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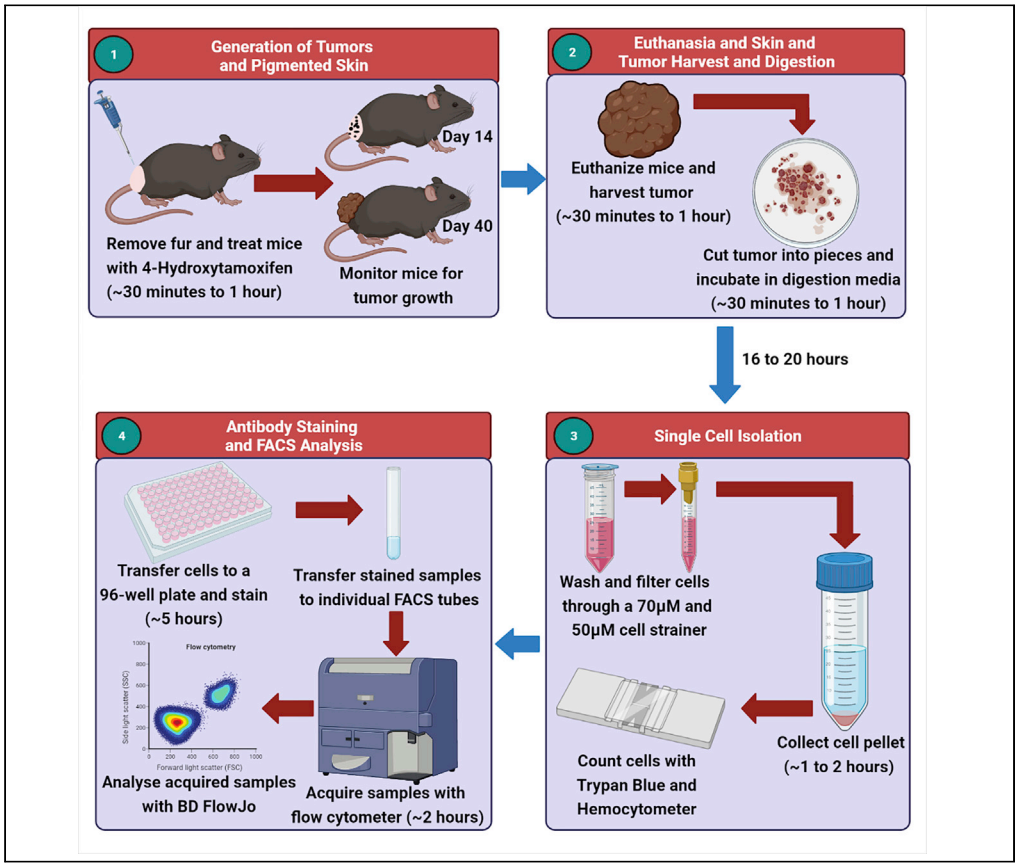
Optimized protocol for immunophenotyping of melanoma and tumor-bearing skin from mouse



While isolating immune cells from spleens and lungs is routinely achieved using flow cytometry, it is challenging to isolate viable immune cells from skin. Here, we describe a step-by-step protocol for skin digestion using a murine melanoma model, which is amenable for detection of low abundant immune cell populations including group 2 innate lymphoid cells.

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Highlights

Optimized skin and melanoma digestion protocol for immune phenotyping

Detection of ILC2s and other immune cells isolated from skin and melanoma samples

Detailed gating strategies to identify ILC2s, T cells, and myeloid cell subsets

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Optimized protocol for immunophenotyping of melanoma and tumor-bearing skin from mouse

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SUMMARY

While isolating immune cells from spleens and lungs is routinely achieved using flow cytometry, it is challenging to isolate viable immune cells from skin. Here, we describe a step-by-step protocol for skin digestion using a murine melanoma model, which is amenable for detection of low abundant immune cell populations including group 2 innate lymphoid cells.

BEFORE YOU BEGIN

Melanoma induction using 4-hydroxytamoxifen

Timing: 30 min–1 h

$BRAF^{V600E}/PTEN^{null/null}$ melanoma transgenic mouse model

The $BRAF^{V600E}/PTEN^{null/null}$ melanoma transgenic mouse model faithfully recapitulates human cutaneous melanoma, with the presence of pigmented lesions (moles) which progress to metastatic melanomas (Dankort et al., 2009, Miller and Mihm, 2006). This melanoma model was designed to allow 4-hydroxytamoxifen (4-HT) inducible melanocyte-targeted $BRAF^{V600E}$ expression and simultaneous *PTEN* inactivation. This is made possible due to the presence of the 4-HT-inducible Cre recombinase-mutated estrogen receptor fusion transgene which is under the control of the tyrosinase promoter (*Tyr::CreER*). Given that this is an inducible melanoma model, not requiring injection of melanoma cells into the mouse, it can serve as a powerful tool to study the immune cells which are present as soon as the pigmented lesions are visible.

