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Isolation of myeloid derived suppressor cells from mouse tumor and determining their migration potential in vitro.

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Alisha DSouza
Editor,
JoVE
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Cambridge, MA 02140

Re: Re-Submission of manuscript Isolation of myeloid derived suppressor cells from mouse tumor and determining their migration potential *in vitro*

Dear Dr. DSouza,

Thank you for reviewing our manuscript titled “Isolation of myeloid derived suppressor cells from mouse tumor and determining their migration potential *in vitro”*.

We have made the necessary changes suggested by the editor and the reviewer. Please find attached our point-by-point address of the concerns.

We believe that this will be of broad interest to the readers, and point out that the data presented in this manuscript will benefit readers with MDSC related assays. The authors declare no conflict of interest associated with the data presented in the manuscript.

Sincerely,

Sharmistha Sarkar

TITLE:

Isolation of Myeloid-derived Suppressor Cells from Mouse Tumor and Determining Their Migration Potential *In Vitro*

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KEYWORDS:
MDSC isolation, migration assay, tumor dissociation, flow cytometry, murine, *in vitro*

SHORT ABSTRACT:
This article outlines the procedures to isolate myeloid-derived suppressor cells from mouse solid tumors and perform an *in vitro* assay with the cells to determine their response migration potential to certain soluble factors like cytokines and chemokines.

LONG ABSTRACT:
The importance of the immune response in cancer and other diseases (like diabetes mellitus, alzheimers, cystic fibrosis) is now known, and the manipulation of the immune system as a therapy to treat cancer is gaining attention. The immune system regulates tumorigenesis both negatively and positively. The myeloid-derived suppressor cells (MDSCs) are a population of immune cells that are increased during cancer, inflammation, and infection. These cells influence the immune response and effectively suppresses the anti-tumor T cell response. They serve as potential targets for therapeutic intervention to effectively use the immune system to inhibit tumorigenesis. To better understand how such intervention can be applied it is important to study these cell types. Using mouse ovarian tumors, we describe the isolation of MDSCs from solid tissue using gentle dissociation techniques. We further describe how MDSCs are isolated from such dissociated tissue based on the expression of cell surface markers with the help of flow cytometry. Additionally, we describe the procedure to perform an *in vitro* MDSC migration assay to determine the migration potential of these cells in response to soluble factors like cytokines and chemokines.

INTRODUCTION:
In recent years a number of studies have focused on understanding the role of immune cells in cancer development and progression. One way by which tumor cells evade the immune system is through the expression of immunosuppressive factors that activate, upregulate, and attract immune suppressive cells like MDSCs in the tumor microenvironment[1].

MDSCs are a population of immature myeloid cells that are generated in the bone marrow. Under normal conditions, these immature cells differentiate into mature myeloid cells like macrophages, monocytes, or dendritic cells[2]. Under pathologic condition, these cells fail to differentiate completely into mature myeloid cells. Instead, they are expanded and activated by factors secreted by activated T cells, tumor cells, and stromal cells. Secretory proteins like stem-cell factor[3], IL-6, granulocyte/macrophage CSF (G-CSF;M-CSF)[4], vascular endothelial growth factor[5], IFNγ[6], ligands for Toll-like receptors (TLRs)[7], IL-4[8], IL-13[9], and transforming growth factor β (TGFβ)[10] promote MDSC proliferation and activation. Upon activation, these cells upregulate expression of factors like arginase 1[11], inducible nitric oxide synthase[12], and reactive oxygen species[13-17], resulting in their remarkable potential to suppress the T-cell response. In addition to inhibiting the adaptive immune response, MDSCs have been reported to negatively influence the innate immune response through macrophages[18] and NK cells[19].

These cells lack markers of mature myeloid cells. In mice, MDSCs are characterized by the expression of the cell surface markers GR1 and CD11b. Based on morphology, they are further characterized as granulocytic ($CD11b^{+}LY6G^{+}LY6C^{low}$) or monocytic ($CD11b^{+}LY6G^{-}LY6C^{hi}$) MDSCs. Both subtypes have immunosuppressive properties, but in mouse tumors, the granulocytic MDSCs are the major population[17]. In humans, MDSC markers are not well characterized. Often, the monocytic MDSCs are identified as $CD11b^{+}$ $CD14^{+}$ $CD33^{+high}$ HLA-$DR^{neg/Low}$ and $CD66b^{neg}$, while granulocytic MDSCs are identified as $CD11b^{+}$ $CD14^{neg}$ $CD33^{+low}$ HLA-DR^{neg} CD66b.

