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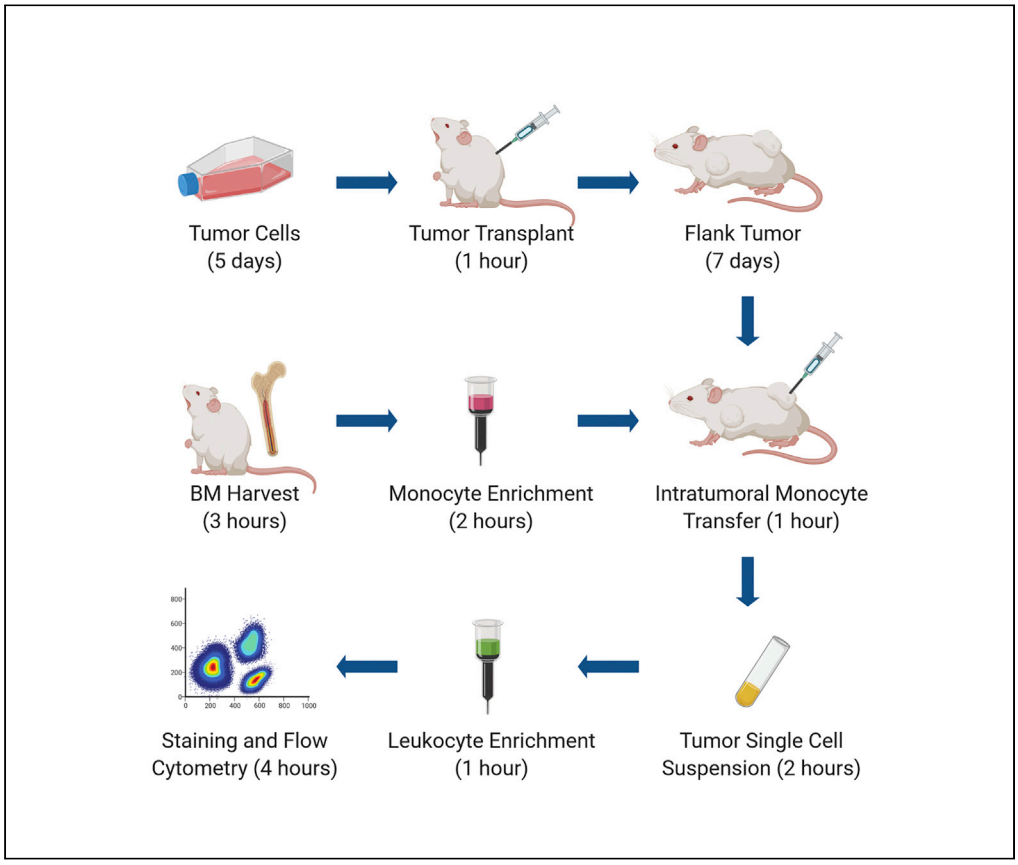
Intratumoral Monocyte Transfer to Examine Monocyte Differentiation in the Tumor Microenvironment



The regulation of monocyte differentiation in the tumor microenvironment is of significant interest to tumor immunologists. Monocytes injected into the circulation may not track into tumors in sufficient numbers, making intratumoral injections a preferred experimental approach. Monocyte enrichment with antibody-based positive selection may activate downstream signaling, while cell sorters expose monocytes to mechanical stress. Here, we describe an approach of intratumoral monocyte transfer that circumvents these limitations by using negative selection and fluorescent reporter mice.

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HIGHLIGHTS

Isolation of bone marrow monocytes from fluorescent reporter mice

Transfer of monocytes directly into tumors generated in syngeneic hosts

Analysis of intratumorally transferred monocytes using flow cytometry

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Intratumoral Monocyte Transfer to Examine Monocyte Differentiation in the Tumor Microenvironment

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SUMMARY

The regulation of monocyte differentiation in the tumor microenvironment is of significant interest to tumor immunologists. Monocytes injected into the circulation may not track into tumors in sufficient numbers, making intratumoral injections a preferred experimental approach. Monocyte enrichment with antibody-based positive selection may activate downstream signaling, while cell sorters expose monocytes to mechanical stress. Here, we describe an approach of intratumoral monocyte transfer that circumvents these limitations by using negative selection and fluorescent reporter mice.

For complete details on the use and execution of this protocol, please refer to Devalaraja et al. (2020).