A challenge in immune phenotyping of melanomas early in the development of this disease is the digestion of the skin and melanoma using enzymes such as collagenase, dispase and liberase. Certain enzymes affect the expression of cell surface markers that decorate immune cells (Autengruber et al., 2012), and may reduce the viability of specific immune cell types in the skin. To overcome these challenges, we developed a protocol for skin tumor processing and digestion, which can be used for leukocyte phenotyping of skin and melanoma. The isolated single cells from the step-by-step digestion protocol presented here can be used for subsequent multiparameter flow cytometry

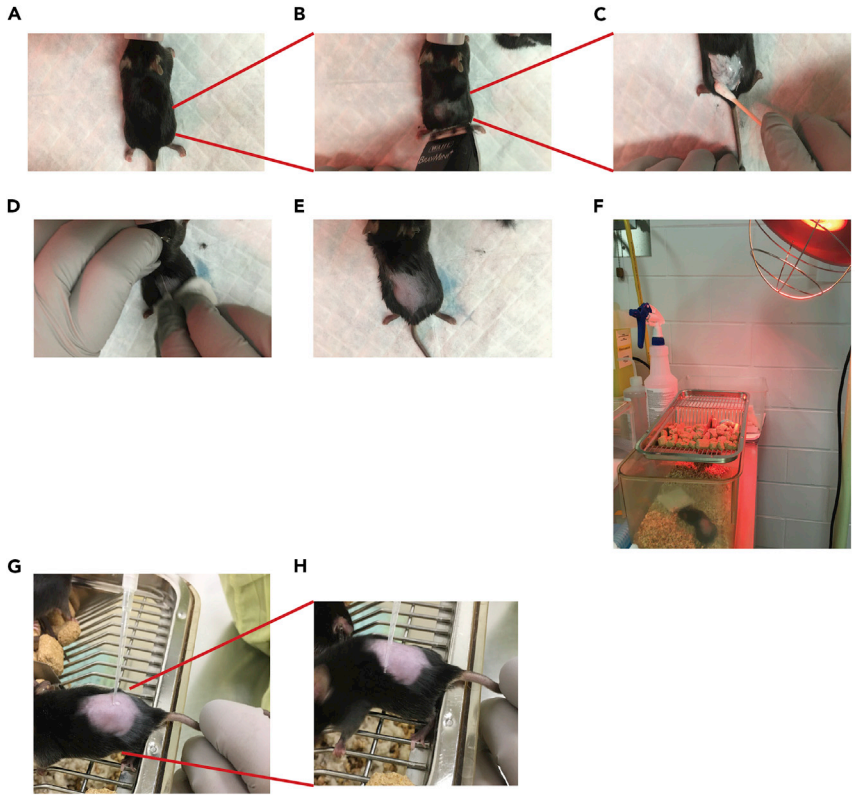

Figure 1. Application of 4-Hydroxytamoxifen (4-HT) for the induction of Melanoma formation

(A) Mice are sedated with 3% Isoflurane.

(B) Mice are shaved at the lower back near the base of tail.

(C) Hair depilatory cream is applied for 2–3 min to remove residual fur.

(D) Wet swab used to remove the cream.

(E) Dry and inspect skin for any residual fur.

(F) Place mice back in the cage with a heating lamp to allow recovery.

(G) Using a pipette tip, add 2μl of 5mM 4-HT to the shaved, exposed skin.

(H) Use the side of a pipette tip to spread 4-HT on the skin and allow it to dry before placing the mouse back into the cage

to enumerate and define distinct functions of numerous cell types, including T cells and myeloid cells, but also for the detection of very low abundant cell types including ILC2s (Salimi et al., 2013).

1. Sedate 6–8 week old male mice with 3% Isoflurane and oxygen (Figure 1A)
2. While sedated, use an electric shaver to shave the fur off the lower back (Figure 1B)
3. Using a cotton swab, apply hair depilatory cream on to the shaved skin and massage the cream for maximum contact. Leave it on for 2–3 min to remove the fur. (Figure 1C)
4. Use a moist cotton gauze to completely remove the depilatory cream (Figure 1D)
5. Use a dry cotton gauze to dry the skin (Figure 1E)
6. Place mouse in cage under heating lamp to recover and proceed to work on the next mouse (Figure 1F)
7. Allow mice to recover for at least 12 h
8. The following morning, take the 4-HT aliquot (5 mM) out of the freezer to thaw on ice
9. While handling the mouse by the base of the tail, allow the mouse to grab the cage grill

Note: The mouse may be sedated with 3% isoflurane if the operator is uncomfortable handling mice while awake

10. Add 2 μL 4-HT (5 mM) to the shaved skin (Figure 1G)
11. Use the side of the same pipette tip to spread the 4-HT around the exposed skin (Figure 1H)
12. Allow the 4-HT to dry until no visible drug is left on the skin
13. Place mouse back into the cage and work on the next mouse
14. Repeat steps 8–13 for the next 2 days, such that the 4-HT is applied for three consecutive days

