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Dear Editors of JoVE,

We submit for your consideration our manuscript entitled **"Subverting Tumor Exploitation of Infiltrating Myeloid Cells as Determined by Flow Cytometry and Adoptive Transfer"**. This is an original manuscript; it is not under consideration for publication anywhere else. All the listed authors have agreed to submit the manuscript. This work provides a protocol to isolate viable intratumoral myeloid cells for functional analyses, and is to be submitted to the Tumor Microenvironment Methods Collection established by Dr. Sofia Sousa.

Though tumor-infiltrating myeloid cells exist within varying activation and differentiation states within the tumor, several subsets have been identified. Unfortunately, the overlapping expression of cell-surface markers used to identify these myeloid cell subsets makes it currently challenging to phenotypically differentiate tumor myeloid cells from other myeloid cells. Similarly, therapy-induced phenotypic changes to tumor-infiltrating myeloid cells may not be easily observed with existing myeloid antibody staining panels. Taken together, the insufficiency of unique surface markers complicates our understanding of myeloid cell biology. To delineate natural, tumor-driven, and therapy-influenced myeloid cells, we recommend evaluating myeloid cells subsets by their function in addition to their phenotypic characteristics.

In comparison to tumor-infiltrating leukocyte (TIL) isolation protocols which favor harsher enzymatic mixes to enhance the reproducible release of various cellular subsets, this method favors more conservative enzymatic digestion. Our results demonstrate that this method produces a high yield of myeloid cells from solid murine tumors. The preservation of receptor integrity and cellular viability facilitates reliable functional analysis of desired myeloid subsets. These improvements to myeloid cell isolation allow us to discern the changing function of intratumoral myeloid cells upon normalization of the TME with Class I histone deacetylase inhibitor (HDACi) MS-275 during adoptive T cell therapy.

The manuscript contains 3 figures for the print version.

Best Regards,

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TITLE:

Functional Analysis of Tumor-infiltrating Myeloid Cells by Flow Cytometry and Adoptive Transfer

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KEYWORDS:

Tumor microenvironment, tumor-infiltrating myeloid cells, tumor dissociation, myeloid cell isolation, flow cytometry, myeloid cell sorting, adoptive cell transfer

SUMMARY:

This protocol provides reliable methods of solid tumor dissociation and myeloid cell isolation in murine intradermal or subcutaneous tumor models. Flow cytometry allows for phenotypic characterization of heterogeneous myeloid populations within the tumor microenvironment and sorting will demonstrate their functionality in the context of adoptive transfer.

ABSTRACT:

The tumor-infiltrating myeloid cell compartment represents a heterogeneous population of broadly immunosuppressive cells that have been exploited by the tumor to support its growth. Their accumulation in tumor and secondary lymphoid tissue leads to the suppression of antitumor immune responses and is thus a target for therapeutic intervention. As it is known that the local cytokine milieu can dictate the functional programming of tumor-infiltrating myeloid cells, strategies have been devised to manipulate the tumor microenvironment (TME) to express a cytokine landscape more conducive to antitumor myeloid cell activity. To evaluate therapy-induced changes in tumor-infiltrating myeloid cells, this paper will outline the procedure to dissociate intradermal/subcutaneous tumor tissue from solid tumor-bearing mice in preparation for leukocyte recovery. Strategies for flow cytometric analysis will be provided to enable the identification of heterogeneous myeloid populations within isolated leukocytes and the characterization of unique myeloid phenotypes. Lastly, this paper will describe a means of purifying viable myeloid cells for functional assays and determining their therapeutic value in the context of adoptive transfer modeling.

INTRODUCTION:

The tumor microenvironment (TME) is comprised of rapidly proliferating neoplastic cells and a surrounding heterogeneous stromal cell compartment. As growing tumors are often poorly vascularized, the TME is a peripheral site uniquely characterized by hypoxia, nutrient deprivation, and acidosis[1]. To survive in this landscape, tumor stress responses and metabolic reprogramming result in the secretion of soluble factors that promote tissue remodeling and angiogenesis as well as the selective recruitment of immune cells[2]. As myeloid cells are one of the most abundant type of hematopoietic cells in the TME, there is increasing interest in examining the role of tumor-infiltrating myeloid cells in the TME.

Myeloid cells are a heterogenous and plastic group of innate immune cells including monocytes, macrophages, dendritic cells, and granulocytes. Although they have critical roles in tissue homeostasis and adaptive immune response regulation, their function can be polarizing depending on the composition of activation signals within the local microenvironment[3]. Tumors take advantage of myeloid cell characteristics through the secretion of soluble factors within the TME. These alternative signals can divert myelopoiesis towards immature differentiation and skew the function of existing tumor-infiltrating myeloid cells[3]. Indeed, myeloid cells within the TME often promote cancer progression and can suppress antitumor immune responses, leading to adverse effects on cancer therapy.

Although therapeutic strategies promoting the depletion of immunosuppressive myeloid cells have been shown to delay tumor growth[4], the lack of target specificity risks the removal of immunostimulatory myeloid cells, which by contrast, aid in the resolution of cancer. These inflammatory myeloid cells can exert profound antitumor effects including direct tumor cell killing and activation of cytotoxic $CD8^+$ T cells[5]. Alternatively, strategies normalizing the composition and function of myeloid cells in the TME have shown therapeutic success[6]; however, the biological mechanisms underlying their re-education towards an antitumor phenotype have still not been fully understood. Ultimately, a comprehensive characterization of tumor myeloid cells is necessary for further improvement of cancer therapy.

Unfortunately, reproducible disaggregation of tumors for myeloid cell isolation is challenging. Tumor-derived myeloid cells are sensitive to *ex vivo* manipulation compared to other leukocyte subsets, and the aggressiveness of tumor processing can lead to enzymatic epitope cleavage and reduced viability of recovered cells[7]. The purpose of this method is to provide a reliable means of tumor dissociation to preserve surface marker integrity for analysis and cellular vitality for functional study. In comparison to tumor-infiltrating leukocyte (TIL) isolation protocols that favor harsher enzymatic mixes to enhance the reproducible release of various cellular subsets, this method favors more conservative enzymatic digestion to maximize myeloid cell recovery. High-level multi-color flow gating strategies are also provided to identify murine tumor myeloid cell subsets for further characterization and/or sorting.

PROTOCOL:

NOTE: All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

1. Tumor harvest and dissociation

1.1. Inoculate 6–8-week-old, female, C57BL/6 mice intradermally/subcutaneously with 1×10^6 B16 melanoma cells as described by Nguyen *et al.*[8] Allow tumors to grow for 12 days before harvesting.

1.2. Euthanize the mouse by cervical dislocation while making sure to not disrupt the tumor when doing so. Spray the mouse down with 70% ethanol before harvesting.

1.3. Using a scalpel and scissors, surgically remove the intradermal/subcutaneous tumor from surrounding tissue (including attached tumor-draining lymph nodes), and place the tumors into a preweighed microfuge tube. Keep on ice.

NOTE: Conduct tumor harvest in an animal-use biosafety cabinet. Use a 15 mL conical tube for larger tumors.

