following procedures in the appropriate biosafety level laboratories based on their infectious nature. Peripheral blood processing may be carried out under BSL2 conditions, while

bronchoalveolar lavage (BAL) and pleural fluid processing should be carried out under BSL3 conditions owing to these site-of-disease samples being highly infectious.

3.1 Peripheral Blood MDSC Isolation Protocol

Peripheral blood mononuclear cells (PBMC) may be isolated using the Ficoll-Paque density centrifugation technique. Peripheral blood has routinely been collected in sodium-heparin (NaHep) vacutainers for the successful isolation of PBMC, although the literature has demonstrated that collection in EDTA vacutainers are more successful for the preservation of MDSC (*see* **Note 1**).

3.1.1 PBMC Isolation

1. Label 1 × 50 mL sterile Falcon tube **per 15 mL peripheral blood** drawn with the unique patient identification number on the lid and the side of the tube.
2. Pipette 15 mL Ficoll-Paque density medium into each 50 mL tube required for the isolation.
3. Slowly invert the NaHep (LiHep and EDTA could serve as alternative blood collection containers) vacutainers five times to mix well and ensure no clotting has occurred.
4. In a separate 50 mL Falcon tube, dilute the peripheral blood in an approximately 1:1 ratio with sterile 1× PBS, **NOT** exceeding 35 mL in total per tube after dilution.
5. Slowly pipette diluted blood over the density medium without disturbing the Ficoll-Paque layer. An interface between the Ficoll-Paque and diluted peripheral blood should be clear.
6. Centrifuge at 400 × *g* for 25 min at room temperature (RT), making sure to turn both the BRAKE and ACCELERATION OFF to ensure the density gradient is not disturbed.
7. Carefully remove the samples from the centrifuge to minimize dispersion of the density bands. The PBMC will band at the interface between the plasma and the density media ($D < 1.077$ g/mL) while red blood cells (RBC) pellet beneath the density media.
8. Aspirate plasma layer and either discard into a waste bottle containing iodine/virkon or store the plasma at –80 °C for later use.
9. Using a plastic Pasteur pipette, carefully remove PBMC and dispense into a sterile 50 mL Falcon tube.
10. *Wash step 1*: Makeup to 50 mL with 1× PBS and centrifuge at 400 × *g* for 10 min at RT.
11. Carefully pour off the supernatant into waste bottle making sure pellet remains. After decanting, resuspend the cell pellet in 1 mL MACS Buffer by gentle pipetting.
12. *Wash step 2*: Makeup to 10 mL in MACS Buffer, then centrifuge at 400 × *g* for 10 min at **4 °C**.

13. Instead of pouring off the supernatant, remove as much of the supernatant as possible using a Pasteur pipette and P1000/P200 pipette.
14. *Optional red cell lysis step*: If a considerable layer of red cells is visible on top of the cell pellet, resuspend pellet in 2.5 mL of sterile 1× ACK Lysis Buffer. Depending on the degree of red cell contamination, incubate for 3–5 min at RT.
15. After incubation, add 10 mL of MACS Buffer and centrifuge at 400 × *g* for 10 min at 4 °C. If the lysis step is performed, another wash step is required by adding 10 mL MACS Buffer to the cells and spinning at 400 × *g* for 10 min at 4 °C.
16. Decant the supernatant and resuspend in 1 mL MACS Buffer (*see* **Note 3**).
17. Prepare a counting solution.
18. Add 10 μL of the cell suspension to the counting solution and mix thoroughly.
19. Transfer 10 μL of this suspension to a hemocytometer for manual counting (*see* **Note 4**).
20. Count the cells using the hemocytometer and a microscope (Fig. 1; *see* **Note 5** and Fig. 9) and document the live cell count, dead cell count, total cell count, and viability (*see* **Notes 6** and **7**).
21. Take a fraction of approximately 5×10^5 cells and place in a 5 mL Falcon tube (suitable for flow cytometry) with 500 μL 4% PFA for purity check should this be required.
22. Fix the cells in the dark for 15 min (RT) and then add 1 mL FACS Buffer.
23. Centrifuge the fixed cells at 400 × *g* for 10 min and decant the supernatant.
24. Continue with the necessary staining for the purity check or cryopreserve the sample for future batched purity checks.
25. Use the remaining cells from **step 20** for MACS isolation (*see* **Note 8**).

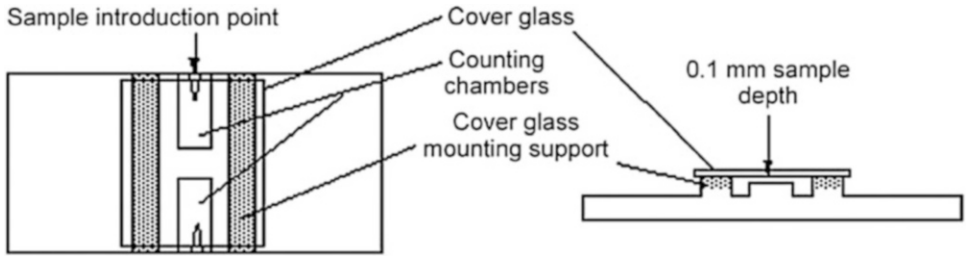

Fig. 1 Hemocytometer design and layout

3.2 Bronchoalveolar Lavage MDSC Isolation Protocol

3.2.1 Total BAL Cell Isolation

1. Collect the bronchoscopy BAL sample into a 50 mL Falcon tube (*see* **Note 9**).
2. Transport sample **on ice** to BSL3 facility (*see* **Note 10**).
3. Once appropriately protected for BSL3 work, sterilize the biosafety hood first with Distel disinfectant solution (10%) followed by 70% EtOH.
4. Filter the BAL sample through 70 μM Cell Strainer, into new labeled 50 mL Falcon tube (*see* **Note 11**).
5. Centrifuge the filtered sample at 300 × *g* at 4 °C for 7 min, within a capped centrifuge bucket.
6. Following centrifugation, pour off the supernatant carefully into a labeled 50 mL Falcon tube.
7. Collect 4 × 2 mL aliquots of the BAL fluid (BALF) supernatant in 2 mL Screw cap tubes and store them along with residual supernatant in the 50 mL Falcon tube at –80 °C (*see* **Note 12**).
8. Resuspend the cell pellet in 1 mL 1× PBS (alternatively, use cRPMI) by gentle pipetting and then top up to 50 mL.
9. Centrifuge at 300 × *g* at 4 °C for 7 min.
10. Decant the supernatant and resuspend in 1 mL MACS Buffer (*see* **Note 3**).
11. Perform a cell count as previously described in Subheading 3.1, **steps 17–24**.
12. Use the remaining cells from **step 11** for MACS isolation (*see* **Note 13**).

3.3 Pleural Fluid MDSC Isolation Protocol

3.3.1 Total Pleural Fluid Cell Isolation

1. Collect Pleural effusion fluid into an anticoagulant-treated tube following aseptic intercostal puncture.
2. Transport sample **on ice** to BSL3 facility (*see* **Note 10**).
3. Once appropriately protected for BSL3 work, sterilize the biosafety hood first with Distel followed by 70% EtOH.
4. Filter the pleural fluid sample through a 70 μM Cell Strainer, into new labeled 50 mL Falcon tube (*see* **Note 11**).
5. Centrifuge the filtered sample at 300 × *g* at 4 °C for 7 min, within a capped centrifuge bucket.
6. Following centrifugation, pour off supernatant carefully into labeled 50 mL Falcon tube (BD).
7. Collect 4 × 2 mL aliquots of the pleural supernatant in 2 mL Screw cap tubes and store them along with residual supernatant in 50 mL Falcon tube at –80 °C (*see* **Note 12**).
8. Resuspend the cell pellet in 1 mL 1× PBS by gently pipetting and then top up to 50 mL.
9. Centrifuge at 300 × *g* at 4 °C for 7 min.

10. Decant the supernatant and resuspend in 1 mL MACS Buffer (*see* **Note 3**).
11. Perform a cell count as previously described in Subheading 3.1, **steps 17–24**.
12. Use the remaining cells from **step 11** for MACS isolation (*see* **Note 14**).

3.4 Adherence of Alveolar Macrophages

When isolating from sample types such as BAL, an adherence step may be necessary to remove cells that are too large for MACS columns or interfere with fluorescence-based end assays. Following adherence, the non-adherent fraction should be kept and used for the MACS isolation of CD3+ T cells as indicated in Subheading 3.5. Subsequently, the CD3- cells of the non-adherent fraction can be pooled with the adherent fraction to ensure all MDSC are successfully isolated. The isolation procedure for MDSC can then be followed.

1. Resuspend the cells from Subheading 3.2.1, **step 12** in 2 mL cRPMI and mix gently.
2. Using a standard 24-well tissue culture plate, clearly label the plate with the date, sample type, and wells to be used.
3. Pipette 500 μL of the cell suspension into four of the wells.
4. Transfer the plate to an incubator 37 °C (5% CO_2) and incubate for 16–24 h.
5. Following incubation, remove the plate and carefully remove the supernatant into a sterile 15 mL Falcon tube (clearly labeled).
6. In a **dropwise** manner, carefully add 500 μL cRPMI to each well and again remove the supernatant into the corresponding sterile 15 mL Falcon tube (*see* **Note 15**).
7. Perform the wash step twice more, making sure to collect the supernatant into the corresponding labeled 15 mL Falcon tube.
8. The collected supernatant now contains the non-adherent cells which can be used for MACS Microbead isolation.
9. Continue with Subheading 3.5.

3.5 MACS Microbead Isolation

Myeloid-derived suppressor cells (MDSC) are isolated from peripheral blood, bronchoalveolar lavage fluid, and pleural fluid samples using the MACS Microbead isolation technique when flow cytometry-based sorting techniques are not available or appropriate (*see* **Note 16**). MDSC are isolated from total cells through a sequence of MACS Microbead isolations (Fig. 2).

The procedure described as follows remains the same for each round of isolations, except the appropriate microbeads are used for the desired cell type to be isolated.

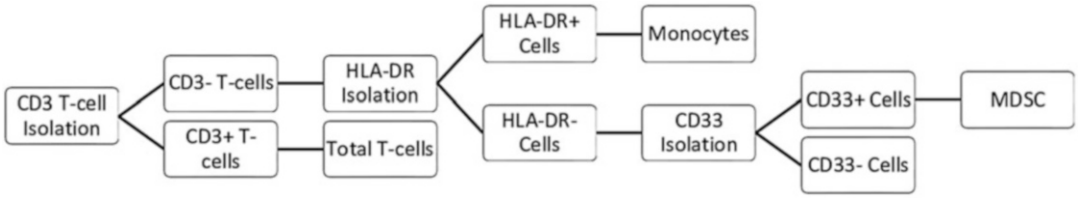

Fig. 2 A flow diagram demonstrating the process flow of MACS Microbead isolations to be done in order to successfully enrich for MDSC

1. Centrifuge cell suspension derived from the isolation performed in either Subheadings 3.1, 3.2, or 3.3, at 300 × *g* for 10 min at 4 °C (*see* **Note 17**).
2. Aspirate the supernatant completely using a pipette.
3. Using the cell count derived during the total cell isolation in either Subheadings 3.1, 3.2, or 3.3, resuspend cell pellet in 80 μL MACS buffer per 10^7 total cells.
4. Add 20 μL of anti-CD3 Microbeads per 10^7 total cells (*see* **Note 18**).
5. Mix well and incubate for 15 min in the refrigerator (2–8 °C).
6. Wash the cells by adding 1 mL MACS buffer per 10^7 cells and centrifuge at 300 × *g* for 10 min at 4 °C.
7. Aspirate the supernatant completely using a pipette.
8. Resuspend up to 10^8 cells in 500 μL of MACS buffer.
9. Place the appropriate column in the magnetic field of a Miltenyi magnet (*see* **Note 19**).
   (a) For ease of reading, the procedure using a LS column will be described from here on.
10. Place a sterile 15 mL Falcon tube in a rack below the column.
11. Prepare the column by rinsing with 3 mL MACS buffer (*see* **Note 20**).
12. Apply the 500 μL cell suspension to the column.
13. Collect flow-through—this will contain the **unlabeled** cells (**negative cellular fraction**).
14. Wash the column with 3 × 3 mL MACS buffer, collecting the flow-through in the unlabeled cell tube.
15. Remove the column from the magnet and place on top of a new sterile 15 mL Falcon tube.
16. Pipette 5 mL MACS buffer into the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column—this fraction will contain the **labeled** cells (**positive cellular fraction**).

17. Centrifuge both cell fractions at 300 × *g* for 10 min at 4 °C and decant the supernatant.
18. Resuspend the pellet in 5 mL MACS buffer.
19. Perform a cell count as previously described in Subheading 3.1, **steps 17–24**.
20. Store the CD3+ fraction for functional characterization and use the CD3- fraction for the HLA-DR isolation (*see* **Notes 21** and **22**).
21. Remember to take a fraction of all isolated cells for purity checks (Subheadings 3.1.1, **steps 21–24**).
22. Repeat the procedure as described above for the HLA-DR isolation (*see* **Note 22**), retaining the negative fraction for the isolation of CD33 positive cells (again using the above-described procedure and taking purity check fractions) (*see* **Note 22**).

3.6 Coculture of MDSC and T Cells for Functional Investigations

The hallmark of MDSC is their T cell-specific suppressive functions, the extent to which has not fully been explored in TB and other high containment infectious diseases. Through coculture experiments of MDSC with other immune cell types, we can elucidate their pathogen-specific and nonspecific suppressive functions through various mycobacterial strain infections, in a contact-dependent and -independent manner. During such coculture experiments, cells may also be stimulated with various bacterial or viral agents, such as lipopolysaccharide (LPS), PPD, or GAG/POL polyprotein, if whole organisms are not available or appropriate for the experimental design.

3.6.1 Standard Coculture

1. Label a 96-well culture plate clearly with the name, date, infection type, and period clearly and have a plate layout template prepared beforehand (*see* **Note 23** and Fig. 10).
2. Centrifuge the tubes containing the MACS enriched MDSC (or other cell types) at 300 × *g* for 10 min at RT. Decant supernatant and resuspend in the appropriate volume cRPMI.
3. Based on the cell concentration obtained from the cell count and the number of wells allocated to use for experimental purposes, the cell number per well is calculated aimed at having a volume of 200 μL per well.
4. An equal number of cells are added to each culture well. Typically, 2×10^5 cells are added per individual well, with a 1:1 ratio for coculture wells, resulting in 4×10^5 cells in those wells (*see* **Note 24**).

For the purposes of this procedure, we have included two T cell stimulation examples using PPD (antigen-specific activation of Mtb-infected samples) and anti-CD3/CD2/CD28 beads (nonspecific activation of T cells).

Stimulation

1. Once the cells have been seeded in the appropriate wells for the experiment, add 10 μL of PPD to all the designated wells (*see* **Note 25**).
2. Mix well by gently pipetting up and down using a multichannel pipette (if necessary).
3. Incubate the plates for an appropriate time at 37 °C (5% CO_2) (*see* **Note 26**).
4. After 43 h of incubation, add 4 μL BFA to all wells and mix well.
5. Return the plates to the incubator for the final 5 h (*see* **Note 27**).
6. After the full 48-h incubation, remove the plate from the incubator and centrifuge at 300 × *g* for 10 min.
7. Carefully remove the supernatant into a pre-labeled, sterile 1.5 mL Eppendorf tube (*see* **Note 28**).
8. Once the supernatant has been removed, add 100 μL of 4% PFA to all wells.
9. Fix the cells for 15 min at RT in the **dark**.
10. Centrifuge the plate at 300 × *g* for 10 min.
11. Carefully discard the supernatant and wash twice with 200 μL cRPMI, centrifuging at 300 × *g* for 10 min after each wash.

T Cell Activation

In order to activate T cells during coculture experiments, the Miltenyi Biotec T cell activation Kit may be used.

1. CD3+ T cells isolated during the process of isolating MDSC (*see* Fig. 2) may be activated and stimulated for proliferation, prior to their addition to the coculture.
2. Centrifuge the isolated CD3+ T cells at 300 × *g* for 10 min at 4 °C.
3. Decant the supernatant, resuspend CD3+ T cells in 900 μL culture medium.
4. During the centrifugation step, resuspend the pre-prepared anti-Biotin MACSiBead Particles thoroughly and add 25 μL MACSiBead Particles per 5 × 10^6 cells to a suitable tube.
5. Add 200 μL culture medium (RPMI supplemented with 10% AB serum) to the anti-Biotin MACSiBead Particles and centrifuge at 300 × *g* for 5 min.

6. Aspirate supernatant and resuspend the MACSiBead Particles in 100 µL fresh culture medium.
7. Add the appropriate volume of prepared anti-Biotin MACSiBead Particles to the labeled tube containing CD3+ T cells and mix thoroughly.
8. Once the beads have been added to these cells, the T cells can be added directly to the MDSC in coculture.

3.6.2 Trans-Well Coculture

For the investigation of contact-independent MDSC-mediated T cell suppression mechanisms, physical separation of MDSC and T cells can be achieved using trans-well coculture plates (*see* **Note 29** and Fig. 11). These plates comprise of 96 wells with an additional 0.4 µm Polycarbonate Membrane Corning Trans-well culture plate insert (Becton Dickinson, New Jersey, USA).

1. Label a 96-well culture plate clearly with the name, date, infection type, and period clearly.
2. Centrifuge the tubes containing the MACS isolated MDSC (or other cell types) at 300 × *g* for 10 min at RT. Decant supernatant.
3. Resuspend cells to concentration of 2×10^5 cells in 100 µL.
4. Add T cells to lower chamber and MDSC to upper chamber.
5. Incubate for 48 h.
6. After the full 48-h incubation, remove the plate from the incubator and centrifuge at 300 × *g* for 10 min.
7. Carefully remove the supernatant from lower chamber into a pre-labeled, sterile 1.5 mL Eppendorf tube (*see* **Note 28**).
8. Once the supernatant has been removed, add 100 µL of 4% PFA to all wells.
9. Fix the cells for 15 min at RT in the **dark**.
10. Centrifuge the plate at 300 × *g* for 10 min.
11. Carefully discard the supernatant and wash twice with 200 µL cRPMI, centrifuging at 300 × *g* for 10 min after each wash.
12. Store cellular fractions for functional characterization.

3.7 Flow Cytometry of Cocultured MDSC and T Cells to Assess Cell Phenotype and Function

3.7.1 Extracellular Staining

1. Prepare the antibody mix for surface staining in FACS Buffer.
2. Add 20 µL antibody mix into each well (50 µL into each tube).
3. Gently mix wells by pipetting up and down ten times or vortex tubes for 5 s.
4. Incubate cells for 30 min at 4 °C in the dark. Resuspend the cells after 15 min and continue incubation.
5. Wash cells by adding 200 µL of FACS Buffer to each well (1 mL into each tube).

6. Centrifuge at the appropriate speed (*see* **Note 30**) for 5 min at RT and discard supernatant.
7. Repeat the cell wash one additional time.
8. After the last wash pellet the cells and discard supernatant.

3.7.2 Fixation and Permeabilization

1. Add 100 μL of Fixation and Permeabilization Solution to each well (*tube*).
2. Gently mix wells by pipetting up and down ten times or vortex tubes for 5 s.
3. Incubate for 20 min at room temperature or 4 °C protected from light (depends on product. Refer to technical datasheet).
4. Add 120 μL of 1× Perm Wash Buffer to each of the wells (1 mL into each tube).
5. Gently mix wells by pipetting up and down ten times or vortex tubes for 5 s.
6. Centrifuge for 5 min at 400 × *g* at RT and discard supernatant.
7. Resuspend cells in 200 μL of 1× Perm Wash Buffer per well (500 μL per tube) and mix wells gently by pipetting up and down ten times or vortex tubes for 5 s.
8. Centrifuge for 5 min at 400 × *g* at RT and discard supernatant.

3.7.3 Intracellular Staining

1. Prepare the antibody mix for intracellular staining in 1× Perm Wash Buffer.
2. Add 20 μL antibody mix into each well (50 μL into each tube).
3. Gently mix wells by pipetting up and down ten times or vortex tubes for 5 s.
4. Incubate cells for 30 min at 4 °C in the dark. Resuspend the cells after 15 min and continue incubation.
5. Wash cells by adding 200 μL of 1× Perm Wash Buffer to each well (1 mL into each tube) and centrifuge for 5 min at 400 × *g* at RT.
6. Repeat the cell wash one additional time.
7. Resuspend pellet in 200 μL FACS Buffer.
8. Keep plate (tubes) covered at 4 °C in the dark until acquisition, which should be performed within 24 h.

3.8 Purity Check of Isolated Cellular Fractions

Purity assessment is critically important in MACS isolation to ensure that cell subsets are contaminated by nontarget cells. The most common method for purity assessment is flow cytometry, in which target cells are labeled with fluorescent markers and analyzed using a flow cytometer, allowing the proportion of each cell type in the sample to be calculated (*see* **Note 31**).

1. Continue with the cells prepared in Subheading 3.1.1, **step 24** or those prepared after cell separation as per Fig. 2 (cells should be at a concentration between 1×10^5 and 1×10^6 cells/mL).
2. Discard the supernatant and resuspend the cells in 100 μL of FACS Buffer (*see* **Note 32**).
3. Separate 50 μL of the above into a second 5 mL tube and use this as the unstained tube.
4. Centrifuge the tubes at 400 × *g* for 5 min and discard the supernatant.
5. Resuspend the cells in the appropriate volume of FACS Buffer and add the appropriate fluorescent-conjugated monoclonal antibody or antibodies (e.g., CD3-FITC, HLA-DR-APC, CD33/CD11b-PacBlue) according to the antibody manufacturer's instructions. The volume will typically be 5–20 μL of antibody per test with a total staining volume of 50 μL.
6. If desired, add a viability stain such as propidium iodide (PI), 7AAD, or Aqua Amine to each sample. This step allows dead cells to be gated out for more accurate flow cytometry analysis.
7. An example of the staining strategy to be used to assess the purity of isolated MDSC is given in Fig. 3.
8. Stain the cells for 30 min in the dark (4 °C).
9. Add 1 mL FACS Buffer to each tube after the incubation.
10. Centrifuge each tube at 400 × *g* for 10 min and discard the supernatant.
11. Resuspend each pellet in 200 μL FACS Buffer and store in the fridge until acquisition (*see* **Note 33**).
12. Acquire the samples on the BD FACSCanto™ II using the FACSDiva™ software (Becton Dickinson, New Jersey, USA) (*see* **Note 34**).