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GATA3 BV711 (clone L50-823)	BD Biosciences	Cat #565449
Mouse monoclonal anti-IgG1,κ Isotype Control BV711 (clone X40)	BD Biosciences	Cat #563044
Mouse monoclonal anti-ST2 PerCP-eFluor 710 (clone RMST2-2)	eBioscience	Cat #46-9335
Mouse monoclonal anti-IgG2,κ Isotype Control PerCP-eFluor 710 (clone eBR2a)	eBioscience	Cat #46-4321-80
Mouse monoclonal anti-CD127 BV650 (clone A7R34)	BioLegend	Cat #135043
Mouse monoclonal anti-IgG2α,κ Isotype Control BV650 (clone RTK2758)	BioLegend	Cat # 400542
Mouse monoclonal anti-KLRG1 AF488 (clone 2F1)	BD Biosciences	Cat #561619
Mouse monoclonal anti-CD45.2 BV785 (clone 30-F11)	BioLegend	Cat #103149
Mouse monoclonal anti-ICOS APC (clone C398.4A)	eBioscience	Cat #17-9949-82
Mouse monoclonal anti-Thy 1(CD90.2) BV510 (clone 53-2.1)	BioLegend	Cat #140319
Mouse monoclonal anti-CD25 PE-Cy7 (clone PC61)	BD Biosciences	Cat #561780
Mouse monoclonal anti-FcεR1 PE (clone MAR-1)	eBioscience	Cat #12-5898-83
Mouse monoclonal anti-CD19 PE (clone eBioID3)	eBioscience	Cat #12-0193-82
Mouse monoclonal anti-CD3ϵ PE (clone 145-2C11)	eBioscience	Cat #12-0031-85
Mouse monoclonal anti-B220 PE (Clone RA3-6B2)	eBioscience	Cat #12-0452-83
Mouse monoclonal anti-TCRβ PE (clone H57-597)	eBioscience	Cat #12-5961-83
Mouse monoclonal anti-TCRγ/δ PE (clone EBioGL3)	eBioscience	Cat #12-5711-82
Mouse monoclonal anti-NK1.1 PE (clone PK136)	eBioscience	Cat #12-5941-82
Mouse monoclonal anti-Gr-1 eF450 (clone RB6-8C5)	eBioscience	Cat #48-5931-82
Mouse monoclonal anti-TER-119 eF450 (clone TER-119)	eBioscience	Cat #48-5921-82
Mouse monoclonal anti-CD11c eF450 (clone N418)	eBioscience	Cat #48-0114-82
Mouse monoclonal anti-CD11b eF450 (clone M1/70)	eBioscience	Cat #48-0112-82
Mouse monoclonal anti-CD45.2 APC-Cy7 (clone 30-F11)	BD Biosciences	Cat #561037
Mouse monoclonal anti-CD3ϵ BV650 (clone 145-2C11)	BD Biosciences	Cat #563044
Mouse monoclonal anti-CD8α PerCP-Cy5.5 (clone 53-6.7)	BD Biosciences	Cat #561109
Mouse monoclonal anti-CD4 PE-Cy7 (clone GK1.5)	BioLegend	Cat #100-422
Mouse monoclonal anti-CD11c BV786 (clone HL3)	BD Biosciences	Cat #563735
Mouse monoclonal anti-F4/80 PE (clone BM8)	eBioscience	Cat #12-4801-80
Mouse monoclonal anti-Ly-6G/Ly-6C AF488 (clone RB6-8C5)	eBioscience	Cat #53-5931-82
Mouse monoclonal anti-Ly-6C APC (clone AL-21)		Cat #560595
Mouse monoclonal anti-FOXP3 PE-eF610 (clone FJK-16s)	eBioscience	Cat #61-5773
Mouse monoclonal anti-Arginase-1 AF700 (clone AlexF5)	eBioscience	Cat #56-3697
Mouse monoclonal anti-CD206 BV711 (clone C068C2)	BioLegend	Cat #141727
Chemicals, peptides, and recombinant proteins		
Isofluorane USP	Fresenius Kabi	Cat #CP0406V2
4-Hydroxytamoxifen (4-HT)	Millipore Sigma	Cat #H6278
1× Dulbecco's phosphate-buffered saline (DPBS)	Wisent Bioproducts	Cat #311-425-CL
Dulbecco's modified Eagle media (DMEM)	Wisent Bioproducts	Cat #319-005-CL
Collagenase P	Roche	Cat #11213865001
Deoxyribonuclease I (DNase I)	Sigma-Aldrich	Cat #D5025-15KU
Fetal bovine serum	Wisent Bioproducts	Cat #080-150
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	Invitrogen	Cat #L10119
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Invitrogen	Cat #L34965
OneComp eBeads™ Compensation Beads	Invitrogen	Cat ##01-1111

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
UltraComp eBeads™ Compensation Beads	Invitrogen	Cat #01-2222
FOXP3 / Transcription Factor Staining Buffer	eBioscience	Cat #00-5523-00
Mouse FcR Blocking Reagent	Miltenyi Biotec	Cat #130-092-575
Experimental models: Organisms/strains		
Male C57BL/6 mouse with the *BRaf*^{V600E}, *Tyr::CreER and Pten*$^{lox4-5}$ genotype (6 to 8 weeks old)	Dankort D. et al., 2009	Gift from Dr Dankort
Software and algorithms		
FlowJo™ v10.7	Becton Dickinson	SCR_008520
Other		
Depilatory cream	Nair	Cat # 6170022261
Electric shaver	Wahl	Cat # 1590-1300
Cotton gauze	AMD Ritmed	Cat # A3007
Cotton swab	Medicom	Cat # 807
P2 micropipette	Gilson	Cat # F144801
2 µL Pipette tips	SARSTEDT	Cat # 70.113
15 mL Tubes	Falcon	Cat # 352096
50 mL Tubes	Falcon	Cat # 352070
100 mm Culture dishes	Ultident	Cat # 229621
6-Well plate	Corning	Cat # 353046
Cell strainer 70 µM	Falcon	Cat # 352350
Cell strainer 50 µM	Sysmex Celltrics	Cat # 04-0042-2317
5 mL Syringe	BD Biosciences	Cat # 309646
Pipette-Aid	Mandel	Cat # TM-155000
25 mL Pipette	SARSTEDT	Cat # 86.1685.001
5 mL Round-bottom tube	Falcon	Cat # 352008
U-bottom 96-well plate	SARSTEDT	Cat # 83.3925
BD LSR Fortessa	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Note: We use 5% FBS for our FACS buffer, however other labs use 2% FBS for their staining, which is acceptable(Duerr, 2016). Furthermore, the amount of FACS buffer prepared can be scaled down according to your needs.