BEFORE YOU BEGIN

Timing: must be prepared 7–14 days prior to experiment

Preparation of Donor Mice for Bone Marrow Collection

In this protocol, we isolate monocytes from bone marrow of 4–16 weeks old LysMCre:Rosa26tdT:Zbtb46GFP mice (C57BL/6 background). Here, tdTomato expression identifies transferred monocytes and their progeny while GFP (expressed from the Zbtb46 locus) identifies dendritic cells (DCs) (Satpathy et al., 2012; Shi et al., 2018). Other combinations of Cre or reporter alleles may be used depending on the experimental design and goals. Nonetheless, the use of genetically engineered mice may present logistical and biological challenges (please refer to "Limitations" section below). This protocol can also be performed using alternative mouse models (e.g., CD45.1 hosts and CD45.2 recipients) or biochemical tools (CFSE or CTV labeling) to identify transferred monocytes and their progeny in recipient tumors. For further details on these alternatives, please refer to the "Limitations" section below.

Recipient Flank Tumor Induction

Generate flank tumors of interest by subcutaneously implanting tumor cells into shaved flanks of recipient C57BL/6 mice (male or female; 6–12 weeks of age).

Note: We use 1 × 10^6 fibrosarcoma (FS) cells that require about 1 week to reach a tumor size compatible with monocyte transfer (Gubin et al., 2018). This time-frame will be different between tumor types.

Note: We recommend that flank tumors be approximately 1–1.5 cm in diameter at the time of monocyte transfer. Hence, tumor cells must be implanted into recipient mice prior to isolation and transfer of donor monocytes, and this period will depend on how long the particular tumor type takes to reach the aforementioned size. For additional details, please refer to Devalaraja et al. (2020).

KEY RESOURCES TABLE

Reagent	Source	Identifier
Antibodies		
Anti-mouse CD11b	Invitrogen	25-0112-82
Anti-mouse Ly6G	BioLegend	127633
Anti-mouse CD11c	BioLegend	117324
Anti-mouse CD45	BioLegend	103138
Anti-mouse MHCII	BioLegend	107635
Anti-mouse F4/80	Invitrogen	MF48005
Anti-mouse Ly6C	BioLegend	128026
Anti-mouse CD3e	BioLegend	100351
Anti-mouse NK1.1	BioLegend	108707
Anti-mouse CD16/32 (Fc Block)	BD Biosciences	553142
Critical Commercial Assays		
Monocyte Isolation Kit (BM), mouse	Miltenyi Biotec	130-100-629
CD45 Microbeads, mouse	Miltenyi Biotec	130-052-301
CompBeads	BD Biosciences	552845
Chemicals, Peptides, and Recombinant Proteins		
DMEM	Thermo Fisher Scientific	10567014
Collagenase B	Sigma Aldrich	11088815001
DNase I	Sigma Aldrich	D4527
Bovine Serum Albumin	Millipore Sigma	A9647
EDTA	thermosphere Scientific	AM9261
7-AAD	BioLegend	420404
Dulbecco's PBS	Millipore Sigma	59331C
RBC Lysis Buffer 10×	BioLegend	420301
Software and Algorithms		
FlowJo	Treestar	https://flowjo.com
Experimental Models: Cell Lines		
C57BL/6 Fibrosarcoma	Robert Schrieber	N/A
Experimental Models: Organisms/Strains		
Mouse (LysMCre:Rosa26LSL-tdT:Zbtb46GFP)	Generated	N/A
Mouse (C57BL/6)	Jackson Laboratories	000664
Other		
LSRFortessa Flow Cytometer	BD Biosciences	N/A
27G Hypodermic Needle	BD Biosciences	305109
18G Hypodermic Needle	BD Biosciences	305196
50 mL Polypropylene Tube	Thermo Fisher Scientific	339652

MATERIALS AND EQUIPMENT

MACS Buffer

Reagents	Final concentration	Amount
10× Dulbecco's Phosphate-Buffered Saline (sterile PBS)	1×	50 mL
Bovine Serum Albumin (BSA)	0.5%	2.5 g
0.5 M EDTA (pH 8.0)	2 mM	2 mL
Distilled water (dH_2O)	N/A	Fill to 500 mL

Filter through polystyrene membrane under sterile conditions; store at 4°C for up to 2 weeks.

- 1× Red Blood Cell (RBC) Lysis Buffer (sterile, dilute 10× to 1× with dH_2O)
- Tumor dissociation buffer

Prepare freshly each time before enzymatic digestion by adding Collagenase B (2 mg/mL) and DNase I (40 U/mL) into DMEM media (5 mL per tumor sample).

Reagents	Final concentration	Amount
Collagenase B	2 mg/mL	10 mg
DNase I	40 U/mL	200 U
DMEM media	N/A	5 mL

STEP-BY-STEP METHOD DETAILS

This protocol is organized into four main sections (see below). Sections 1 and 2 are performed on day 0, whereas sections 3 and 4 are performed on a subsequent day (e.g., day 3, day 5). The buffers listed above may be prepared prior to the first day of experimentation.