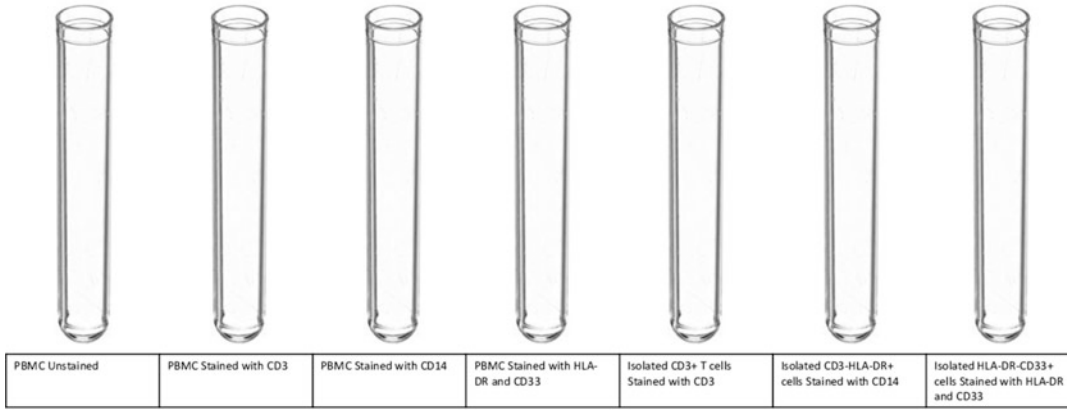

Fig. 3 Purity check staining example

13. Export the .fcs files for each tube and analyze using the third-party software FlowJo® (FlowJo LLC, Oregon, USA).
14. Analyze the files by following the workflow described in Fig. 4.
15. Use the gating strategy to determine single stain frequencies representative of the isolated cell population purity. Sample purity is the percentage of cells positive for the relevant staining antibody in the gated population.
16. A second dot plot can be created of the data displaying FSC-A vs. the viability stain. Dead cells will be positive for the viability marker and should be excluded by the gate.
17. An example of such gating is given in Fig. 5.

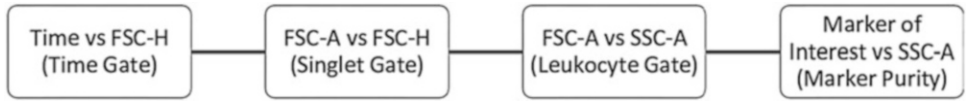

Fig. 4 The gating strategy employed to identify single markers and their purity. All created plots should be in dot plot format

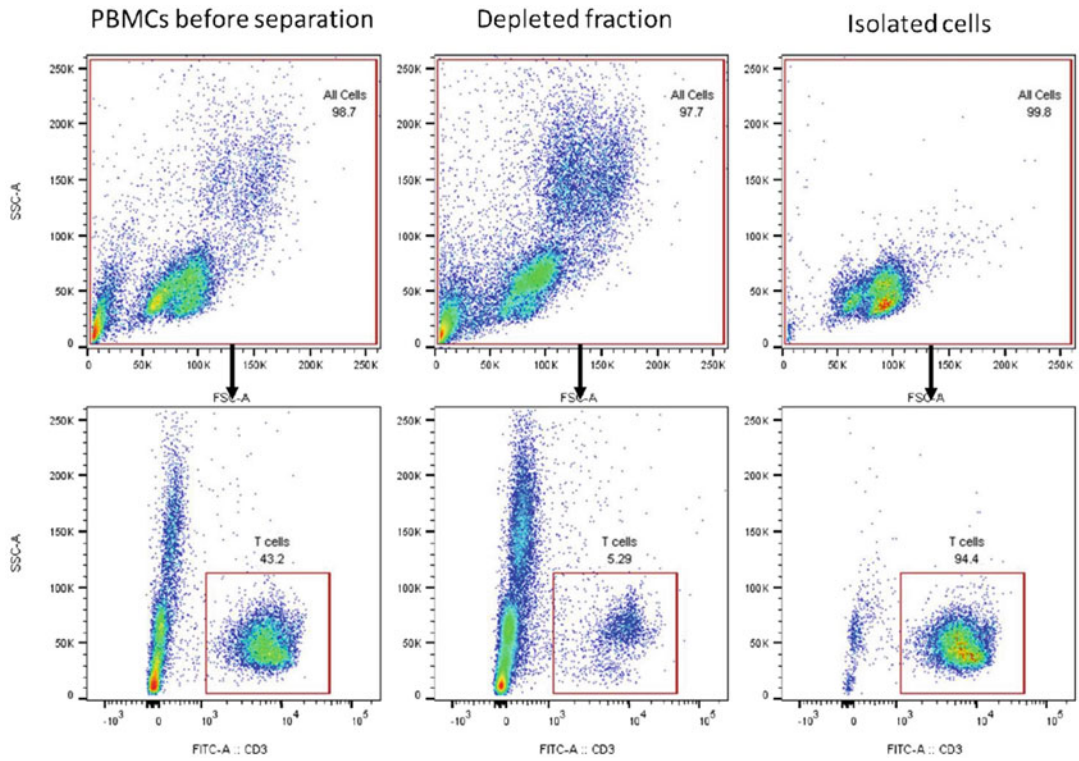

Fig. 5 Example of gating strategy demonstrating the purity of CD3+ T cells within the PBMC fraction before separation (43.2% CD3+ T cells, CD3 negative cells) from the depleted fraction of the MACS Microbead isolation (5.29% CD3+ T cells), and the isolated CD3 positive cell fraction of the MACS Microbead isolation (94.4% CD3+ T cells)

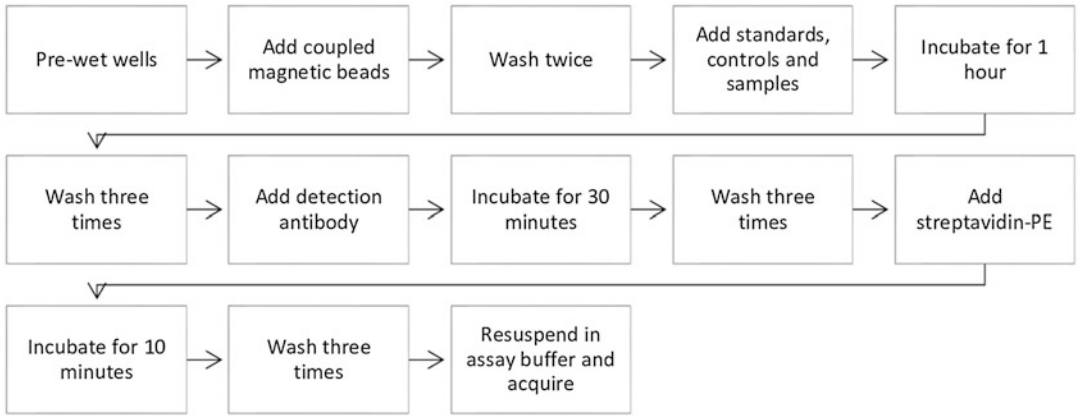

Fig. 6 A flow diagram demonstrating the assay workflow for basic Luminex Immunoassays, specifically the Bio Rad Acute Phase Assay, for cytokine and chemokine concentration determination

3.9 Luminex Immunoassay

Various Luminex kits are available for purchase by various manufacturers. For a simplified representation of a Luminex immunoassay, we have chosen to briefly describe a Bio Rad Acute Phase Assay kit which comes with a 4- and 5-Plex Multiplex assay (Luminex, Bio Rad Laboratories, Hercules, CA, USA) (*see* **Note 35**). A simplified assay workflow is provided in Fig. 6.

3.9.1 Preparation of Samples

1. Allow samples to thaw at RT for 1 h prior to the start of the assay.
2. Once fully thawed, centrifuge all samples at 13,200 rpm for 10 min (*see* **Note 36**).
3. Dilute the samples in two separate 96-well plates, one for the 4-Plex and one for the 5-Plex, placing the samples in the wells designated in the plate layout.
4. For the 5-Plex assay, dilute 3 μL of sample in 297 μL of serum-free diluent and mix thoroughly to achieve a dilution of 1:100.
5. For the 4-Plex assay, dilute 3 μL of the 1:100 dilution (*see* **step 4**) further in the second 96-well plate with 297 μL of serum-free diluent to achieve a dilution of 1:10,000.

3.9.2 Preparation of Standards

1. Prepare the standard/control diluent (provided) for the 4-Plex kit by adding 25 mL serum-free diluent to a sterile 15 mL Falcon tube.
2. Prepare the standard/control diluent for the 5-Plex kit by adding 1 mL of serum-based diluent to 24 mL of serum-free diluent to a sterile 15 mL Falcon tube and vortexing for 10 s.
3. Reconstitute the lyophilized standards (provided) by adding 500 μL of the appropriate standard/control diluent as prepared in **steps 1** and **2** (*see* **Note 37**).
4. Gently vortex the reconstituted standards for 3 s and incubate on ice for 1 h.

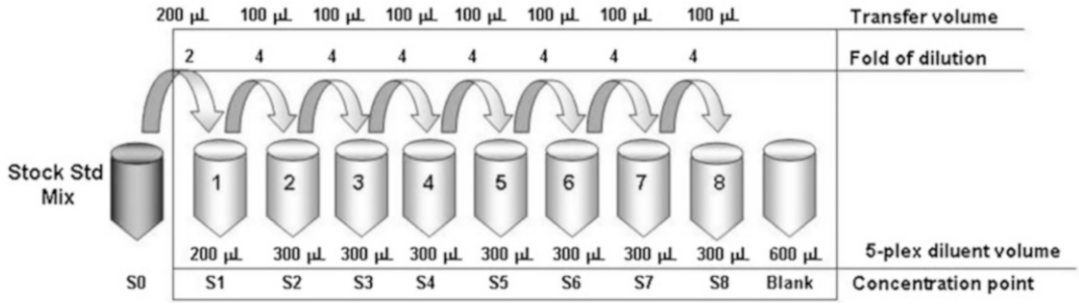

Fig. 7 Diagram of the 5-Plex Standard dilution Series to be made

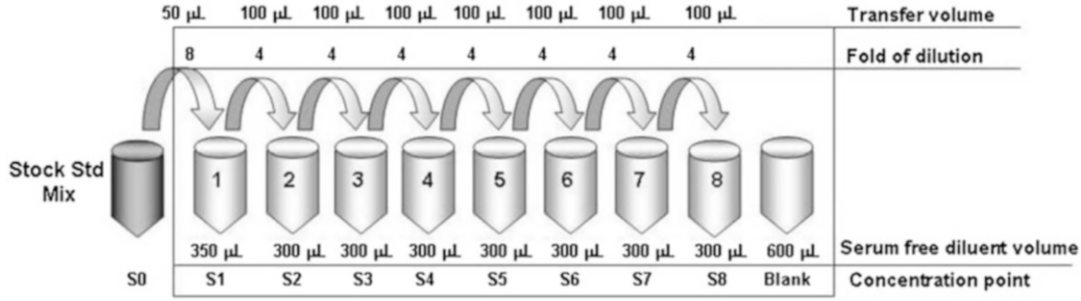

Fig. 8 Diagram of the 4-Plex Standard Dilution series to be made

3.9.3 Preparation of Controls

1. Reconstitute the first control vial with 1 mL of the 5-Plex standard/control diluent, and the second vial with 2 mL of the 4-Plex standard/control diluent.
2. Vortex each vial for 3 s.
3. Incubate each of the controls on ice for 1 h.

3.9.4 Preparation of Dilution Series

1. Prepare two sets of nine 1.5 mL Eppendorf tubes—one set for the 4-Plex and one set for the 5-Plex kits.
2. Label each tube according to its dilution and create the dilution series using Figs. 7 and 8 as a guide for the 5-Plex and 4-Plex, respectively. Use a clean pipette tip for each dilution and vortex each tube before preparing the next dilution (*see* **Notes 38–40**).

3.9.5 Preparation of Coupled Magnetic Beads

1. Vortex the coupled magnetic beads for 30 s at medium speed.
2. Dilute the magnetic beads by adding 125 µL of the beads (25×) to 5880 µL assay buffer in a sterile 15 mL Falcon tube covered with aluminum foil. Keep the beads on ice until use.

3.9.6 Assay Procedure

1. Before use, equilibrate the diluted standards, samples, and controls to RT for 20 min.
2. Pre-wet a clean 96-well plate with 100 μL of assay buffer (*see* **Note 41**) and wash the plate twice using the Bio-Plex Pro plate washer (*see* **Note 42**).
3. Vortex the coupled magnetic beads for 20 s.
4. Add 50 μL of the coupled magnetic beads to each well to be used (check plate template).
5. Wash twice.
6. Vortex the diluted standards and controls for 3 s.
7. Add 50 μL of the diluted standards and controls to the appropriate wells according to the plate template.
8. Mix the samples by pipetting and add 50 μL of each to the appropriate wells according to the plate template.
9. Cover the plate with a sealing tape and then foil.
10. Incubate the plate for 1 h at RT on a plate shaker (*see* **Note 43**).
11. During the incubation step, centrifuge the detection antibodies (10×) for 30 s to collect the full volume at the bottom of the vial.
12. Dilute the detection antibodies to a 1× concentration by adding 150 μL of the detection antibodies (10×) to 1350 μL of detection antibody diluent in a sterile 15 mL Falcon tube.
13. Following the incubation in **step 10**, remove the foil and plate sealer, and wash the plate three times.
14. Vortex the detection antibodies for 3 s and add 12.5 μL to each well.
15. Return the plate sealer and foil and incubate the plate for 30 min at RT on a plate shaker.
16. During the incubation step, centrifuge the streptavidin-PE (100×) vial for 30 s to collect the full volume at the bottom of the vial.
17. Dilute the streptavidin-PE to a 1× concentration by adding 30 μL of streptavidin-PE (100×) to 2970 μL of assay buffer in a sterile 15 mL Flacon tube.
18. Following the incubation in **step 15**, remove the foil and plate sealer, and wash the plate three times.
19. Vortex the streptavidin-PE for 10 s and add 25 μL to each well.
20. Return the plate sealer and foil and incubate the plate for 10 min at RT on a plate shaker.
21. Remove the foil and plate sealer and wash the plate three times.
22. Add 125 μL of assay buffer to each well.

23. Return the plate sealer and foil and incubate the plate for 30 s at 900 rpm on a plate shaker.
24. Keep the foil and plate sealer on the plate until ready for acquisition.

3.9.7 Acquisition

1. Switch on the Bio-Plex 200 system 30 min prior to use (*see* **Note 44**).
2. Calibrate the Bio-Plex 200 using the Bio-Plex Calibration kit and the manufacturer's instructions.
3. When the instrument is ready, remove the foil and plate sealer and load the plate.
4. Set the instrument to acquire and analyze the samples using the low RP1 target value using the 100 beads per region option.
5. Run the plate and export the results to excel once acquisition is complete.

4 Notes

1. Literature has shown that EDTA vacutainers are more successful in preserving MDSC, both the monocytic and granulocytic subsets, than sodium heparin vacutainers which are more routinely used for PBMC isolations [8–10].
2. BSA can be substituted for FBS in the preparation of MACS Buffer at the same concentration.
3. Depending on the density of the cell pellet, this volume can be increased to 5 mL to ensure that the 1:10 dilution in trypan blue and 1× PBS does not result in too many cells to count. This volume should then be accounted for in the calculation of the total cell count (*see* **Note 7**).
4. Make sure to count only PBMC (lymphocytes) and low-density neutrophils, not any other cells that may have separated with the PBMC fraction, such as platelets. Platelets appear similar in shape and color to PBMC, but are significantly smaller in size. Low-density neutrophils are considered as part of the granulocytic subset of MDSC and should this be counted [11, 12].
5. Using the Grid System displayed in Fig. 9, cells should be counted in each of the four outer counting blocks (labeled 1–4 in Fig. 9). The average of the four counts should then be used for further calculations. The average is taken to ensure that the cell count is a true representation of the population.
6. The adjustment of the microscope phase settings is used to distinguish between live and dead cells on the hemocytometer. Using Phase 1, live cells appear off-white with intact membranes. Once cells begin to die the membrane is compromised,

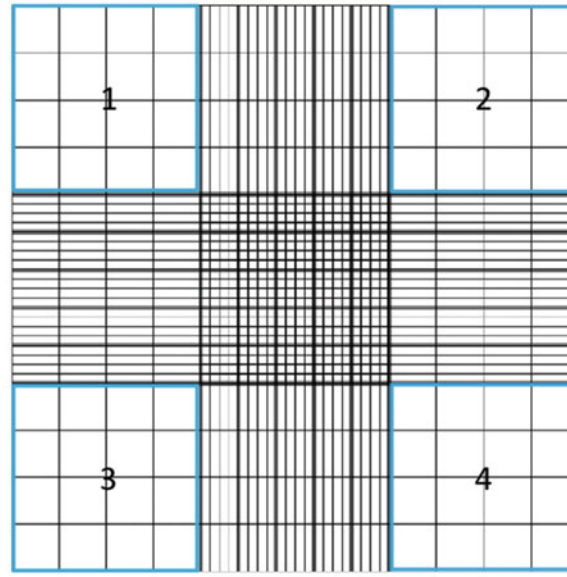

Fig. 9 Hemocytometer grid counting technique for accurate representations of the sample population

allowing for the entry of the Trypan Blue solution into the cell. As a result, dead or dying cells will appear blue under the Phase 0 setting of the microscope.

7. Cell counts can be calculated as follows:

   For example:

   To determine the cell number/mL:

   Average number of live cells counted = 33
   Average number of dead cells counted = 3
   Dilution factor (df) = 10
   Resuspension Volume (RV) = 5 mL

$$\text{Total Number of PBMC} = (\text{Live Cells} + \text{Dead Cells}) \times 10^4 \times \text{df} \times \text{RV}$$

   ∴The total number of PBMC is 1.8×10^7

   In order to determine the cell count/mL, simply divide the total number of PBMC by the resuspension volume.

   To determine the viability of the cells isolated:

$$\text{Viability} = \left(\frac{\text{Live Cell Count}}{\text{Total Cell Count}}\right) \times 100$$

   ∴The viability of the PBMC isolated in this example is 91.7%.

8. Traditionally, at least 1×10^6 PBMC are expected from 1 mL of peripheral blood; therefore, from an isolation using 32 mL of peripheral blood, a yield of approximately 3.2×10^7 PBMC

should be achieved. Some patients who have autoimmune diseases, for example, may have significantly lower yields, but this should not be a problem for infectious disease like active TB disease. The viability achieved should be no less than 90% for cells to be used for cell culture work, and no less than 85% for other downstream assays. Factors that could reduce the viability of isolated PBMC include leaving the cells in contact with Ficoll-Paque for too long (e.g., not removing the PBMC layer immediately after the density centrifugation step).

9. Person's collecting BAL samples during bronchoscopy procedures should always wear a BSL3 grade face mask to protect themselves from inhaling any possible contaminants or pathogens, regardless of the status of the patient. Bronchoscopies always have the potential to generate aerosols which may be harmful to the clinicians and person collecting the sample, especially when a patient diagnosis is undetermined, and the procedure is exploratory.
10. Each and every sample collected from the site of disease (including BAL, pleural fluid, and pericardial fluid) should be processed under BSL3 conditions owing to the potentially highly infectious nature of the sample. These samples are collected from the site of disease when a patient is diagnosed with active *Mycobacterium tuberculosis* infection which leads to Tuberculosis in the site in question. These samples could also be collected from the suspected site of disease in patients who lack a definitive diagnosis. Patients recruited with other lung diseases may potentially have secondary bacterial infections accompanying their primary disease or infection, and as such, aerosols pose a threat to person who process the sample outside of containment facilities like the BSL3.
11. The 70 μM strainer removes artifacts from the BAL sample like mucus or large cell clumps.
12. Stored fluid, be it from BAL, pleural fluid, or pericardial fluid, may be used for downstream applications like proteomics analyses, Luminex, and ELISA.
13. Approximately 1.8×10^7 total BAL cells can be expected to be isolated from 50 mL of BAL on average, ranging from 1.5×10^6–6.8×10^7 (viability of 98%). The largest proportion of these cells are alveolar macrophages (±90%), while neutrophils and lymphocytes can also be present.
14. On average, pleural fluid from patients with pleural TB have approximately 2×10^6 cells/mL, with 50–90% of these cells being lymphocytes [13].
15. Ensure that standard tissue culture plates are used and not those designated as low-adherence plates. Plates with

low-adherence properties will not separate the adherent cells from the non-adherent cells due to protective coating of the plate surfaces.

16. When isolating MDSC from human BAL samples, often times flow cytometry-based isolation techniques are detrimental to experimental outcomes. This is due to the extremely high autofluorescent nature of carbon-loaded macrophages. One of the risk factors associated with increased susceptibility to TB includes cigarette smoking and exposure to biomass fuel inhalation. Particularly in South Africa, a large proportion of study participants (whether diagnosed with active TB disease or other lung diseases) are known smokers or live in poorly ventilated dwellings which causes the accumulation of carbon within the alveolar macrophages of the lungs. These carbon-loaded macrophages become extremely autofluorescent and make flow cytometry near impossible. In addition to this, not all BSL3 facilities are fortunate enough to contain a flow cytometry-based sorting machine like the FACSJazz™ which makes MACS Microbead isolations a favorable alternative.
17. When performing MACS Microbead isolations, centrifugation steps should ALWAYS be performed at 4 °C. Microbeads should be kept in the fridge until the moment of use, and buffers should be kept on ice throughout the duration of the isolation. Failure to use cold reagents and centrifugation temperatures may quickly result in nonspecific binding of Microbead targets, further resulting in impure isolated cellular populations.
18. For subsequent isolations, use anti-HLA-DR Microbeads (Catalog number: 130-046-101) and anti-CD33 Microbeads (Catalog number: 130-045-501) instead of anti-CD3 microbeads (Catalog number: 130-050-101).
19. Column choices are event-specific and should be decided upon following cell counts (of PBMC, BAL, or pleural fluid). The most common column types are the MS and LS columns, the former used for smaller cell numbers and the latter used for larger cell numbers (Table 1). Column capacity may decrease when separating cells larger than lymphocytes (e.g., alveolar macrophages), so provision should be made to use two columns instead of one. Based on previous experience, should a sample contain many alveolar macrophages, as found in a BAL sample, the column does not get blocked. However, should a BAL sample contain many giant, multinucleated cells the columns have been known to get blocked. In this instance, overnight adherence of the sample should occur prior to the MACS Microbead isolation, whereby the giant cells may be excluded from the non-adherent fraction and the isolation on this fraction may occur without the risk of the column getting blocked.

Table 1
Miltenyi biotech column specifications

	MS columns		LS columns	
	Max number of labeled cells	Max number of total cells	Max number of labeled cells	Max number of total cells
Manual use	1×10^7 cells	2×10^8 cells	1×10^8 cells	2×10^9 cells

Table 2
Average cell yield and viability from 18 mL peripheral blood and 50 mL BAL fluid

	Average cell numbers						
	Total PBMC	CD3+	CD3-	HLA-DR+	HLA-DR-	CD33+	CD33-
Blood							
Expected (cell/mL)	2×10^6	1.3×10^6	0.7×10^6	0.05×10^6	1.5×10^6	0.27×10^6	0.83×10^6
Expected (% PBMC)	100	62	38	2.5	75	13.5	41.5
Viability (%)	95	96	96	92	93	90	91
BAL							
Expected (cell/mL)	4×10^5	0.28×10^5	3×10^5	0.76×10^5	0.68×10^5	0.28×10^5	0.58×10^5
Expected (% BAL)	100	7	75	19	17	7	14.5
Viability (%)	98	98	95	96	96	93	93

20. Always allow for the columns to empty completely before adding more buffer or a cell suspension.
21. Depending on the design of the experiment, sorted cellular fractions that are not required for the isolation of MDSC may be stored or discarded.
22. The average cell yields and viability for CD3+ isolated T cells and isolated MDSC for both peripheral blood and BAL fluid samples are given below in Table 2.
23. A standard coculture 96-well plate layout example is given below in Fig. 10.
24. MDSC and T cells should be cocultured in a 1:1 ratio. It should, however, be taken into consideration that the

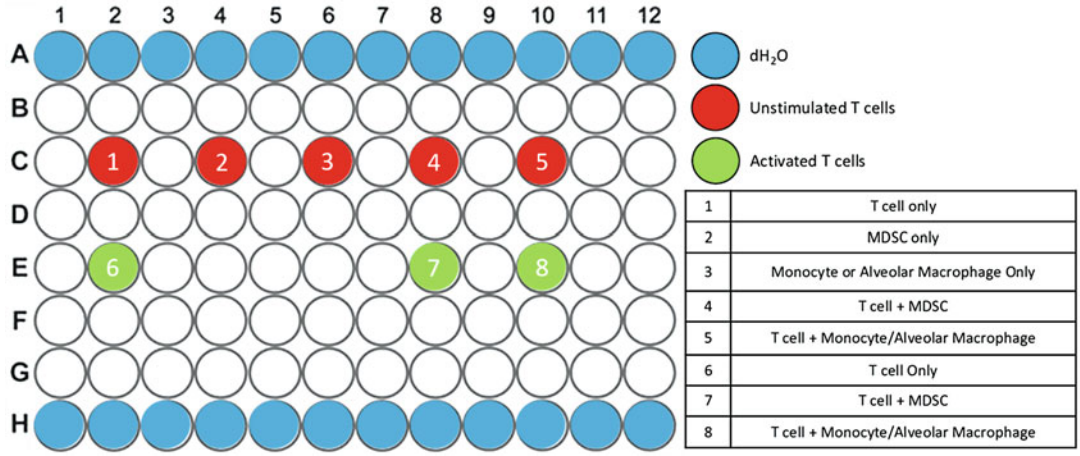

Fig. 10 Representative 96-well coculture plate layout

suppressive potential of MDSC might differ for various disease types or stages. Therefore, individual experiments should conduct a titration to determine the most appropriate coculture ratio.

