Video Article

Preparation of Myeloid Derived Suppressor Cells (MDSC) from Naive and Pancreatic Tumor-bearing Mice using Flow Cytometry and Automated Magnetic Activated Cell Sorting (AutoMACS)

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Abstract

MDSC are a heterogeneous population of immature macrophages, dendritic cells and granulocytes that accumulate in lymphoid organs in pathological conditions including parasitic infection, inflammation, traumatic stress, graft-versus-host disease, diabetes and cancer[1-7]. In mice, MDSC express Mac-1 (CD11b) and Gr-1 (Ly6G and Ly6C) surface antigens[7]. It is important to note that MDSC are well studied in various tumor-bearing hosts where they are significantly expanded and suppress anti-tumor immune responses compared to naïve counterparts[7-10]. However, depending on the pathological condition, there are different subpopulations of MDSC with distinct mechanisms and targets of suppression[11,12]. Therefore, effective methods to isolate viable MDSC populations are important in elucidating their different molecular mechanisms of suppression *in vitro* and *in vivo*.

Recently, the Ghansah group has reported the expansion of MDSC in a murine pancreatic cancer model. Our tumor-bearing MDSC display a loss of homeostasis and increased suppressive function compared to naïve MDSC [13]. MDSC percentages are significantly less in lymphoid compartments of naïve vs. tumor-bearing mice. This is a major caveat, which often hinders accurate comparative analyses of these MDSC. Therefore, enriching Gr-1$^+$ leukocytes from naïve mice prior to Fluorescence Activated Cell Sorting (FACS) enhances purity, viability and significantly reduces sort time. However, enrichment of Gr-1$^+$ leukocytes from tumor-bearing mice is optional as these are in abundance for quick FACS sorting. Therefore, in this protocol, we describe a highly efficient method of immunophenotyping MDSC and enriching Gr-1$^+$ leukocytes from spleens of naïve mice for sorting MDSC in a timely manner. Immunocompetent C57BL/6 mice are inoculated with murine Panc02 cells subcutaneously whereas naïve mice receive 1XPBS. Approximately 30 days post inoculation; spleens are harvested and processed into single-cell suspensions using a cell dissociation sieve. Splenocytes are then Red Blood Cell (RBC) lysed and an aliquot of these leukocytes are stained using fluorochrome-conjugated antibodies against Mac-1 and Gr-1 to immunophenotype MDSC percentages using Flow Cytometry. In a parallel experiment, whole leukocytes from naïve mice are stained with fluorescent-conjugated Gr-1 antibodies, incubated with PE-MicroBeads and positively selected using an automated Magnetic Activated Cell Sorting (autoMACS) Pro Separator. Next, an aliquot of Gr-1$^+$ leukocytes are stained with Mac-1 antibodies to identify the increase in MDSC percentages using Flow Cytometry. Now, these Gr1$^+$ enriched leukocytes are ready for FACS sorting of MDSC to be used in comparative analyses (naïve vs. tumor- bearing) in *in vivo* and *in vitro* assays.

Video Link

The video component of this article can be found at https://www.jove.com/video/3875/

Protocol

Prior to starting, prepare the following solutions:

3% Staining Media (SM):

-3% Fetal Bovine Serum (FBS) in 1X Phosphate Buffer Saline (PBS)

MACS Buffer (MB):

- 0.5% Albumin from Bovine Serum (BSA) in 1XPBS

1. Harvest Spleens from Mice

1. Subcutaneously inject 6-8 week of age C57BL/6 mice (Harlan) with 1.5 x 10^5 murine Panc02 cells suspended in 100 µl 1x PBS (tumor-bearing; TB). Control mice (Naïve) receive 100 µl 1XPBS.

2. Approximately 4 weeks post injection, euthanize mice by carbon dioxide asphyxiation.
3. Harvest spleens from mice by blunt dissection using forceps and scissors then weigh using a balance. Place spleens in separate, labeled 50 ml conical tubes containing 1 x PBS.

2. Generate a Single-cell Suspension of Leukocytes from Spleens

All procedures should be performed in a sterile environment under a Biological Safety Hood and cells and antibodies kept on ice.