Flow cytometer

The panels used in this protocol were designed to be used on the BD LSRFortessa flow cytometer with the following configuration: 2 channels for the blue 488 nm laser (Alexa Fluor 488, PerCP-Cy5.5, and PerCP-eFluor710), 3 channels for the red 640 nm laser (APC, Alexa Fluor 700, APC-Cy7, and Near IR), 5 channels for the violet laser 405 nm (Aqua, eFluor450, Brilliant Violet 510, Brilliant Violet 650, Brilliant Violet 711, Brilliant Violet 785, and Brilliant Violet 786), 3 channels for the yellow-green 561 nm laser (PE, PE-eFluor610, and PE-Cy7).

STEP-BY-STEP METHOD DETAILS

Tumor harvest, digestion, and single-cell isolation

Timing: 2 Days

Pigmented lesions will start to form on the backs of mice between days 12–15 after the last 4-HT treatment (Figures 2A and 2B). The pigmented lesions will progress to a measurable melanoma between days 24–28 (Figure 2C). The humane endpoint is set at day 50 (Figure 2D). In our experience, 100% of the BRAF^{V600E}/PTEN$^{null/null}$ mice painted with 4-HT develop melanoma and outgrow at a

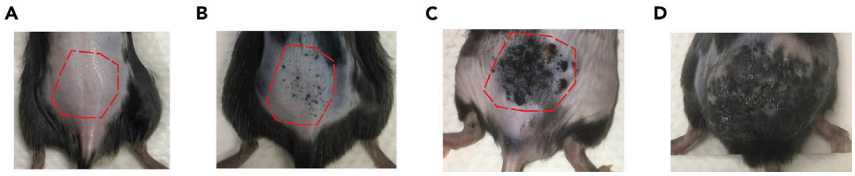

Figure 2. Melanoma progression following 4-HT treatment

(A) Day 7 following 4-HT treatment. During this timepoint, no pigmented lesions yet visible.

(B) Day 12 – 14 following 4-HT treatment. During this timepoint, pigmented lesions will start to form.

(C) Day 24 – 28 following 4-HT treatment. During this timepoint, measurable tumors will start to form.

(D) Any time after Day 40 following 4-HT treatment, mice are monitored closely for human endpoints.

similar rate (Huang et al., 2021). An advantage of this optimized digestion protocol is that we can isolate viable immune cells on day 7, prior to the generation of pigmented lesions on the backs of mice (Figures 2A). The latter enables us to characterize potential rare immune cell populations such as ILC2s, which are known to be "first-responder" immune cells at the site of inflammation.

Prior to harvesting the skin or tumor, it is recommended to shave the mice. We determined that cutting the skin tumors with a razor into thin strips and then into 1–2 mm^2 squares resulted in optimal cell viability and numbers.

By optimizing the skin digestion protocol, we determined Collagenase P to be the optimal digestion enzyme. We also tested Collagenase IV, Collagenase D, Dispase I, and Liberase TM but found that the obtained cell viabilities and counts were very low (~30% viable cells). Cell viability and yield further improved when tissues were digested for 16–20 h at 4°C, compared to 1 h at 37°C (Figures 3A–3D). It is important to note that this is different from our routinely used lung digestion protocol which employs Liberase TM and DNase for 45 min at 37°C. We opted to not to include a red blood cell (RBC) lysis buffer, as we observed it to decrease cell viability. However, all of our staining procedures included intracellular protein detection using fixation and permeabilization reagents that lyse RBCs. As such, removal of RBC was ensured.

1. Euthanize 4-HT treated mice using approved institutional guidelines, typically through 5% isoflurane and carbon dioxide asphyxiation and confirmation of death by cervical dislocation (Figure 4A)
2. Shave off any hair from the tumor and peripheral area (Figure 4B)
3. Using a pair of scissors and forceps, cut the tumor around the periphery, minimizing the amount of non-tumor bearing skin (Figure 4C)

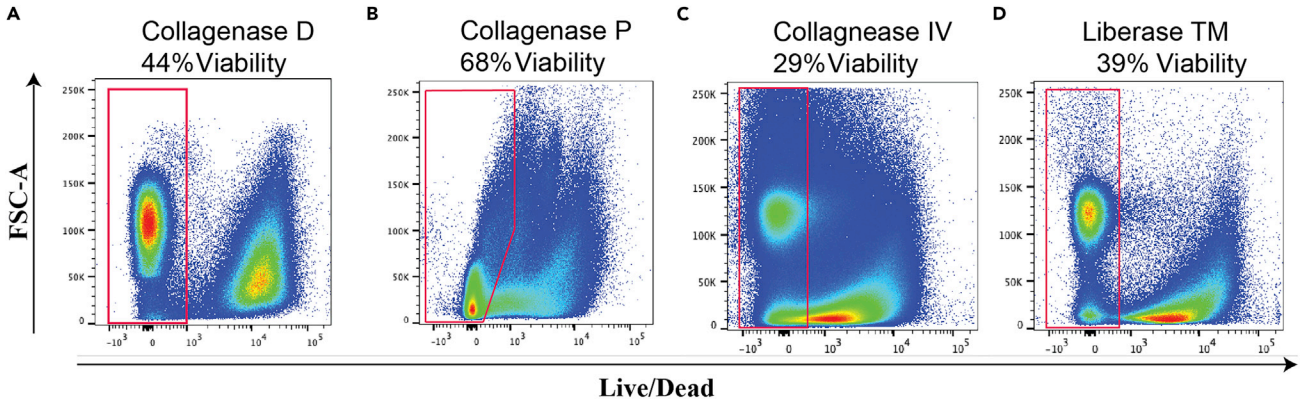

Figure 3. Single cell suspensions of melanomas were prepared using distinct enzymatic treatments and cell viabilities were determined by flow cytometric analysis.