A number of functional studies in mouse tumor models have established the importance of MDSCs in tumor development and T cell suppression. Hence, they are potential targets for therapeutic intervention and it is critical to study this subset of immune cells to understand immune suppression in cancer. We need to identify new factors that may promote or inhibit MDSC expansion and activation, which can be utilized for therapy. To perform such tests, we need to isolate MDSCs in a simple and timely manner to obtain live, non-contaminated cells. Here, we outline the methods to isolate a viable MDSC population from mouse tumor. This method can be used to isolate MDSC from any other tissue following the same method. Cell types other than MDSCs can also be isolated by this method using the appropriate cell-specific markers. We also outline methods to determine MDSC migration potential towards a cytokine gradient. Here we use tumor necrosis factor α (TNFα) as an example. Other cytokines can also be tested. Instead of conditioned media from cell lines, regular media supplemented with specific cytokines in varying concentrations can be used to test the role of those cytokines in MDSC migration from a particular tumor type.

PROTOCOL:

All procedures were performed under the guidance of University of Texas at MD Anderson IACUC review board.

1. Reagent Preparation

1.1 Preparation of medium for migration assay

1.1. Prepare 500 mL of complete RPMI media for the migration assay: RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% pen-strep (penicillin 500 U/mL and streptomycin 500 µg/mL).

1.2 Preparation of buffers for staining MDSCs

1.2.1. Prepare the tumor dissociation buffer with 100 U/mL Collagenase type IV and 100 µg/mL DNase in RPMI with 10% FBS. Store the buffer on ice.

1.2.2. Prepare the 1X RBC lysis buffer by diluting the 10X stock 1:10 in sterile water.

1.2.3. Prepare the FACS buffer by adding 1% heat-inactivated FBS to 1X PBS.

1.2.4. Add 1 µL of amine reactive viability dye (binds and fluoresces non-viable or necrotic cells more strongly than live cells) to 1 mL of FACS buffer to stain for dead cells. Store the solution in the dark on ice or at 4 °C.

1.2.5. Prepare the staining buffer by adding 20 µL each of the following antibodies (α-FITC-GR1, α-BV510-CD11b, α-APC-Cy7-CD45, α-PE-Cy7-Ly6C, and α-APC-Ly6G) in 940 mL of FACS buffer with cell viability dye. Keep the antibody cocktail covered on ice or 4 °C.

1.3 Generation of murine tumors

Note: When syngeneic mice are injected with a murine ovarian cancer cell line 333 derived from TPP[20] mice, they will spontaneously form tumors 8–12 weeks post injection. These cells can be modified to stably express PRKCI by lentiviral infection[21].

1.3.1. Grow 333 cells in DMEM with glutamine supplementation on 15 cm tissue culture plates in a tissue culture incubator at 37 °C and 5% CO_2.

1.3.2. To passage the cells: aspirate the media, add 5 mL of 2.5% trypsin with EDTA, and incubate for 1–2 min at 37 °C and 5% CO_2. When the cells detach from the tissue culture plate, neutralize trypsin with 5 mL of DMEM and collect the cells in a 15 mL tube. Centrifuge the cells at 300 x g for 5 min at room temperature.

1.3.3. Discard the supernatant and resuspend the cell pellet in 10 mL of HBSS media. Count the cells using trypan blue (4%, 1:1 cell suspension to dye ratio) and a cell counter.

1.3.4. Aliquot the cell suspension according to cells required and concentration of cells. Inject intraperitoneally, 10 million cells per mice into 4–6 week old syngeneic BL/6 mice. Let the tumors grow for 8–12 weeks.

2. Isolation of Single Cells from Tumor Tissue and Staining Cells for FACS

2.1. When the mice develop tumors 8–12 weeks post injection (determined by bloated abdomen cavity), euthanize the mice by CO_2 asphyxiation.

2.2. Pin down all four limbs of a mouse on a polystyrene foam board with the abdominal cavity facing up. Cut open the abdominal cavity using sharp scissors and expose the tumor tissue. If the tissue is isolated from a large tumor, avoid using the center mass of the tumor if it appears necrotic.