1. Assemble the cell dissociation sieve by inserting the mesh screen into the opening of the cup towards the bottom. Then, insert the retaining ring into the threaded area with the slotted side up and use the ring key to tighten the retaining ring, thus holding the screen in place. Place the assembled sieve in a Petri dish containing 10 ml 1 x PBS.
2. Pool spleens and use glass pestle to grind spleens against the mesh screen of the cell dissociation sieve and into the Petri dish. Repeat for each treatment group of mice.
3. Filter cell suspension into a 50 ml conical tube using a 70 µm cell strainer and a 5 ml serological pipette. Centrifuge at 12,000 rpm (250-300 x g) for 5 min.
4. Remove supernatant and resuspend pellet in 5 ml 1 x RBC lysis buffer per spleen. Pipet up and down vigorously. Incubate at room temperature for 5 min. Stop the reaction by adding 20 ml of 1 x PBS. Pipet up and down vigorously. Centrifuge at 12,000 rpm (250-300 x g) for 5 min.
5. Remove supernatant and resuspend pellet in 20 ml sterile 1 x PBS. Pipet up and down vigorously.
6. Count cells using trypan blue and a hemacytometer and resuspend at desired concentration in 3% SM so that 50 µl is equivalent to $5x10^5$-$1x10^6$ cells ($1x10^7$ cells/ml - $2x10^7$ cells/ml).

3. Cell-surface Staining /Immunophenotyping of MDSC using Flow Cytometry

1. Label the wells of a 96-well V bottom plate for control and experimental samples and single stains for compensation controls (No Stain, NS; Mac-1-FITC; Gr-1-APC and DAPI).
2. Add $5x10^5$-$1x10^6$ cells equivalent to 50 µl/well of splenocytes to their respective wells in the 96-well V-bottom plate. Centrifuge plate at 12,000 rpm (250-300 x g) for 5 min.
3. Prepare "Master Mix" (MM) of Mouse BD Fc Block (Rat anti-mouse CD16/32 monoclonal antibody) diluted in 3% SM, in a 1.5 ml microcentrifuge tube on ice. As a starting point, use 1 µg Fc Block in 3% SM for a final volume of 50 µl, per well.
4. Carefully remove supernatant from each well of the 96-well V-bottom plate by quickly inverting the plate over and back, over a waste container or sink without disruption of the cell pellets.
5. Vortex, briefly centrifuge Fc Block MM for 5 seconds and add 50 µl to all pellets in the 96-well V-bottom plate. Mix well by gently pipetting up and down, leaving samples in their wells. Incubate plate for 15 min in the dark on ice. Centrifuge plate at 12,000 rpm (250-300 x g) for 5 min.
6. Prepare "Master Mix" (MM) of fluorochrome-conjugated antibodies diluted in 3% SM in a 1.5ml microcentrifuge tube on ice, while samples incubate with Fc Block. Antibodies should be titrated to determine optimal dilutions for staining procedures. As a starting point, combine a 1:25 dilution of Mac-1-FITC and 1:20 dilution of Gr-1-APC in 3% SM for a final volume of 50 µl, per sample well.
7. Carefully remove supernatant from each well of the 96-well V -bottom as previously described.
8. Vortex, briefly centrifuge MM of fluorescent-conjugated staining antibodies for 5 seconds and add 50 µl to control and experimental pellets. Mix well by gently pipetting up and down. For single-stain compensations, add a 1:25 dilution of Mac-1-FITC, 1:20 dilution of Gr-1-APC and 75 ng/ml of DAPI, in 3% SM for a final volume of 50 µl per well to their respective wells. Add 50 µl 3% SM to unstained well (No Stain). Mix well and incubate cells in 96-well V-bottom plate for 30 min in the dark on ice.
9. Label FACS tubes (5 ml, 12 mm x 75 mm polystyrene round bottom tubes) to correspond to each well in the 96-well V-bottom plate. Add 200 µl 3% SM to each FACS tube.
10. Centrifuge plate at 12,000 rpm (250-300 x g) for 5 min and remove supernatants. Wash pellets by adding 100 µl 3% SM to each pellet and mix well by gently pipetting up and down. Centrifuge plate at 12,000 rpm (250-300 x g) for 5 min. Repeat wash step once more.
11. Carefully remove supernatant from each well of the 96-well V-bottom as previously described. Resuspend each pellet in 100 µl 3% SM and mix well.
12. Transfer 100 µl resuspended pellet from each well of the 96-well V-bottom plate to their respectively labeled FACS tube.
13. Prior to Flow cytometry analysis, add 75 ng/ml DAPI to control and experimental samples and DAPI single stain compensation control.
14. Perform flow cytometric data acquisition of MDSC percentages. Perform compensation using the negative (no stain control) and the single positive controls. Set up a dot plot that displays the forward (FSC) versus side scatter (SSC) in log scale so that leukocyte populations of interest can be identified. Draw a large gate on all leukocytes, excluding debris and clumps with lowest forward and side scatter. From this parent gate, create a new dot plot that displays SSC versus DAPI and gate on DAPI- (live) cells. Select this newly gated population and create a dot plot that displays Mac-1 versus Gr-1 and gate on your double positive (Mac-1^+ Gr-1^+) MDSC.