1.4. Weigh the tumors, and add 500 µL of RPMI-1640 medium with 10% fetal bovine serum (FBS) to each tube, using scissors to cut the tumors into small pieces within the tube or in a 6-well plate.

NOTE: The tumor pieces should be small enough to be mixed by an electric pipettor once the digestion medium has been added.

1.5. Prepare the dissociation mix by dissolving collagenase type IV at 0.5 mg/mL and DNase at 0.2 mg/mL in RPMI-1640 medium with 10% FBS and 5 mM calcium chloride.

NOTE: Dissociation mix must be prepared fresh to maximize collagenase activity.

1.6. Transfer the minced tumor suspension to a 15 mL conical tube, and add 10 mL of dissociation mix per 0.25 mg of tumor. Place the tube in a temperature-controlled orbital shaker for 30 min at 37 °C with 200 rpm agitation. Neutralize the collagenase activity by adding two volumes of cold RPMI-1640 medium with 10% FBS and 2 mM ethylenediamine tetraacetic acid (EDTA), and refrigerate for 10 min at 4 °C.

1.7. Briefly vortex and pipette the suspension into a 40 µm strainer on a 50 mL conical tube. Use a syringe plunger and neutralizing media to disaggregate the residual tumor tissue, and wash it through the strainer. Centrifuge the suspension for 5 min (500 × *g*, 4 °C), discard the supernatant, and resuspend the pellet in phosphate-buffered saline (PBS) with 2% FBS and 1 mM EDTA.

2. TIL enrichment and flow cytometric staining (FACS)

2.1. To enrich TILs for myeloid cell characterization, use a magnetic cell separation kit designed for biotin-positive selection with biotinylated CD45.2 antibodies according to the

manufacturer's instructions (see the **Table of Materials**).

2.2. Resuspend the cells in 200 µL of FACS buffer (PBS with 0.5% w/v bovine serum albumin (BSA)), and transfer them to a 96-well U-bottom plate.

NOTE: Do not exceed a staining concentration of 1×10^8 cells/mL. Adjust the volume, and split samples into multiple wells to compensate for high cell numbers.

2.3. Centrifuge the plate for 5 min (500 × *g*, 4 °C), and discard the supernatant. Add 50 µL of Fc block solution (1:200 dilution of purified rat anti-mouse CD16/CD32 [see the **Table of Materials**) in FACS buffer, final concentration of 2.5 µg/mL), and resuspend the cells by pipetting. Incubate for 10 min at 4 °C.

2.4. Add 50 µL of FACS buffer containing 2x concentration of surface-staining antibody (1:50 dilution of CD45.2, NK1.1, CD11c, F4/80, CD8a, Ly6C, CD11b, CD4, Ly6G) and fixable viability stain (FVS, 1:500 dilution), and mix the cells by pipetting. Incubate for 20 min at 4 °C.

NOTE: Cover the plate with aluminum foil to minimize light exposure. Antibodies should be titrated prior to the experiment to empirically determine the optimal dilution.

2.5. Wash the cells twice by adding 200 µL of FACS buffer to each well, centrifuging the suspension (5 min, 500 × *g*, 4 °C), and discarding the supernatant.

2.6. Add 100 µL of fixation/permeabilization solution (see the **Table of Materials**) to each well, mix the cells by pipetting, and incubate for 20 min at 4 °C.

2.7. Add 100 µL of 1x permeabilization buffer (see the **Table of Materials**) to each well, centrifuge the plate for 5 min (500 × *g*, 4 °C), and discard the supernatant.

2.8. Wash the cells by adding 200 µL of 1x permeabilization buffer to each well, centrifuging the suspension (5 min, 500 × *g*, 4 °C), and discarding the supernatant.

NOTE: The experiment can be paused overnight after resuspending the cells in permeabilization buffer. Store the sample at 4 °C and protected from light. Resume after briefly mixing the cells before centrifuging.

2.9. Add 100 µL of permeabilization buffer containing 1x concentration of intracellular staining antibody (1:100 dilution of nitric oxide synthase 2 (NOS2), arginase 1 (Arg1)), and mix the cells by pipetting. Incubate for 20 min at 4 °C.

NOTE: Cover the plate with aluminum foil to minimize light exposure. Antibodies should be titrated prior to the experiment to empirically determine the optimal dilution.

2.10. Add 100 µL of 1x permeabilization buffer to each well, centrifuge the plate for 5 min (500

× *g*, 4 °C), and discard the supernatant.

2.11. Wash the cells by adding 200 µL of 1x permeabilization buffer to each well, centrifuging the suspension (5 min, 500 × *g*, 4 °C), and discarding the supernatant.

2.12. Resuspend the cells in 300 µL of FACS buffer. Filter the sample through a 5 mL round-bottom polystyrene tube with 40 µm strainer cap before performing flow cytometry analysis.

3. Tumor myeloid cell sorting for functional studies

3.1. After identifying the desired myeloid cell populations by flow cytometry analysis, pre-enrich bulk myeloid cells for sorting with a magnetic cell separation kit designed for CD11b- or CD11c-positive selection according to the manufacturer's instructions (see the **Table of Materials**).

3.2. Using surface-staining antibodies specific for the desired myeloid cell subsets (1:100 dilution of CD11b, Ly6C, Ly6G), stain the pre-enriched cells as described in steps 2.2–2.5.

NOTE: Include fixable viability stain to ensure the sorting of live myeloid cells. Do not exceed a staining concentration of 1×10^8 cells/mL. Adjust the volume, and split the samples into multiple wells to compensate for high cell numbers.

3.3. Resuspend the cells in cold sorting buffer (PBS with 1% w/v BSA, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mM EDTA). Filter the sample through a 5 mL round-bottom polypropylene tube with a 40 µm strainer cap. Keep the cells on ice, and cover the tube with aluminum foil.

NOTE: Adjust the concentration of cells/volume to the desired instrument specification for sorting.

3.4. Prepare a sample collection tube with capture medium (5 mL round-bottom polypropylene tube containing PBS with 50% FBS).

NOTE: Coat tubes with 5 mL capture medium overnight prior to sorting. Discard all but 1–2 mL of the capture medium on the next day before running the sample.

3.5. Modify the sorter instrument settings to decrease sample pressure and prevent perturbations in droplet formation. Equip the 130 µm nozzle tip, and utilize the 10 psi setting. Run the sample at a low flow rate with periodic sample agitation at 100 rpm, ensuring that the deposition of droplets is in the center of the tube. After the sort, keep the sample on ice.

3.6. Incubate the sample for 10 min at 4 °C. Centrifuge the tube for 5 min (500 × *g*, 4 °C), discard the supernatant, and resuspend the pellet in the desired medium for functional assays or adoptive transfer.

4. Adoptive transfer of purified tumor myeloid cells

4.1. Inoculate 6–8-week-old, female, C57BL/6 mice intradermally with 1×10^6 B16 melanoma tumor cells resuspended in 30 µL of PBS.

NOTE: Inoculate mice in advance such that tumor growth does not exceed 100 mm^3 by the time of adoptive transfer.