2.3. Aseptically collect the freshly dissected murine intraperitoneal tumor from the mice in a 50 mL tube with 2.5 mL/g of tumor dissociation buffer to tissue.

Note: This protocol can be used for subcutaneous xenograft tumors or for the isolation of single cells from most mouse tissue. The percentage of MDSCs in tumors varies and needs to be determined for the specific tumor type. To collect enough MDSCs from the isolation, multiple tumor samples from the same experimental background can be combined.

2.4. In a tissue culture hood, transfer the tissue to a culture dish with a small amount of tumor and dissociation buffer and chop the tumor with a razor blade into smaller pieces, about 2 mm in size.

2.5. Transfer the chopped tissue back into the tube and incubate the partially dissociated tissue at 37 °C with continuous rotation on a rotator at approximately 12 rpm for 45 min.

2.6. After incubation, centrifuge the dissociated tissue in the tube at 300 x g for 5 min at room temperature. Discard the supernatant.

2.7. Resuspend the dissociated tissue in 25 mL of RPMI and filter through a 70-µm cell strainer placed on a 50 mL tube to collect single cells from the dissociated tissue.

2.8. Centrifuge the single cell suspension at 300 x g for 5 min at room temperature and discard the supernatant.

2.9. Add 25 mL of 1X RBC lysis buffer to the tube and incubate on ice for 5 min to lyse RBCs in the cell suspension.

2.10. Centrifuge the cells at 300 x g for 5 min at room temperature and discard the red supernatant. If the cell pellet still appears red or dark brown, repeat the RBC lysis step (step 2.9).

2.11. Add 25 mL of PBS to the cell pellet and centrifuge at 300 x g for 5 min at room temperature. Discard the supernatant.

2.12. Resuspend the isolated single cells in 1 mL of FACS buffer and proceed to stain the immune cells.

3. Staining and Isolation of MDSCs from Single Cells Isolated from Mouse Tumor

3.1. Aspirate two 50 µL aliquots of the single cell suspension into centrifuge tubes for the controls. To one tube, add 100 µL of FACS buffer. To the other tube, add 100 µL FACS buffer containing cell viability dye (see step 1.2.4). Keep the controls on ice until use.

3.2. Centrifuge the remaining cell suspension at 300 x g for 5 min and then incubate the cell pellet with FACS staining buffer (see step 1.2.5) containing anti-CD45 (4 µg/mL), anti-Gr1 (10 µg/mL), anti-Cd11b (4 µg/mL), anti-Ly6C (4 µg/mL), and anti-Ly6G (4 µg/mL) antibodies.

3.2.1. Incubate the cell suspension with the antibody solution on ice in the dark for 30 min.

3.2.2. Centrifuge the antibody conjugated cells at 300 x g for 5 min at 4 °C and discard the supernatant. Keep the tubes covered with aluminum foil to avoid light exposure.

3.2.3. Resuspend the cells in FACS buffer, count them using a cell counter, centrifuge the cells at 300 x g for 5 min at 4 °C, and discard the supernatant. Repeat this step once more and discard the supernatant.

3.2.4. Resuspend the stained cells in 1 mL of FACS buffer and keep on ice covered with aluminum foil before FACS.

3.3. For the color compensation controls for flow cytometry, take 100 µL of compensation beads in a centrifuge tube and add 1 mL of FACS buffer. Centrifuge the beads at 300 x g for 5 min and discard the supernatant.

3.3.1. Resuspend the beads in 150 µL of FACS buffer and divide them equally into three parts (50 µL/tube). To each part, add anti-FITC-GR1 (1.6 µg/mL), anti-BV510-CD11b (0.6 µg/mL), and anti-APC-Cy7-CD45 (0.6 µg/mL) for the staining. Incubate for 15 min on ice in the dark.

3.3.2. Centrifuge the beads at 300 x g for 5 min and discard the supernatant.

3.3.3. Add 1 mL of FACS buffer and centrifuge the beads at 300 x g for 5 min. Discard the supernatant.

3.3.4. Resuspend the beads in 500 µL of FACS buffer and store on ice before flow cytometry and cell sorting.

4. Flow cytometry and Cell Sorting of the MDSCs from Mouse Tumor

Note: See **Figure 1**. Here, we performed flow cytometry and cell sorting in a core facility.