Section 1: Isolation of monocytes from mouse bone marrow (BM) section 2: Transfer of monocytes into mouse tumors

Section 3: Generation of single-cell suspensions from mouse tumors section 4: Antibody staining of single-cell suspensions for flow cytometry

Section 1: Isolation of Monocytes from Mouse BM

Timing: 3+ h, depending on number of monocytes required

Monocytes were isolated from BM of mice using the Mouse BM Monocyte Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions with slight modifications as described below.

Note: Perform all steps on ice and under sterile conditions

1. Spray hindlimbs of euthanized mouse with 70% ethanol (EtOH).
2. Remove overlying skin and muscle from the femur and tibia.
3. Using sterile surgical tools, amputate femur from the ileum, separate femur from tibia, separate tibia from hind foot. To ensure that the maximum amount of bone marrow is extracted, carefully preserve epiphyses of femur and tibia.

4. Cut proximal epiphyses of bones and flush the bone shafts with 5 mL MACS buffer into sterile petri dishes using a 27G needle.
5. Using an 18G needle, disaggregate and transfer flushed bone marrow in MACS buffer into 50 mL conical tube.
6. Centrifuge at 300 × *g* for 8 min at 4°C. Bone marrow cells will form red pellet; aspirate supernatant.
7. Resuspend cell pellet in 2 mL of 1× RBC Lysis Buffer and incubate for 5 min on ice to lyse red blood cells.

⚠ CRITICAL: Although RBC lysis is not a necessary part of the protocol, we have found it to be useful in preventing clogging of the magnetic columns used in subsequent steps (see below). Nonetheless, it is important to keep this incubation period ≤ 5 min as extended exposure to this lysis buffer negatively affects cell viability.

8. Quench by adding 10 mL MACS buffer.
9. Centrifuge at 300 × *g* for 8 min at 4°C. Cell pellet should now be white in color.
10. Resuspend cell pellet in MACS buffer and take an aliquot for cell counting.
11. Follow the Miltenyi Mouse BM Monocyte Isolation Kit protocol exactly as described in the manufacturers protocol (https://www.miltenyibiotec.com/US-en/products/monocyte-isolation-kit-bm-mouse.html; click "Data sheet") to magnetically label bone marrow cells and perform column-based enrichment of monocytes.

Note: We have closely adhered to the above protocol to isolate bone marrow monocytes and have consistently achieved ≥ 75% purity of enriched monocytes. We have shown a representative flow cytometry plot comparing Ly6C+ monocytes in total BM cells and after enrichment in Figure 1A. The fraction of this monocyte population in the BM that expresses tdTomato is also shown in Figure 1B.

Note: The estimated purity of enriched monocyte is a conservative one based on positive staining with markers such as Ly6C (Figure 1). Cells negative/low for these markers may include subsets of monocytes.

Note: Anticipate isolation of ~2 × 10^6 monocytes for every mouse (4–16 weeks female or male) used in the experiment. However, this number may vary based on the bone marrow isolation technique as well the age, weight, sex, and genotype of the donor mouse. In general, monocytes make up ~10% of the cellular composition of mouse bone marrow.

12. If desired, expose the isolated monocytes to drugs or other external factors (and the appropriate controls). For example, in Devalaraja et al. 2020, we incubated isolated monocytes with an irreversible retinoic acid receptor (RAR) antagonist or DMSO for 1 h to examine the impact of RAR signaling on monocyte differentiation in tumors.
13. Thoroughly wash monocytes twice with 1× PBS and resuspend 5 × 10^5 monocytes in 50 μL of 1× PBS. Monocytes are now ready to be transferred into tumors.

Section 2: Transfer of Monocytes into Mouse Tumors

Timing: ~1 h, depending on the number of tumors

Note: Flank tumors must be generated prior to transfer of monocytes (please refer to the "Before You Begin" section for additional details on establishing flank tumors).

14. Draw monocytes up into a 27G needle (with 0.5 inch length) and remove any air bubbles that may be present in the syringe. This needle will be used to directly inject the flank tumor.