4.2. Resuspend the sorted tumor myeloid cells in PBS with 25 mM HEPES and 1 mM EDTA at a concentration of 2×10^6 cells/mL. Filter the sample through a 5 mL round-bottom polystyrene tube with a 40 µm strainer cap. Keep on ice.

4.3. Induce and maintain mice under anesthesia with 3% isoflurane. Apply ophthalmic ointment to prevent ocular dryness/injury.

4.4. Load a 31 G syringe with 50 µL of cell suspension. Dislodge air bubbles by gently flicking the syringe. Clean the injection site using an alcohol swab.

4.5. Using sterile forceps, lift the skin at the base of the tumor. Insert the needle into the subcutaneous space at a slight upward angle to enter the tumor from below the skin. Use the forceps to pinch the skin surrounding the needle, and slowly dispense the syringe volume. Continue to pinch the skin with the forceps while removing the needle slowly, and use a cotton swab to clean potential leakage.

NOTE: Proceed with any additional therapeutic treatments if desired.

4.6. Allow the mice to recover from anesthesia.

REPRESENTATIVE RESULTS:

The results demonstrate that this method produces a high yield of myeloid cells from solid murine tumors. The preservation of receptor integrity and cellular viability facilitates reliable functional analysis of the desired myeloid subsets. These improvements to myeloid cell isolation allowed the discernment of the changing function of intratumoral myeloid cells upon normalization of the TME with the class I histone deacetylase inhibitor (HDACi), MS-275, during adoptive T cell therapy. TIL isolation protocols typically do not take the steps to maximize myeloid cell yield[9]. As a result, enzymatic digestion is typically too harsh and leads to a loss of sample viability. When processed B16 melanoma tumors were treated with collagenase type I for 1 h (a commonly used condition) before positively enriching for $CD45.2^+$ cells by magnetic selection, the morphology (forward Scatter–area (FSC-A) *vs*. side scatter–area (SSC-A)) and myeloid cell subgating (CD11b *vs.* CD11c) indicated that the yield of myeloid cells ($CD11b^+$ or $CD11c^+$) and non-myeloid cells ($CD11b^-$ $CD11c^-$) was extremely low (**Figure 1**). To reduce the potentially excessive specific activity of collagenase, the duration of tumor digestion was decreased to 30 min. While there was a slight improvement in myeloid cell recovery, the overall yield was still low and there was no improvement in non-myeloid cell recovery.

Because lot variation may introduce proteases with specific activity high enough to cause excessive cell death, a separate lot of collagenase type I was requested from a different commercial supplier for comparison. Interestingly, the overall yield of myeloid and non-myeloid cells was much higher, with a slight enhancement in myeloid cell number upon the addition of FBS. Although FBS was added to stabilize the myeloid cells to collagenase-induced damage, this raised the question as to whether FBS was also neutralizing the tryptic activity of the collagenase type I preparation, which could be impairing cell recovery. As collagenase type I preparations have collagenase, caseinase, clostripain, and tryptic activities[10], to reduce protease exposure, the digestion was attempted using collagenase type IV, which has higher collagenase-specific activity and lower tryptic activity. This condition resulted in a greater increase in myeloid cell yield, with the addition of FBS resulting in the highest yield. Interestingly, collagenase type I and type IV, with or without FBS, did not markedly change the overall yield of non-myeloid cells.

With these optimized enzymatic digestion conditions, leukocytes were isolated from murine B16 melanoma tumors, and flow cytometry was used to phenotype the different myeloid cell populations within the TME based on their expression of surface markers. Tumors were harvested and processed and the leukocytes isolated using a CD45.2 magnetic selection kit. The cells were then stained using a carefully designed panel of cell surface markers (**Figure 2**). The gating strategy described here starts with a morphological assessment of the cells using FSC-A *vs.* SSC-A. This allows the exclusion of cellular debris based on their small size. FSC-H *vs.* FSC-W was used to select single cells and exclude the doublets. Total live leukocytes were then gated based on CD45.2 and viability staining. Lymphocytes were excluded based on NK1.1, CD4, and CD8 staining; note that in BALB/c mice, Asialo-GM1 and/or DX5 can be used to exclude natural killer (NK) cells as NK1.1 is not expressed on BALB/c-derived NK cells. CD11b was then plotted against CD11c to identify tumor-associated dendritic cells (TADCs)/conventional dendritic cells (cDCs).

Cells that are negative for CD11c represent the bulk myeloid cells, which can be further separated based on Ly6C and Ly6G staining. Cells that express intermediate levels of Ly6C and high levels of Ly6G represent the neutrophils. This population shares the same phenotype as the granulocytic myeloid-derived suppressor cells (G-MDSCs). $CD11b^{+}$ cells that stain negative for Ly6G, but positive for Ly6C can be divided into $Ly6C^{hi}$, $Ly6C^{int}$, and $Ly6C^{lo}$. $Ly6C^{int/hi}$ cells express lower levels of F4/80 and represent inflammatory monocytes. However, $Ly6C^{hi}$ cells also share the same phenotype with monocytic myeloid-derived suppressor cells (M-MDSCs). Finally, $Ly6C^{int}$ cells express high levels of F4/80 and are usually associated with tumor-associated macrophages (TAMs). While this characterization may not fully identify the myeloid cell subsets of interest, it provides a useful gating strategy to sort myeloid cell populations within the TME for further functional or genomic analyses. Within the context of immunotherapy, adoptive T-cell therapy incorporating epigenetic modifying drugs, such as the class I histone deacetylase inhibitor (HDACi) MS-275, can affect the TME to promote sustained tumor regression, while its absence results in tumor relapse[8].

Although microarray analysis of bulk tumor RNA suggests a role for tumor-infiltrating myeloid cells, this phenotypic characterization did not indicate major surface marker changes during MS-275 treatment[8]. Interestingly, functional markers present in the flow cytometry staining panel

identified a certain myeloid cell subset (CD11b^{+} Ly6C^{hi} Ly6G^{-}) differentially producing nitric oxide synthase 2 (NOS2) and arginase 1 (Arg1), which are implicit readouts of polarizing or divergent functional programming (**Figure 3A**). By sorting CD11b^{+} Ly6C^{hi} Ly6G^{-} cells from differentially treated, tumor-bearing mice, more extensive functional studies could be performed to understand their role. Using carboxyfluorescein succinimidyl ester (CFSE) labelling to monitor lymphocyte proliferation[11], sorted myeloid cells derived from naïve and vaccinated mice were found to suppress T cell proliferation *in vitro,* while cells derived from vaccinated + MS-275-treated mice had reduced immunosuppressive function (**Figure 3B**). Adoptive transfer of these cells revealed that they instead possessed antitumor capability and promoted sustained regression of tumors during vaccination and prolonged mouse survival (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative data showing the effectiveness of varying dissociation conditions. In C57BL/6 mice (n=3 per group), untreated intradermal B16F10-gp33 tumors were processed and dissociated under various enzymatic conditions before CD45.2 selection. Shown above are the flow cytometry gating strategies used to demonstrate cellular yield differences (FSC-A *vs*. SSC-A) illustrates tumor-infiltrating leukocyte yield implicitly by cell size/granularity discrimination. (CD11b *vs.* CD11c) allows for the quantification of myeloid (CD11b^{+} or CD11c^{+}) or non-myeloid (CD11b^{-} CD11c^{-}) cells. Error is defined by standard error of the mean. Abbreviations: CD = cluster of differentiation; SSC-A = side scatter-area; FSC-A = forward scatter area; FBS =fetal bovine serum.