4.1. Sort the stained MDSCs using fluorescence-activated cell sorting (FACS). For color compensation, use the compensation beads stained with individual antibodies (step 3.3). Use the cells stained with only the viability dye for the compensation of dead and live cells (step 3.1).

4.2. Use the unstained cells (step 3.1) as a negative control before running the stained samples.

4.3. For sorting the samples, gate on the total events based on forward and side scatter of the density plots to remove all doublets and cellular debris.

4.4. Exclude all dead cells based on the positive viability dye staining.

4.5. Gate on live single cells positive for CD45 staining (total leukocytes), followed by cells double positive for $CD11b^+$ and $Gr1^+$ to collect MDSCs. $CD11b^+/Gr1^+$ MDSCs can be further gated for monocytic and granulocytic markers with Ly6C and Ly6G as shown in **Figure 1**. For the purpose of the MDSC migration assay, $CD11b^+/Gr1^+$ MDSCs are sufficient.

4.6. Sort the cells into a collection tube with complete RPMI media.

5. MDSC Migration Assay

5.1. Centrifuge the sorted MDSCs at 300 x g for 5 min at room temperature. Discard the supernatant.

5.2. Resuspend the cell pellet with 10 mL of RPMI media with pen-strep and without FBS. Centrifuge at 300 x g for 5 min at room temperature and discard the supernatant. Repeat this step once.

5.3. Prepare a cell suspension of the sorted cells at 0.3–0.5 x 10^6 cells per 0.5 mL of RPMI media with pen-strep and without FBS.

5.4. Count the cells using trypan blue (4%, 1:1 cell suspension to dye ratio) and a cell counter.

5.5. Add 500 mL/well of complete RPMI media (with pen-strep and FBS; see step 1.1) of a 24-well plate. Add desired concentrations of the cytokine to be tested. As a control, add neutralizing antibody corresponding to the cytokine tested as one set of conditions. Replicate each condition in at least 3 wells.

5.6. Gently place a membrane-insert into each well to avoid the formation of any bubbles under the membrane.

5.7. Add 500 mL of cell suspension containing 0.3–0.5 x10^6 sorted MDSCs into each membrane insert and incubate the plate for 6 h at 37 °C, 5% CO_2 in a tissue culture incubator.

5.8. Count the migrated MDSCs in the bottom chamber of the transwell system by counting the absolute number of FITC positive cells using flow cytometry.

REPRESENTATIVE RESULTS:

Here, we present results obtained from the isolation of MDSCs from mouse ovarian tumors[20]. Following the procedure described above, we isolated single cells and stained them for MDSCs. MDSCs in the tumors were labeled with APC-Cy7-CD45, FITC-GR1, PE-CD11b. To elucidate the MDSC population, these cells can be further stained with Ly6C and Ly6G, as shown in the gating strategy in **Figure 1**. Labeled cells were sorted by flow cytometry. Labeled cells were first gated for total events based on the forward and side scatter of the density plots to remove all doublets and cellular debris. All dead cells were excluded based on viability dye staining. Live cells were then gated for CD45 staining for total leukocytes, followed by $CD11b^{+}Gr1^{+}$ double positive cells to isolate MDSCs (**Figure 1**). In our tumors, we obtained a yield of about 45% MDSCs in the total CD45 population. Further gating of $CD11b^{+}Gr1^{+}$ for Ly6G and Ly6C gives the granulocytic and monocytic populations, respectively. For the purpose of the MDSC migration assay, $CD11b^{+}Gr1^{+}$ double positive cells were used irrespective of their granulocytic or monocytic nature. All fluorophores were compensated using compensation beads, and dead cell compensation was performed with a cell sample that was stained with cell viability dye. Statistical significance was tested using Student's *t*-test.

We have shown in previous work that the MDSCs from the ovarian tumors respond to TNFα[20]. Hence for our migration assay here we have used TNFα as the tested cytokine. Complete RPMI media was added to the wells of a 24-well plate. The different conditions were: 1) TNFα, and 2) TNFα + anti-TNFα neutralizing antibody to test if TNFα induced migration can be rescued by the neutralizing the antibody. Each condition of the experiment was performed in triplicate. MDSCs sorted by flow cytometry were washed with serum-free media and plated at 0.5 x 10^6 cells/well in serum-free media in the upper chamber of permeable membrane well inserts. For TNFα neutralization, complete RPMI media was pre-treated with TNFα and its neutralizing antibody. Cells were allowed to migrate to the bottom of the well for 6 h at 37 °C, 5% CO_2. Migrated cells were then analyzed by flow cytometry. Migrated FITC positive cells were gated to count the absolute number of cells migrated through the membrane insert. **Figure 2** shows the increased migration of MDSCs in media with TNFα compared to untreated media; this suggests that TNFα promotes migration of these MDSCs isolated from mouse tumors. Neutralization of TNFα by antibody reduced the MDSC migration.