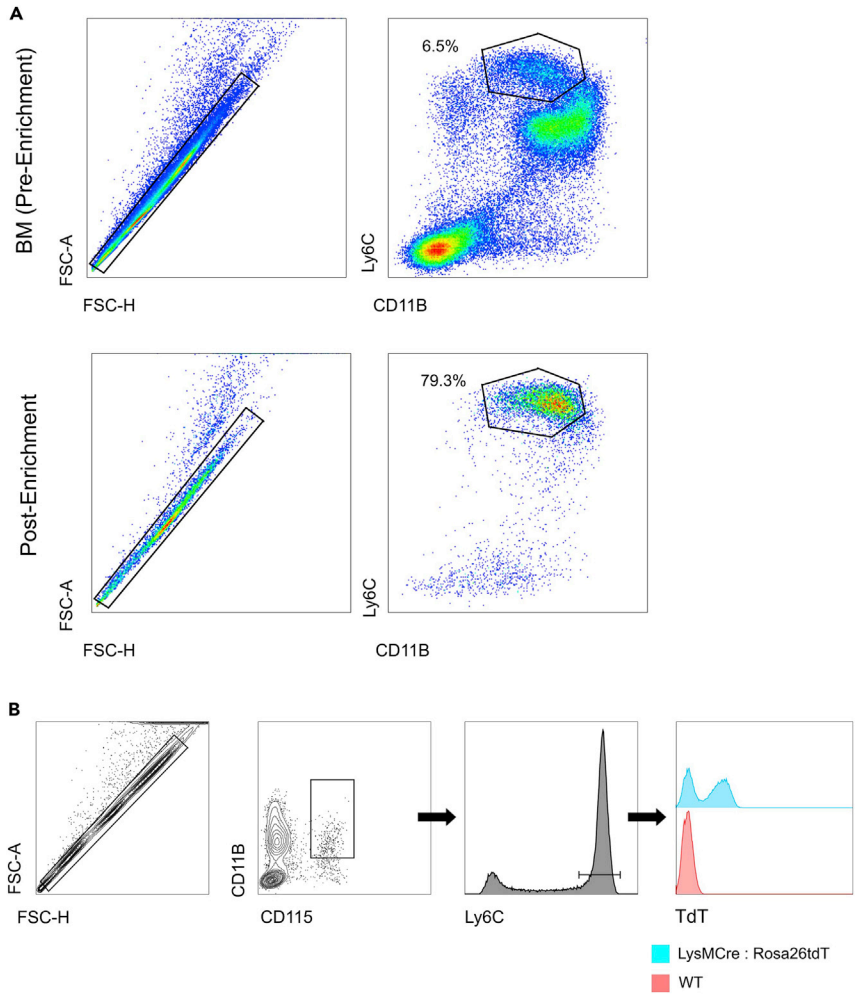

Figure 1. Purity of Monocytes Enriched with Negative Selection Magnetic Beads

(A) Flow cytometry plots showing frequency of Ly6C+ monocytes in pre- and post-enriched bone marrow (BM) cell suspensions obtained using the MACS Miltenyi Monocyte Isolation Kit.

(B) Flow cytometry plots and histograms showing the expression of tdTomato within CD115+ Ly6C+ monocytes from BM of LysMCre:Rosa26tdT mice. Arrows: plots before each arrow show the gating for cells shown on plots after the arrow.

15. Induce general anesthesia using isoflurane gas for the recipient mouse and ensure that mouse remains under anesthesia for the duration of the procedure.
16. To prevent monocytes from "clumping" within the syringe, place the syringe on a slow-moving shaker at 15°C–25°C while the mouse is being prepared for injection.
17. Inject 50 μL of monocytes/PBS into flank tumor.

Note: We have found that performing the injection at a 45° angle with the bevel up minimizes both the physical disruption to the tumor architecture (FS tumors) and the number of monocytes lost during the injection procedure. As mentioned before, flank tumors would ideally be approximately 1–1.5 cm in diameter at the time of monocyte transfer; larger tumors generally have increased intratumoral hemorrhage, fluid extravasation, and pressure, making injection of additional volume more difficult. We scruff the mouse to ensure the skin is held taut, and the needle traverses the skin covering the top of the tumor to reach the center of the tumor for intratumoral injection. To minimize the amount of post injection tumor fluid loss, swiftly withdraw the needle and replace needles between mouse injections.

⚠ CRITICAL: We have attempted iterations of this procedure using varying numbers of monocytes and have found that transfer of 5×10^5 per mouse tumor is sufficient to track *in situ* monocyte differentiation at various timepoints post injection. However, pre-treatment of monocytes with drugs etc. (see above in section 1) may affect viability. Therefore, for protocols incorporating such treatments, the optimal number of monocytes needed for subsequent analyses will have to be determined empirically.

Section 3: Generation of Single-Cell Suspensions from Mouse Tumors

Timing: 2+ h, depending on the number of tumors

Note: Time between monocyte transplant and tumor harvest depends on the intended purpose of the analyses. We were able to detect the monocytes and their progenies up to 10 days after transfer.

18. Spray euthanized mouse with 70% EtOH and remove layer of skin overlying the flank tumor.
19. Excise entire flank tumor from underlying muscle and adipose tissue.
20. Place tumor into petri dish, mince tumor into small pieces (1–3 mm) and transfer tissue to container containing a magnetic stir bar.
21. Add 5 mL of tumor dissociation buffer containing Collagenase B (2 mg/mL) and DNase I (40 U/mL) in DMEM media.