(A) Collagenase D, (B) Collagenase P, (C) Collagenase IV at 30°C for 1 h, (D) Liberase TM

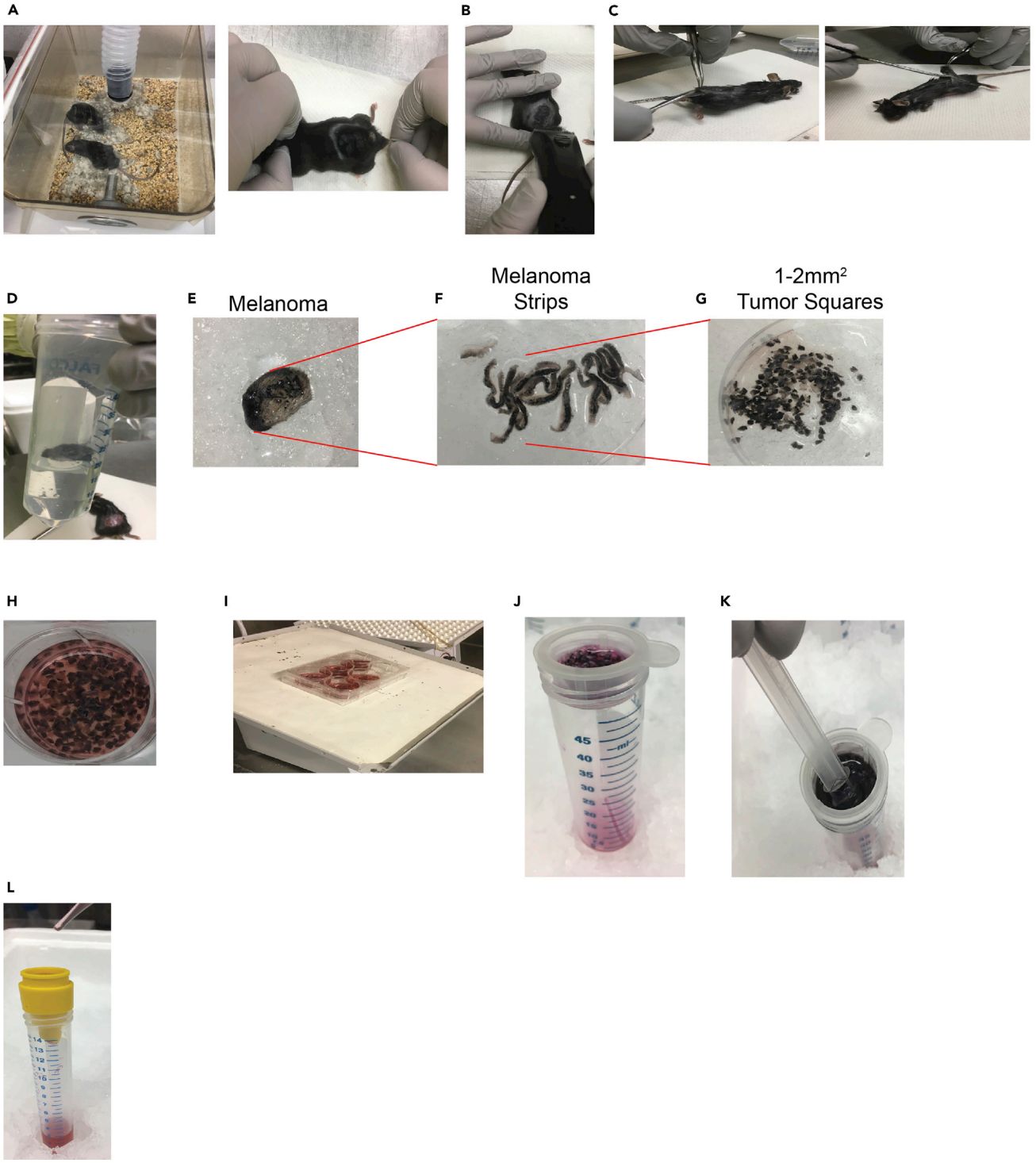

Figure 4. Skin and melanoma harvest

(A) Euthanize mice according to institutional guidelines, typically by sedation with 5% Isoflurane, followed by asphyxiation and physical confirmation of death by cervical dislocation.

(B) Shave fur from tumor sites and peripheral area.

(C) Harvest tumors using scissors and forceps.

(D) Place tumors in cold PBS.

(E) Place tumors in 10cm dishes on ice.