FIGURE LEGENDS:

Figure 1. Gating strategy for sorting CD45+GR1+CD11b+ myeloid-derived suppressor cells. First, single cells are gated and then live cells that lack viability dye stain are gated. Following that, CD45+ cells are gated from the live cells. Once CD45+ cells are identified, Gr1+ and Cd11b+ cells are gated for MDSCs. These cells have been used for the MDSC migration assay. Further gating of MDSCs can be performed for granulocytic and monocytic MDSCs using Ly6G and Ly6C markers as shown in this figure.

Figure 2. Migration of MDSCs. MDSC isolated from ovarian tumors show increased migration with complete RPMI media with TNFα. The increased migration is partially rescued by pretreating media with TNFα and its neutralizing antibody. The *p* value in each case is represented by "*". (**= 0.0026, ****= <0.0001). The experiment was performed in triplicate for each condition.

DISCUSSION:

We have described the methodology to isolate MDSCs from mouse ovarian tumor. The same method can be utilized for isolating MDSCs or other immune cells from any solid normal tissue or solid tumor using cell-specific markers. Additionally, depending on the nature of the tissue the incubation time with the dissociation buffer will need to be optimized.

The isolation of viable immune cells from tumor tissue depends on performing the different isolation steps from dissecting tumors to obtain the sorted MDSCs in a timely manner. Extended incubations in digestion buffer can lead to more cell death. It is also critical to maintain aseptic conditions to avoid contamination of the cells.

While performing the migration assay setup, it is critical to ensure that no bubbles are created below the membrane insert chamber as that will inhibit migration and produce erroneous results. Finally, we have used TNFα as an example cytokine for the assay. Other cytokines like IL-6, IL-1A, and IL-1B can also be tested. Instead of regular media, conditioned media from cell lines can also be used to test whether the cells secrete any soluble factor that promotes MDSC migration from a particular tumor type. Finally, this method of MDSC isolation can also be used for further *in vitro* assays like the T cell proliferation assay[20]. One limitation of this method is it is tedious when there are a large number of samples as each step needs to be performed within an optimal time limit. Prolonged steps may affect the quality of the cells and the data obtained.

DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure 1

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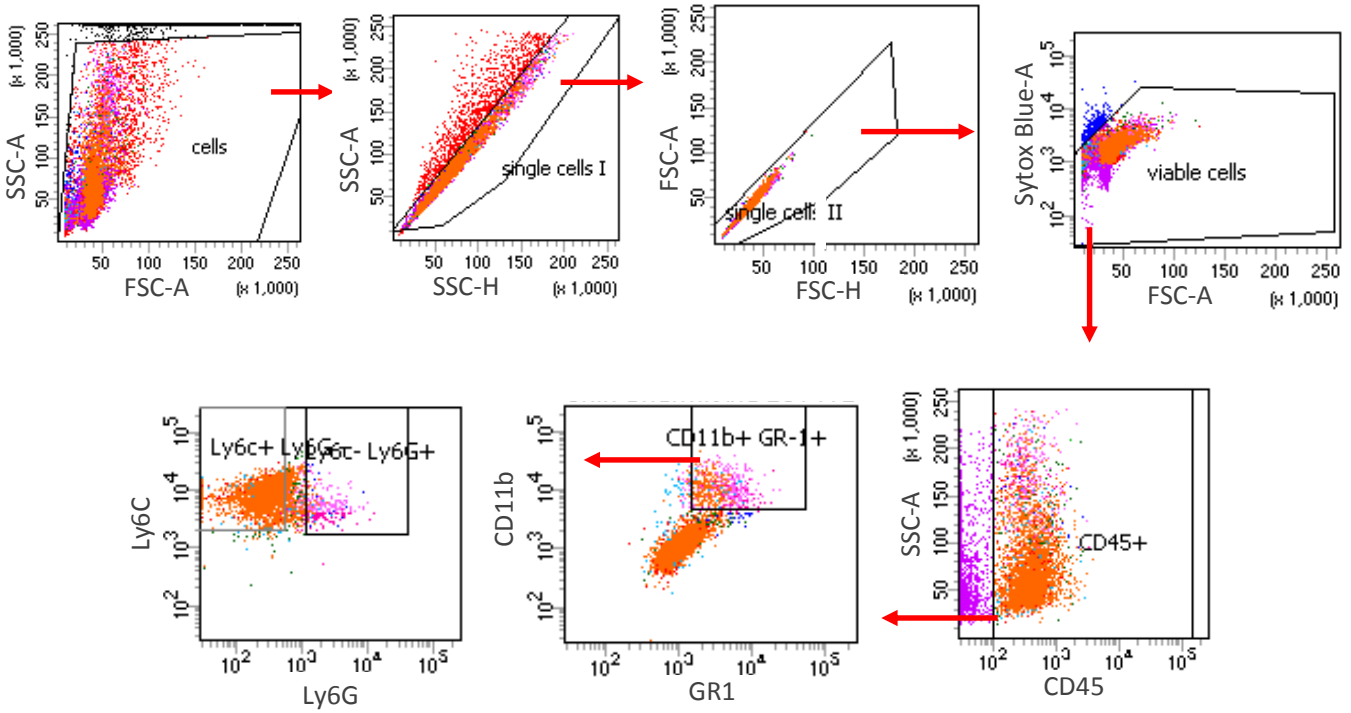