4. Magnetic Enrichment of Gr-1^+ Leukocytes

1. Aliquot $1x10^7$ remaining unstained leukocytes into appropriately labeled FACS tubes and centrifuge at 12,000 rpm (250-300 x g) for 5 min.
2. Prepare MM of Gr-1-PE antibody in a 1.5 ml microcentrifuge tube. For up to 10^7 cells, use a 1:10 dilution of Gr-1-PE antibody in 50 µl MB, per sample. For greater cell numbers, scale up volumes accordingly. Briefly centrifuge for 5 seconds, add to leukocytes in FACS tubes and incubate for 15 min at 4 °C in the dark.
3. Add 2 ml of MB to FACS tubes, centrifuge at 12,000 rpm (250-300 x g) for 5 min and discard supernatant.
4. Prepare MM of Anti-PE MicroBeads in a 1.5 ml microcentrifuge tube. For up to 10^7 cells, use a 1:4 dilution of anti-PE MicroBeads in 200 µl MB, per sample. For greater cell numbers, scale up volumes accordingly. Briefly centrifuge for 5 seconds, add to leukocytes in FACS tubes and incubate for 15 min at 4 °C in the dark.
5. Add 2 ml of MB to FACS tubes, centrifuge at 12,000 rpm (250-300 x g) for 5 min and discard supernatant. Resuspend pellet in 3 ml of SB. Filter through a 70 µm strainer into a new, labeled 50 ml conical tube.

6. Prepare and prime Auto MACS Pro Separator. Refill all bottles with the appropriate solutions and empty the waste bottle, if necessary. Turn instrument on and examine status of fluid containers and column(s) after initialization. All symbols should be green. On the menu, select "Separation" from the upper menu bar followed by "Wash Now" from the lower menu bar. Select "Rinse" from the pop-up option followed by "Run" to start the priming process. Once the priming process is successfully completed, the instrument will then display that it is "Ready for Separation" under the Status menu.
7. Choose suitable chilled tube rack for tube sizes and place 50 ml conical tube with magnetically labeled cells in row A, 50 ml conical tube for negative fraction collection in row B and 15 ml conical tube for positive fraction collection in row C.
8. Choose "POSSEL_S" cell separation program for positive selection of labeled target cells in sensitive mode from samples. Magnetically labeled target cells are retained on the automats column; unlabeled cells are released into the negative fraction collection tube in row B. On automated retraction of the magnet, the labeled target cells will be released into the positive collection tube in row C of the tube rack.

5. Post-Sort Analysis of Gr-1$^+$ Enriched Leukocytes

1. Recount Gr-1$^+$ and Gr-1$^-$ fractions using trypan blue and a hemacytometer. Resuspend cells at desired concentration in 3% staining medium so that 50 µl is equivalent to 5x10^5-1x10^6 cells (1x10^7 cells/ml - 2x10^7 cells/ml) and transfer 50 µl to correspondingly labeled FACS tubes.
2. Prepare a MM of Mac-1 only, at a 1:25 dilution in 3% SM for a final volume of 50 µl per sample. Stain cells and prepare single stain compensations of Gr-PE, Mac-1 FITC and DAPI for flow cytometry analysis. Add 200 µl 3% SM and DAPI viability dye as previously described.
3. Perform flow cytometric data acquisition to determine Gr-1$^+$ and Gr-1$^-$ percentages and to also compare MDSC percentages pre- and post-autoMACS enrichment.