Figure 4. *Continued*
(F) Cut tumors into strips.
(G) Cut strips into 1–2 mm^2 squares.
(H) Place tumors squares into a 6-well plate and add 5 mL digestion media.
(I) Place plate containing tumor squares on a rocker in a cold room and incubate for 16–20 h.
(J) Place tumors and digestion media through a 70μM cell strainer.
(K) A 3 mL or 5 mL syringe plunger is used to mash the tumor on the strainer and wash the strainer twice with DMEM containing 5% FBS.
(L) Filter the tumor and media through a 50μM cell strainer, wash, and count cells with Trypan Blue and a hemocytometer.

Note: The tumour bearing skin will typically separate from the body easily. If not, place the scissors under the tumour and open the scissors repeatedly to separate the tumour from the body by removing the connective tissue

4. Harvest the tumor bearing skin and weigh it
5. Place the tumor in a 50 mL conical tube containing FACS buffer (Figure 4D) (Table 1)
6. Place tube on ice and work on the next mouse
7. Place tumor on a 100 mm dish on ice (Figure 4E)
8. Using a single-use blade, cut the tumor into small strips (Figure 4F)
9. From the strips, cut the tumor into approximately 1–2 mm^2 squares (Figure 4G)

⚠ CRITICAL: We have tried mincing the tissues with scissors to form a paste but determined that this results in a great reduction in cell viability. Instead, we established that cutting tissues into strips and squares yields a cell preparation with significantly increased viability.

10. Place the cut tumor in a 6-well plate with 5 mL tumor digestion media (Figure 4H) (Tables 2 and 3)
11. Place the 6-well plate on a rocker in a cold room for 16–20 h (Figure 4I)
12. The following morning transfer the contents of each well to an individual 50 mL conical tube overlaid with a 70 μM cell strainer (Figure 4J)
13. Add 2 mL DMEM with 10% FBS to the wells to collect any remaining single cells and transfer to the same cell strainer (Table 2)
14. Use a 5 mL syringe plunger to further crush the tissue pieces on the 70 μM strainer, 3 times (Figure 4K)
15. Rinse the cell strainer with 5 mL DMEM plus 10% FBS between each tissue crush
16. Place the 50 mL conical tube in an ice box while working on the next samples
17. Once all samples have been processed as in steps 12–15, place the 50 mL tubes in a centrifuge and spin at 450 × *g* for 5 min at 4°C
18. Decant the supernatant and resuspend the pellet with 5 mL DMEM with 10% FBS
19. Transfer the resuspended contents to a 15 mL conical tube containing a 50 μM cell strainer (Figure 4L)
20. Place the tubes in a centrifuge and spin at 450 × *g* for 5 min at 4°C
21. Decant the supernatant and resuspend the pellet with 1 mL DMEM with 10% FBS
22. Count cells using a hemocytometer and trypan blue

⚠ CRITICAL: We have observed cell viability between 60%–70% with our protocol. Having said that, we may occasionally observe viability between 40%–50% and we still acquired data from those samples. However, we do not recommend staining samples with

Table 1. Fluorescence-Activated Cell Sorting (FACS) Buffer Recipe

Reagent	Final concentration	Amount
1× Dulbecco's Phosphate-Buffered Saline (DPBS)	N/A	475 mL
Fetal Bovine Serum (FBS)	5%	25 mL
Total	**n/a**	**500 mL**

Store at 4°C for up to 1 month.

Table 2. Dulbecco's Modified Eagle Media (DMEM) with 10% FBS Recipe		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Media (DMEM)	N/A	450 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Total	**N/A**	**500 mL**

Store at 4°C for up to 1 month.

viabilities lower than 40% viability, as we have experienced difficulties identifying rare cell populations such as ILC2s.

Characterization of ILC2 cells over the time course of melanoma progression

Timing: 5 h

After acquiring the single cells from the digested tumor, ILC2s are characterized by flow cytometry using specific markers to the receptors and transcription factors that they express such as CD45, ST2/IL-33R, CD127/IL-7Rα, and GATA3. In addition, ILC2s do not express lineage-specific markers such as TCRβ, TCRγ/δ, Gr-1 (myeloid differentiation marker), NK1.1 (marker of NK cells), FcεR1 (high affinity IgE receptor) and B220/CD45R (marker of B cells), and thus these are useful to discriminate between lineage-positive and lineage-negative populations. To effectively distinguish the various populations, Fluorescence Minus One (FMO) controls, isotype controls and an unstained control are used for proper gating. The titrations listed below are optimized for our institute's flow cytometer (BD LSRFortessa) and further titration may be required for your institute's flow cytometer.

Note: 1- Titration of antibodies will be required to determine optimal antibody dilutions for your institute's flow cytometer. 2- *NK1.1 is used only when immune phenotyping tissue from C57BL/6 mice, while CD49b/DX5 is used for BALB/c mice.

23. Prepare antibody lineage cocktail in FACS buffer
24. Add ILC2 extracellular surface antibodies to the cocktail
25. Prepare the GATA3 antibody dilution in FACS buffer in a separate tube for intracellular staining
26. Aliquot 2–4 million cells per sample per well in a 96-well plate for staining with the ILC2 extracellular surface antibody cocktail

Note: Remaining cells from samples once allocated for ILC2 staining can be used for FMO and unstained controls

Note: 2 sets of cells per sample are required for test and isotype controls

27. Wash cells with cold 1× DPBS to remove DMEM at 450 g at 4°C for 5 min.
28. Decant the supernatant in a quick downward motion
29. In the dark, add 50 μL of 1/2000 viability dye to each well, except to the unstained control well.
30. Incubate for 30 min on ice in the dark