⚠ CRITICAL: Prepare fresh solution of Collagenase B and DNase I media to maximize enzymatic activity.

22. Place container with tissue, media and stir bar onto stir plate in 37°C incubator and let stir at low to medium speed for 30–45 min.

Note: Tumors with a dense stromal component such as fibrosarcoma may require 45 min of dissociation, whereas tumors with low fibrotic content such as B16-F10 melanoma may require less dissociation time. The optimal time will depend on the tumor type and will need to be determined empirically.

23. Pass dissociated suspension through a 70 μm filter (placed on top of a 50 mL conical tube). Wash filter with 10 mL of DMEM media.
24. Centrifuge at 300 × *g* for 8 min at 4°C. Aspirate supernatant.
25. Resuspend cell pellet in 2 mL of 1× RBC Lysis Buffer and incubate for 5 min on ice to lyse red blood cells.
26. Quench cells by adding 10 mL of MACS buffer.
27. Centrifuge at 300 × *g* for 8 min at 4°C. Aspirate supernatant.

Note: Given that transferred monocytes comprise a rare population in tumors, we recommend enriching for intratumoral leukocytes using a column-based positive selection kit to select for CD45+ cells.

28. Follow the Miltenyi Mouse CD45 MicroBeads protocol (https://www.miltenyibiotec.com/US-en/products/cd45-microbeads-mouse.html#130-052-301; click "Data sheet") exactly as described in the manufacturers protocol to select for CD45+ cells in mouse tumors.
29. Centrifuge the CD45 enriched fraction at 300 × *g* for 8 min at 4°C. Aspirate supernatant.
30. Resuspend cell pellet in 1 mL MACS buffer and take an aliquot for cell counting.
31. Resuspend 1–2 × 10^6 cells in 100 μL of MACS buffer and add anti-mouse CD16/32 Fc Block (1:200 final dilution).

⚠ CRITICAL: Cells should be kept on ice and are now ready to be stained with fluorescently tagged antibodies for flow cytometry.

Section 4: Antibody Staining of Single-Cell Suspensions for Flow Cytometry

Timing: 1+ h, depending on the number of samples

This portion outlines the use of primary-fluorophore conjugated antibodies to examine the differentiation of transferred monocytes.

32. Prepare cocktail of antibodies (see table below). The volume of mix for one sample is 3.75 μL.

Note: This cocktail includes markers to gate on monocytes and their progeny macrophages and dendritic cells. The user can include additional markers of monocyte, macrophage, or dendritic cell subsets depending on experimental goals. This cocktail also included markers to gate out unwanted cell populations in our analyses (e.g., neutrophils, NK cells, T cells). It is also important to avoid antibodies conjugated to either FITC or PE to avoid spectral overlap with endogenous GFP or tdTomato (monocytes were transferred from LysMCre:Rosa26tdT:Zbtb46GFP hosts).

Fluorophore	Marker	Company	Catalog #	Final Dilution	Volume
FITC	GFP	N/A	N/A	N/A	0 μL
PercpCy5.5	Ly6G	BioLegend	127633	1:400	0.25 μL
PercpCy5.5	CD3e	BioLegend	100351	1:200	0.5 μL
PercpCy5.5	NK1.1	BioLegend	108707	1:200	0.5 μL
APC	F4/80	Invitrogen	MF48005	1:200	0.5 μL
APC-Cy7	CD11c	BioLegend	117324	1:200	0.5 μL
BV421	MHCII	BioLegend	107635	1:400	0.25 μL
BV510	CD45	BioLegend	103138	1:200	0.5 μL
BV605	Ly6C	BioLegend	128026	1:200	0.5 μL
PE	tdTomato	N/A	N/A	N/A	0 μL
PE-Cy7	CD11b	BioLegend	25-0112-82	1:400	0.25 μL

33. Add the indicated volume of antibody cocktail to cells (prepared in step 31), vortex briefly, and incubate in the dark at 4°C for 30 min.
34. Wash with 1 mL of MACS buffer (add directly to the 100 μL mix) and centrifuge at 300 × *g* for 8 min at 4°C.
35. Aspirate supernatant and resuspend cells in 200 μL of MACS buffer.
36. Add 5 μL of 7AAD (cell viability dye, compatible with detection in the PercpCy5.5 channel) to each sample and keep samples in the dark. Samples are now ready to be analyzed via flow cytometry.
37. Run samples and collect data on a flow cytometer.

Note: Fluorescently labeled cells can be fixed in formaldehyde to perform flow cytometry at a later time. However, 7AAD cannot be used in this case. The use of alternative cell viability markers that are compatible with fixation is recommended.