6. Representative Results

Here we show representative results for autoMACS enrichment of Gr-1$^+$ leukocytes from pooled, naïve spleens for subsequent FACS sorting of MDSC (**Figure 1**). 1x10^6 naïve leukocytes were stained with Mac-1-FITC and Gr-1-APC antibodies to identify MDSC percentages using a BD LSRII instrument, prior to autoMACS sorting. 1x10^7 naïve leukocytes were then stained with anti-Gr-1-PE antibodies and PE-MicroBeads for enrichment of Gr-1$^+$ leukocytes using an autoMACS Pro Separator. Post-autoMACS enrichment, Gr-1 percentages in Gr-1$^+$ and Gr-1$^-$ collected fractions were evaluated using flow cytometry. 1x10^6 Gr-1$^+$ leukocytes were removed and stained with Mac-1-FITC antibody to analyze and compare MDSC percentages of enriched, pooled naïve leukocytes to non-enriched, pooled tumor-bearing leukocytes by flow cytometry.

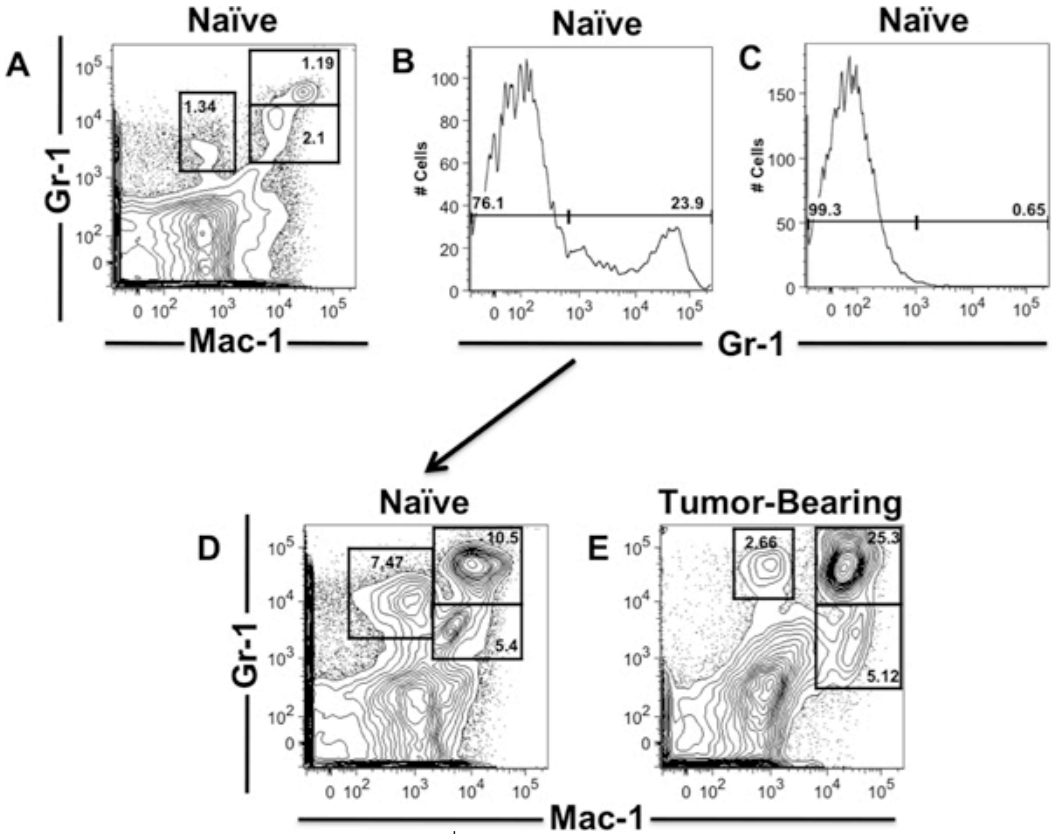

Figure 1. AutoMACS enrichment of Naïve Gr-1$^+$ leukocytes for MDSC FACS Sorting. Spleens were harvested from pancreatic tumor-bearing and naïve mice and processed into single-cell suspensions. Flow cytometry analysis of naïve leukocytes surface stained with Mac-1 and Gr-1 fluorescent-conjugated antibodies, prior to autoMACS enrichment (A). Flow cytometry analysis of Gr-1$^+$ (B) and Gr-1$^-$ (C) fractions post-autoMACS enrichment of Gr-1$^+$ cells from pooled naïve leuckocytes stained with Gr-1-PE antibodies and anti-PE MicroBeads. Flow cytometry analysis of MDSC and Gr-1$^+$ percentages post-autoMACS enrichment of Gr-1$^+$ cells from pooled naïve leukocytes (D) compared to non-enriched

pooled leukocytes from tumor-bearing mice (E) (naïve mice, n=5; tumor-bearing mice, n=3). MDSC and Gr-1 percentages are gated in the representative contour plots and histograms.

Discussion

This is a detailed method for processing and immunophentyping MDSC populations that is applicable to different lymphoid tissues from various animal models. In particular, autoMACS enrichment can be used for the isolation of various leukocyte populations including Gr-1 depletion of splenocytes [4], purification of myeloid subsets from splenocytes and lymph nodes [5], isolation of bone marrow neutrophils [14] and purification of $CD8^+$ T cells from spleen and lymph nodes [15]. Regardless of the cell population of interest, generating single-cell suspensions of leukocytes from the desired lymphoid tissues is required for optimal surface staining, flow cytometry analysis, autoMACS enrichment and FACS sorting. In this protocol, we used mechanical dissociation to create a single cell suspension of leukocytes. However, using enzyme digestion such as Collagenase D is also an adequate alternative to this protocol to increase the yield of leukocytes from spleens or other lymphoid organs [5].