Figure1

Figure 2

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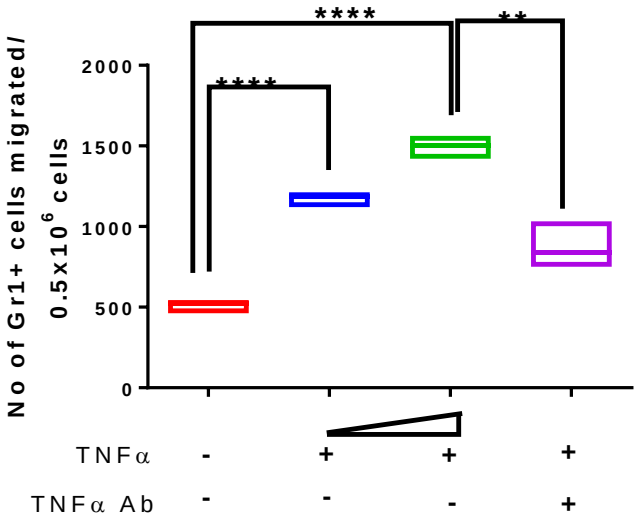

Figure2

Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Collagenase IV	Thermo Fisher	17104019	Tumor dissociation
Cell strainer	Falcon	352350	Cell strain
RBC lysis buffer	Biolegend	420301	Cell culture medium
DMEM+Glutamax	Gibco	10569010	Cell culture medium
RPMI	Gibco	11875093	Cell culture medium
FBS	Gibco	10082147	Serum for Cell culture
Phosphate Buffered Saline (PBS)	Sigma	D8537-500ML	PBS
Trypsin	Invitrogen	25200-056	Cell dissociation
Penstrep	HyClone	SV30010	Antibiotic
Sterile water	Invitrogen	10977-015	For dilution of buffers
BV605-CD11b	Biolegend	101237	Antibody for MDSC labeling
FITC-GR1	Biolegend	108405	Antibody for MDSC labeling
APC-Cy7-CD45	Biolegend	103115	Antibody for lymphocyte labeling
PE-Cy7-Ly6C	Biolegend	128017	Antibody for MDSC labeling
APC-Ly6G	Biolegend	127613	Antibody for MDSC labeling
TNFα	Sigma	T7539	Cytokine
TNFα neutralizing antibody	Biolegend	506309	neutralizing antibody
Ghost Dye Violet 450	Tonbo Biosciences	13-0863	Cell viability dye
UltraComp Beads	Invitrogen	01-2222-42	Compensation beads
Cell culture inserts	Corning	353097	Migration chamber
LSR Fortessa X-20	BD Biosciences	LSR Fortessa X-20	Fluorescent Cell analyzer
BD FACSAria Fusion	BD Biosciences	BD FACSAria Fusion	Fluorescent Cell sorter
Cell countess	Cell countess	Cell countess	Cell countess
Flow Jo			Software for Analysis of flow data
Prism			Plotting of graph and statistical analysis
C57BL/6 mice	Taconic	B6-F	mice for tumor generation

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Article Title: Isolation of MDSC from mouse tumors & determining their migratory potential

Signature: Sharmistha Sarkar Date: 11/18/2017

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NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

• **Protocol Detail:** Please add more details to the following protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.).
All the points mentioned below has been taken care of except 12.