Figure 2: Representative flow cytometry analysis of tumor-infiltrating myeloid cells. Following tumor processing and CD45.2 selection, enriched cells were surface-stained as described in the protocol. Shown above is the gating strategy used to exclude the lymphocytes and identify the individual subsets of myeloid cells within the tumor microenvironment. Debris (SSC-A *vs*. FSC-A) and doublets (FSC-H *vs.* FSC-W) were excluded, and CD45.2^{+} live cells were determined using the fixable viability stain FVS510 (CD45.2 *vs*. FVS). CD4^{+}, CD8^{+}, NK1.1^{+} cells were gated out. CD11b$^{hi/lo}$ CD11c^{+} cells represent cDCs. CD11b^{+} CD11c^{-} were then subgated based on Ly6C and Ly6G expression. Three populations were identified: (i) Ly6C^{int} Ly6G^{+} (neutrophils/G-MDSCs), (ii) Ly6C^{hi} Ly6G^{-} F4/80$^{lo/int}$ (monocytes/M-MDSCs), (iii) Ly6C$^{lo/int}$ Ly6G^{-} F4/80^{hi} (macrophages/TAMs). Abbreviations: CD = cluster of differentiation; SSC-A = side scatter-area; FSC-A = forward scatter area; FSC-H = forward scatter-height; FSC-W = forward scatter-width; FVS = fixable viability stain; cDCs = conventional dendritic cells; Ly = lymphocyte antigen; G-MDSCs = granulocytic myeloid-derived suppressor cells; M-MDSCs = monocytic myeloid-derived suppressor cells; TAMs= tumor-associated macrophages.

Figure 3: Functional analyses of purified tumor-infiltrating myeloid cells. In C57BL/6 mice (n=3–5 per group), intradermal B16F10-gp33 tumors were either unvaccinated or administered adoptive T cell therapy in the presence or absence of the HDAC inhibitor, MS-275. Five days posttreatment, tumors were processed and positively enriched for CD11b^{+} cells. The cells were surface-stained to subgate on the desired tumor-infiltrating myeloid cell subset (CD11b^{+} Ly6C^{hi} Ly6G^{-}). (**A**) These cells were further stained intracellularly for markers that can delineate the

polarity of functional activation, and the data are presented as the frequency of $CD11b^+$ $Ly6C^{hi-}$ $Ly6G^-$ cells that produce NOS2 or Arg1. Alternatively, the surface-stained $CD11b^+$-enriched myeloid cells were sorted to obtain a pure $Ly6C^{hi}$ $Ly6G^-$ cell population. (**B**) These purified cells were peptide-pulsed and cocultured with CFSE-labeled, naïve TCR-transgenic T cells in varying ratios. CFSE dilution, as determined by flow cytometry, is shown as a representative histogram and quantified by cellular division index (1:1). (**C**) Separately purified $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells derived from vaccinated + MS-275-treated tumor-bearing mice were adoptively transferred into new tumor-bearing mice (n=5) in conjunction with vaccination and tumor regression, and survival curves were monitored. Error is defined by the standard error of the mean. *** p=0.0004, * p=0.0479, **** p<0.0001. This figure has been modified from Nguyen *et al*.[8]. Abbreviations: CD = cluster of differentiation; HDAC = histone deacetylase inhibitor; NOS2 = nitric oxide synthase 2; Arg1 = arginase 1 ; CFSE = carboxyfluorescein succinimidyl ester; TCR = T-cell receptor; Ly = lymphocyte antigen; NS= not significant.

DISCUSSION:

Although tumor-infiltrating myeloid cells exist in varying activation and differentiation states within the tumor, several subsets have been identified including tumor-associated DCs (TADCs), tumor-associated neutrophils, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs)[12]. Unfortunately, the overlapping expression of cell-surface markers used to identify these myeloid cell subsets makes it currently challenging to phenotypically differentiate tumor myeloid cells from other myeloid cells[13]. Similarly, therapy-induced phenotypic changes in tumor-infiltrating myeloid cells may not be easily observed with existing myeloid antibody staining panels. Taken together, the insufficiency of unique surface markers complicates the understanding of myeloid cell biology. To delineate natural, tumor-driven, and therapy-influenced myeloid cells, the evaluation of myeloid cells subsets must be done according to their function in addition to their phenotypic characteristics.

The methods described herein to harvest and dissociate tumor tissue allow for the isolation of myeloid cells with preserved viability and surface marker integrity. As tumor-infiltrating myeloid cells are sensitive to *ex vivo* manipulation[7], the emphasis of the protocol is on less aggressive mechanical and enzymatic dissociation. However, depending on the tumor tissue type, different samples require variably aggressive treatment to generate a single-cell suspension. For more collagen-rich tumor models (*i.e.,* CMS5 fibrosarcoma), a syringe plunger could be used in addition to scissors to more fully mechanically disaggregate tumor tissue prior to enzymatic treatment. Conversely, less collagen-rich tumor models (*i.e.,* B16 melanoma) do not necessarily require enzymatic treatment. As demonstrated in these results, the type of collagenase preparation and the lot from which it was derived can significantly influence the variety and potency of proteases that cells are exposed to during enzymatic dissociation.

While non-myeloid leukocytes (*i.e*., lymphocytes) do not seem to be as sensitive, these data suggest that myeloid cells, in particular, may be very susceptible to excessive protease exposure (**Figure 1**), resulting in damage to membrane proteins and decreased viability. As a result, the use of collagenase type IV over collagenase type I is recommended for its increased collagenase-specific proteolytic activity and the incorporation of FBS into the digestion mix to neutralize

residual tryptic activity. Furthermore, utilizing purified collagenase products, as opposed to crude preparations from commercial sources, can increase the reproducibility of cellular recovery. Once the digestion is complete, the addition of EDTA and incubation at 4 °C is mandatory to chelate the Ca^{2+} ions and reduce the temperature needed for collagenase activity[14].

To characterize myeloid cell infiltrate within a heterogeneous TIL population, high-level gating strategies have been provided (**Figure 2**). The polychromatic flow cytometry panel should be designed in such a way that 1) dead and non-myeloid cells are excluded, 2) myeloid cell subsets can be identified, and 3) altered functionality can be implicitly observed across groups of samples (**Figure 3**). Depending on the number of myeloid cell subsets of interest and the depth of functional characterization, these criteria may not be fully accommodated within one staining panel. To free up channels for the panel, markers could be strategically pooled into a single dump channel. Although this method allows for high-dimensional analysis of tumor-infiltrating myeloid cells, it is still constrained by a relatively low parameter limit. More recent technologies using heavy metal reporter ions, such as mass cytometry, enable up to 40 independent parameters within a single panel, which will allow better study of the cellular and functional diversity of myeloid cells within the TME[15].