25. For functional investigations, PPD stimulations should always be compared to an unstimulated control well as a reference standard, so be sure to always include one unstimulated well for every PPD stimulated condition.
26. The incubation period should be optimized or adjusted for each individual experiment depending on the experimental design and needs. Incubation times could be 24, 48, or 72 h.
27. Brefeldin A is used in culture when intracellular cytokine production is to be assessed. BFA is a protein transport inhibitor which leads to the accumulation of cytokines within the Golgi complex/endoplasmic reticulum during cell activation, preventing their extracellular release and allowing for cell-specific cytokine production analysis.
28. The supernatants of the unstimulated and stimulated cells can be used for downstream assays like Luminex or ELISA, and demonstrate the extracellular cytokines and/or chemokines being produced by the cells during coculture.
29. A trans-well coculture plate allows for the separation of two cell types to determine whether these cells have contact-dependent or -independent requirements for interaction (Fig. 11).
30. When deciding the speed to centrifuge cells, it is required that fresh/viable cells (unfixed) be centrifuged at 250 × *g* and fixed cells be centrifuged at 400 × *g*.
31. Users should be appropriately trained to work on any flow cytometer, and as such should be familiar with their use and

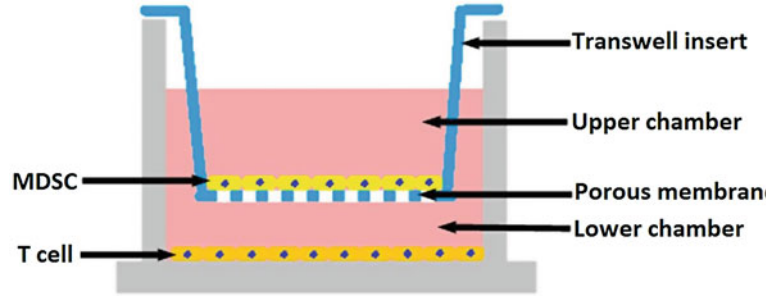

Fig. 11 Trans-well coculture plate design. T cells are loaded in the lower chamber, while MDSC are loaded in the upper chamber, above the trans-well insert with its porous 0.4 μm membrane (Adapted from https://figshare.com/articles/_Illustration_of_the_transwell_co_culture_system_and_the_chemotaxis_assay_/320967/1)

setup as this will not be outlined in this chapter. Antibodies used should first be titrated to determine the correct concentration to suit the staining of the sample type of interest. Only once the appropriate concentration has been determined and the purity checks be completed.

32. Depending on the number of markers being investigated during the purity check, cells should be resuspended in 50 μL FACS Buffer per single stain condition to be investigated. For example, the PBMC fraction needs to be split into four tubes (unstained, stain with CD3, stain with CD14, combined stain with HLA-DR and CD33); therefore, the cells should be resuspended in 200 μL before being split into separate 5 mL Falcon flow tubes.
33. Acquisition of cells should preferably be on the same day as the staining occurred; however, should the cells be fixed they can be run the following day. Fresh (unfixed cells) should most certainly be acquired on the same day.
34. Based on the user's desires, the flow rate, number acquired events, and stopping gate may all be set to meet the user's needs. It is recommended however that the flow rate be set to medium and not fast to ensure a consistent stream of single cells through the flow cell. The general number of acquired events ranges from 10,000 for unstained control samples, to 100,000 for rare cell populations, to all cells where the entire population frequency is to be determined and acquisition time is not a problem. Generally, 100,000 events are acquired for purity check samples.
35. The following sample types may be used for Luminex Immunoassays: serum, plasma, or culture supernatant. Take care to take the sample type into consideration when preparing the standards and controls (*see* **Note 37**).

36. The supernatants of samples used for Luminex are centrifuged in order to pellet any precipitate within the sample. Make sure to avoid this pellet when preparing the sample dilutions as the precipitate can interfere with the assay readout.
37. Lyophilized standards should be reconstituted with the appropriate standard/control diluent **only** when serum or plasma samples are used. However, when the sample types are culture supernatants, the standards must be reconstituted in the **culture medium** used (e.g., cRPMI) instead of the serum-free and serum-based diluents.
38. The S0 standard to be used for the 5-Plex and 4-Plex is the standard/control diluent prepared for each in Subheading 3.9.2. All standards, controls, and dilution series are reconstituted/prepared on the same day as the assay and are kept on ice until ready for use.
39. The blank (0 pg/mL) should be made up of the appropriate standard/control diluent for the assay (or culture media where culture supernatants are used) as this is used to format the software to automatically subtract the background median fluorescent intensity (MFI) values from all other values to obtain a representative value lacking background influence.
40. For accurate results, all standard dilutions, blanks, and controls should be run in duplicate, followed by the diluted samples of interest which can be run in duplicate or singlet depending on the study-specific criteria or available space on the 96-well plate (Fig. 12).
41. When performing Luminex Immunoassays, avoid making bubbles in the 96-well plate as this will interfere with the sample labeling and the instrument.
42. During the Luminex Immunoassay, all wash steps refer to washing using the Bio-Plex Pro plate washer. Manual washing is not performed because the magnetic beads allow for the collection of the labeled targets at the bottom of the plate during washing.
43. Luminex Immunoassays should be performed in the dark wherever possible. This includes wrapping the magnetic beads in foil, and the 96-well plate during every incubation step as the reagents used are highly light sensitive.
44. The Bio-Plex 200 system is a suspension array system, using the dual-laser, flow-based microplate reader system (Bio Rad). The beads from each sample are acquired individually and analyzed using the Bio-Plex Manager™ Software version 6.1 according to the recommended settings. The system requires a 30 min warm-up to ensure the lasers are warm and ready for use.

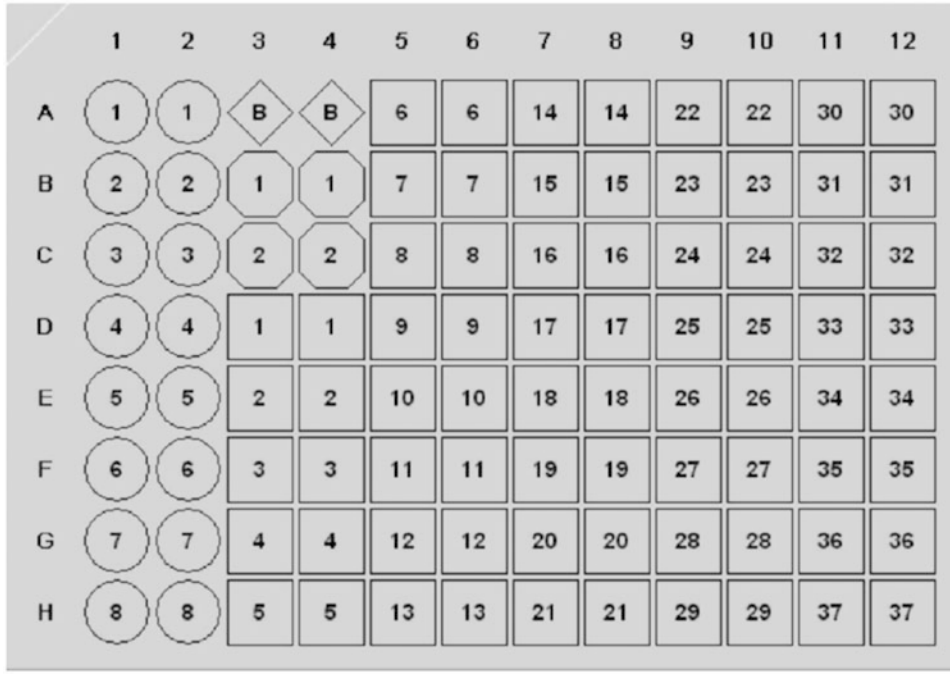

Fig. 12 96-Well plate layout template for the organization of standards (circles), controls (blanks—diamonds; controls—octagons), and samples (squares). Depending on the Bio-Plex 200 system, duplicates may also be placed vertically instead of horizontally as depicted in the example

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Chapter 13

High-Dimensional Analysis of Circulating and Tissue-Derived Myeloid-Derived Suppressor Cells from Patients with Glioblastoma

Tyler J. Alban, Defne Bayik, Alvaro G. Alvarado, Harley I. Kornblum, and Justin D. Lathia

Abstract

We will first describe analysis of MDSC subsets from patient tumors with multicolor flow cytometry. The key components of this methodology are to obtain viable single cell suspensions and eliminate red blood cell contamination.

Key words Myeloid-derived suppressor cells (MDSCs), CyTOF, Flow cytometry, Glioblastoma (GBM), Peripheral blood, Patient tumor, Immune analysis

1 Immuno-phenotyping of MDSC in GBM with Flow Cytometry

Elevated levels of myeloid-derived suppressor cells (MDSCs) have been identified across multiple cancers including glioblastoma (GBM), and much of our work has sought to identify if the increased circulating MDSCs in GBM patients were specific to GBM or a variety of primary and metastatic brain tumors [1–3]. After development of these protocols on patient peripheral blood samples and tumor samples, we utilized these techniques in a phase 0/1 clinical trial aimed at targeting MDSCs via low-dose chemotherapy [4]. Furthermore, we have now expanded on these techniques to capture transcriptional information of MDSCs from GBM patients by using cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq). In this approach, we identify MDSCs by protein expression, similar to standard practice in flow cytometry and CyTOF, and subsequently allow for the analysis of their RNA expression profile. Lastly, it is important to note that our studies primarily focus on banked blood and tissue samples that

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have been frozen prior to analysis removing the PMN-MDSC population; however, these protocols can be performed on fresh samples if available, allowing for comparisons of PMN-MDSCs and M-MDSCs.

1.1 Introduction

Recent studies have established that the frequency of MDSCs differ between peripheral circulation and tumor tissue in patients with breast and cervical cancer [5, 6]. Furthermore, functional assessment of MDSCs isolated from different organs in murine models indicates that the tumor microenvironment can influence immunosuppressive properties [7–9]. Therefore, it is important to analyze MDSC subset pattern at the site of tumor to evaluate their contribution to tumorigenesis. Monocytic (M)-MDSC and polymorphonuclear myeloid-derived suppressor cells (PMN)-MDSC subsets express different surface receptors that could be used to determine their levels in human tumor tissue. For phenotypic characterization of these cells in human patients, we designed a marker panel based on the published guidelines [10] and included LOX-1 to distinguish between PMN-MDSCs and granulocytes as demonstrated in Fig. 1.

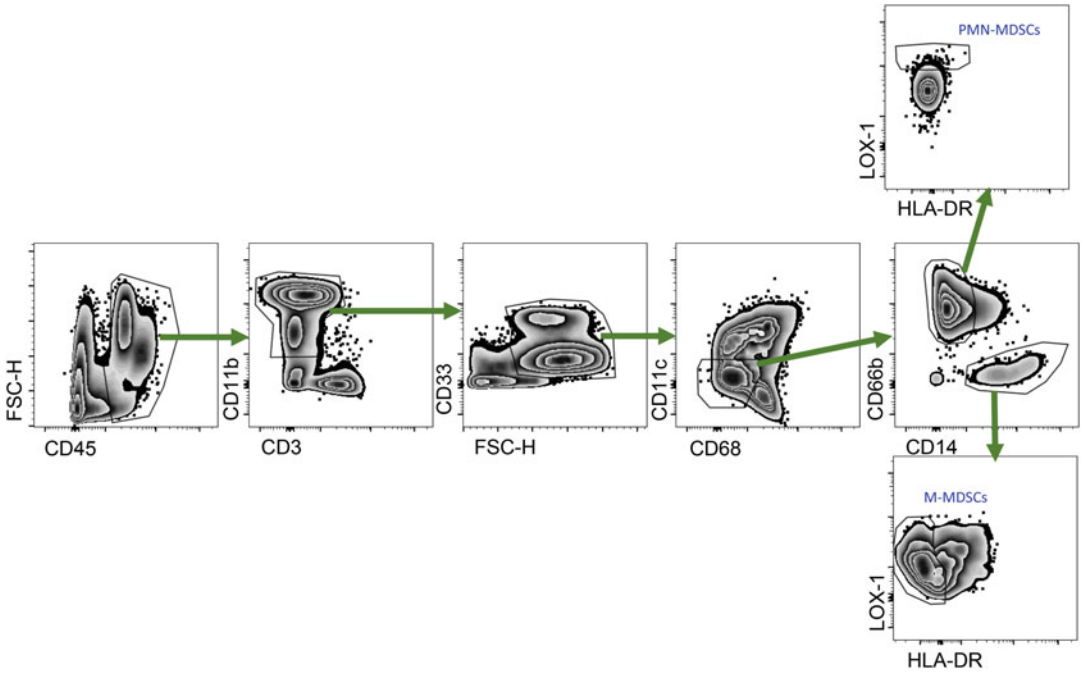

Fig. 1 Flow cytometry gating strategy for PMN-MDSCs and M-MDSCs from GBM patients samples via flow cytometry. M-MDSC: CD45+CD3-CD33+CD11b+CD68-CD11c-CD14+CD66b-HLA-DR- and PMN-MDSC: CD45+CD3-CD33+CD11b+CD68-CD11c-CD66b+CD14-LOX1

1.2 Materials

1. FACS Buffer: PBS, 2% BSA, 2 mM EDTA.
2. Collagenase IV.
3. 10× Red Blood Cell Lysis (RBC) Buffer: ammonium chloride, potassium carbonate, EDTA.
4. LIVE/DEAD™ Fixable Blue Dead Cell.
5. FcR blocking reagent.
6. Brilliant Stain Buffer.
7. Anti-human CD68 antibody.
8. Anti-human CD3 antibody.
9. Anti-human LOX1 antibody.
10. Anti-human CD11b antibody.
11. Anti-human CD33 antibody.
12. Anti-human CD11c antibody.
13. Anti-human HLA-DR antibody.
14. Anti-human CD45 antibody.
15. Anti-human CD14 antibody.
16. Anti-human CD66b antibody.
17. Intracellular Fixation Buffer.
18. Permeabilization Buffer.
19. Multicolor Flow Cytometer.

1.3 Methods

1.3.1 Digestion

1. Transfer fresh tumors to a 10 cm Petri dish.
2. Cut 1 mm pieces with a razor blade.
3. Transfer tumor pieces to a 50 mL falcon and add 5 mL Collagenase IV (*see* **Note 1**).
4. Incubate at 37 °C on a rotator for 1 h.
5. Place a 40 μ strainer on a new 50 mL falcon.
6. Pour Collagenase IV/tumor mixture to the strainer.
7. Use the back of a 1 mL syringe to smash the big places on the strainer.
8. Wash the strainer with 30 mL PBS.
9. Centrifuge at 400 × *g* for 5 min.
10. Aspirate the supernatant.
11. Prepare 2 mL 1× RBC buffer by diluting in Q water.
12. Resuspend the tissue in 2 mL 1× RBC buffer.
13. Incubate at room temperature for 4–5 min
14. Add 30 mL PBS to inhibit the reaction.
15. Centrifuge at 400 × *g* for 5 min.
16. Aspirate the supernatant.

1.3.2 Staining

1. Resuspend the cell pellet in 500 μL PBS supplemented with 1:1000 diluted LIVE/DEAD™ Fixable Blue Dead Cell.
2. Incubate on ice for 10 min.
3. Add 5 mL of PBS to inhibit the reaction.
4. Centrifuge at 400 × *g* for 5 min.
5. Aspirate the supernatant.
6. Resuspend the pellet with 500 μL FACS buffer supplemented with 1:100 diluted human FcR blocking reagent.
7. Incubate on ice for 15 min.
8. Add 5 mL of FACS to inhibit the reaction.
9. Centrifuge at 400 × *g* for 5 min.
10. Aspirate the supernatant.
11. Prepare the antibody cocktail in 1× Brilliant Stain Buffer with FACS buffer (*see* **Note 2**).
12. Resuspend the pellets in 500 μL antibody cocktail.
13. Incubate for 20 min on ice protecting from light.
14. Add 5 mL of FACS to inhibit the reaction.
15. Count the number of cells.
16. Centrifuge at 400 × *g* for 5 min.
17. Aspirate the supernatant.
18. Resuspend the cells in 500 μL fixation buffer (*see* **Note 3**).
19. Store cells in a refrigerator (*see* **Note 4**).
20. Before analysis, add 5 mL FACS buffer.
21. Centrifuge at 400 × *g* for 5 min.
22. Resuspend the pellets in FACS buffer at a concentration 1–2 million/mL.
23. Set up the compensation panel in the multicolor flow cytometer (*see* **Note 5**).
24. Gate on the target MDSC population from Single cells/Live cells based on the following strategy: As demonstrated in Fig. 1.
    (a) M-MDSC: $CD45^+CD3^-CD33^+CD11b^+CD68^-CD11c^-CD14^+CD66b^-HLA\text{-}DR^-$.
    (b) PMN-MDSC: $CD45^+CD3^-CD33^+CD11b^+CD68^-CD11c^-CD66b^+CD14^-LOX1^+$.
25. Acquire desired number of MDSC subsets with the flow cytometer.

1.4 Notes

1. For GBM tissue, we have optimized Collagenase IV digestion to generate single cells with 90% viability. Maximum tumor size processed with this procedure was 2 cm. DNase I can be included in the cocktail to reduce clumping. Depending on the tumor type, dissociation method should be optimized.
2. We recommend to perform antibody dilution to determine the optimum concentration for each antibody. Fluorescence-minus-one or isotype control staining should be applied to assess the specificity of the signal for each antibody. This will be important for the calculation of the Brilliant Stain Buffer volume in the staining cocktail. Our optimized panel contains the following fluorophores:

   PerCP-Cy5.5 anti-human CD68 antibody, APC-Cy7 anti-human CD3 antibody, APC anti-human LOX1 antibody, PE-Cy7 anti-human CD11b antibody, PE/Dazzle 594 anti-human CD33 antibody, V650 anti-human CD11c antibody, V570 anti-human HLA-DR antibody, V500 anti-human CD45 antibody, Pacific Blue anti-human CD14 antibody, and PE anti-human CD66b antibody.

   These are subject to change depending on investigator needs. When more than one violet fluorophore is used, Brilliant Stain Buffer should be included to reduce the overlap between the dye conjugates. We have determined that CD3 is sufficient for our purposes to exclude lymphocytes. However, CD19 and CD56 can be included on the same channel for lineage gating.
3. For live analysis, cells should be resuspended in FACS buffer at a concentration of 1–2 million cells/mL. They should be kept on ice during analysis.
4. Samples can be stored in fixation buffer for up to 3 days. Subsequent intracellular marker staining can be performed following permeabilization of the cells. Cells should be washed with 2 mL of 1× permeabilization buffer, divided into two fractions and resuspended in 250 μL permeabilization buffer containing the antibodies of interest or corresponding isotype controls. Cells should be incubated for 20 min at room temperature protecting from light. Following another wash step with permeabilization buffer, pellets should be resuspended in PBS.
5. We use compensation beads freshly stained with individual fluorophores. However, peripheral blood mononuclear cells can be stained with target fluorophores to use for compensation.

2 High-Dimensional Phenotyping of Human Circulating and Tissue-Derived MDSC in Glioblastoma by Mass Cytometry Time of Flight (CyTOF)

Abstract: *In this section, we will outline the necessary methods for the staining and analysis of MDSC populations from patient peripheral blood mononuclear cells (PBMCs) or tissue by CyTOF. This powerful method allows for the staining of both cell surface and intracellular markers, thus enabling the examination of several functional markers once the populations have been correctly identified.*

Keywords: *Mass cytometry time of flight (CyTOF); Glioblastoma (GBM); Immune profiling; Myeloid-derived suppressor cells (MDSCs)*

2.1 Introduction

Mass cytometry time of flight (CyTOF) allows for the assessment of protein expression of several markers on a cell-by-cell basis. It uses antibodies conjugated with heavy metals and each cell is atomized when it passes through the cytometer so that the metals bound to it can be read by mass spectrometry. Recent approaches for the treatment of cancer have focused on immune checkpoints and immunotherapies targeting key components that regulate immune function. In order to analyze the abundance of these populations and to be able to assess the functionality of the immune populations identified (e.g., expression of costimulatory molecules, exhaustion markers), we developed a 28 human antibody panel, as shown in Fig. 2.

2.2 Materials

1. Maxpar® Cell Staining Buffer (Fluidigm).
2. CyTOF ready conjugated antibodies.
3. Cell-ID™ Cisplatin (Fluidigm).
4. Maxpar® Fix and Perm Buffer (Fluidigm).
5. Cell-ID™ Intercalator-Ir (Fluidigm).
6. 4% PFA.
7. Methanol.
8. RPMI.
9. Fresh Milli-Q water.

2.3 Methods

2.3.1 Tissue Preparation

1. Transfer fresh tumors to a 10 cm Petri dish.
2. Cut 1 mm pieces with a razor blade.
3. Transfer tumor pieces to a 50 mL falcon and add 5 mL Collagenase IV.
4. Incubate at 37 °C on a rotator for 1 h.
5. Place a 40 μm strainer on a new 50 mL falcon.
6. Pour Collagenase IV/tumor mixture to the strainer.
7. Use the back of a 1 mL syringe to smash the big places on the strainer.