1) 2.1: What are Enzyme A, B, D?
   2) 3.1: What kind of tumor? What is the mouse strain, age, sex, weight? What size of the tumor? Are these subcutaneous tumors? Are they xenograpfts? How are they dissected? Is the animal anesthetized/euthanized? Mention the method. What is the size of tumor?
   3) 3.2: What is the recommended size of pieces?
   4) 3.3: Mention dissociator speed, duration and temperature in generic terms.
   5) 3.4: What is the rotation speed?
   6) 3.5: Mention dissociator speed, duration and temperature in generic terms.
   7) 4.1: Mention all antibody concentrations.
   8) 4.3: Unclear how the washing steps are performed.
   9) 4.5: It sounds like there may be a missing centrifugation step. Mention all antibody concentrations.
   10) 4.5: check double numbering
   11) 4.6: Mention centrifuge speed and duration.
   12) 5.1 to 5.4: Unclear what is done here. If this is to be filmed more specific details are needed, e.g. button clicks and menu selections.

These steps involve using flow cytometry instrument, normally only done by a trained personnel. The steps cannot be precisely wriiten as it depends on what instrument is being used and what is the associated program with it.

13) Section 5: Step numbers are repeated.
14) 6.1, 6.2: Penstrep %?
15) 6.2: How are cells counted?
16) 6.4: Mention e.g. of cytokines you tested and their concentrations.
17) 7.4: When and how are the cells counted?
18) 7.6: Mention step numbers where this is described.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

• **Results:** Please mention the statistical tests performed and report sample sizes.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:**:
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• **Figure/Table Legends:**:
1) Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.
2) Define **, ***, ****.

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• Please define all abbreviations at first use.

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Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The manuscript "Isolation of myeloid derived suppressor cells from mouse tumor and determining their migration potential in vitro" describes the experimental procedure to isolate

MDSCs from mouse solid tumors by flow cytometry sorting and perform an in vitro MDSC migration assay. Its introduction clearly indicates the importance of MDSC studies in cancer field. The protocol is generally well-written and easily understandable. However, some detailed explanation may be needed in some parts of the protocol and figures.

Major Concerns:
Protocol 2.1:
The author does not explain what Enzyme D, R and A are and their functions in tumor tissue dissociation. There is no material information too.
Response: We have edited the text to avoid using commercially available reagents. The exact composition of Enzymes D, R and A are not known.

How long can the dissociation buffer be stored? It needs to be clarified since enzymes are easily degraded.
Response:The dissociation buffers need to be made fresh..

It would be better to add the method for murine ovarian tumor injection, such as the number of cells for implantation and tumor growth rates.
Response: Details added to text.

There is no detailed information about how and when tumors necropsied and processed.
Protocol 2.4:
Response: Details added to text.

The protocol says that the staining buffer with antibodies can be stored for long term. But antibody can be degraded and we generally avoid to keep the antibody dilution for long time. It needs to be clarified. It also does not indicate the concentration of each antibody.
Response: The staining buffer is stable for up to 1 week if stored appropriately. This is useful only if staining experiments are done on a regular basis. We have edited the text to avoid such confusion.

Minor Concerns:
Protocol 3.7: What is the volume of RPMI medium for resuspension?
Response: We used 25ml. We have added the information to the text.

Protocol 3.12: It would be better to indicate the approximate number of single cells in 1gm of tumor tissue for antibody dilution and flow cytometry sorting analysis.
Response: We have added the information to the text.

Protocol 5.4: It needs to indicate the approximate number of MDSCs in 1gm of tumor tissue after sorting. It gives ideas to set up MDSC migration assay.
Response: It is difficult to determine the number of MDSCs as it depends on the tumor type.