Flow cytometry is an important technique for immunophenotyping leukocytes. Therefore, adequate preparation for flow cytometry and enrichment also requires the use of appropriate buffers for cell suspension and the dilution of antibody reagents. Many protocols alternate between FBS and BSA for preparing staining media and MACS buffer. However, these reagents are also commercially available from companies such as eBioscience, BD Pharmingen and Miltenyi Biotec. As explained by Miltenyi Biotec and eBioscience, both BSA and FBS prevent non-specific binding when staining leukocytes with fluorochrome-conjugated antibodies. More importantly, low percentages of these animal serum proteins maintain viability and prevent clumping of leukocytes [16]. However, from our experience, BSA works better for optimal staining and better resolution for autoMACS enrichment than FBS and is recommended in the described protocol.

In this protocol, we characterized murine MDSC using CD11b-FITC and Gr-1-APC fluorescent-conjugated antibodies and flow cytometry. It is imperative to titrate these antibodies prior to use to reduce high background fluorescence staining to prevent non-optimal results. In addition, when choosing antibodies for multicolor flow cytometry analysis, possible spectral overlap of the fluorochromes is also another critical factor to be considered [17,18]. Therefore, compensation controls in the form of single color stains for each fluorochome-conjugated antibody of interest or compensation beads, can correct for issues of spectral overlap [18]. Another important consideration for accurate flow cytometry results is the use of viability dyes. Dead cells can non-specifically bind to antibodies, thus creating false positive results [19]. In this protocol, we use the nucleic acid stain, DAPI as a viability dye to exclude dead cells from our analyses as it best complements the panel of fluorochrome-conjugated antibodies used with minimal spectral overlap. However, other viability dyes such as 7-Aminoactinomycin D (7-AAD) and propidium iodide (PI) may be used with the described staining methods depending on the panel of antibodies used. Currently, there are also a number of fixable dead cell stains available that allow stained cells to be fixed and permeabilized without losing the ability to discriminate viability (Invitrogen). Overall, other staining techniques are also important for accurate flow cytometry analysis. For example, in this protocol, the use of 96-well V-bottom plates allows for small, concentrated volumes of cells and reagents to be used for the analysis of MDSC and leukocyte percentages using flow cytometry. The use of the Master Mix (MM) approach is also a significant technique for equal immunofluorescence labeling of leukocytes in small volumes using either 96-well V-bottom plates or FACS Tubes. These techniques reduce the risk of cross contamination of samples, maintain sterile antibodies and reduce the amount of reagents and antibodies used.