Table 3. Tissue Digestion Media Recipe		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Media (DMEM) + 10% FBS	N/A	5 mL
50 mg/mL Collagenase P	0.5 mg/mL	50 μL
1 mg/mL Deoxyribonuclease I (DNase I)	10 μg/mL	50 μL
Total	**N/A**	**5.1 mL**

Store at 4°C for up to 1 month

31. Add 100 μL cold 1× DPBS to each well
32. Centrifuge at 450 × *g* for 5 min at 4°C
33. Decant the supernatant in a quick downward motion
34. Add 10 μL of Fc receptor blocking reagent to each well
35. Incubate for 30 min on ice in the dark
36. Without washing the plate, add 25 μL of the ILC2 antibody cocktail directly to the wells
37. Incubate for 30 min on ice in the dark
38. Add 100 μL cold FACS buffer to each well
39. Centrifuge at 450 × *g* for 5 min at 4°C
40. Decant the supernatant in a quick downward motion
41. Add 100 μL of FOXP3 Fixation/Permeabilization solution (1:4 dilution with Fixation/Permeabilization Diluent) to each well
42. Incubate for 30 min on ice in the dark
43. Add 100 μL cold 1× permeabilization buffer to each well
44. Centrifuge at 600 × *g* for 5 min at 4°C
45. Decant the supernatant in a quick downward motion
46. Add 10 μL of Fc receptor blocking reagent to each well
47. Incubate for 30 min on ice in the dark
48. Without washing the plate, add 50 μL of the diluted GATA3 antibody directly to the wells
49. Incubate for 30 min at room temperature (20°C–25°C) in the dark
50. Add 150 μL cold 1× permeabilization buffer to each well
51. Centrifuge at 600 × *g* for 5 min at 4°C
52. Decant the supernatant in a quick downward motion
53. Resuspend in 150 μL cold 1× permeabilization buffer to each well
54. Incubate for 5 min at room temperature (20°C–25°C) in the dark
55. Centrifuge at 600 × *g* for 5 min at 4°C
56. Decant the supernatant in a quick downward motion
57. Resuspend in 200 μL cold 1× FACS buffer to each well
58. Wrap plate in aluminum foil to protect from light and keep at 4°C

⚠ CRITICAL: We optimized our staining panel according to the available fluorophores. If your lab or institute is in a region where it may be difficult to purchase the indicated markers with the above-mentioned fluorophores, we recommend using different fluorophores and adjust and optimize accordingly.

⚠ CRITICAL: If your institute's flow cytometer has an UV laser, we recommend using BUV-conjugated antibodies (e.g.,: Mouse Anti-GATA3 BUV395, BD #565448) and titrate accordingly.

⚠ CRITICAL: For endpoint tumors (Day 40), we typically harvest at least 20 million cells. While 2–4 million cells are recommended for the staining, for the Day 14 immune phenotyped samples, no measurable tumor has yet formed. Thus, it is primarily pigmented skin and low cell numbers are recovered. We have added 200,000 cells for our staining and have acquired between 100,000 and 150,000 events via FACSDiva. The end-users of this protocol can pool the skin samples should they wish to stain an increased number of cells.

Characterization of T cells and myeloid cells over the time course of melanoma progression

Timing: 5 h

After acquiring the single cells from the digested tumor, T-cells and myeloid cells are characterized by flow cytometry using specific markers to the receptors and transcription factors that they express, such as CD45, CD3ϵ, CD11b, CD11c, F4/80 and FOXP3. In this panel we identify: $CD3^+$ cells, $CD4^+$

Table 4. List of antibodies used to characterize ILC2s

Antibody	Clone	Fluorochrome	Dilution	Company and Catalog #
ILC2-Specific Markers				
GATA3	L50-823	Brilliant Violet 711	1/100	BD #565449
IgG1, κ Isotype Control	X40	Brilliant Violet 711	1/100	BD #563044
ST2	RMST2-2	PerCP-eFluor710	1/50	eBioscience #46-9335
IgG2, κ Isotype Control	eBR2a	PerCP-eFluor710	1/50	eBioscience #46-4321-80
CD127	A7R34	Brilliant Violet 650	1/200	BioLegend #135043
IgG2α, κ Isotype Control	RTK2758	Brilliant Violet 650	1/200	BioLegend # 400542
KLRG1	2F1	Alexa Fluor 488	1/50	BD #561619
CD45.2	30-F11	Brilliant Violet 785	1/2000	BioLegend #103149
ICOS	C398.4A	APC	1/50	eBioscience #17-9949-82
Thy 1 (CD90.2)	53-2.1	Brilliant Violet 510	1/400	BioLegend #140319
CD25	PC61	PE-Cy7	1/100	BD #561780
Viability Dye	-	Near IR	1/2000	Invitrogen #L10119
Lineage Markers				
FcεR1	MAR-1	PE	1/50	eBioscience #12-5898-83
CD19	eBioID3	PE	1/100	eBioscience #12-0193-82
CD3ε	145-2C11	PE	1/400	eBioscience #12-0031-85
B220	RA3-6B2	PE	1/200	eBioscience #12-0452-83
TCRβ	H57-597	PE	1/200	eBioscience #12-5961-83
TCRγ/δ	EBioGL3	PE	1/400	eBioscience #12-5711-82
NK1.1*	PK136	PE	1/50	eBioscience #12-5941-82
Gr-1	RB6-8C5	eFluor450	1/500	eBioscience #48-5931-82
TER-119	TER-119	eFluor450	1/50	eBioscience #48-5921-82
CD11c	N418	eFluor450	1/50	eBioscience #48-0114-82
CD11b	M1/70	eFluor450	1/500	eBioscience #48-0112-82

Lineage markers are included as these provide a lineage-negative population to discriminate ILC2s.