A.

label	signal_di	target	Clone #	Cat #
209Bi	226	CD11b (Mac-1)	ICRF44	3209003B
170Er	590	CD3	UCHT1	3170001B
167Er	204	CD27	L128	3167006B
165Ho	1000	CD61	VI-PL2	3165010B
164Dy	133	CD15 (SSEA-1)	W6D3	3164001B
163Dy	60	CD56 (NCAM)	NCAM16.2	3163007B
146Nd	508	CD8a	RPA-T8	3146001B
159Tb	500	CD11c	Bu15	3159001B
158Gd	142	CD33	WM53	3158001B
169Tm	100	CD45RA	HI100	3169008B
89Y	800	CD45	HI30V	3089003B
153Eu	290	CD7	CD7-6B7	3153014B
151Eu	150	CD14	M5E2	3151009B
150Nd	70	CD161	HP-3G10	3159004B
149Sm	333	CD66a	CD66a-B1.1	3149008B
148Nd	70	CD16	3G8	3148004B
147Sm	300	CD20	2H7	3147001B
145Nd	70	CD4	RPA-T4	3145001B
143Nd	575	CD25 (IL-2R)	2A3	3149010B
142Nd	232	CD19	HIB19	3142001B
141Pr	216	CD196 (CCR6)	11A9	3141014A
139La	1000	CD107a (LAMP1)	H4A3	3151002B
174Yb	3000	HLA-DR	L243	3174001B
155Gd	167	CD279 (PD-1)	EH12.2H7	3155009B
176Yb	200	CD127 (IL-7Ra)	A019D5	3176004B

B.

PMN-MDSCs (minimum CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14-, CD11b +, CD15+) (better CD45+, CD66a-, CD3-, CD19-, CD56-, HLADR$^{low/-}$, CD11b+, CD33+, CD14-, CD15+)

M-MDSCs (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, HLADR$^{low/-}$, CD11b+, CD33+, CD14+, CD15-)

e-MDSCs (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, HLADR-, CD33+)

Classical monocytes (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14high, CD16-, HLA-DR+)

Intermediate monocytes (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14high, CD16+)

Non-classical monocytes (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14low/+, CD16+)

Myeloid dendritic cells (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14-, CD11b+, CD11c+, HLA-DR+)

Monocyte-derived dendritic cells (CD45+, CD66a-, CD3-, CD19-, CD20-, CD56-, CD14+, CD11b+, CD11c+, HLA-DR+,)

Natural killer cells 1 (CD45+, CD66a-, CD3-, CD19-, CD20-, CD14-, CD11c-, CD56-, CD16+)

Natural killer cells 2 (CD45+, CD66a-, CD3-, CD19-, CD20-, CD14-, CD11c-, CD56+, CD16-)

Granulocytes (CD3-, CD20-, CD 14-, CD11c-, CD45-, CD66a+)

Naïve CD8+ T cells (CD45+, CD66a-, CD3+, CD8a+, CD45RA+, CD27+, CD127+)

Effector T killer cells (CD45+, CD66a-, CD3+, CD8a+, CD45RA+, CD27-)

Activated T killer cells (CD45+, CD66a-, CD3+, CD8a+, HLA-DR+)

Cytotoxic T cells (CD45+, CD66a-, CD3+, CD8a+, CD107a+)

Memory T killer cells (CD45+, CD66a-, CD3+, CD8a+, CD45RA-, CD27+)

DP T cells (CD45+, CD66a-, CD3+, CD8a+, CD4+, CD27+, CD161+)

Naïve CD4+ T cells (CD45+, CD66a-, CD3+, CD4+, CD45RA+, CD25-, CD127+, CD27+)

Activated T helper cells (CD45+, CD66a-, CD3+, CD4+, HLA-DR+)

Effector T helper cell (CD45+, CD66a-, CD3+, CD4+, CD45RA+/-, CD25+, CD127-, CD27-)

Effector regulatory T helper cells (CD45+, CD66a-, CD3+, CD4+, CD45RA-, CD25+, CD127-)

Resting regulatory T helper cells (CD45+, CD66a-, CD3+, CD4+, CD45RA+, CD25+, CD127-)

Memory T helper cells (CD45+, CD66a-, CD3+, CD4+, CD45RA-, CD25+, CD127+, CD27+)

Th17 cells (CD45+, CD66a-, CD3+, CD4+, CD161+, CD196+)

Naïve B cells (CD45+, CD66a-, CD3-, CD19+, CD20+, HLA-DR+, CD27-)

Plasma B cells (CD45+, CD66a-, CD3-, CD19+, CD20-, HLA-DR-, CD27+)

Memory B cells (CD45+, CD66a-, CD3-, CD19+, CD20+, HLA-DR+, CD27+, CD196+)

Platelets (CD45-, CD61+)

Fig. 2 CyTOF antibody panel and population identification list. Antibodies along with catalog numbers, clones, and heavy metal tags used are presented (**a**). While the population identifications including PMN-MDSCs and M-MDSCs along with other populations which can be identified using the panel (**b**)

8. Wash the strainer with 30 mL PBS.
9. Centrifuge at 400 × *g* for 5 min.
10. Aspirate the supernatant.
11. Prepare 2 mL 1× RBC buffer by diluting in Milli-Q water.
12. Resuspend the tissue in 2 mL 1× RBC buffer.
13. Incubate at room temperature for 4–5 min.
14. Add 30 mL PBS to inhibit the reaction.
15. Centrifuge at 400 × *g* for 5 min.
16. Aspirate the supernatant.

2.3.2 Viability Staining

1. Resuspend the cell pellet in 1 mL PBS and add 1 μL of Cell-ID Cisplatin.
2. Vortex and incubate for 10 min at 37 °C.
3. Add 5 mL of media to inhibit the reaction.
4. Centrifuge at 400 × *g* for 5 min.
5. Aspirate the supernatant and resuspend in 1 mL of media.
6. Add 1 mL of 4% PFA.
7. Mix gently and incubate for 15 min at room temperature.
8. Add 10 mL of Cell Staining Buffer.
9. Centrifuge at 400 × *g* for 5 min.
10. Aspirate the supernatant.

2.3.3 Cell Surface Staining (Antibody Panel in Fig. 2)

1. Aliquot surface antibody mix to corresponding tubes. Volume will depend on the number of antibodies used (generally, 1 μL per antibody).
2. Resuspend cells in Cell Staining Buffer. Volume will depend on the number of samples and antibody mix. Staining total volume should be 100 μL.
3. Vortex samples at low speed.
4. Incubate for 30 min at room temperature.

2.3.4 Intracellular Staining

1. Add 2 mL of Cell Staining Buffer.
2. Centrifuge at 400 × *g* for 5 min.
3. Aspirate the supernatant.
4. Resuspend cells in minimal volume by vortexing at low speed.
5. Incubate for 5 min on ice.
6. Add 1 mL of cold (ice temperature) methanol.
7. Vortex at low speed and incubate for 20 min on ice.
8. Add 2 mL of Cell Staining Buffer.
9. Centrifuge at 800 × *g* for 5 min.

10. Aspirate the supernatant.
11. Resuspend cells in Cell Staining Buffer. Volumes will depend on antibody mix. Staining total volume should be 100 μL.
12. Add aliquots of intracellular antibody mix.
13. Vortex samples at low speed.
14. Incubate for 30 min at room temperature.
15. Add 2 mL of Cell Staining Buffer.
16. Centrifuge at 800 × *g* for 5 min.
17. Aspirate supernatant.
18. Repeat **steps 15–17**.
19. Resuspend cells in 1 mL of 1:1000 Cell-ID Intercalator diluted in Fix and Perm Buffer.
20. Vortex at low speed and incubate for 60 min at room temperature.
21. Add 2 mL of Cell Staining Buffer.
22. Centrifuge at 800 × *g* for 5 min.
23. Aspirate supernatant.
24. Resuspend in 2 mL of Fresh Milli-Q water.
25. Centrifuge at 800 × *g* for 5 min.
26. Aspirate supernatant.
27. Keep cells as a pellet in minimal volume on ice until ready for acquisition.
28. Acquire expression data using mass spectrometry.

2.3.5 Data Gating and Processing (Workflow Is Presented in Fig. 3)

1. Normalize CyTOF samples between runs using beads and the Nolan lab bead normalizer package (https://github.com/nolanlab/bead-normalization/wiki/Installing-the-Normalizer).
2. Using the output files from normalization use FlowJo to gate for live cells by first gating for intact cells (DNA1:Ir191^{+}/DNA2:IR193^{+}), then singlets (Cell_length^{low}/DNA1:Ir191^{+}), and lastly (Live-Dead:ln115^{-}). The final populations from this gating are single, live cells.
3. After gating for Live/single cells the next step is to gate for CD45+ cells which will be the focus of the downstream analysis. Gate the CD45+ population using FlowJo and save a separate FCS file for this population along with sample ID.
4. Using the live CD45+ cells from each sample these FCS files are then utilized as the starting sample for Nowicka et al., F1000

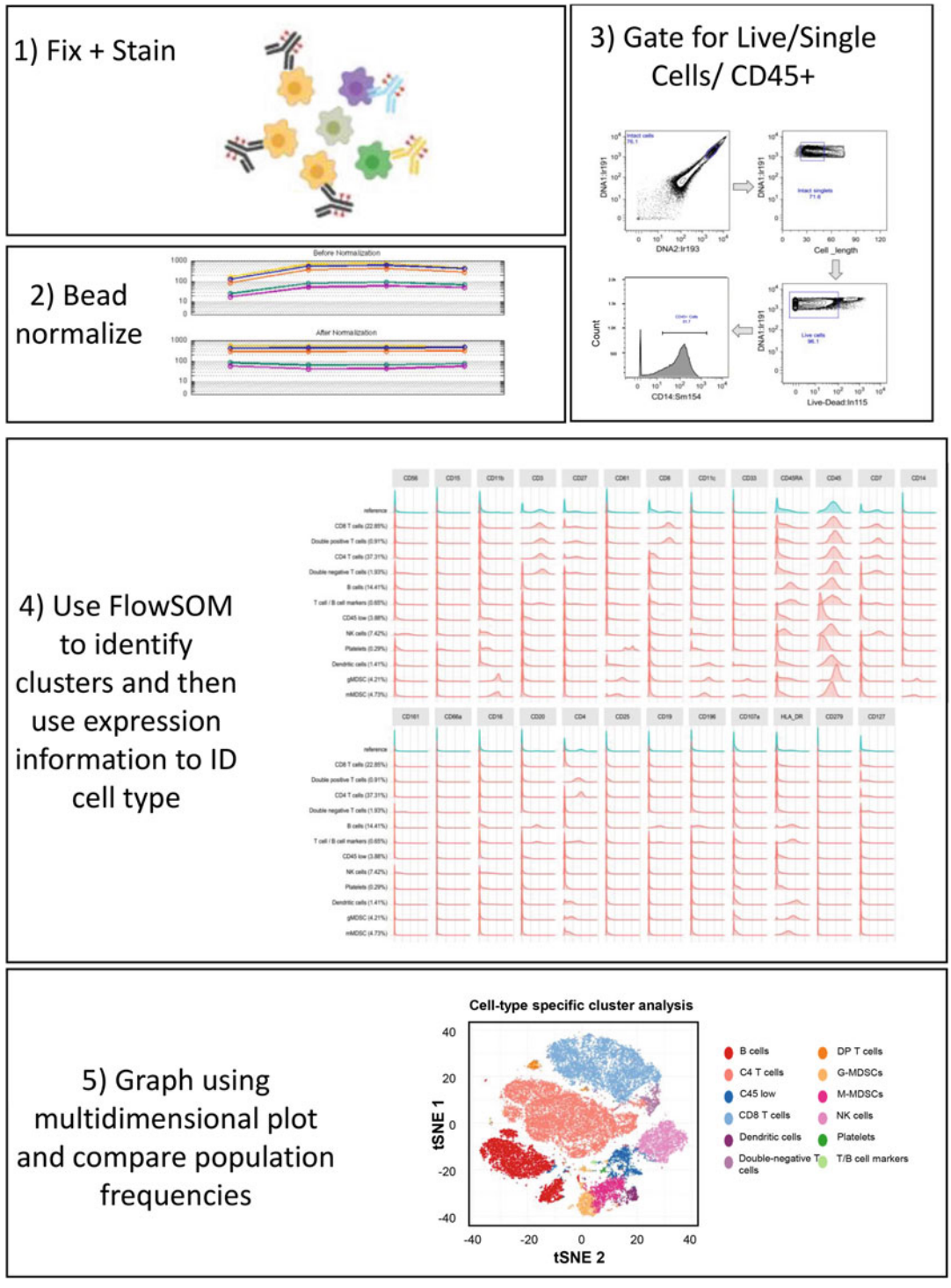

Fig. 3 CyTOF analysis figure demonstrating the workflow of normalizing, cleaning, identifying clusters, and comparing samples based on multidimensional plots

methods 2017 [11] workflow for sample comparison and analysis.

5. In this workflow, cells are clustered together based on the abundance of each antibody detected on the cell surface in an unbiased manner.
6. To name the clusters generate a heatmap based on the median marker intensities of each cluster and then determine the cell type. MDSCs can be identified on the heatmap by standard MDSC markers if present in the sample.

2.4 Notes

1. Antibodies to be used should be tested independently if they have not been validated for mass cytometry use. Fluidigm has a nice catalog of already conjugated antibodies but sometimes other proteins of interest are not available. We recommend looking for antibodies with the least amount of preservatives and clones that have proven efficacy in flow cytometry applications. Kits for conjugation to a variety of metals are available from Fluidigm.
2. We recommend antibody cocktails to be prepared the same day to prevent antibody crosslinking and false signals downstream. Always keep the antibody cocktails on ice or at 4 °C.
3. We aliquot the cisplatin reagent in volumes of 10 μL and keep it at −20 °C and always thaw it on ice before adding to the samples. Similarly, intercalator aliquots are kept at −20 °C and diluted fresh on fix and permeabilization buffer.
4. We typically start with 1 million cells per sample. This easily provides acquisition of 400–500 K cells. For downstream analysis, a complexity of 100 K cells allows for distinction of 25 immune populations based on the markers included in this panel. On other applications, we have worked with acquisitions as low as 25 K and obtained distinct clusters albeit with a lower resolution.
5. Depending on the cell number, the pellet will be very difficult to observe. This is especially true after methanol fixation. Extra care should be considered on these steps and we recommend stopping the aspiration a few millimeters above the bottom of the tube. Increased centrifuge speed aim at increasing cell recovery and ensuring pellet is not aspirated.
6. Incubation with the intercalator can be longer depending on equipment availability. If acquisition will not be performed the same day, then incubation should be at 4 °C. We have kept samples as long as 72 h with no issues. Alternatively, cells can be stained and the final pellets resuspended in 100 μL of water and kept for longer times. We have seen a decrease in signal after 1 week.

7. Milli-Q water should be as pure as possible. Metal accumulation will result in higher signals at the time of acquisition.
8. Always coordinate with the person doing the acquisition. Experts will be specific in how and in which volume you should provide them with your samples.
9. The number of clusters generated is a user preference in the Nowicka et al. workflow and if too few clusters are requested then multiple cell types can end up in the same cluster. This is easy to spot when you see T cell markers and myeloid markers in the same cluster. The opposite can also happen where you have generated too many clusters and you can have 5 T cell clusters that look identical. This may take some trial and error to determine the number of clusters that truly represent unique cell populations.

3 Identification of Circulating M-MDSC Using Cellular Indexing of Transcriptomes and Epitopes by Sequencing CITE-seq in Patients with Glioblastoma

Abstract: *In this section, we describe the analysis of the immune system and myeloid-derived suppressor cells (MDSCs) from patient-derived peripheral blood mononuclear cells (PBMCs). In this technique, we stain viable PBMCs with oligonucleotide tagged antibodies and subsequently perform single cell RNA-sequencing to identify MDSCs by protein expression while capturing their transcriptional profile.*

Keywords: *Myeloid-derived suppressor cells (MDSCs); Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq); 10× Genomics; Feature barcoding; Peripheral blood mononuclear cells (PBMCs); Single cell RNA-sequencing*

3.1 Introduction

Myeloid-derived suppressor cells (MDSCs) are primarily characterized by protein expression; however, the field of single cell RNA-sequencing has greatly expanded into immunology where cell types are now being determined by their gene expression rather than their protein expression [12, 13]. To define cell types by protein expression while still gaining transcriptional information, we propose the use of oligonucleotide barcoded antibodies for protein expression identification combined with single cell RNA-sequencing using the 10× genomics single sequencing platform [14, 15]. With this approach, MDSCs can be identified in the traditional sense via protein expression and subsequently their gene expression can be analyzed. In this section, we outline the use of CITE-seq to identify MDSC populations in GBM patient PBMCs, which are known to have a robust population of MDSCs [4, 16, 17].

3.2 Materials

1. Ficoll-Paque PLUS (GE Life Sciences).
2. Sepmate Tube (Catalog #85450 STEMCELL).
3. BD Vacutainer (REF: 366480G).
4. FBS (Fetal Bovine Serum).
5. DMSO (DIMETHYLSULFOXIDE).
6. RPMI 1640 Medium.
7. Phosphate Buffered Saline (PBS).
8. Phosphate Buffered Saline (PBS) 1% BSA.
9. 37° Water Bath.
10. 2 mL Eppendorf DNA LoBind Microcentrifuge Tubes (Fisher Scientific).
11. FcR blocking reagent (Miltenyi Biotec).
12. 10× genomics chromium controller (10× genomics).
13. Chromium™ Single Cell 3′ GEM, Library & Gel Bead Kit v3 (10× genomics).
14. Chromium™ Chip B Single Cell Kit, 48 rxns.
15. Chromium™ i7 Multiplex Kit, 96 rxns.
16. Chromium™ Single Cell 3′ Feature Barcode Library Kit, 16 rxns.
17. TotalSeq antibodies (Biolegend).
18. CellRanger Software (10× genomics).
19. Seurat R package (Satija lab https://satijalab.org/seurat/).

3.3 Methods

3.3.1 PBMC Isolation and Storage

1. Collect 5–10 mL of blood using green top BD vacutainer (REF: 366480G).
2. Spin collection tube at 500 × *g* for 15 min and remove and freeze serum in 2–4 0.5 mL aliquots and store at –80 °C.
3. Fill the bottom chamber of the Sepmate tube (Catalog # 85450 STEMCELL) with 15 mL of RT Ficoll (17-1440-02 GE life sciences).
4. Mix 10 mL of blood with 10 mL of RPMI 2%FBS and then pipette the mixture quickly down the side of the Sepmate tube to layer it on top of the Ficoll without mixing the two layers.
5. Gently place in the centrifuge and spin at 1200 × *g* for 15 min.
6. Pour off top layer into a new tube rapidly not to pour the Ficoll.
7. Add 15 mL of RPMI 2% FBS to the isolated supernatant from **step 4** and mix.
8. Centrifuge at 300 × *g* for 15 min.
9. Remove supernatant and save the pellet.

10. Add 5 mL of RPMI 2% FBS to the pellet and resuspend.
11. Spin at 200 × *g* for 15 min to remove platelets.
12. Discard supernatant and resuspend in 1–5 mL of media to count the cells.
13. Resuspend the cells at 2–4 million cells per mL in freezing media (90% FBS 10% DMSO) and freeze in an Isopropyl alcohol and a mechanical freeze chamber for 48 h at −80 °C before placing into the liquid nitrogen storage. In this method, freezing the samples prior to staining depletes PMN-MDSCs as previously demonstrated [18]. If fresh cells are utilized for this experimental protocol instead of frozen cells then the PMN-MDSCs may also be analyzed and note that no other steps will need to be changed.

3.3.2 Staining (Antibody Panel Table 1)

1. Remove cells from liquid nitrogen storage and rapidly thaw in a 37 °C water bath.
2. Transfer cells into a 5 mL round bottom tube with 3 mL RPMI.
3. Centrifuge cells at 400 rcf for 5 min and then remove supernatant.
4. Suspend pellet in 500 μL PBS and place in 2 mL DNA LoBind centrifuge tube.
5. Centrifuge cells at 400 rcf for 5 min and subsequently remove supernatant.
6. Suspend pellet in 50 μL of FCR block buffer (FCR block buffer = 100 μL Human FCR block with 900 μL of PBS 1% BSA). Do Not Use EDTA as it is incompatible with the system.
7. Incubate on ice for 10 min.
8. Prepare antibody mix-Depending on the total number of antibodies desired in the cocktail this step will vary. In general 1μl of each antibody will be used per sample and then FACS buffer will be added to a final volume of 50 μl per sample. Example of 32 Total-SeqB antibodies for 8 samples = (32 antibodies X 8 samples = 256 μl, Next add 170 μl FACS buffer to equal a total volume of 426 μl. From the 426 μl mixture of antibody and FACS buffer use 50 μl per sample.
9. Add 50 μL of each antibody mixture to each tube for a total volume of 100 μL during staining.
10. Incubate on ice for 30 min.
11. Add FACs buffer (PBS 1% BSA) 1.4 mL total volume.
12. Centrifuge 400 rcf 5 min and then remove supernatant.
13. Add 1.5 mL of FACs buffer for total of 1.5 mL volume.
14. Count cells.

Table 1
Total-seq B antibodies utilized for CITE-seq experiment. Each antibody has a unique oligo tag as labeled in the table and there are three isotype controls for normalization in the data analysis section

Category	Barcode	Specificity	Clone	Reactivity	Barcode sequence
TotalSeq™-B	161	CD11b	ICRF44	Human	GACAAGTGATCTGCA
TotalSeq™-B	390	CD127 (IL-7Rα)	A019D5	Human	GTGTGTTGTCCTATG
TotalSeq™-B	355	CD137 (4-1BB)	4B4-1	Human	CAGTAAGTTCGGGAC
TotalSeq™-B	81	CD14	M5E2	Human	TCTCAGACCTCCGTA
TotalSeq™-B	392	CD15(SSEA-1)	W6D3	Human	TCACCAGTACCTAGT
TotalSeq™-B	83	CD16	3G8	Human	AAGTTCACTCTTTGC
TotalSeq™-B	50	CD19	HIB19	Human	CTGGGCAATTACTCG
TotalSeq™-B	148	CD197 (CCR7)	G043H7	Human	AGTTCAGTCAACCGA
TotalSeq™-B	100	CD20	2H7	Human	TTCTGGGTCCCTAGA
TotalSeq™-B	85	CD25	BC96	Human	TTTGTCCTGTACGCC
TotalSeq™-B	154	CD27	O323	Human	GCACTCCTGCATGTA
TotalSeq™-B	7	CD274 (B7-H1, PD-L1)	29E.2A3	Human	GTTGTCCGACAATAC
TotalSeq™-B	88	CD279 (PD-1)	EH12.2H7	Human	ACAGCGCCGTATTTA
TotalSeq™-B	386	CD28	CD28.2	Human	TGAGAACGACCCTAA
TotalSeq™-B	34	CD3	UCHT1	Human	CTCATTGTAACTCCT
TotalSeq™-B	101	CD335 (NKp46)	9.00E+02	Human	ACAATTTGAACAGCG
TotalSeq™-B	72	CD4	RPA-T4	Human	TGTTCCCGCTCAACT
TotalSeq™-B	391	CD45	HI30	Human	TGCAATTACCCGGAT
TotalSeq™-B	63	CD45RA	HI100	Human	TCAATCCTTCCGCTT
TotalSeq™-B	87	CD45RO	UCHL1	Human	CTCCGAATCATGTTG
TotalSeq™-B	84	CD56(NCAM) Recombinant	QA17A16	Human	TTCGCCGCATTGAGT
TotalSeq™-B	147	CD62L	DREG-56	Human	GTCCCTGCAACTTGA
TotalSeq™-B TotalSeq™-B	146	CD69	FN50	Human	GTCTCTTGGCTTAAA
TotalSeq™-B	5	CD80	2D10	Human	ACGAATCAATCTGTG
TotalSeq™-B	6	CD86	IT2.2	Human	GTCTTTGTCAGTGCA
TotalSeq™-B	55	CD8a	RPA-T8	Human	GCTGCGCTTTCCATT
TotalSeq™-B	159	HLA-DR	L243	Human	AATAGCGAGCAAGTA
TotalSeq™-B	90	Mouse IgG1, κ isotype Ctrl	MOPC-21	N/A	GCCGGACGACATTAA

(continued)

Table 1
(continued)

Category	Barcode	Specificity	Clone	Reactivity	Barcode sequence
TotalSeq™-B	91	Mouse IgG2a κ isotype Ctrl	MOPC-173	N/A	CTCCTACCTAAACTG
TotalSeq™-B	92	Mouse lgG2b. κ isotype Ctrl	MPC-11	N/A	ATATGTATCACGCGA
TotalSeq™-B	89	TIGIT (VSTM3)	A15153G	Human	TTGCTTACCGCCAGA

15. Centrifuge 400 rcf for 5 min.
16. For sequencing 10,000 PBMCs per sample suspend the pellet to 1200 cells/μL in 0.01% PBS. If desired cell number is less, refer to 10× genomics user guide Chromium Single Cell 3′ Reagent Kits v3 with Feature Barcoding technology for Cell Surface Protein Rev. C (CG000185 Rev. C) page 29.