⚠ CRITICAL: To set up appropriate voltages on the cytometer, it is critical to use compensation controls (commercial compensation beads or cells stained with individual antibodies), unstained samples, single stained samples and fluorescence minus one (FMO) samples. Please

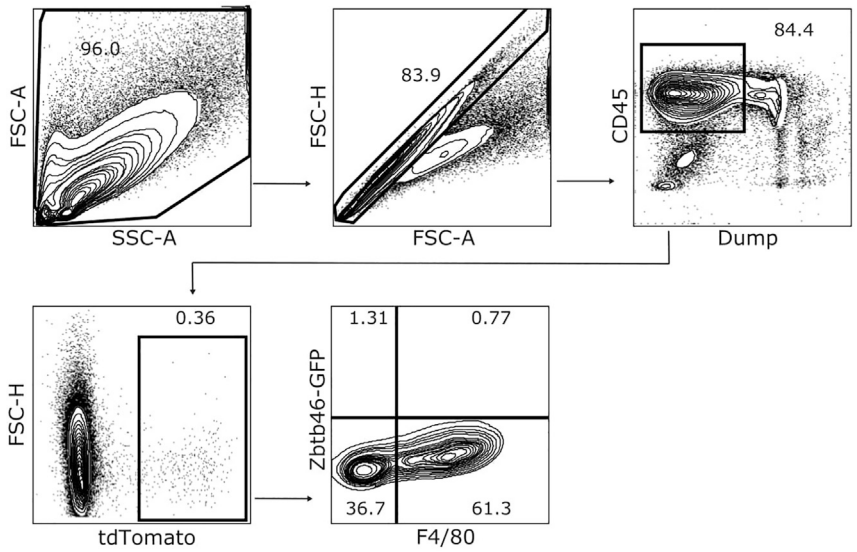

Figure 2. Representative Flow Cytometry Plots of Intratumorally Transferred Monocytes from Bone Marrow of LysMCre:Rosa26tdT:Zbtb46GFP Mice

Arrows: Plots before each arrow show the gating for cells shown on plots after the arrow. Cells were pregated on Ly6G− CD3e− NK1.1− live singlets and intratumorally transferred monocytes were subsequently identified as CD45+ tdT+.

refer to Cossarizza et al. for thorough explanation of compensation setup and the use of rigorous controls in flow cytometry (Cossarizza et al., 2017). In our analyses, we use the BD™ CompBeads, which are polystyrene microparticles that bind any immunoglobulin containing rat or hamster κ light chain. The beads are incubated with individual fluorochrome-conjugated antibodies following manufacturer's recommendations. FITC and PE conjugated antibodies were used to compensate for GFP and tdTomato in our experiments. Nonetheless, cells expressing these fluorescent proteins or other compatible fluorochrome-conjugated antibodies can be used to compensate for GFP and tdTomato depending on the configuration or the cytometer and availability of reagents.

EXPECTED OUTCOMES

Using the above methodology, we have tracked the differentiation of monocytes in tumors at various time points post transfer. Our gating scheme to identify transferred monocytes is the following:

- Gate on singlets using FSC-A, SSC-A, FSH-H, SSC-H.
- Gate out dead cells by 7AAD positivity.
- Gate out neutrophils, T cells, and NK cells using the dump gate of Ly6G, CD3e, and NK1.1.
- Select hematopoietic cells by gating on CD45.
- Gate on transferred monocytes using tdTomato expression (LysMCre:Rosa26tdT) (Figure 2).
- Finally, examine monocyte differentiation using monocyte, macrophage, and dendritic cell markers such as Ly6C, CD11b, F4/80, CD11c, MHCII, and Zbtb46.

Note: Despite using a CD45 enrichment step, we still recommend the use of CD45 to gate on leukocytes to enhance specificity.

Note: The markers for monocyte differentiation above is a limited set used in our particular analyses and we show a representative analyses in Figure 2. However, monocytes and their progenies can take on many different phenotypic and functional forms. The user can perform a more comprehensive marker analyses depending on experimental goals.

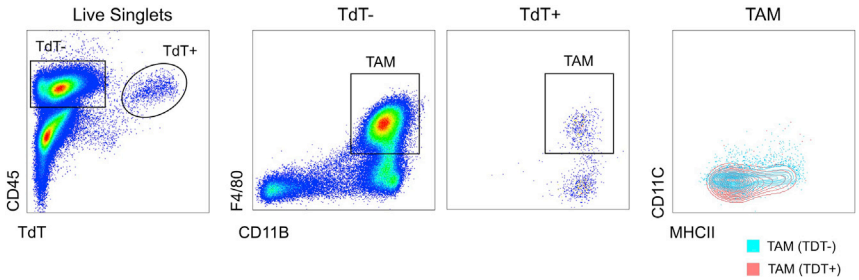

Figure 3. Representative Flow Cytometry Plots Comparing Endogenous and Transferred Myeloid Cells in Tumors

Cells were pregated on live singlets and identified as either F4/80+ tdT− (endogenous) or F4/80+ tdT+ (transferred) tumor-associated macrophages (TAM). The expression of CD11c and MHCII within each TAM population is compared in the final plot.