However, the cost of instrumentation limits the ubiquity of its use. Flow cytometry is more accessible and produces reliable characterization data, although the mixing and matching of markers across multiple panels necessitates panel validation as an extremely important step prior to the experiment. Upon identification of tumor-infiltrating myeloid subsets of interest, several researchers[16] describe techniques to sort these cells for functional assays and studies. Sorted cells can be reliably analyzed for their T cell-suppressive capacity *in vitro* as well as their antitumor capability *in vivo* after adoptively transferring them into mouse models of tumor relapse (**Figure 3**). Overall, the significance of the described methods is that, in comparison to other TIL isolation protocols, the conditions of tumor harvest, dissociation, cell enrichment, and cell sorting were tailored towards reproducibly acquiring a high yield of the myeloid cell compartment over all other leukocytes and without compromising their function.

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DISCLOSURES:

No conflicts of interest declared.

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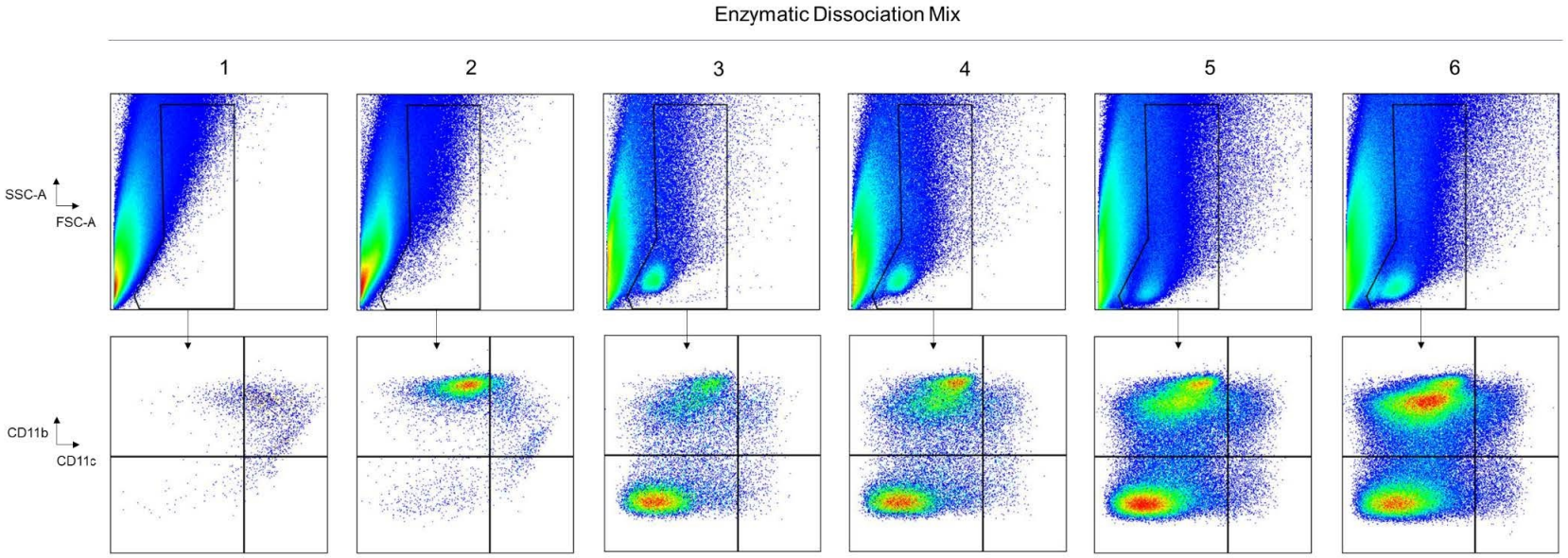
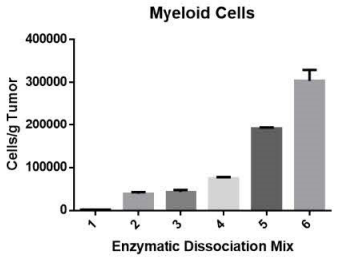
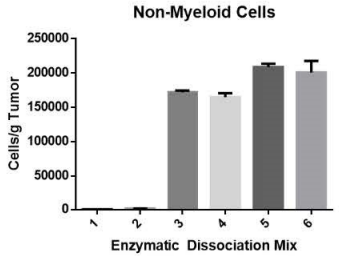
12 Schupp, J. et al. Targeting myeloid cells in the tumor sustaining microenvironment. *Cellular Immunology.* **343**, 103713 (2019).
13 Gabrilovich, D. I., Ostrand-Rosenberg, S., Bronte, V. Coordinated regulation of myeloid cells by tumours. *Nature Reviews Immunology.* **12** (4), 253–268 (2012).
14 Seglen, P. O. Preparation of isolated rat liver cells. *Methods in Cell Biology.* **13**, 29–83 (1976).
15 Roussel, M. et al. Mass cytometry deep phenotyping of human mononuclear phagocytes and myeloid-derived suppressor cells from human blood and bone marrow. *Journal of Leukocyte Biology.* **102** (2), 437–447 (2017).

Figure 1







Collagenase	I	I	I	I	IV	IV
Incubation Time	1 h	30 min	30 min	30 min	30 min	30 min
Lot Number	1	1	2	2	1	1
10% FBS	-	-	-	+	-	+

Figure

Click here to access/download;Figure;Figure 2 - Myeloid Characterization (revised).pdf