3.3.3 Library Preparation

1. After staining and resuspending counted cells to 1200 cell/μL the cells are loaded in the 10× genomics chromium controller as per user guide Chromium Single Cell 3′ Reagent Kits v3 with Feature Barcoding technology for Cell Surface Protein Rev C (CG000185 Rev C) and the standard workflow protocol is followed yielding a gene expression library and an antibody barcode library, which are used for sequencing.

3.3.4 Sequencing

1. For the gene expression library, sequencing is performed at 25,000 reads per cell using 150 bp paired-end reads. Antibody barcode library is sequenced at 5000 reads per cell using 150 bp paired-end reads.

3.3.5 Analysis

1. BCL files are converted to FASTQ files using Cellranger software using the Cellranger mkfastq command.
2. Sample FASTQs are then aligned to the reference Human Genome GRCh38—hg38 using Cellranger count command.
3. After Cellranger analysis the data is quality controlled and aligned to identify transcript quantities and antibodie quantities on a per cell basis. This data is available in the output of Cellranger as count matrix's, which are used downstream as input for the R package Seurat. Within Seurat the Transcript counts are read in as the assay "RNA" and the antibody counts are seperately read into the program as the assay "Protein".

4. Seurat is used to normalize the data using SCTransform and clusters generated using uMAP with a resolution of 0.2.
5. Post clustering the protein expression is used to name the clusters by generating average protein expression per cluster. The average protein expression per cluster is used to generate a heatmap to identify monocytic MDSCs as those who are CD14 positive, HLA-DR negative, CD11b positive, and negative for all other immune markers in the panel. PMN-MDSC cannot be analyzed with this protocol, because of their sensitivity to freeze-thaw procedures (PMID 23160385).

3.4 Notes

1. These protocols have not been optimized for single cell suspensions of tumor cells although in theory a mechanical or gentle dissociation method that does not use Trypsin or papain to digest prior to staining should work similarly but would require a greater sequencing depth for both the gene expression and the antibody libraries.
2. The use of EDTA in any step will result in failure due to incompatibility with the 10× genomics kit.
3. When determining the protein expression per cluster by heatmap normalize by clusters because if normalized by protein marker CD45 tends to negate the true expression of other markers due to its high intensity.
4. These studies were performed using Cellranger 3.1.0 and Seurat version 3.0.
5. Isotype control antibodies are included to subtract background as some cluster types such as platelets tend to non-specifically bind antibodies but can be characterized via RNA expression.
6. If cell populations are identified using the RNA and informatics approaches they will likely not include a reference MDSC population, and thus will not be able to identify an MDSC. In these types of analyses, MDSCs typically show up as being identified as M2 macrophage or Monocyte.
7. TotalSeq A and C antibodies are also compatible with antibody staining; however, we have not tested these directly.
8. In using this technique on frozen PBMCs as presented here you may not be able to identify PMN-MDSCs and because there is a Ficoll gradient performed the neutrophils will also be absent in the analysis. However, if desired this protocol is compatible with live unfrozen cells and can be performed to analyze both subsets.

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Chapter 14

Single-Cell Transcriptome Analysis Workflow for Splenic Myeloid-Derived Suppressor Cells from Murine Breast Cancer Models

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Abstract

Single-cell transcriptomics is a powerful tool to study previously unrealized cellular heterogeneity at the resolution of individual cells. Most of the previous knowledge in cell biology is based on data generated by bulk analysis methods, which provide averaged readouts that usually mask cellular heterogeneity. This approach is challenging when the biological effect of interest is limited to a subpopulation within a cell type. This may particularly apply immune cell populations as these cells are highly mobile and swiftly respond to changes in cytokines or chemokines. For example, in cancer certain subset of myeloid immune cells may acquire immunosuppressive features to suppress antitumor immune responses, and thus described as myeloid-derived suppressor cells (MDSCs). Advances in single-cell RNA sequencing (scRNAseq) allowed scientists to overcome this limitation and enable in-depth interrogation of these subsets of immune cells including MDSCs. Here, we provide a detailed protocol for using scRNAseq to explore MDSCs in the context of splenic myeloid cells from breast tumor-bearing mice in comparison to wildtype controls to define the unique molecular features of immunosuppressive myeloid cells.

Key words Single-cell RNA sequencing, Cellular heterogeneity, Breast cancer, Neutrophils, Monocytes, Myeloid-derived suppressor cells

1 Introduction

In the past decade, scientific studies have widely used bulk profiling methods to explore gene expression at population level. This method provides average gene expression across thousands of cells that generally masks cellular heterogeneity. The recent development of robust single-cell RNA sequencing (scRNAseq) technologies has now enabled us to explore large scale gene expression at the single-cell resolution, and thus advanced our understanding of how biological systems function particularly in the areas of immunology, neurobiology, stem cell biology, and cancer research [1, 2]. This approach is particularly useful to define changes in

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poorly defined cell populations when there is a lack of specific cell surface receptors for prospective enrichment. Our recent work utilized scRNAseq to reveal the cellular and molecular properties of myeloid-derived suppresser cells (MDSCs) in breast cancer and demonstrating distinct MDSCs clusters that stand out from normal spectrum of myeloid cells [3]. Here, we discuss tissue dissociation and single-cell isolation, cell enrichment, quality control approaches optimized for scRNAseq analysis of myeloid cell populations to diminish batch effects and technical variation that may overshadow true biological insights. We also provide details on computational analysis pipelines and settings used to analyze immune cell single-cell transcriptomics datasets.

2 Materials

2.1 Tissues and Reagents

1. Spleens from FVB/n and Transgenic PyMT (MMTV-PyMT) mice were purchased from The Jackson (JAX) Laboratory.
2. RPMI (Corning, 10-040-CV).
3. Fetal bovine serum (FBS) (Omega Scientific, FB-12).
4. 1×PBS (Corning® DPBS (Dulbecco's Phosphate-Buffered Saline) MT21031CV).
5. FACS buffer (1×PBS, 3% FBS).
6. EasySep™ Mouse MDSC ($CD11b^+Gr1^+$) Isolation Kit, Stem cell Technologies, Cat.No.19867.
7. Fc-receptor blocking with anti-mouse FcγR (CD16/CD32) (BioLegend, 101301).
8. SYTOX Blue viability dye (Life Technologies, S34857).
9. Anti-mouse-CD45 (30-F11) (BioLegend).
10. Anti-mouse-CD11b (M1/70) (BioLegend).
11. Anti-mouse-Gr1 (Rb6-8C5) (BioLegend).
12. 70-μm cell strainer (Fisher Scientific, 22363548).
13. Automated cell counter Countess™ II (ThermoScientific, AMQAX1000).
14. 5 mL culture tubes with closures (VWR, 211-0061).
15. 5 mL polystyrene round bottom with cell-strainer cap (Thermo Scientific, 352235).
16. FlowJo software v 10.0.7 (Tree Star, Inc).
17. BD FACSAria™ Fusion.

2.2 Single-Cell RNA Sequencing Reagents

1. Chromium™ Single Cell 3′ Library & Gel Bead Kit v2, 16 rxns (10× Genomics 120237).
2. Chromium Single Cell A Chip Kit, 48 rxns (10× Genomics 120236).

3. Chromium i7 Multiplex Kit, 96 rxns (10× Genomics 120262).
4. TempAssure PCR 8-tube strip (USA Scientific 1402-4700).
5. DNA LoBind Tubes, 1.5 mL (Eppendorf 022431021).
6. DynaBeads MyOne Saline Beads (Thermo Fisher 37002D).
7. Nuclease-Free Water (Thermo Fisher AM9937).
8. Low TE Buffer 10 mM Tris–HCL pH 8.0, 0.1 mM EDTA (Thermo Fisher 12090-015).
9. Ethanol (Sigma 459836-500ML).
10. SPRIselect Reagent Kit (Beckman Coulter B23318).
11. 10% Tween 20 (Bio-Rad 1610781).
12. Glycerin 50% (v/v) Aqueous Solution (Ricca Chemical Company 3290-32).
13. Qubit Fluorometer/dsDNA HS Assay Kit (Thermo Fisher Q33216/Q32854).
14. Illumina Library Quantification Kit (KAPA Biosystems KK4824).
15. 2100 Bioanalyzer Laptop Bundle/High Sensitivity DNA Kit (Agilent G2943CA/5067-46726).
16. Chromium Controller (10× Genomics 1000202).
17. C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad 1851197).
18. *See* **Note 1**.

2.3 Computational Analysis

1. Recommended computing requirements (Mac, Linux, or Windows OS installed), 8 GB RAM or higher.
2. R software suite installed version 3.5.0 or higher.
3. R software packages installed (Seurat version 2.3.1 or higher, Monocle version 2.8.0 or higher).

3 Methods

3.1 Tissue Dissociation and Single-Cell Preparation for scRNAseq

1. Spleens were collected from tumor-bearing mice and FVB/n in small volume of RPMI with 3%FBS under sterile conditions.
2. Place spleen in a 70-μm cell strainer in the top of a 50 mL conical tube and gently disaggregate using the plunger of a 5-mL syringe and push through the filter (Fig. 1 (*1*)).
3. Wash the 70-μm cell strainer with 5 mL of RPMI with 3%FBS to create a cell suspension of splenocytes into the 50 mL conical tube.
4. Repeat **step 3** one more time to collect all splenocytes into the 50 mL conical tube.

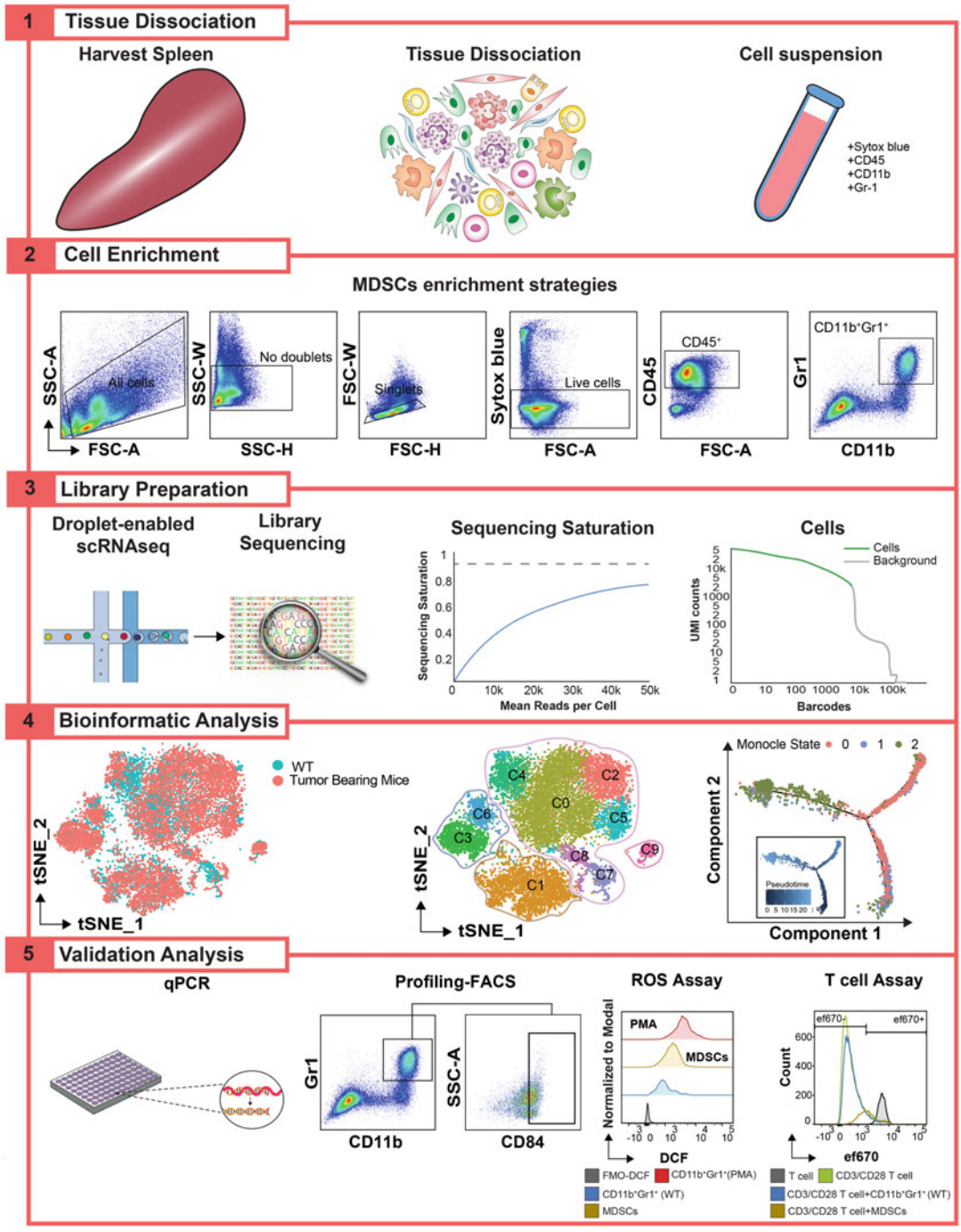

Fig. 1 Illustration of scRNAseq protocol (*1*) Tissue dissociation and single-cell suspension (*2*) MDSCs gating and enrichment strategies (*3*) Library preparation and sequencing for scRNAseq include sequencing saturation and cell calling plot (*4*) tSNE plots for; combined WT and tumor-bearing mice, and various distinct clusters of $CD11b^{+}Gr1^{+}$ cells. Monocle analysis on subset of MDSCs clusters resulted in branched trajectory with three distinct Monocle states (color code for each state is indicated) (*5*) qPCR, FACS profiling, ROS, and T cell suppression assays for validation

5. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
6. Lyse red blood cells by adding a volume of 5 mL of RBCs lysis buffer (for each spleen) and incubate at room temperature for 5 min.
7. Quench cells with at least three times of RBCs lysis buffer volume with RPMI with 3%FBS and gently pipet up and down.
8. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
9. Resuspend cell pellet in 10–15 mL of RPMI with 3%FBS.
10. Count the cells with trypan blue solution (10 μL of cells + 10 μL of trypan blue) loading samples into Countess™ II and record live/dead and viability.
11. Transfer 1×10^8 live cells from spleen tumor-bearing mice and FVB/n to new 15 mL conical tube and add 10 mL of FACS buffer to wash the cells.
12. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
13. Preform EasySep™ Mouse MDSC ($CD11b^+Gr1^+$) Isolation Kit to enrich for MDSCs *(if you are using this kit ignore **steps 6 and 7 of the isolation kit protocol**)*. Follow the manufacturer protocol.
14. Repeat **step 10,** to count the cells post MDSC isolation kit.
15. Desired number of cells should be transferred into FACS tubes (culture tubes with closures 12 × 75 MM, 5 mL) for staining.
16. Centrifuge cells at 500 × *g* at 4 °C for 5 min and discard the supernatant.
17. Incubate cells with blocking reagent (1:100) FcγR (CD16/CD32) in FACS buffer at room temperature for 10 min.
18. Add mixture of antibodies containing anti-CD45 (1:100), anti-CD11b (1:100), and anti-Gr-1 (1:100) and incubate at 4C for 20–30 min.
19. Wash cells 1–2 times with FACS buffer and centrifuge cells at 500 × *g* at 4 °C for 5 min and discard the supernatant.
20. After labeling the cells, resuspend cells with 500 μL of FACS buffer.
21. Transfer cells into FACS tubes 5 mL tube polystyrene round bottom with cell-strainer cap.
22. Add 0.5 μL of SYTOX Blue viability dye to the cells.
23. Process your samples to BD FACSAria™ Fusion to sort your MDSCs.
24. FACS gating strategies are illustrated in Fig. 1 (*2*).
25. Sorting MDSCs in FACS buffer.

3.2 Cell Enrichment

Tissue dissociation and single-cell preparation for scRNAseq is described in Subheading 3.1. There are numerous methods for enriching a specific cell population and depleting unwanted cells of specific tissue types. Magnetic beads have been utilized to enrich CD11b/Gr1-positive MDSC populations (Stem cell technologies, Cat.No.19867). Fluorescence-activated cell sorting (FACS) is a high throughput method widely used to enrich specific cell types, such as neutrophils or monocytes. The following steps demonstrate how MDSC-containing myeloid populations should be gated in FACS for isolation and subsequent scRNAseq (Fig. 1 (*2*))

1. All cells should be gated based on SSC-A vs. FSC-A.
2. To avoid doublets, two gating strategies should be performed:
   (a) SSC-W vs. SSC-H.
   (b) FSC-W vs. FSC-H.
3. Gate for live/dead cells.
4. Gate for $CD45^+$ positive cells.
5. Gate for $CD11b^+Gr\text{-}1^+$ double positive cells.
6. Sorting MDSCs in FACS buffer.
7. Quality control (*see* Subheading 3.3).

3.3 Quality Control

After isolating MDSC-containing myeloid cells by FACS, quality control measurements should be executed prior to droplet-enabled scRNAseq performance. A useful metric can be acquired using microscopic imaging of cells and assessing viability using the countess platform (Fisher Scientific). Also, FACS is an additional valuable metric to measure cell viability and purity. The following steps are useful measurements prior to subjecting isolated myeloid cells to droplet-enabled scRNAseq

1. Take small aliquot of sorted MDSCs and mix them with trypan blue solution (10 μL of MDSCs + 10 μL of trypan blue).
2. Load MDSCs into Countess™ II and record live/dead and viability of cells after FACS sorting.
3. Take small aliquot of sorted MDSCs ~10 μL and do post sort analysis in flow cytometry to evaluate MDSCs population and their viability.
4. Perform functional characterization of MDSCs such as T cell suppression and reactive oxygen species (ROS) assays and other biochemical and molecular parameters associated with MDSC characterization that has been described in recent review [4] to validate MDSCs prior scRNAseq.
5. After MDSCs viability, purity, and characterization were confirmed, pure sorted MDSCs will be introduced into droplet-enabled scRNAseq.

3.4 Single-Cell RNA Sequencing

Single-Cell RNA libraries were prepared according to: Chromium Single Cell 3′ Reagent Kits v2 User Guide (10× Genomics CG00052 Rev. B) (*see* **Note 1**). In short, libraries were prepared by the following procedure:

1. Wash FACS isolated cells in 0.04% BSA in PBS solution.
2. Resuspend cell pellet to achieve approximately 1000 cells/μL.
3. Count the cells with trypan blue solution (10 μL of cells + 10 μL of trypan blue) by loading samples into Countess II. Note actual cell concentration for chip loading.
4. Prepare Single-Cell A Chip according to 10× Chromium protocol and load cells for Targeted Cell Recovery of 10,000 cells.
5. Generate Gel Beads in Emulsion (GEMs) using the Chromium Controller.
6. Collected GEMs were processed according to 10× Chromium protocol into cDNA libraries.
7. Check cDNA libraries concentration using Qubit dsDNA HS Assay Kit and Qubit Fluorometer.
8. Check fragment distribution using High Sensitivity DNA kit and 2100 Bioanalyzer.
9. Quantify library for Illumina indexed fragments using Illumina Library Quantification Kit.

3.5 Library Sequencing

1. Single Cell 3′ libraries were sequenced on the Illumina HiSeq 2500, using Rapid Run. (*see* **Note 2**).
2. Sequencing run was performed using the following cycles for each read:
   (a) Read1 26 cycles
   (b) Read2 98 cycles
   (c) i7 Index 8 cycles
   (d) i5 Index 0 cycles
3. Approximately 50,000 reads per cell were targeted for our sequencing depth. (*see* **Note 3**).
4. FastQ files were aligned using Cell Ranger Count 2.1.0.
5. Aligned libraries were aggregated to normalization based on mapped reads per cell using Cell Ranger Aggr 2.1.0.

3.6 Quality Control

Sequencing data should be checked to ensure appropriate read depth and cell calling before downstream analysis is performed. Useful metrics to consider can be obtained from the Cell Ranger alignment software (10× Genomics), such as sequencing saturation, which may vary for each specific cell type that is sequenced. For proper read depth, the balance between mean reads per cell and library complexity is important. The sequencing saturation curve in

Fig. 1 (*3*) shows observed library complexity for the projected mean reads per cell. As the sequencing saturation approaches 1, a larger portion of the converted mRNA has been sequenced. Cells that have lower complexity (such as neutrophils) will approach this saturated library complexity faster, and therefore will require lower mean reads per cell. Cell types with higher gene expression complexity will require more increased reads per cell to capture more of the converted mRNA.

Cell calling by Cell Ranger is done by comparing UMI counts and barcodes detected. It is important to note that for a library with many different cell types including cells with high and low library complexity, cell calling may be skewed. Neutrophil granulocytes in particular commonly have lower numbers of genes detected. In the presence of cells with higher gene expression, complexity of these neutrophils may be improperly called as an empty droplet and filtered out from further analysis (*see* **Note 4**).

3.7 Bioinformatic Analysis

Here, we present a general overview of the independent steps encompassing useful computational analysis approach to study scRNA-seq libraries from MDSC-containing immune cell populations. For detailed instructions please refer to the original Seurat (https://satijalab.org/seurat/vignettes.html) and Monocle (http://cole-trapnell-lab.github.io/monocle-release/) vignettes.

1. Load R, R packages and their dependencies (e.g., Seurat, Monocle).
2. Read in Cell Ranger filtered gene matrix for each library (Wild Type and PYMT mouse cells) into R environment.
3. For Each: Create Seurat object with gene matrix, trimming out genes not expressed in at least three cells, and trimming out cells expressing less than 500 unique genes (*see* **Note 5**).
4. Calculate percent of counts corresponding to genes on mitochondrial genome per cell (percent.mito).
5. Remove cells from analysis that have a percent.mito above 8% or those that have more than 5000 unique genes expressed (*see* **Note 6**).
6. Scale Seurat Object.
7. Find highly variable genes to use for canonical correlation analysis (CCA) (*see* **Note 7**).
8. Take intersect of highly variable genes and perform CCA out to 30 components for the two objects.
9. Select number of components to align subspace (*see* **Note 8)** and align the two objects.
10. Perform dimensionality reduction (tSNE) and clustering, keeping the number of components used as input consistent with those used to align the subspace (Fig. 1 (*4*)).