The autoMACS Pro Separator allows for efficient and automated isolation of cell populations from multiple samples, in any species. In this protocol, indirect magnetic labeling of leukocytes using Gr-1-PE antibodies and magnetic PE MicroBeads is used for autoMACS enrichment of $Gr\text{-}1^+$ leukocytes. This procedure can be modified for autoMACS separation using MicroBeads to other fluorochrome-conjugated, biotinylated and unconjugated primary antibodies for the enrichment of various cell populations [20]. However, in this procedure, we highly recommend using PE-conjugated antibodies and anti-PE-microbeads as opposed to FITC for example, as the PE molecule is said to have multiple binding sites, thus resulting in stronger and better magnetic labeling (Miltenyi Biotec). Another possible modification to this protocol is the use of direct magnetic labeling as there are a wide variety of MACS MicroBeads for the isolation of human, mouse and rat leukocytes (Miltenyi Biotec). However, indirect magnetic labeling using a fluorochrome-conjugated primary antibody allows for flow cytometry evaluation of enriched or depleted leukocyte fractions without the need for additional staining. There are also a variety of MACS Separators available but the autoMACS Pro Separator System is advantageous as it allows for high-speed, automated sorting of up to six samples for gentle selection of highly viable leukocytes, in comparison to manual MACS Separators. However, manual MACS Separators are appropriate alternatives to the autoMACS Pro Separator, if available. To further enrich naïve $Gr\text{-}1^+$ leukocytes, a potential modification to this protocol would be to re-run the positively collected fraction using a new autoMACS column on the autoMACS Pro Separator. Enrichment of $Gr\text{-}1^+$ tumor-bearing leukocytes using the autoMACS Pro Separator is not recommended in this protocol since this results in significant reduction in the recovery of Gr-1 fractions as these cells tend to stick to the columns during enrichment in comparison to naïve leukocytes. Therefore, this protocol strongly recommends pooling tumor-bearing leukocytes for subsequent FACS sorting of viable MDSC.

Pre-enriched, pooled naïve leukocytes and pooled tumor-bearing leukocytes can used for future applications such as low-speed FACS sorting for the isolation of MDSC populations. This protocol is specifically designed to circumvent tedious and timely FACS sorting of MDSC from naïve mice, which is due to significantly lower percentages of MDSC. AutoMACS enrichment of $Gr\text{-}1^+$ leukocytes from naïve mice then allows for prompt, convenient and efficient FACS sorting of viable and functional MDSC for their use in *in vivo* and *in vitro* experiments. Direct sorting of lowly expressed cell populations such as naïve MDSC is a very inefficient process with lengthy sort times, high costs and reduced cell recovery and viability. Preparation and autoMACS enrichment of $Gr\text{-}1^+$ leukocytes from naïve mice takes approximately 45 minutes and increases MDSC percentages greater than 4-fold (**Figure 1**). This enrichment reduces the time of FACS sorting from approximately 12 hours for non-enriched leukocytes to approximately 20-30 minutes for the isolation of at least $1x10^6$ viable, enriched MDSC from pooled naïve leukocytes. However, FACS sorting of viable MDSC from non-enriched, pooled leukocytes from tumor-bearing mice will require less time due to an increased abundance of MDSC percentages in response to tumor burden. It is important to note that sorted MDSC and other leukocytes can then be used in both functional assays such as mixed leukocyte reactions (MLR)[4] and *in vivo* adoptive transfer experiments [21] as well as in non-functional assays including qRT-PCR [22], Western Blot [23] and cytospin/microscopy analyses [14]. Overall, this protocol can be modified for immunophenotyping and the enrichment of immunosuppressive and other leukocytes from lymphoid tissues or peripheral blood from mice, rat and humans to be used in a plethora of *in vivo* and *in vitro* assays.

Disclosures

No conflicts of interest declared.

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