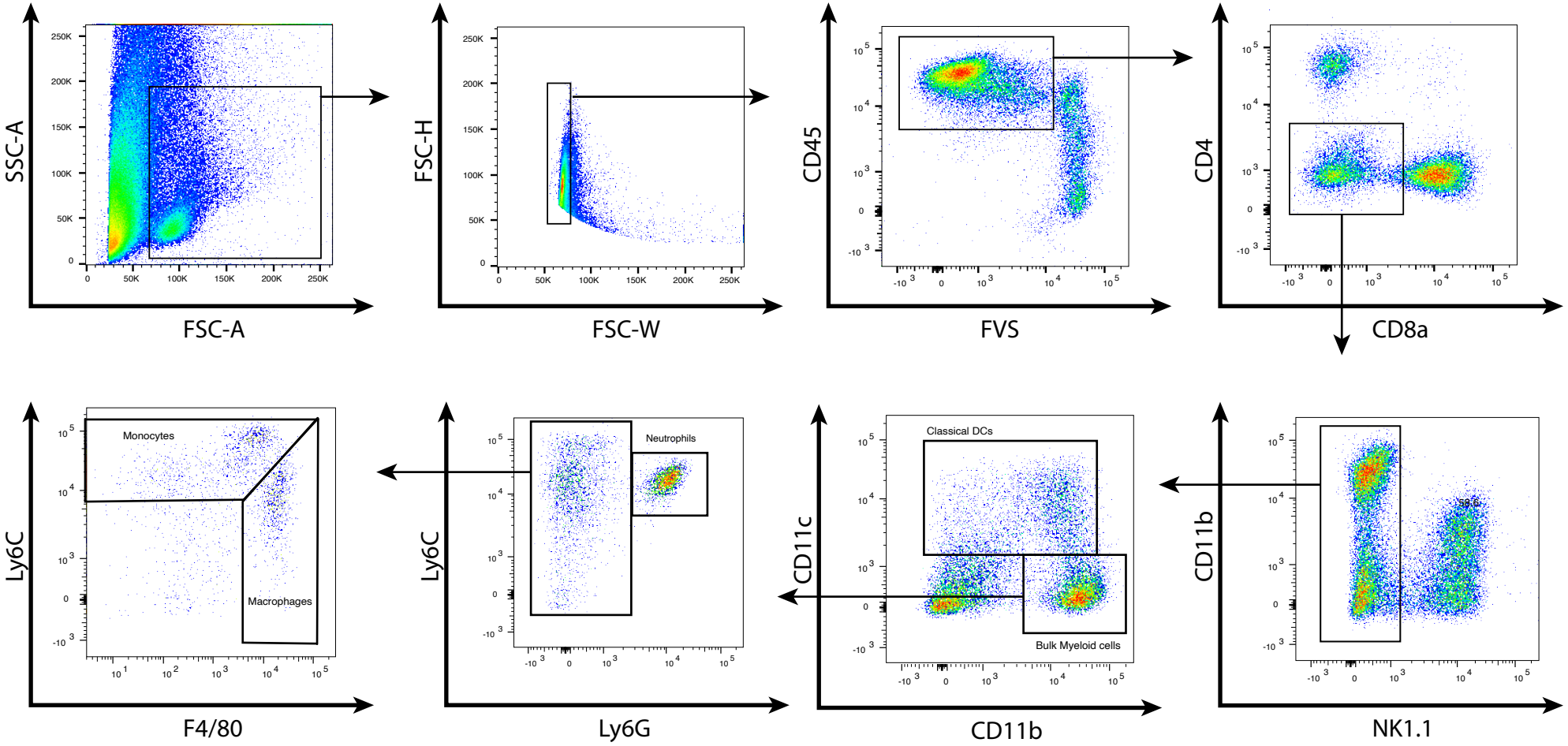


Figure 3

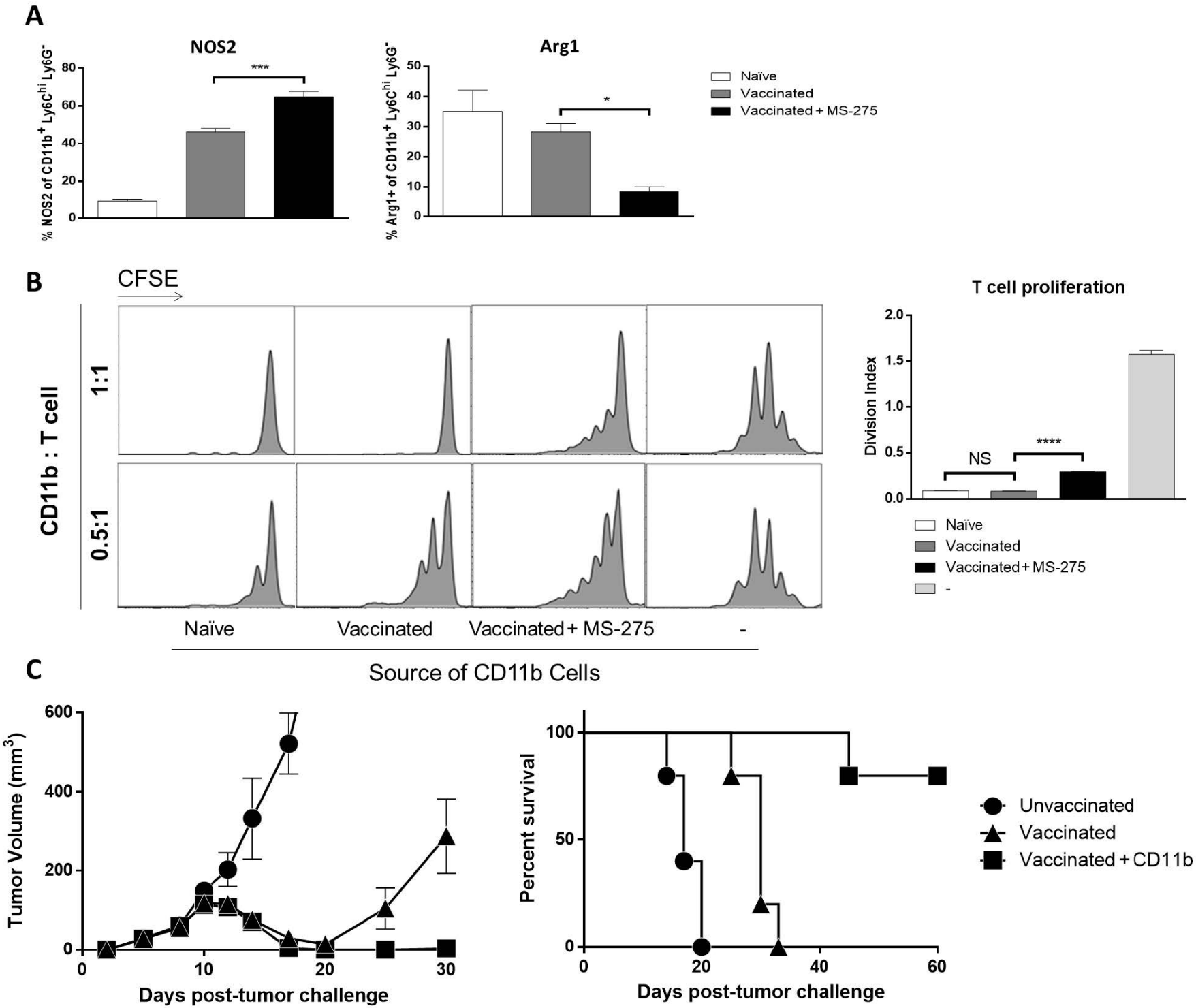


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials complete.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
Alexa Fluor 700 Mouse Anti-Mouse CD45.2	BD Biosciences	560693	1:100
APC-Cy7 Mouse Anti-Mouse NK-1.1	BD Biosciences	560618	1:100
Biotin Mouse Anti-Mouse CD45.2	BD Biosciences	553771	
BV421 Hamster Anti-Mouse CD11c	BD Biosciences	562782	1:100
BV650 Rat Anti-Mouse F4/80	BD Biosciences	743282	1:100
BV711 Rat Anti-Mouse CD8a	BD Biosciences	563046	1:100
Collagenase, Type IV, powder	Gibco	17104019	
DNase I	Roche	10104159001	
EasySep Mouse CD11b Positive Selection Kit II	Stemcell technologies	18970	
EasySep Mouse CD11c Positive Selection Kit II	Stemcell technologies	18780	
EasySep Release Mouse Biotin Positive Selection Kit	Stemcell technologies	17655	
FITC Rat Anti-Mouse Ly-6C	BD Biosciences	553104	1:100
Fixable Viability Stain 510	BD Biosciences	564406	1:1000
Fixation/Permeabilization Solution Kit (BD Cytofix/Cytoperm)	BD Biosciences	554714	
PE Rat Anti-CD11b	BD Biosciences	557397	1:100
PE-Cy7 Rat Anti-Mouse CD4	BD Biosciences	552775	1:100
PerCP-Cy5.5 Rat Anti-Mouse Ly-6G	BD Biosciences	560602	1:100
Perm/Wash (BD Perm/Wash)	BD Biosciences	554723	
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block)	BD Biosciences	553141	
iNOS Monoclonal Antibody (CXNFT), APC	Thermo Fisher	17-5920-82	1:100
Human/Mouse Arginase 1/ARG1 Fluorescein-conjugated Antibody	R&D Systems	IC5868F	1:100