Figure 1: It would be better to show all steps of sorting strategy. It does not show the plots of total events and negative controls. It needs to be clear that the gates of CD45, CD11b and GR1 positive cells is reasonable. It also needs to show the percentages or numbers of MDSCs by

graphs.
Response: We have added the gatings to the figure.

Reviewer #2:
This manuscript is interesting. Data analysis and results are also perfect. In this manuscript, the authors have discussed the methodology to isolate MDSCs from mouse ovarian tumor. The same method can be utilized for isolating MDSCs or other immune cells from any solid normal tissue or solid tumor using cell specific markers.
Although the manuscript is well written, few minor revisions needs to be done before publication.

1. Did you treat the cells with Mitomycin -C to check the migration.
Response: the cells were not treated with Mitomycin-C
2. Please also stain the migrated cell and quantify by manual method or by absorbance based method.
Response: The cells were quantified my flow cytometry based method.

Reviewer #3:
In this manuscript (JoVE57585), Sarkar describes a protocol to isolate myeloid-derived suppressor cells from tumors and assess their migration capacity. As noted in the introduction, similar methods have been reported for isolation of other types of immune cells, including for MDSCs. Although migration assay provides a slightly different perspective, the value of this section is weakened by the lack of some important steps that would verify the robustness of the method.
The manuscript needs editing for clarity and the proper use of scientific language/terminology.
There are some knowledge gaps that should be corrected in the introduction.
Throughout the manuscripts the term MDSCs is spelled out as myeloid derived suppressor cells. It should be corrected as myeloid-derived suppressor cells.
Response: We have edited the text.

In Figure 1, it would benefit the readers to show granulocytic and monocytic MDSCs on the same flow plot (likely needed to show additional plots) and re-label axes with the antibody (not channel).
Response: We have modified the figure to accommodate the changes suggested.

Line 22: Although immunological mechanism that associate with different disease states might remain elusive, the importance of immune system in cancer is known and appreciated. It is unclear what the other diseases are.
Response: We have added a few examples of other non-cancer diseases where immune system plays a role.

Line 26: For precision it should be emphasized MDSCs negatively influence "anti-tumor" or "pro-inflammatory" immune response.
Response: We have edited the text accordingly.

Line 38-44: There are no citations in this section.

Line 48: Unlike macrophages and dendritic cells, monocytes are immature cells. Could you please cite the respective articles reporting MDSC differentiation into these cell types?
Response : We have added the reference.

Line 51: COX2 is not a secretory molecule.
Response : We have corrected that.

Line 65: "Commonly CD14-CD11b+ cells are characterized as MDSCs" This statement is wrong. Monocytic MDSCs are positive for both markers, while granulocytic MDSCs express CD15 instead of CD14.
Response: We apologize for the error. We have corrected it in our revision.

Step 2.3) Addition of EDTA could be important to ensure single cell suspensions.
Response: We have not added additional EDTA in the solution.

Although CD11b, Gr1 are commonly used to analyze MDSCs as a bulk population, there is an ongoing debate about how to discriminate between MDSCs and neutrophils with this staining profile. It would be best if the author could show the Ly6C and Ly6G staining pattern of the sorted cells to address the issue of MDSC subsets.
Response : We have added the flow gating but the experiment has been done with Gr1+CD11b+ cells.

Line 169: It is important to emphasize the approximate cell density appropriate for cell sorting.
Response : We have added the information.

Line 194: Can the author please comment on the yield of sorting process? Despite increased frequencies in tumors, 0.3-0.5 10^6 sorted MDSCs seems to be a difficult number to reach.
Response : We have used intraperitoneal tumors for the purpose. In this case we get enough tumor to perform the experiment. About 25% of all viable cells are MDSCs in this tumor type. For other tumors cells from multiple tumors can be pooled together if genetic background of tumors and host mice remain the same, for a general idea about the nature of MDSCs in those tumor type.

Did the author confirm the viability of MDSCs immediately after post-sorting and 6 hours later? Immature myeloid cells have very short half-life and unless exposed to stimulants, they start dying within hours. The lack of transmigration in untreated or anti-TNFa containing groups could simply be accumulation of more dead cells in the upper chamber.
Response : We appreciate the criticism. The cell viability was determined while counting cells post sorting and before plating into inserts for migration assay.
6 hours post migration cells were stained with cell viability dye for flow cytometry. We have added that information to the protocol.