11. Find marker genes for resultant clustering, iterating through clustering resolutions until good separation of cell types and states is achieved (*see* **Note 9**).
12. Explore differential expression between cell types and states of interest in the analysis (*see* **Note 10**).
13. Identify candidates for pseudotemporal analysis of cell types and states and subset the cell groups into their own objects.
14. Using Monocle, create a CellDataSet (Monocle object) of cells of interest. Maintain metadata from Seurat analysis and convert format to a phenodata object for Monocle (Fig. 1 (*4*)).
15. Preprocess Monocle object by calculating size factors and dispersion for genes.
16. Select genes to use for pseudotemporal ordering of cells and reduce dimensions (*see* **Note 11).**
17. Select the origin of pseudotime (*see* **Note 12**).
18. Analyze gene expression changes through pseudotime using Monocle's differential expression test or use Seurat's marker gene functionality to interrogate state expression.

3.8 Validation Analysis

To validate the scRNAseq data do the following (Fig. 1 (*5*)):

1. Quantitative PCR (qPCR): $CD11b^{+}Gr1^{+}$ cells from spleens of WT and tumor-bearing mice should be sorted by FACS and subject to (qPCR) [3].
2. FACS: profile your findings by FACS [3].
3. Preform ROS assay [3].
4. Preform T cell suppression assay [5].

4 Notes

1. Reagents and methods are specific to the methods used in the recently published article by Alshetaiwi et al. [3]. We recommend following 10× Genomics Chromium Single Cell User Guide for latest material list. Chromium Single Cell 3′ Reagent Kits User Guide (v2 Chemistry) https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v2-chemistry.
2. Sequencing can be performed on any next-generation sequencing instrument capable of performing the necessary cycles described.
3. Initial sequencing can be done at lower depth (e.g., approximately 5000 reads per cell) to first estimate the number of cells captured. This will allow for calculation of optimal sequencing depth to obtain appropriate number of reads per cell in a second sequencing run.

4. Manually increasing cell calling by Cell Ranger will include droplets with lower RNA contents. This might alleviate the problem of improper calling of lower complexity cells. More stringent analysis and validation will be needed to ensure cell calling is accurate. Validation such as checking marker genes from any new clusters that may form will be necessary to ensure detection of an actual cell type. If new cluster does not have any distinct marker genes that correspond to known cell types, it might be made up of droplets that contain ambient RNA.
5. These cutoffs represent approximate guidelines for trimming out lowly captured genes and cell barcodes that did not have many unique genes expressed. These can be adjusted depending on cell type, as some cell types may naturally express less genes than others and thus can be empirically revisited.
6. The numbers used here are a recommendation to eliminate cells that elicit stress response due to cell isolation and capture, as well as potential doublets cells with the highly unique gene cutoff. As before, these are subject to empirical adjustments depending on the source of these cells.
7. Seurat's default settings to select variable genes for downstream clustering analysis is a valuable starting point; however, the selection settings may be adjusted to move forward with genes representing pathways in which much of the expected heterogeneity of the dataset is captured.
8. This is a selection that often needs to be reiterated during an analysis. A useful functionality within Seurat (the MetageneBicorPlot) can be helpful in this context. In more recent versions of the Seurat Package, the data integration workflow seeks to combine multiple Seurat objects into a single analysis. The goal is to have generalizable commentary on the presence of cell types and states among different batches/conditions.
9. The clustering resolution is another parameter that requires multiple iterations. A useful metric to select the appropriate resolution can be gauged based on the distinctness of marker genes identified for the resultant clusters. These are then visualized via heatmap to assess how exclusive their expression is found across the clusters; if the resolution is too high there will be too many clusters and the marker gene expression is not exclusive enough, while if the resolution is too low not enough clusters are detected and additional potentially important biological diversity may remain undefined.
10. The metadata annotating cells in the analysis are increasing in dimensionality based on batch/condition/tissue/clustering, etc., and this information can be leveraged when performing tests for differential expression between groups. Depending on

the question at hand, a test can be designed to compare between cell types within a condition, between conditions, or across batches. Organization is critical here.

11. The genes used for pseudotemporal ordering can be selected in a variety of ways. Analysis using pseudotime is a way to linearly cluster cells based on a spectrum of similarity of expression for a given set of genes. These genes can be differentially expressed genes as calculated using Monocle, marker genes for cell types from Seurat, or genes curated that have an association with a phenotype with which the cells are to be stratified. Each will produce different results, and the interpretation of the trajectory is dependent on what was used as input to order the cells.
12. Pseudotime as a calculation in Monocle is contingent on the choice of an origin. Typically, this will be informed with prior knowledge of what a more immature cell type/state expresses, and so where those genes are highly expressed in the trajectory calculated will be chosen as the origin. In other cases of a stratification based on acquisition of a phenotype or expression program, researchers can choose to pick those cells that have not acquired expression of those genes as the start.

Acknowledgments

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Chapter 15

Intravital 2-Photon Microscopy of Diverse Cell Types in the Murine Tibia

Anja Hasenberg, Lucas Otto, and Matthias Gunzer

Abstract

Intravital imaging allows the visualization of fluorescently labeled structures like cells, blood flow, and pathogens in a living organism. Nowadays, numerous methods for imaging in several organs are available. In this chapter, we present a method for intravital 2-photon microscopy of the murine tibial bone marrow. It enables the observation of hematopoietic cells including cells of the innate and adaptive immune system under physiological conditions. Motility analyses within this complex environment led to insights into their migratory potential as well as their interactions with other cells or blood vessels.

Key words Intravital 2-photon microscopy, Murine tibia, Bone marrow, Cell migration

1 Introduction

The concept of 2-photon microscopy was already postulated in 1931 by Maria Goeppert-Mayer [1]. However, due to the lack of appropriate technical equipment, it was applied in the field of biomedical research not before 1990 [2]. The major advantage of this technique is the sample illumination with low energy near infrared photons that lead to reduced bleaching and an improved tissue penetration, a characteristic that is indispensable for intravital imaging. To achieve this, a pulsed highly energetic light source, typically a mode-locked Titan-Sapphire laser, is necessary. The generated high photon flux per individual pulse ensures that during illumination two photons hit all fluorophores in the area of maximum focus of the optical system at almost the same time to excite fluorescence. This allows the using of red-shifted photons which can penetrate deeply into biological tissues to excite, e.g., dyes that normally need high-energy blue or green photons for optimal single-photon excitation. Hence, this approach displays a unique

Anja Hasenberg, Lucas Otto, and Matthias Gunzer contributed equally to this work.

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possibility to observe vital cells in the tissue of a living mammalian under physiological conditions [3–6]. For this reason, various protocols for intravital 2-photon microscopy were established in the last two decades which enable imaging in multiple murine organs like lymph nodes [7–9], liver [10–12], lungs [13, 14], or brain [15, 16]. We generated a method to realize microscopy in the bone marrow of murine tibiae [17–19]. The tibia, as a long bone, is distinct from flat bones such as the calvaria on which other established methods of bone marrow imaging typically focus [20–23].

The bone marrow displays a complex 3-D environment, densely filled with almost all cells of the hematopoietic system as well as their progenitors [24, 25]. It is traversed by blood vessels that provide the route of cell emigration during homeostasis but also allow the quick mobilization of high cell numbers during an immune response [26–28]. Moreover, special regions like different types of niches, which can influence cell behavior in distinct ways, are present in the bone marrow [29, 30]. After thinning the compact bone with an electric grinder it is possible to observe fluorescently labeled cells and vessels in the bone marrow up to depths of approximately 100 μm. We already visualized and analyzed different cell types like early hematopoietic progenitor cells with respect to their localization to the endosteum [31] as well as the migration speed of neutrophils under normal conditions and after stimulation with the hematopoietic cytokine Granulocyte-Colony Stimulating Factor (G-CSF) [32, 33]. Furthermore, an antiviral immune response by virus-specific $CD8^+$ T cells was monitored in this way. Single cytotoxic $CD8^+$ T cells were tracked and their speed was calculated [17]. However, our general approach can be applied to observe any type of cell or structure that resides in the bone marrow and can be visualized with 2-photon excitation. Therefore, it enables to study responses to various treatments, infections, or cancer.

2 Materials

2.1 Ketamin-Xylazine Narcosis

1. Xylazine for small animals (f.c. 2 mg/mL).
2. Ketamin for small animals (f.c. 20 mg/mL).
3. Sodium chloride (NaCl) solution (0.9%) or alternative: Dulbecco's phosphate-buffered saline (PBS).

2.2 Isoflurane Narcosis

1. Forene [100% V/V] Isoflurane (1-Chloro-2,2,2-trifluoroethyl Difluoromethyl Ether) (AbbVie, North Chicago, IL, USA).
2. Medical grade oxygen (100%) (Air Liquide, Düsseldorf, Germany).

3. Small animal anesthesia, Univentor 400 with isoflurane syringe (Fig. 1a) (Zetjun, Malta).
4. Small animal respirator with connecting hoses, Minivent Type 845 (Fig. 1b) (Hugo Sachs Electronic, Havard Apparatus, March-Hugstetten, Germany).
5. Contrafluran isoflurane filter (ZeoSys, Berlin, Germany).

2.3 Intubation

1. Intubation stage for the mouse (Fig. 1c) (custom made, fine mechanics, University Duisburg-Essen, Germany).
2. Elastic band for mouse fixation.
3. Hook-and-loop tape for mouse fixation.
4. Laryngoscope for intubation, self-made from a lab scoop (Fig. 2a) (Fine Science Tools, Heidelberg, Germany).
5. Permanent venous catheter, Introcan-W 22G, 0.9 × 25 mm (Fig. 2a).
6. Bright and focused lamp (KL1500 LED, Schott, Mainz, Germany).

2.4 Preparation

1. PBS or alternatively NaCl solution (0.9%).
2. Preparation stage (Fig. 1d) (custom made, fine mechanics University Duisburg-Essen, Germany).
3. Stereo microscope S8 AP0 with Dual Arm (Leica Microsystems, Mannheim, Germany).
4. Electric grinder, Dremel 300 (300-1/55) (Dremel, Racine, WI, USA).
5. Aluminum oxide grinding stone 997 (Dremel).
6. Cautery instrument 18010-00 (Fine Science Tools).

2.5 Intravital 2-Photon Microscopy

1. Imaging chamber with preparation stage (Fig. 1d) (custom made, fine mechanics, University Duisburg-Essen, Germany).
2. NaCl solution (0.9%) or alternatively PBS.
3. Heating bath, Proline P5C (Lauda, Lauda-Königshofen, Germany).
4. Bepanthen eye- and nose-cream (Bayer, Leverkusen, Germany).
5. 2-photon microscope, TCS SP8 (Leica microsystems) equipped with a Chameleon Vision II Laser (Coherent, Santa Clara, CA, USA).

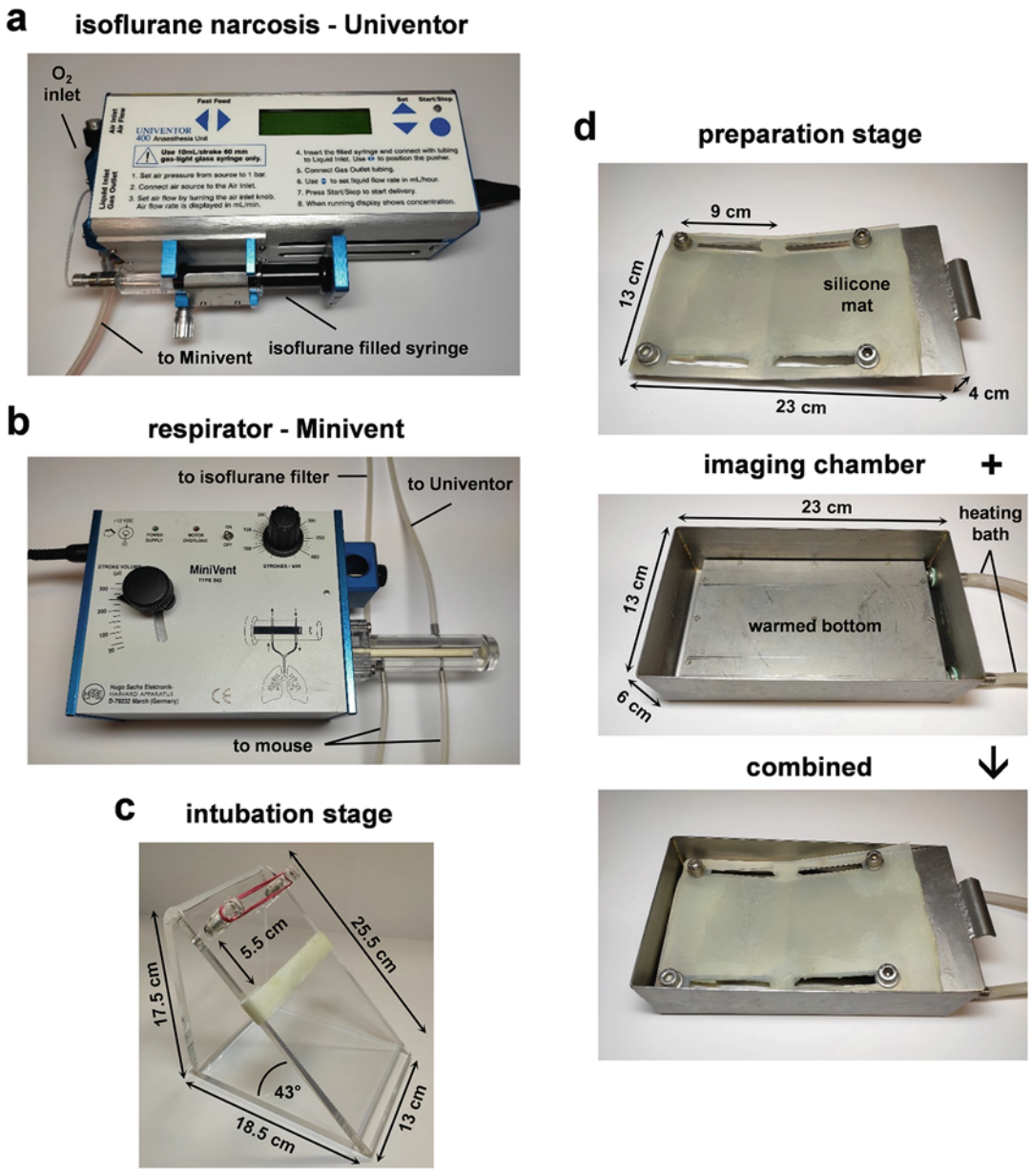

Fig. 1 Anesthesia and imaging equipment. (**a**) Isoflurane and oxygen are mixed by the Univentor for inhalation narcosis. Narcosis gas is provided to the Minivent via the indicated tube. (**b**) Stroke volume (μL) and the number of strokes/min are adjusted at the Minivent to regulate the breathing of the intubated animal. (**c**) Mouse intubation is performed with the help of an intubation stage (*see* Fig. 2). (**d**) The preparation stage consists of a silicon mat fixed on a metal stage (left). To warm the mouse with PBS, the imaging chamber is connected to a water heating bath that pumps pre-warmed water through the bottom of the chamber (middle). For imaging, the preparation stage is placed into the imaging chamber and filled with PBS (right, *see* Fig. 4)

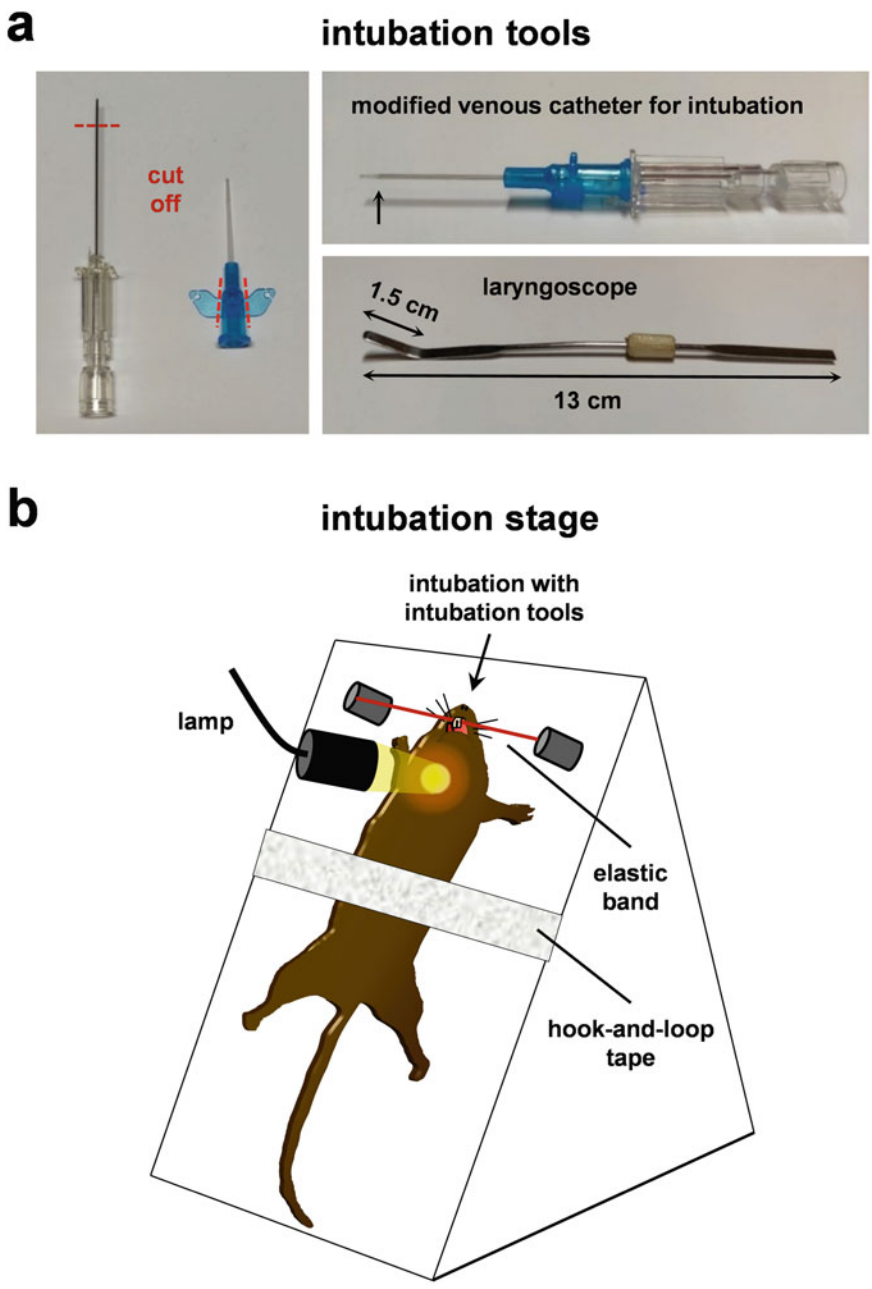

Fig. 2 Mouse intubation. (**a**) The mouse intubation tools are a modified venous catheter and laboratory scoop (laryngoscope). (**b**) The intubation procedure is performed on an intubation stage where the mouse is positioned on an elastic band with its front teeth and is fixed with a hook-and-loop tape at the abdomen. A lamp illuminates the throat making the trachea visible as a bright point in the throat. After opening the mouth by the laryngoscope the modified venous catheter can be inserted into the trachea

3 Methods

3.1 Adjust Materials and Machines

1. Prepare the permanent venous catheter for the intubation (Fig. 2a and *see* **Note 1**).
2. Place the imaging chamber on the microscopic table, fill it with PBS or NaCl (0.9%) and start the heating bath for temperature adjustment, whereby drift during the microscopy is reduced.
3. Fill the syringe of the Univentor with isoflurane under a flow hood and clamp it into the machine (Fig. 1a and *see* **Note 2**).
4. Connect the Univentor (Fig. 1a) with the oxygen supply and the Minivent (Fig. 1b) as well as both with the isoflurane filter.
5. Start the 2-photon microscope and the laser to have them ready to use.

3.2 Intubation

1. Anesthetize the mouse by an i.p. injection of a Ketamin (100 µg/g body weight)/Xylazine (10 µg/g body weight) mixture, dissolved in NaCl solution (0.9%) or PBS (*see* **Note 3**).
2. After 5–10 min ensure deep narcosis by food pad reflexes. Put eye- and nose-cream on the mouse eyes to prevent their desiccation.
3. Place the mouse on the intubation stage by hanging it at the elastic band with the front teeth and fix it with the hook-and-loop tape over the abdomen (Fig. 2b).
4. Place an external lamp in front of the mouse to illuminate the thorax from outside (Fig. 2b).
5. Open the mouth of the mouse with a laryngoscope and put the tongue to one side to allow an unrestricted look into the throat.
6. Carefully overstretch the head with the laryngoscope to expose the trachea that should be visible as a bright point in the throat. Open-close movement of the vocal cords should be visible due to the breathing (*see* **Note 4**).
7. Insert the permanent venous catheter into the trachea and immediately remove the metal part out of the plastic cover (*see* **Note 5**).
8. Connect the Minivent respirator immediately to the catheter. A proper thorax movement that coincides with the Minivent settings confirms a successful intubation (*see* **Note 6**).
9. Start the Univentor, use 1.0–1.5% Isoflurane mixed with O_2.
10. Move the mouse to the preparation stage (Fig. 1d and *see* **Note 7**).

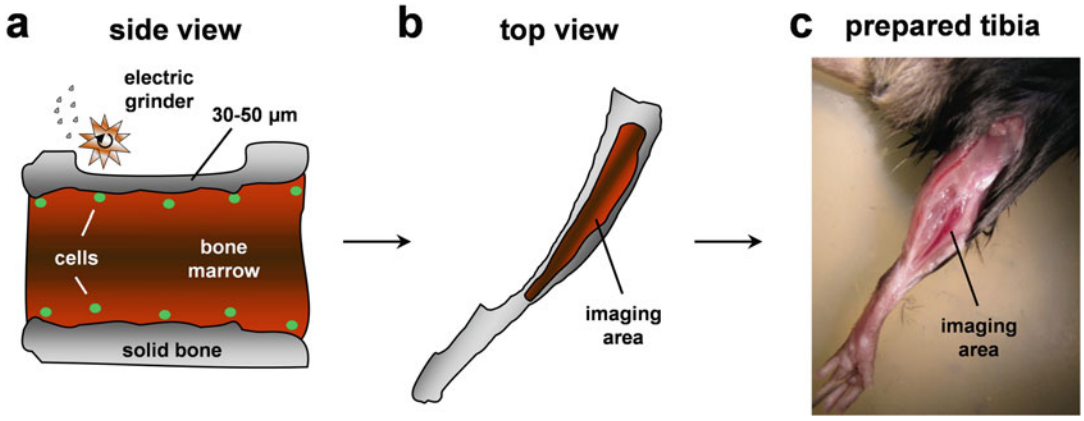

Fig. 3 Thinning of the tibia. (**a**) The virtual side view of the tibial bone within the final imaging area demonstrates the careful bone-thinning procedure by an electric grinder (Dremel), which results in a final bone surface thickness of 30–50 µm. Additionally, the thinned area becomes more reddish. The complete imaging area, from the top view perspective, is shown schematically (**b**) and in real (**c**) after the thinning procedure. (The figure is adapted from the original publication. This research was originally published in *Blood*. Kohler A, Schmithorst V, Filippi MD, Ryan MA, Daria D, Gunzer M, Geiger H (2009) Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. Blood 114(2):290–298. doi:https:/doi.org/10.1182/blood-2008-12-195644 © the American Society of Hematology)

3.3 Preparation

1. Fix the paws, except the one that will be used for the imaging, on the imaging stage with sterile needles to prevent any shift of the animal during the preparation.
2. Fix the intubation catheter and tube with needles and tape to ensure the ventilation during the preparation.
3. Damp the fur of the hind leg with ethanol and cut the skin over the tibia with scissors.
4. Place the leg under the stereo microscope and proceed with all the following steps.
5. Cut through the muscle straight in the middle of the tibia with a scalpel and avoid harming bigger vessels (*see* **Note 8**). Push aside muscles and skin and fix it with needles at both sides of the bone.
6. To avoid drying put a drop of pre-warmed (37 °C) PBS on the bone.
7. Thin out the bone with the electric grinder and the recommended grindstone (Fig. 3). Hold the bone at the ankle joint with a pair of tweezers. Slightly turn it to bring the flat side of the bone parallel to the grindstone and start drilling. As soon as dust of the bone or debris of removed bone (white slime) appears, stop the drilling. Clean and damp the bone with PBS (*see* **Note 9**) before proceeding with the drilling.