February 9, 2021

Dear Dr. Alisha D'Souza
Senior Review Editor, JoVE

Thank you for your consideration of our manuscript now entitled "Isolation of Tumor-infiltrating Myeloid Cells for Flow Cytometry and Adoptive Transfer" that is under review for publication in JoVE (JoVE61511). We appreciate the constructive comments from the editor and three reviewers which proved very insightful and allowed us the opportunity to strengthen our methodology regarding the recovery of functional tumor-infiltrating myeloid cells for characterization and functional study.

We appreciate you for providing us the additional time to complete the revisionary changes that were suggested by the editor and reviewers. Please do not hesitate in contacting me regarding any questions you may have about the manuscript, and we look forward to future correspondence with you about the outcome of this submission.

Editorial Comments:

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your 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. Some examples:
1) Section 1: It is unclear which type of mouse/mouse strain this is. Please mention tumor cell line, implantation method, etc. It sounds like they are xenografts. Please cite a reference for how the tumor was implanted or describe the steps in brief. Mention animal age, sex, etc.
2) 2.1: Steps need to be describe if we are to film this.
3) 2.7: What is the solution composition?
4) 2.8: What is permeabilization buffer composition?
5) 2.10: mention antibody and dilution. List in the table of materials along with RRIDs.
6) 3.1: There is a step missing between 2.13 and 3.1 which should describe flow cytometry sorting. Unclear how the analysis is performed.
7) 3.2: Vague. Mention antibodies, concentration etc. Mention referenced step numbers.

Author Response

1) The authors have made the changes accordingly and the original paper for tumor implantation was cited.
2) The step was removed from the highlights since the protocol is described in manufacturer instructions

3) The solution composition is proprietary, the authors have added "(see table of materials)" to draw the reader's attention to the commercial product.
4) The permeabilization buffer composition is proprietary, the authors have added "(see table of materials)" to draw the reader's attention to the commercial product.
5) The authors have mentioned the antibodies and listed them in the table of materials as requested.
6) Flow cytometry sorting is described throughout Step 3. Flow cytometry staining (Step 2) precedes the sorting step (Step 3). Steps 3.1 and 3.2 describe more targeted methods of pre-enrichment and staining of desired subsets prior to sorting due to having identified those subsets through the characterization done in Step 2. To maintain a proper flow for the video, steps 2.13, 3.1, and 3.2 were removed from the highlighted section.
7) The authors have made the changes accordingly by adding antibodies, concentrations and referenced step numbers.

• 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. Please highlight ~2.5 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.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Section 3 appears to be core of manuscript but is vague and unhighlighted. Please highlight steps in this section. Please ensure that the title best represents your highlighted portions.

Author Response
The authors agree and have made corresponding adjustments to the highlighted sections to more accurately reflect a detailed and cohesive narrative. Highlighted sections have been added for Section 3 and an additional section (Section 4) has been added to more accurately reflect the overall methodology.

• 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 (3-6 paragraphs): 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.

Author Response
The authors have made best efforts to ensure that the discussion follows the described requirements set by the editor. Additional statements have been added to troubleshoot the issue of collagenase lot variability by suggesting the purchase of highly purified collagenase preparations rather than more commonly available crude preparations of collagenase.

• References: Please spell out journal names.

Author Response
The authors have corrected the citations to reflect full journal names as requested.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Eppendorf, Falcon, EasySep Mouse Biotin Positive Selection Kit, BD FACSAria III, etc
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Author Response
The authors have removed commercial sounding language and trademark symbols as requested.

Reviewer #1 (Comments to the Author)
This manuscript describes an optimized protocol for isolating myeloid cells from solid tumors. The authors highlight the need for a specialized protocol for isolating tumor-infiltrating myeloid populations in order to maintain cell surface proteins and myeloid cell function. Overall, this is an interesting article and many researchers in the field would be interested in learning more about this method. The article is clearly written in a non-biased manner and the methods are described in a straightforward, concise manner with adequate detail. Overall, this is a well-written paper on an important method.

Reviewer Comment #1
Minor Concerns:
- The use of some brand names like "Eppendorf tube" and "Falcon tube" should be minimized. Could these terms be replaced with "microfuge tube" and "conical tube"?

Author Response
All trademarked terms have been replaced by their generic counterparts in the manuscript.

Reviewer Comment #2
- In Step 2.2 and 3.2, the concentration or numbers of cells that are stained in each sample should be clarified.

Author Response
The manuscript has been updated to reflect the concentration of cells that were stained by flow cytometry.

Reviewer #2 (Comments to the Author)
The manuscript by Nguyen et al. describes a protocol to isolate myeloid cells from mouse tumors for functional assays. The authors provide a comparison a cell yields obtained with different tumor dissociation protocols that could be very useful for the scientific community. The manuscript also describes a simple flow cytometric gating strategy to identify major myeloid cell populations in in B16 melanoma tumors. The authors then demonstrate that the protocol described here allows for the assessment of functional alterations in myeloid cells induced by antitumor treatments.

Reviewer Comment #1
Major Concerns:
1. Although adoptive transfer of myeloid cells is mentioned in the title, a detailed description of the adoptive myeloid cell transfer procedure is missing. Thus, the title in its current form is somewhat misleading. Adoptive myeloid cell transfer should be either left out from the title or the description of the method should be added to the text. I do believe this information would be very valuable for the community. In case it is not described in the text, the protocol used for adoptive myeloid cell transfer should be cited in the text.

Author Response
The title of the manuscript has been edited to more accurately reflect the methodology being provided and a section describing adoptive myeloid cell transfer has been included (**4. Adoptive Transfer of Purified Tumor Myeloid Cells**).

Reviewer Comment #1
Minor Concerns:
1. Line 98: It could be mentioned that the tumor-draining lymph node can often be found attached to the tumor when the tumor is injected into the flank. If this lymph node is not removed before tumor dissociation, immune cells from the lymph node will contaminate the tumor sample and bias the results.

Author Response
The authors agree with the reviewer regarding this potential issue and have added an additional step to ensure that contaminating draining lymph nodes are not included during tumor harvest.