Note: In our model, tdTomato was driven by the Rosa26 promoter, thereby generating a bright fluorescence signal 1–2 logs above background. This allowed for a clear and accurate identification of transferred monocytes *via* flow cytometry. Given the low frequency of transferred monocytes compared to cells within the recipient tumor, it is advisable to perform this experiment with a model in which transferred monocytes will be clearly distinguishable above background.

Note: In our model systems, we found that the majority of intratumorally transferred monocytes differentiated into macrophages (defined by F4/80 expression), whereas very few differentiated into dendritic cells (defined by expression of dendritic cell marker Zbtb46) (Figure 2). Furthermore, we find that endogenous tumor-associated macrophages (tdTomato-negative tumor macrophages) displayed similar surface expression profile to macrophages generated from transferred monocytes (tdTomato-positive tumor macrophages). This is shown in Figure 3. Nonetheless, this differentiation of monocytes may depend on the type of tumor and the outcome may be different in non-sarcoma tumor models.

Note: We have harvested and analyzed sarcoma flank tumors at several time points post-monocyte transfer. In this model, we found that the frequency of transferred tdT+ cells remains relatively similar from 3 days to 10 days. However, we observed a precipitous drop in the frequency of tdT+ cells at 11 days post transfer (data not shown). This may suggest that the lifespan of transferred monocytes and their progeny is around 10 days. An alternative explanation is the migration of monocytes or monocyte-derived dendritic cells to tumor draining lymph nodes.

Although this protocol describes monocyte transfer & differentiation in flank tumors, we envision that this protocol may be adapted to transfer monocytes into other sites, such as orthotopic tumors, spontaneous tumors, normal tissue, peritoneum, etc.

LIMITATIONS

One limitation of this protocol is the use of LysMCre:Rosa26tdT:Zbtb46GFP mice as monocyte donors. Though these mice were useful for us to clearly identify transferred monocytes using tdTomato and subsequently track their differentiation using the widely used dendritic cell marker Zbtb46, this strategy was accompanied with some logistical and biological limitations (Abram et al., 2014).

Logistically, breeding LysMCre:Rosa26tdT:Zbtb46GFP mice is resource and time intensive. Viable alternatives include using CD45.1 hosts and CD45.2 recipients (and vice versa), or using CFSE or CTV to label monocytes once isolated from host bone marrow. However, we have observed that labeling monocytes with CFSE may impact their survival and differentiation in the tumor microenvironment (data not shown). Additionally, because the LysM-Cre is not specific to monocytes (also targets the granulocytic lineage), it will be important to perform these studies with additional Cre drivers

that may more specifically mark monocytes. These include well-known myeloid Cre drivers such as CSF-1R-Cre or CX3CR1-Cre, or additional newly characterized mouse models such as the Ms4A3-Cre to fate map monocytes (Liu et al., 2019).

Another limitation of this protocol is the imprecise injection of monocytes into flank tumors. This may contribute to inter-experiment variability and compromise the reproducibility of intratumoral monocyte transfer experiments. Though we have not attempted to utilize delivery systems outside of a standard 27G needle, more sophisticated delivery approaches will likely improve the precision of intratumoral delivery (Solorio et al., 2010). We have observed, however, that intratumoral delivery of 5×10^5 monocytes in 50 μL to flank tumors between 1 and 1.5 cm in diameter has resulted in reproducible outcomes in multiple experiments spanning a range of time points.

Lastly, as we use a negative selection magnetic-based monocyte isolation kit, the purity of isolated monocytes is lower than FACSorting. We have attempted FACSorting bone marrow monocytes (positive selection) and subsequent transfer into fibrosarcoma flank tumors. However, when we analyzed transferred monocytes at 3d, we observed a significantly lower frequency of live intratumoral monocytes. This suggested that FACSorting may compromise the viability of bone marrow monocytes, either due to extrinsic fluidic pressure associated with cell sorting or the use of positive-selection antibodies. Isolating monocytes using magnetic-based negative selection is likely less cytotoxic (Sutermaster and Darling, 2019).

TROUBLESHOOTING

Problem 1

Impure monocyte isolation (step 11).

Potential Solution

As this is a negative selection kit (i.e., enriches for monocytes by antibody-labeling other hematopoietic lineages and magnetically separating them from monocytes), it is important to strictly adhere to the recommended maximum limit of cells in the antibody incubation, magnetic labeling, and magnetic column separation steps. This will ensure maximal purity of the enriched monocyte population.