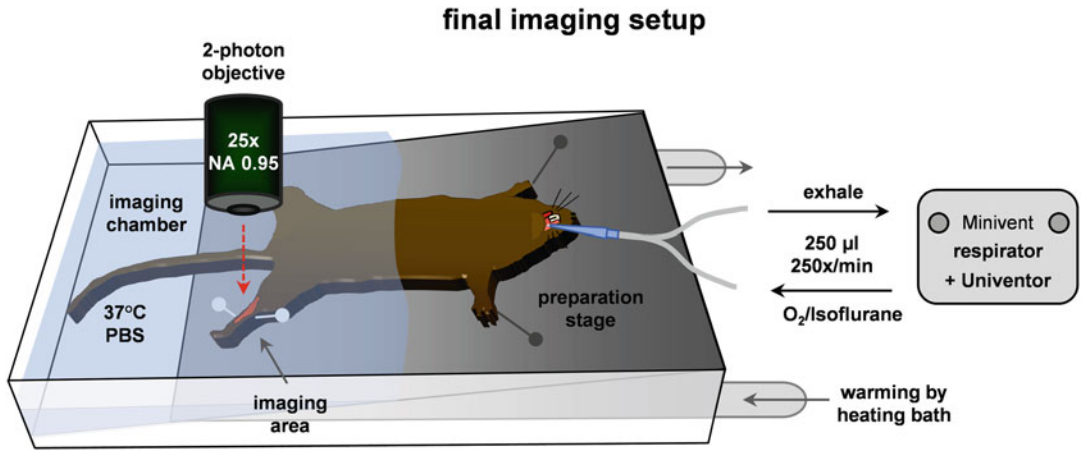

Fig. 4 Final setting for intravital imaging. The readily operated animal is fixed on the preparation stage, which is placed in the PBS-filled and warmed (37 °C) imaging chamber. During the microscopy, the mouse is constantly ventilated and narcotized through a respirator (Minivent) and an O_2/isoflurane mixing device (Univentor). (The figure is adapted from the original publication. This research was originally published in *Blood*. Kohler A, Schmithorst V, Filippi MD, Ryan MA, Daria D, Gunzer M, Geiger H (2009) Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. Blood 114(2):290–298. doi:https:/doi.org/10.1182/blood-2008-12-195644 © the American Society of Hematology)

8. During the preparation, carefully observe the bone through the stereo microscope. The red color of the bone marrow will become more and more intensive during the bone-thinning procedure (Fig. 3 and *see* **Note 10**).
9. If there are any bleedings after the drilling procedure stop them by using a cautery.
10. Place the preparation stage with the mouse into the pre-warmed and PBS-filled imaging chamber under the microscope (Figs. 1d and 4 and *see* **Note 11**).

3.4 Intravital 2-Photon Microscopy

1. Align the microscope as required for the fluorescent structures, e.g., wavelength and filter settings (*see* **Note 12**).
2. Find your structure of interest by using the fluorescent lamp of the 2-photon microscope (*see* **Note 13**).
3. Start the microscopy with defined settings (*see* **Note 14**).
4. Try to record at least three movies at three different regions of the marrow (*see* **Note 15**).
5. Control the mouse breathing and the Univentor after each movie (*see* **Note 16**).
6. Process and reconstruct the raw data from the microscope by using a microscopy image analysis software like Imaris (Bitplane AG, Zurich, Switzerland) (Fig. 5) and perform diverse analyses, e.g., cell tracking and volume analysis.

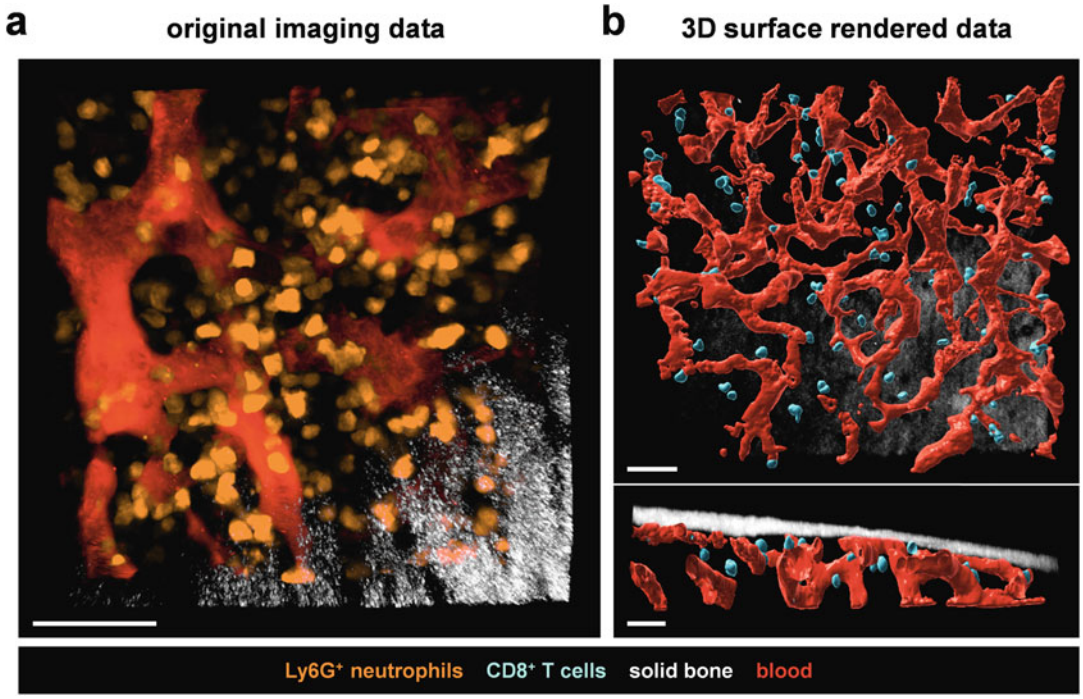

Fig. 5 View into the murine tibial bone marrow. The tibial bone marrow was subjected to intravital microscopy in (**a**) a Catchup$^{IVM\text{-}red}$ mouse, expressing tdTomato specifically under the Ly6G promotor, to visualize Ly6G$^+$ neutrophils and in (**b**) a Friend retrovirus infected C57BL/6 mouse to depict cytotoxic CD8$^+$ T cells during an ongoing adaptive immune response. The solid bone was visualized by the second harmonic generation (SHG) signal and the blood via i.v. injected Qdots. Ly6G$^+$ neutrophils and CD8$^+$ T cells were visible based on endogenous fluorescence. (**b**) Shows exemplarily processed imaging data (3D rendering by IMARIS) from different perspectives for further analysis, e.g., cell tracking and volume analysis. Scale bars, 50 μm. (The figure (**a**) is adapted by permission from Springer Nature: Springer Nature. Nature Methods: Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes, Hasenberg A, Hasenberg M, Mann L, Neumann F, Borkenstein L, Stecher M, Kraus A, Engel DR, Klingberg A, Seddigh P, Abdullah Z, Klebow S, Engelmann S, Reinhold A, Brandau S, Seeling M, Waisman A, Schraven B, Gothert JR, Nimmerjahn F, Gunzer M, Nat Methods 12(5):445–452. doi:https:/doi.org/10.1038/nmeth.3322, © 2015)

4 Notes

1. Cut off the top of the sharp metal guide rod with a pair of scissors and insert it back into the plastic cover. Make sure that the metal part is completely capped and no sharp borders from the cutting damage the plastic cover. The metal guide rod will give the required stability to allow the intubation (Fig. 2a).
2. Handle with care to prevent inhalation of isoflurane.
3. The injection volume is 5 μL/g mouse weight. Prepare the solution fresh each time. Advisable is the administration of additional analgesics 30 min after Ketamin-Xylazine injection (e.g., Buprenorphine).

4. Do not try any intubation without seeing the bright point in the throat. Adjust the position of the lamp until it becomes visible.
5. If there is no lung movement in accordance with the Minivent settings or a noise like an air pump arises, immediately remove the permanent venous catheter and intubate again, because this indicates that the catheter was inserted into the esophagus instead of the trachea.
6. Set the Minivent to a frequency of 250 breaths/min and a volume of 250 μL/breath.
7. While moving the mouse from the intubation stage to the preparation stage, take care that the catheter is not removed from the trachea. Once the mouse is placed on the preparation stage, check that the ventilation is still functional.
8. Try to cut as less muscle as possible to avoid bleeding. For the observation of frequently occurring cell types cutting a little muscle near the ankle joint is sufficient. Accordingly, the preparation of the whole tibia is recommended to observe rare cells (Fig. 3b, c).
9. Keep the bone wet and clean during the drilling. We recommend to prepare a small dish with PBS before starting the preparation. After slowly moving the grindstone over the bone 3–5 times, use a tissue or your gloved finger and PBS to gently clean the bone. Afterwards, continue with the rotation of drilling and cleaning. While doing so the dust is removed and the process of thinning can be assessed. Importantly, it also prevents overheating of the bone due to the drilling.
10. Try to thin the bone uniformly over its length and do not break the bone marrow cavity open. Once this happens, blood and marrow flow out and no microscopy is possible anymore. We strongly recommend to train this step with isolated bones properly to gain experience. The bones can be placed several times under the microscope during the training to verify the progress and to avoid breaches of the marrow cavity.
11. Take care of the intubation catheter during the movement and check the ventilation after everything is well-positioned on the microscopic table.
12. The optimal wavelength for the used fluorescent dyes should be evaluated beforehand. For a combination of eGFP and tdTomato, we recommend an excitation wavelength of 950 nm at the 2-photon microscope, which allows the simultaneous excitation of both fluorophores. In addition, the bone can be displayed with second harmonic generation (SHG) at exactly half of the excitation light without any staining. If blood flow or vessels should be observed, i.v. injection of either Qdots or fluorescently labeled dextran can be performed (Fig. 5).

13. In the beginning, screen the complete thinned area with the fluorescent lamp because the thinning is not evenly good everywhere. Search for the region with the brightest fluorescence to perform the imaging if the structure of interest is uniformly distributed in the marrow (e.g., vessels or frequent cells).
14. If migration speed of cells should be analyzed the time between two frames or z-stacks has to be restricted to allow tracking, depending on the speed of the cells. For G-CSF mobilized neutrophils as well as for $CD8^+$ T cells a time interval of 30–60 s is recommended. Try to image at least 10 min to gain a certain time period for tracking, if possible 30 min should be reached. Define appropriate settings before starting the real measurement. For a better comparability, it is advisable to use the same settings (frame size, scan speed, dimensions of the z-stack in x, y, z, number of steps in z, etc.) for all movies and all experiments.
15. Thermal expansion might lead to focus shift. Therefore, define the frame and z-stack fast and immediately start the measurement. Moreover, blood can flow into the field of view, which necessitates cleaning of the bone before the imaging can be continued.
16. The percentage of isoflurane might slightly change over time. Therefore, control it regularly to guarantee a constant narcosis. Optional, a capnograph type 340 (Hugo Sachs Electronic, Havard Apparatus) can be used for better animal monitoring.

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Chapter 16

Isolation of Bovine Neutrophils by Fluorescence- and Magnetic-Activated Cell Sorting

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Abstract

Flow cytometry and magnetic bead technology enable the separation of cell populations with the highest degree of purity. Here, we describe protocols to sort bovine neutrophils from blood, the labeling and sorting, including gating strategies. We also provide advice to preserve neutrophil viability and detail a protocol to measure phagocytosis and oxidative species production.

Key words Neutrophils, Bovine, Cell sorting, Magnetic isolation, Phagocytosis, Chemiluminescence

1 Introduction

Fluorescence-activated cell sorting (FACS) allows the purification of individual cells based on size, granularity, and fluorescence, with a high degree of purity (95–100%). Labeled cells in suspension pass inside a stream in front of a laser. The stream is fractioned in cell-containing droplets. The fluorescence detection system detects cells of interest and the instrument applies a charge to the corresponding droplet. An electrostatic deflection system facilitates the collection of the charged droplets into appropriate collection tubes [1]. Magnetic isolation of cells is based on antibody-coupled magnetic beads and represents another method regularly employed nowadays for cell purification [2]. This technology employs antibodies bound to magnetic microbeads to separate either labeled cells (positive selection) or unlabeled cells (negative selection) upon passage of the cellular suspension through a magnetic field. This method is highly robust, necessitates short processing time, requires a simple platform, and is cost-effective [3–5]. Cell purification based on FACS or magnetic beads is routinely used in laboratories to enrich specific

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cellular populations [6]. Reagents as well as separation procedures have been established for various cell types and cell populations, and commercial kits are widely available for humans and laboratory rodent species [7–9]. However, for other mammalian species methods allowing enrichment for highly pure cell subsets await validation.

Neutrophils are renowned for being difficult to work with; they are fragile and highly reactive cells with a short lifetime. In most available publications, bovine neutrophils are collected after centrifugation of blood samples and elimination of plasma, buffy coat, and the upper third of the red cell portion [10, 11]. This method makes it possible to work with cell preparations enriched in neutrophils (up to 70–80%); however, they also contain mononuclear cells and eosinophils, another granulocyte subset. For studies focusing on cellular functionality, e.g., cytokine release, as well as omics studies including RNA Seq, reliable isolation methods are required to avoid erroneous results due to contaminants [12]. Analysis of highly pure cell populations, such as neutrophils, will enable accurate conclusions about their roles at steady state and during disease. We describe in the following a protocol to prepare and label bovine blood cells and sort neutrophils by FACS. In our hands, sorted bovine neutrophils are viable in culture for up to 24 h and are competent for essential functions such as bactericidal activity or oxidative species production. This method also allows RNA extraction with satisfactory yield and quality (data not shown). We further describe a protocol to enrich bovine neutrophils from peripheral blood using magnetic cell isolation technology. Bovine neutrophils isolated with magnetic beads show robust phagocytosis and oxidative burst.

2 Materials

2.1 Cell Preparation and Labeling for FACS Sorting

1. Vacutainer K2E EDTA tubes (BD Vacutainer).
2. D-PBS.
3. Ethylenediaminetetraacetic acid (EDTA).
4. Bovine Serum Albumin, heat inactivated (BSA, Sigma SLBG2412), *see* **Note 1**.
5. Horse serum, heat inactivated.
6. Fetal Calf Serum (FCS).
7. Hydroxyethyl piperazineethanesulfonic acid (HEPES).
8. RPMI 1640 medium, supplemented with 2 mM of L-Glutamine, alternatively RPMI 1640 GlutaMAX™ which is already supplemented with L-Glutamine.

9. Red Blood Cell Lysing Buffer (Sigma R7757), *see* **Note 2**.
10. Antibodies:
    (a) Mouse IgM isotype control (diluted at 1 μg/mL).
    (b) Mouse IgM anti-bovine G1 antibody (Kingfisher, clone CH138A, 1 μg/mL), *see* **Note 3**.
    (c) Goat anti-Mouse IgM Alexa594 (ThermoFisher ref. A21044, 10 μg/mL).
    (d) Fixable Viability Dye eFluor™ 780 (ThermoFisher, reference 65-0865-14, used at 0.1 μL/mL).
11. Türk's solution.
12. Centrifuge.
13. Class II biological safety cabinet to provide a sterile working environment.
14. Malassez cell-counting chamber.

2.2 Cell Sorting

MoFlo Astrios^{EQ} Flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) equipped with four lasers: violet (405 nm), blue (488 nm), yellow/green (561 nm), and red (640 nm) and placed under a class II biological safety cabinet.

2.3 Cytospin and May-Grünwald Giemsa Staining

1. SuperFrost™ Plus Microscope Slides.
2. Coverslips.
3. EUKITT mounting medium (Sigma).
4. Cytospin™ Cytocentrifuge with accessories.
5. Kit RAL 555 for May-Grünwald Giemsa coloration (RAL Diagnostics).
6. Optical microscope.

2.4 Cell Preparation and Labeling for Magnetic Isolation

1. S-Monovette tubes 9 mL K3E 1.6mgEDTA/mL, (SARSTEDT).
2. 10× PBS
3. Erylysis buffer: 156 mM NH4Cl (Roth), 12 mM $NaHCO_3$ (Merck), 0.8 mM EDTA (Invitrogen) in Millipore H_2O.
4. D-PBS supplemented with 1% FCS (PAN Biotech).
5. BD IMag buffer (P-BSA): D-PBS, 0.5% Albumin Fraction V (Roth), 2 mM EDTA, *see* **Note 4**.
6. Blocking solution: D-PBS, 5% goat serum (Gibco), Fc Receptor block clone 24G2 diluted at 30 μg/mL, and Rat serum (Sigma) diluted at 16 μg/mL.
7. BD IMag™ Cell separation magnet, *see* **Note 5**.

8. Antibodies and beads:
   (a) Mouse IgG1 anti-bovine MHC-II antibody isotype IgG1, clone CAT82A, diluted at 0.5 μg/mL in buffered saline, (Monoclonal Antibody Center, Washington State University), *see* **Note 6**.
   (b) Anti-mouse IgG1 Magnetic particles-DM (BD Biosciences).
   (c) Mouse IgM anti-bovine G1 antibody, clone CH138A, diluted at 0.5 μg/mL in buffered saline, (Monoclonal Antibody Center, Washington State University).
   (d) Goat anti-mouse IgM biotin (Southern biotech), diluted at 2 μg/mL in buffered saline.
   (e) Streptavidin beads (BD Biosciences).
9. Centrifuge 5810R (Eppendorf).
10. Trypan Blue.

2.5 Cytospin and Kwik-Diff Staining

1. Cut edges frosted microscopic slides (VWR).
2. Filter cards (VWR).
3. Cell-counting chamber (BRAND).
4. T62.2 Cytocentrifuge with accessories (MLW electronic).
5. Optical microscope.

2.6 Phagocytosis

1. Sterile U-bottom polypropylene 96-well plate, *see* **Note 7**.
2. Assay medium: RPMI 1640 medium supplemented with 2% FCS, 50 μM 2-Mercaptoethanol, 2 mM L-Glutamine, 10 mM HEPES.
3. *E. coli* bioparticles Fluorescein conjugate (Invitrogen), *see* **Note 8**.
4. Polystyrene 96-well V-bottom plates (Roth).
5. Class II biological safety cabinet to provide a sterile working environment.
6. Incubator set at 37 °C with 5%CO_2.
7. 4% Paraformaldehyde (PFA)
8. D-PBS.
9. FACS tubes.
10. Flow cytometer.

2.7 Chemiluminescence

1. Luminol (Sigma).
2. Peroxidase from horseradish (HRP, Sigma).
3. Phorbol myristate acetate (PMA, Sigma).
4. Dimethyl sulfoxide (DMSO, Sigma).

5. RPMI 1640 medium with sodium carbonate, without L-glutamine and Phenol red and supplemented with 1% FBS (Biochrom), *see* **Note 9**.
6. 96 Flat bottom non-treated sterile white microwell plate, *see* **Note 10**.
7. TECAN SPARK plate reader.

3 Methods

3.1 FACS Sorting of Bovine Neutrophil

1. Collect bovine blood from the jugular vein into vacutainer K2 EDTA tubes (10 mL).
2. Centrifuge the tube(s) at 1000 × *g* for 10 min at 20 °C, *see* **Note 11**.
3. Remove the plasma layer and the buffy coat.
4. Lyse red blood cells by adding four volumes of Red Blood Cell Lysing Buffer (Sigma R7757) for 1 volume of blood, directly in the vacutainer tube. Invert slowly 5–6 times and incubate 5 min at room temperature.
5. Transfer the cells into a 50 mL conical bottom tube and add D-PBS 2 mM EDTA to 50 mL.
6. Centrifuge at 300 × *g* for 10 min at 20 °C.
7. Remove supernatant and wash the cells another time with 50 mL PBS 2 mM EDTA.
8. Resuspend the cells in 5 mL of D-PBS.
9. To quantify cell number, dilute 10 μL of cell suspension into 90 μL of Türk's (lysis of residual red blood cells) and count the cells in a Malassez cell-counting chamber.
10. In order to set the cell sorter parameters, put 1×10^6 cells/tubes in 4 Eppendorf tubes, respectively for unlabeled cells, IgM isotype control, IgM anti-bovine G1 antibody, and Fixable Viability Dye eFluor™ 780.
11. Put the rest of the cells in a 15 mL tube.
12. Centrifuge at 300 × *g* for 10 min at 4 °C.
13. Remove the supernatant and resuspend in PBS containing 10% of horse serum and 2 mM of EDTA: 50 μL into the Eppendorf tubes and 1 mL per 1×10^7 cells into the 15 mL tube.
14. Label the cells by adding either anti-bovine G1 antibody or IgM isotype control in the tubes. Incubate 30 min at 4 °C.
15. Centrifuge at 300 × *g* for 10 min at 4 °C.
16. Remove supernatant and wash the cells in D-PBS 2 mM EDTA.
17. Repeat **step 16** to wash another time.

18. Remove the supernatant and resuspend in D-PBS containing 10% of horse serum and 2 mM of EDTA: 50 µL into the Eppendorfs tubes and 1 mL per 1×10^7 cells into the 15 mL tube.
19. Label the cells by adding the secondary antibody Goat anti-mouse anti-IgM Alexa Fluor 594, and the Fixable Viability Dye eFluor 780 in different tubes for 30 min at 4 °C.
20. Repeat **steps 15–17** to wash the cells twice.
21. Resuspend the cells in RPMI medium 10 mM of HEPES, 0.5 mg/mL of bovine serum albumin (*see* **Note 12**) adjust the cell concentration to $10–15 \times 10^6$ cells/mL.
22. Proceed with the FACS sorting. Ensure fluidics stability, good droplet formation, and cellular viability after sorting; all crucial points for good sorting performance. Maintain room temperature stability during the whole duration of the sorting.
23. Fill the sheath tank with sheath liquid (Isoflow Sheath Fluid, Beckman Coulter) and pressurize the tank 1 day before the sorting to degas the liquid and to allow it to reach room temperature.
24. For a high-speed sorting, use a 90-µm nozzle (*see* **Note 13**). Sonicate the nozzle for 3 min before use.
25. Select the sheath pressure of 40 Psi (current value for a nozzle of 90 µm on a MoFlo Astrios^{EQ}).
26. Adapt the settings of the cell sorter for each day of sorting. The drop drive frequency and amplitude must respectively be around 67,000 Hz and 50 Volts. The drop delay is between 34 and 37 drops. Use «IntelliSort» to maintain the drop delay along with the sort.
27. For sort decision settings, use «Purify» mode (only positive cells per drop) and choose a droplet envelope of «1–2 Drops» (the maximum of positive events are sorted).
28. Set sorting speed between 15,000 and 18,000 cells/s (sorting efficiency between 70% and 90%).
29. Use a cooling water bath to maintain samples and recovery tubes at a temperature under 10°.
30. Collect sorted cells in 1.5 mL Eppendorf tubes, 3×10^5 cells per microtubes, *see* **Note 14**.
31. *See* Fig. 1 for the flow cytometry sorting strategy. Granulocytes are defined by their size and granularity by the R1 gate, and dead cells are excluded by the Fixable Viability Dye eFluor780 staining in gate R2. In R1, not R2 gate, neutrophils highly expressed the G1 marker (R3 gate), eosinophils are $G1^{low}$ or ^{int} (R4) depending on the animal (left vs. right panel).
32. Sorted neutrophils can be cultured in RPMI Glutamine 1 mg/mL BSA 10 mM HEPES.