Reviewer Comment #2
2. Line 103: Make sure to show in the video how the samples should look like after thorough cutting. Failure to cut the tumor to small enough pieces will drastically reduce cell yields.

Author Response
The authors have provided additional information to clarify that the tumor pieces should be small enough to be mixed by electric pipettor once the digestion media has been added. This will also be reflected in the video.

Reviewer Comment #3
3. Line 168: 2.10 and 2.11 describe the same step twice.

Author Response
This is an error that has been corrected to represent two distinct steps.

Reviewer Comment #4
4. Figure 1: In conditions 1 and 2 (first two columns) fluorescence compensation between CD11b and CD11c stains seems to be insufficient. Were the same compensation settings used in conditions 1-2 as in conditions 3-6?

Author Response
The same compensation settings were used in conditions 1-2 as in conditions 3-6 since the samples were analyzed in parallel. However, live/dead staining was not originally utilized during optimization which could explain the presence of ungated debris/dead cells in quadrant 2 of Figure 1: condition 1 and 2. Those conditions in particular produced little to no yield in terms of tumor-infiltrating myeloid cell recovery due to lot variation and inherent tryptic activity. These conditions were abandoned for more effective enzymatic digestion reagents as can be observed in the same figure.

Reviewer Comment #5
5. Figure 2: Regarding the gating strategy in Figure 2, please note that type 1 conventional DCs can be CD11b(low) and may be outgated this way. I would suggest gating DCs first after the CD4- CD8- gate, by gating DCs as CD11c+ F4/80- MHCII+ cells (or if that's not possible with the current staining panel, CD11c+ F4/80- cells) and then continue with gating the additional cell

subsets. Macrophages are often negative for Ly6C, for this reason, the Ly6C/Ly6G gate should be extended to include Ly6C- cells as well for the next gating step.

Author Response
We have altered our gating strategy accordingly to include CD11b^{lo} cells and have changed the title of the DC label to be "classical DCs". This is in accordance with the phenotypic population definitions found in literature[1]. We have also expanded the macrophage gate to include the Ly6C -ve cell, although the majority of TAMs we observe have an intermediate expression of Ly6C. The corresponding changes can be observed in the revised version of Figure 2 and as red highlighted text in the manuscript.

1. Durai V, Murphy KM. Functions of Murine Dendritic Cells. Immunity. 2016;45(4):719-36)

Reviewer Comment #6
6. Figure 3B: The source of the protocol used to assess T-cell proliferation should be cited to direct readers who wish to perform this functional assay.

Author Response
A citation has been provided to readers who wish to perform a T cell proliferation assay.

Quah, B. J. & Parish, C. R. The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. J Vis Exp. (44), doi:10.3791/2259, (2010).

Reviewer Comment #7
7. A similar protocol optimized for isolating tumor-associated macrophages and DCs has been published by Laoui et al. This protocol could be cited to direct readers to additional information on this topic: Laoui et al. (2014). Purification of Tumor-Associated Macrophages (TAM) and Tumor-Associated Dendritic Cells (TADC). Bio-protocol 4(22): e1294. DOI: 10.21769/BioProtoc.1294.

Author Response
The authors agree with the reviewer and have included this citation to provide readers with additional information regarding similar protocols.

Reviewer #3 (Comments to the Author)
The manuscript "Subverting Tumor Exploitation of Infiltrating Myeloid Cells as Determined by Flow Cytometry and Adoptive Transfer" by Nguyen et al. described methods for dissociation intradermal/subcutaneous tumors, preparation for leukocyte recovery and identification of heterogeneous myeloid populations within isolated leukocytes. This article also described a

protocol for purifying viable myeloid cells for functional assays and determining their therapeutic value in the context of adoptive transfer modeling. In general, the methods are practical to isolate, identify and characterize the heterogeneous myeloid populations. Furthermore, the protocol for purifying viable myeloid cells is practicable.

Reviewer Comment #1

Minor Concerns:

1. The title of the manuscript is not appropriate for this methods article. Additionally, there are no steps about adoptive transfer.

Author Response

The title of the manuscript has been edited to more accurately reflect the methodology being provided and a section describing adoptive myeloid cell transfer has been included (**4. Adoptive Transfer of Purified Tumor Myeloid Cells**).

Reviewer Comment #2

2. Whether collagenase type Ⅱ from the different commercial supplier affect the yield of myeloid cells? If so, how to choose the reagent?

Author Response

We have found that lot variation occurred more frequently when utilizing crude collagenase preparations of collagenase, which is a common practice for tissue dissociation. Depending on the preparation there is variability in the number of contaminating proteases which will affect cell recovery. We recommend purchasing purified collagenase preparations to enhance the reproducibility of tissue dissociation. We have incorporated this concept into the Discussion section which is highlighted in blue.

Reviewer Comment #3

3. As mentioned in the discussion section, "Less collagen-rich tumor models (i.e. B16 melanoma) do not necessarily require enzymatic treatment.", Why B16 melanoma was used to establish the methods in this article? It would be supportive to use different collagen-rich tumor models.

Author Response

The authors agree that in B16 melanoma, the addition of enzymatic treatment does not improve the success of tissue dissociation to the extent that one would see in CMS5 fibrosarcoma. However, the protocol (initially tested in B16) was designed as a standardized “best-practice” method in order to be efficacious in many tumor models and was validated in more collagen-rich tumors such as CMS5 fibrosarcoma and MC38 colon carcinoma. All three tumors are extensively studied by the authors and it is from personal experience that B16 may not necessarily require enzymatic treatment (due to being less collagen-rich), though enzymatic treatment of B16 does not harm

cellular recovery. Conversely, tissue dissociation of CMS5 and MC38 absolutely require enzymatic treatment to maximize cellular recovery.

Reviewer Comment #4
4. step 1.7, at what temperature the samples were refrigerated?

Author Response

The samples were refrigerated at 4°C and the protocol has been updated to reflect that temperature.

Reviewer Comment #5
5. in 1.10, Why steps 2.5 and 2.6 were repeated?

Author Response
This is an error that has been corrected to represent two distinct steps.

Reviewer Comment #6
6. How many cells were used in 2.2?

Author Response
The manuscript has been updated to reflect the concentration of cells that were stained by flow cytometry.

Reviewer Comment #7
7. Step for removal of erythrocyte was missing

Author Response
Typically for harvest and processing of intradermal/subcutaneous tumors, the tissue is not heavily vascularized and as such it is standard practice to not have an erythrocyte removal step with ACK (Ammonium-Chloride-Potassium) lysing buffer as one would normally do with spleens or blood samples.

Reviewer Comment #8
8. What is the advantage using CD45.2 magnetic selection kit over sorting with flow cytometry?

Author Response
The advantage of using CD45.2 magnetic selection over flow sorting can be described in several ways. There is improved cost and time effectiveness in purchasing a commercial kit compared to utilizing the flow sorter for a significant amount of time. Furthermore, while flow sorting increases purity which is useful for *in vivo* applications and/or co-incubation studies, it is unnecessary for

flow-based characterization. CD45.2 magnetic selection allows for nearly comparable purity and contaminants can be identified through exclusion marker staining.