Problem 2

Imprecise intratumoral injection (step 17).

Potential Solution

As flank tumors grow in size, they tend to become more vascularized that may be associated with higher intratumoral pressures, making it more difficult to inject external substances into the tumor bed. Thus, we recommend using flank tumors that are less than 1.5 cm in diameter for monocyte injections. In our fibrosarcoma model where we generate tumors with implantation of 1×10^6 tumor cells, the tumors reach 1–1.5 cm in diameter in approximately 7 days. We do not recommend troubleshooting imprecise intratumoral injections by using less than 50 μL of volume, as this will likely magnify issues with injection volume accuracy and may significantly alter the number of transferred monocytes both within and between experiments.

Problem 3

Low cell viability after generation of single-cell suspension from tumors (step 30).

Potential Solution

Low cell viability during generation of tumor single-cell suspension may have multiple etiologies, including biological causes such as a highly necrotic tumor, and technical causes such as over digestion of tissue or inadequate temperature control. To troubleshoot the technical issues, we recommend decreasing Collagenase B and/or DNase I concentration, reducing digestion time in the 37°C incubator, performing all steps on ice, and reducing RBC lysis time.

Problem 4

Shortcomings in the use of surface markers to distinguish macrophages from dendritic cells (step 37).

Potential Solution

It can be difficult to distinguish macrophages from dendritic cells based on surface markers alone (Broz et al., 2014). To accurately identify dendritic cells, we used Zbtb46-GFP mice as monocyte hosts. An alternative to using this genetically engineered mouse may be to optimize and use commercially available antibody against Zbtb46 or use additional expanded list of surface markers.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dr. Malay Haldar (mhaldar@pennmedicine.upenn.edu).

Materials Availability

This study did not generate any unique materials or reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conception and Design, S.D. and M.H.; Methodology, S.D. and M.H.; Data Acquisition, S.D. and M.H.; Analysis and Data Interpretation, S.D. and M.H.; Writing – Review & Editing, S.D. and M.H.; Supervision, S.D. and M.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Abram, C.L., Roberge, G.L., Hu, Y., and Lowell, C.A. (2014). Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J. Immunol. Methods *408*, 89–100.

Broz, M.L., Binnewies, M., Boldajipour, B., Nelson, A.E., Pollack, J.L., Erle, D.J., Barczak, A., Rosenblum, M.D., Daud, A., Barber, D.L., et al. (2014). Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. Cancer Cell *26*, 638–652.

Cossarizza, A., Chang, H.-D., Radbruch, A., Akdis, M., Andrä, I., Annunziato, F., Bacher, P., Barnaba, V., Battistini, L., Bauer, W.M., et al. (2017). Guidelines for the use of flow cytometry and cell sorting in immunological studies. Eur. J. Immunol. *47*, 1584–1797.

Devalaraja, S., To, T.K.J., Folkert, I.W., Natesan, R., Alam, M.Z., Li, M., Tada, Y., Budagyan, K., Dang, M.T., Zhai, L., et al. (2020). Tumor-derived retinoic acid regulates intratumoral monocyte differentiation to promote immune suppression. Cell *180*, 1098–1114.e16.

Gubin, M.M., Esaulova, E., Ward, J.P., Malkova, O.N., Runci, D., Wong, P., Noguchi, T., Arthur, C.D., Meng, W., Alspach, E., et al. (2018). High-dimensional analysis delineates myeloid and lymphoid compartment remodeling during successful immune-checkpoint cancer therapy. Cell *175*, 1014–1030.e19.

Liu, Z., Gu, Y., Chakarov, S., Bleriot, C., Kwok, I., Chen, X., Shin, A., Huang, W., Dress, R.J., Dutertre, C.-A., et al. (2019). Fate mapping via Ms4a3-expression history traces monocyte-derived cells. Cell *178*, 1509–1525.e19.

Satpathy, A.T., KC, W., Albring, J.C., Edelson, B.T., Kretzer, N.M., Bhattacharya, D., Murphy, T.L., and Murphy, K.M. (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J. Exp. Med. *209*, 1135–1152.

Shi, J., Hua, L., Harmer, D., Li, P., and Ren, G. (2018). Cre driver mice targeting macrophages. Methods Mol. Biol. *1784*, 263–275.

Solorio, L., Patel, R.B., Wu, H., Krupka, T., and Exner, A.A. (2010). Advances in image-guided intratumoral drug delivery techniques. Ther. Deliv. *1*, 307–322.

Sutermaster, B.A., and Darling, E.M. (2019). Considerations for high-yield, high-throughput cell enrichment: fluorescence versus magnetic sorting. Sci. Reports *9*, 227.