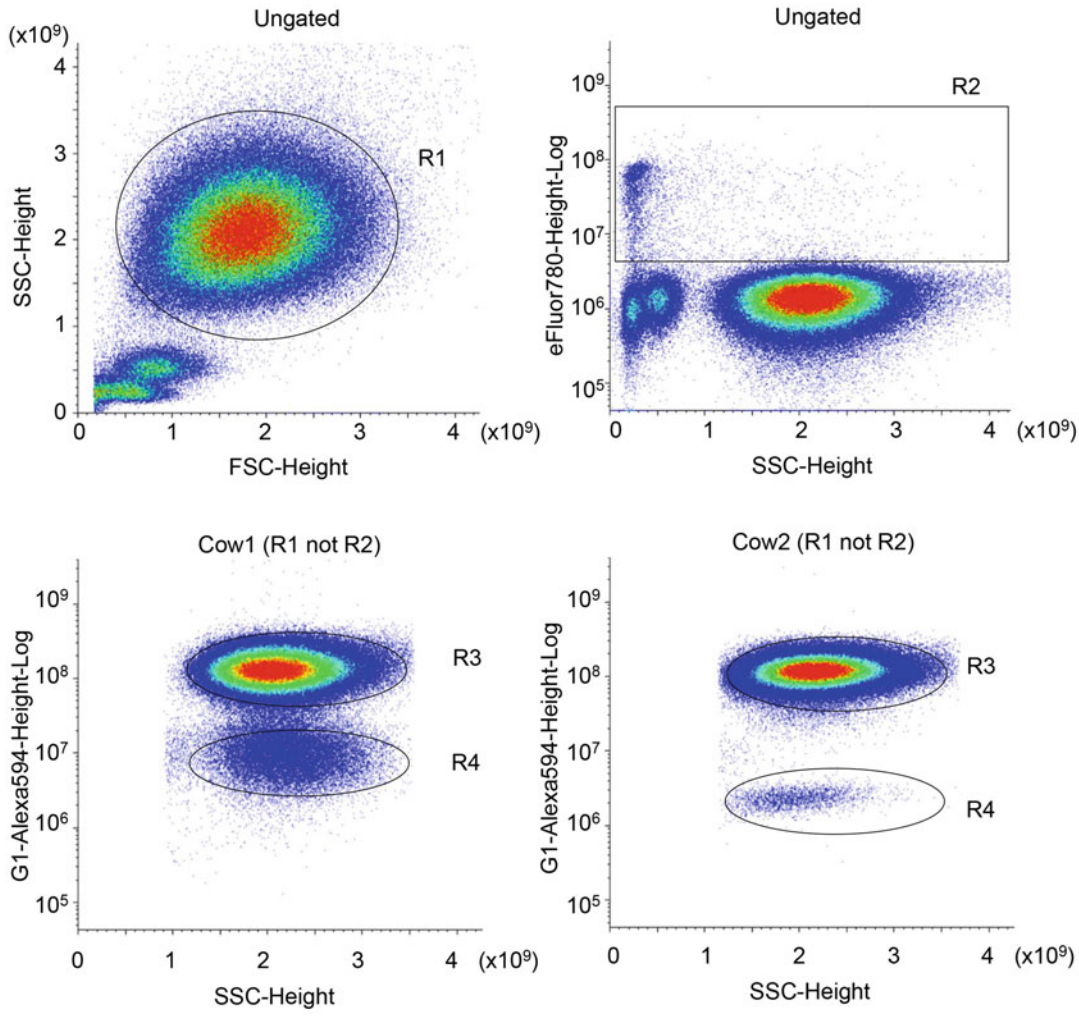

Fig. 1 Bovine neutrophils cell sorting strategy. Bovine blood cells are labeled with the Fixable Viability Dye eFluor780 marker and the mouse anti-granulocyte G1 antibody, revealed by a Goat and Mouse IgM Alexa 594 secondary antibody. Cells are sorted with a MoFlo Astrios^{EQ} cytometer. Granulocytes are defined by their size and granularity by the R1 gate, and dead cells are excluded by the Fixable Viability Dye eFLuor780 staining in gate R2. In R1, not R2 gate, neutrophils highly expressed the G1 marker (R3 gate), eosinophils are G1^{low} or ^{int} (R4) depending on the animal (left vs. right panel)

3.2 Cytospin and May-Grünwald Giemsa Staining

1. Resuspend between 5×10^4 and 2×10^5 cells in 200 µL of RPMI medium.
2. Prepare cytocentrifuge with a labeled slide, chamber, and blotter for each sample.
3. Add the totality of each cell suspension to the slide chamber.
4. Centrifuge at 1000 rpm for 3 min.
5. Carefully remove slides from cytocentrifuge and allow drying overnight.

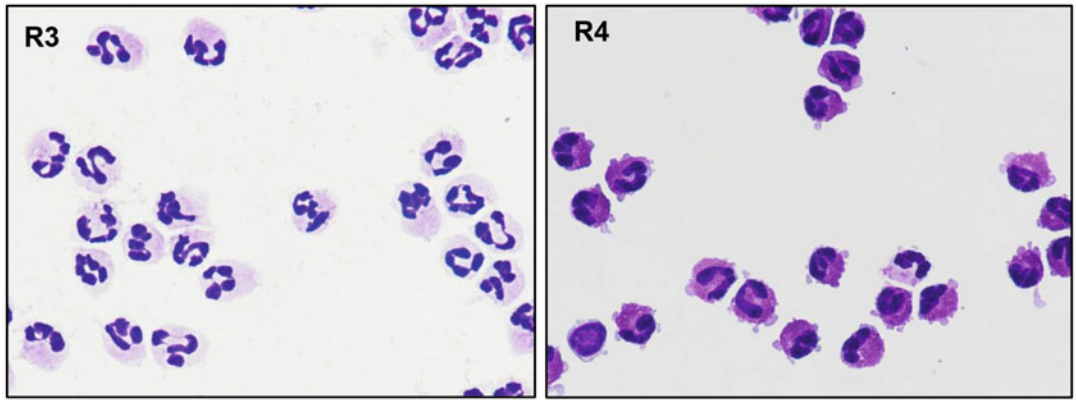

Fig. 2 Observation of sorted neutrophils and eosinophils. Sorted cells in R3 (neutrophils) and R4 (eosinophils) gates depicted in Fig. 1 are cytocentrifuged and stained with May-Grünwald-Giemsa according to the manufacturer's instruction (Magnification ×60)

6. Stain the cells with the May-Grünwald Giemsa staining according to the manufacturer's recommendations. Allow drying for at least 2 h.

   For optical mounting, use EUKITT mounting medium (few μL), add a coverslip and allow drying at least 30 min before observation with a microscope (Fig. 2).

3.3 Magnetic Isolation of Bovine Neutrophil

1. Collect bovine blood from jugular vein of a healthy cow in 9 mL vacutainer tubes containing 1.6 mg EDTA/mL blood.
2. Centrifuge the vacutainer tubes at 1000 × *g* for 15 min, *with brake*.
3. After centrifugation, remove plasma and top 1/3rd layer of cells and collect lower 2/3rd pellet.
4. Perform erylysis by transferring the fractions to 50 mL falcon tube and adding 9× the volume of erylysis buffer.
5. Invert the tubes slowly until the solution becomes clear in approximately 1 min and reconstitute the osmolarity using 1:10 10× sterile PBS.
6. Fill the tubes with D- PBS and centrifuge at 300 × *g* for 10 min at RT.
7. Repeat erylysis step if the RBC lysis is not successful, *see* **Note 15**.
8. Remove the supernatant and wash the pellet once with D-PBS and centrifuge at 300 × *g* for 5 min to remove any remaining erylysis buffer.
9. Resuspend the resulting pellet in 1000 μL blocking solution and count total leukocytes on Neubauer chamber using trypan blue as a sample diluent.

10. Adjust the cells to 2×10^7/mL using PBS and take required amount of cells in a FACS tube considering approximately half of the cells as neutrophils.
11. Centrifuge the FACS tube at $453 \times g$ for 3 min at RT.
12. Resuspend the resulting pellet in 100 μL P-BSA.
13. Label the cells by adding the primary anti-bovine MHC-II antibody in 100 μL volume and incubate the tube for 10 min on ice.
14. Wash by adding 1000 μL P-BSA and centrifuge at $453 \times g$ for 3 min at RT.
15. Discard supernatant and add 100 μL P-BSA and 10 μL anti-mouse IgG1 magnetic particles (BD Biosciences; 10–50 μL/10^7 cells is recommended), *see* **Note 16**.
16. Incubate the tube for 15 min on ice.
17. Adjust the final volume to 2 mL with P-BSA and place the tube in the BD IMag cell separation magnet for 10 min.
18. Aspirate the supernatant carefully using 1 mL pipette with the tube still in the magnet.
19. Transfer the supernatant into a new FACS tube (negative selection). The magnetically labeled unwanted cells remain attached to the original tube by the magnet field.
20. Place the tube containing the supernatant in the magnetic field for 4 min to get rid of any remaining magnetically labeled cells.
21. Repeat **steps 18–20** two more times and centrifuge the tube at $453 \times g$ for 3 min at RT, *see* **Note 17**.
22. Label the cells by adding the anti-bovine G1 antibody in 100 μL volume in 200 μL P-BSA and incubate for 10 min on ice.
23. Wash by adding 1000 μL P-BSA and centrifuge at $453 \times g$ for 3 min at RT.
24. Resuspend pellet in 200 μL P-BSA containing anti-mouse IgM biotin antibody and incubate on ice for 10 min.
25. Add 10 μL streptavidin magnetic particles (BD Biosciences), recommended is 10–50 μL/10^7 cells, incubate the tube further for 15 min on ice, and afterward adjust the final volume to 2 mL with P-BSA and place the tube in the magnet for 10 min.
26. Aspirate the supernatant carefully without disturbing the tube using a 1 mL pipette (positive selection) and wash the magnetically labeled cells by adding 1000 μL P-BSA and leave the tube in the magnet for 5 min.
27. Place the supernatant in the magnetic field to recover any remaining labeled cells.

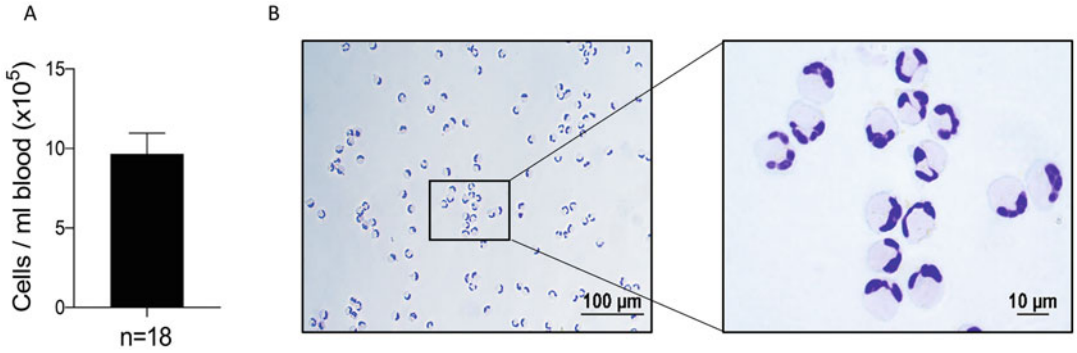

Fig. 3 Magnetically isolated bovine neutrophils with yield and purity. The yield (**a**) after isolation is calculated by dividing the total number of live neutrophils by the starting amount of blood. The isolated neutrophils are cytocentrifuged and stained with Kwik-Diff stain according to the manufacturer's instruction to evaluate the purity of isolated cells (**b**). Images are acquired using the microscope Nikon Type 120c with 20× and 100× objectives

28. Repeat **steps 26** and **27** for at least two more times.
29. Resuspend the magentically labeled cell pellet in 1000 µL P-BSA.
30. Estimate the yield by counting the cells by trypan blue staining on Neubauer chamber (Fig. 3a depicts cell yields), *see* **Note 18**.

3.4 Cytospin and Kwik-Diff Staining

1. Resuspend 5×10^5 cells in 750 µL PBS.
2. Prepare a cytospin cuvette with a labeled object slide, filter paper, cuvette, holder assembled together.
3. Load the cell suspension to the slide chamber.
4. Centrifuge at 900 rpm for 3 min, *see* **Note 19**.
5. Carefully remove slides from cytocentrifuge and air-dry sample slide.
6. Stain the slide with Diff-Quik according to manufacturer's instructions.
7. Allow the slide to dry for few hours before visualization under microscope (Fig. 3b).

3.5 Phagocytosis Assay

1. Seed 5×10^5 cells in 100 µL assay medium/well in a U-bottom polypropylene 96-well plate in triplicates.
2. Incubate the plate for 30 min at 37 °C and 5% CO_2.
3. Prepare *E. coli* bioparticles according to manufacturer's instructions at MOI of 1.
4. Add 50 µL *E. coli* bioparticles to respective wells for 10 min.
5. Transfer the cells to a V-bottom plate at the end of the assay.
6. Centrifuge the plate at $453 \times g$ for 3 min to remove any extracellular particles.

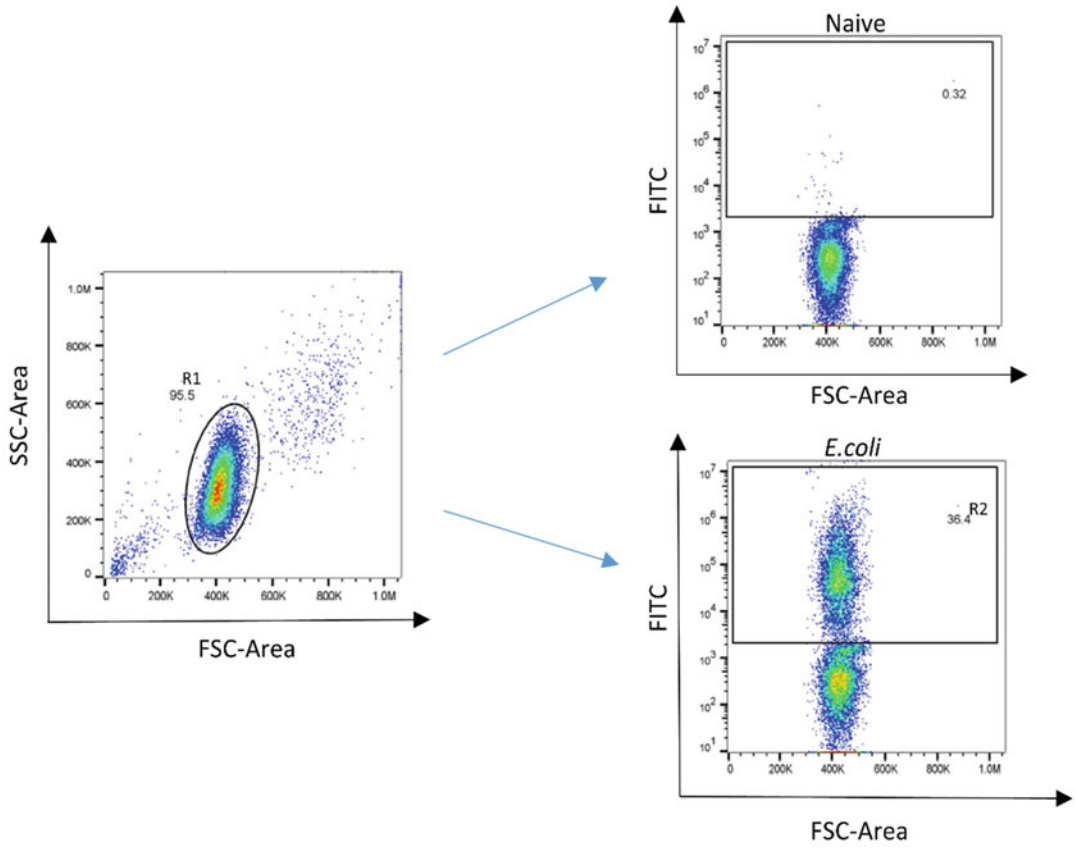

Fig. 4 Phagocytosis of *E. coli* particles by magnetically isolated bovine neutrophils. Neutrophils are stimulated with *E. coli* particles at MOI 1 for 10 min. Plots display the gating strategy with neutrophils gated in R1, and cells that phagocytosed FITC-labeled *E. coli* in R2. Data representative of $n = 3$

7. Add 100 µL D-PBS and centrifuge at 453 × *g* for 3 min.
8. Discard the supernatant and resuspend the pellet in D-PBS or fix the sample with 100 µL 4% PFA and incubate at RT in dark for 20 min, *see* **Note 20**.
9. Centrifuge the plate at 453 × *g* for 3 min and remove PFA carefully in a separate waste.
10. Resuspend the pellet in 300 µL D-PBS.
11. Transfer the cells to FACS tubes and acquire on flow cytometer (*see* Fig. 4).

3.6 Chemiluminescence Assay

Reactive oxygen species (ROS) production in isolated human neutrophils can be measured using luminol and horseradish peroxidase (HRP)-based chemiluminescence assay [13, 14].

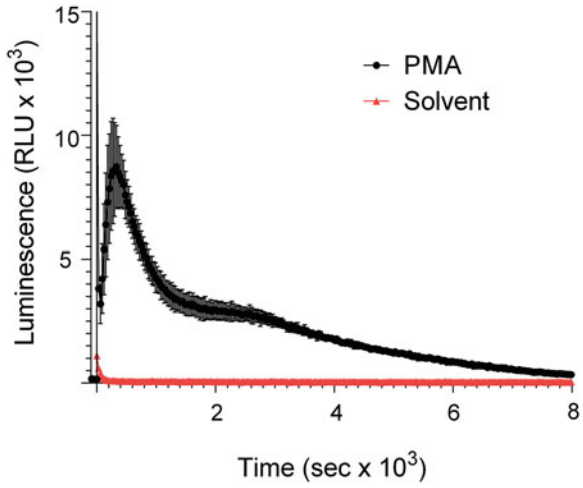

Fig. 5 ROS burst by magnetically isolated bovine neutrophils. Neutrophils are stimulated with 1 μM PMA and subjected to ROS luminol assay to quantify the ROS production. Release of ROS is monitored by reading luminescence over time with a maximum burst observed at 500 s. The graph is representative of $n = 4$

1. Seed 5×10^5 cells in 100 μL in assay medium containing 1% bovine serum and 50 μM luminol and 1.2 U/mL HRP, in a flat bottom non-treated 96 white well plate in triplicates.
2. Incubate at 37 °C and 5% CO_2 for 30 min.
3. Preheat plate reader (TECAN SPARK) to 37 °C and 5% CO_2 and preset the reading parameters.
4. Prepare the stimuli (PMA 1 μM, in DMSO) in assay media.
5. Remove the plate from the incubator, place it in the plate reader, and measure 10 cycles of baseline at 1000 ms/cycle.
6. Pipette PMA and DMSO control in 100 μL starting with DMSO control and ending with PMA addition, *see* **Note 21**.
7. Immediately put the plate in the machine and measure further in continuous rounds, for at least 450 cycles (*see* Fig. 5).

4 Notes

1. Use a cell-culture approved BSA with extremely low endotoxin level (≤1.0 EU/mg) to prevent neutrophil activation.
2. If Red Blood Cell Lysing Buffer is stored at 4 °C, allow the buffer to reach room temperature prior to use. If too cold, the solution will not work optimally!
3. Clone CH138A is an IgM. In the case of multiple staining, we observed better results when cells are first labeled with the other antibodies, then the CH138A. One possible explanation

is the high expression of G1 on neutrophils and the presence of sterically hindered pentameric IgM. Kingfisher Biotech also commercializes a Mouse IgG1 anti-G1 antibody (Clone MM20A). We found similar results with this clone and recommend the dilution 1 μg/mL.

4. We tested different buffer compositions for optimal conditions and in our hands BD IMag buffer containing D-PBS supplemented with 0.5% Albumin Fraction V and 2 mM EDTA helps in reducing cell clumps.
5. The Cell Separation Magnet contains a strong permanent magnet; therefore, persons wearing cardiac pacemakers should not handle the magnet and it should be stored away from any electronic equipment.
6. This antibody is most important to increase purity of the cells as it stains all the MHCII+ cells leaving the neutrophils (MHCII-) unlabeled.
7. Use polypropylene plates in case cells need to be removed for further analysis. These plates minimize adherence of the cells to the well.
8. *E. coli* bioparticles are provided as lyophilized powder, for reconstitution and exact particle numbers carefully check the manufacturer's instructions.
9. For chemiluminescence assay use medium without phenol red to avoid any interference in measurements due to pH changes.
10. We use 96 flat-bottom white well plate for chemiluminescence assay as it gives maximum reflection and reduces autofluorescence and autoluminescence caused due to surrounding wells.
11. Work at room temperature and centrifuge between 18 °C and 20 °C to prevent neutrophil activation by temperature changes.
12. Do not increase BSA concentration to avoid foaming.
13. The smaller the nozzle, the higher is the drop drive frequency and consequently the sorting speed. Sonicate the nozzle just before the startup of the cell sorter and visually inspect the nozzle with a binocular stereo microscope to verify the absence of salt crystals, cellular debris, or agglutinates inside. Resonicate the nozzle until clean if needed.
14. For cell recovery, we superpose a 1.5 mL Eppendorf tube containing 400 μL of RPMI 10 mM HEPES, 2 mM EDTA, and 0.5 mg/mL BSA, above a tube of 5 mL. This preserves cell viability by reducing the falling distance and avoiding the cells to fall on the tube's wall.
15. First erylysis step should be followed as indicated in the method; in our hands, we did not require a second erylysis step.

16. The amount of magnetic particles we used were sufficient for labeling 2×10^7 bovine leukocytes.
17. Washing steps are crucial to increase purity in this step to avoid carrying over undesirable cells before proceeding toward next steps.
18. The yield of isolated neutrophils depends on the breed and age of cows used for blood donation; we sampled German Holstein cows with age ranging from 4 to 6 years.
19. The speed and time of the centrifugation should be considered to avoid splashing the cells on the glass slide. This might lead to cell disruption.
20. PFA is carcinogenic and should be discarded as a separate waste.
21. The positive control PMA should be pipetted in the end as ROS burst is within seconds and the plate should be immediately placed in the machine for measurements.

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