T-cells, $CD8^+$ T-cells, regulatory T-cells (Tregs), monocytic-myeloid-derived suppressor cells (M-MDSCs) and granulocytic-MDSCs (G-MDSCs), non-lymphoid tissue dendritic cells, myeloid dendritic cells, and macrophages. To effectively distinguish the various populations, Fluorescence Minus One (FMO) controls, isotype controls and an unstained control are used for proper gating. This protocol applies to C57BL/6 mice, but as already specified in Major Step 2, it can be applied to BALB/c mice with one modification: instead of using the NK1.1 antibody, the CD49b/DX5 antibody is instead used for staining immune cells from BALB/c mice (Table 4). The titrations listed below are optimized for our institute's flow cytometer (BD LSRFortessa) and further titration may be required for your institute's flow cytometer.

⚠ CRITICAL: We optimized our staining panel according to the available fluorophores. If your lab or institute is in a region where it may be difficult to purchase those markers with the above-mentioned fluorophores, we recommend using different fluorophores and adjust and optimize accordingly.

⚠ CRITICAL: If your institute's flow cytometer has an UV laser, we recommend using BUV-conjugated antibodies (e.g.,: CD45 BUV395, BD #264279) and titrate accordingly.

59. Prepare extracellular antibody cocktail (Excluding FOXP3 and Arginase-1) in FACS buffer
60. Prepare FOXP3 and Arginase-1 antibody dilutions in FACS buffer in a separate tube for intracellular staining
61. Aliquot 2–4 million cells per sample per well in a 96-well plate

Note: Remaining cells from samples once allocated for staining can be used for FMO and unstained controls

Table 5. List of antibodies used to characterize T-cells and Myeloid Cells

Antibody	Clone	Fluorochrome	Dilution	Company and Catalog #
CD45.2	30-F11	APC-Cy7	1/800	BD #561037
CD3ϵ	145-2C11	Brilliant Violet 650	1/12.5	BD #563044
CD8α	53-6.7	PerCP-Cy5.5	1/50	BD #561109
CD4	GK1.5	PE-Cy7	1/100	BioLegend #100-422
CD11b	M1/70	eFluor450	1/2000	eBioscience #48-0112-82
CD11c	HL3	Brilliant Violet 786	1/500	BD # 563735
F4/80	BM8	PE	1/100	eBioscience #12-4801-80
Ly-6G/Ly-6C	RB6-8C5	Alexa Fluor 488	1/2000	eBioscience #53-5931-82
Ly-6C	AL-21	APC	3/400	BD #560595
FOXP3	FJK-16s	PE-eFluor610	1/100	eBioscience #61-5773
Arginase-1	A1exF5	Alexa Fluor 700	1/100	eBioscience #56-3697
CD206	C068C2	Brilliant Violet 711	1/200	BioLegend #141727
Viability Dye	-	Aqua	1/250	Invitrogen #L34965

(***Note:*** Titration of antibodies required to determine an optimal dilution for your institute's flow cytometer)

Note: 2 sets of cells per sample are required for test and isotype controls

62. Wash cells with cold 1× DPBS to remove DMEM at 450 g at 4°C for 5 min.
63. Decant the supernatant in a quick downward motion
64. In the dark, add 50 µL of 1/250 viability dye to each well, except to the unstained control well.
65. Incubate for 30 min on ice in the dark
66. Add 100 µL cold 1× DPBS to each well
67. Centrifuge at 450 × *g* for 5 min at 4°C
68. Decant the supernatant in a quick downward motion
69. Add 10 µL of Fc receptor blocking reagent to each well
70. Incubate for 30 min on ice in the dark
71. Without washing the plate, add 25 µL of the antibody cocktail directly to the wells
72. Incubate for 30 min on ice in the dark
73. Add 100 µL cold FACS buffer to each well
74. Centrifuge at 450 × *g* for 5 min at 4°C
75. Decant the supernatant in a quick downward motion
76. Add 100 µL of FOXP3 Fixation/Permeabilization solution (1:4 dilution with Fixation/Permeabilization Diluent) to each well
77. Incubate for 30 min on ice in the dark
78. Add 100 µL cold 1× permeabilization buffer to each well
79. Centrifuge at 600 × *g* for 5 min at 4°C
80. Decant the supernatant in a quick downward motion
81. Add 10 µL of Fc receptor blocking reagent to each well
82. Incubate for 30 min on ice in the dark
83. Without washing the plate, add 50 µL of the diluted FOXP3 and Arginase-1 antibody directly to the wells
84. Incubate for 30 min at room temperature (20°C–25°C) in the dark
85. Add 150 µL cold 1× permeabilization buffer to each well
86. Centrifuge at 600 × *g* for 5 min at 4°C
87. Decant the supernatant in a quick downward motion
88. Resuspend in 150 µL cold 1× permeabilization buffer to each well
89. Incubate for 5 min at room temperature (20°C–25°C) in the dark
90. Centrifuge at 600 × *g* for 5 min at 4°C
91. Decant the supernatant in a quick downward motion
92. Resuspend in 200 µL cold 1× FACS buffer to each well
93. Wrap plate in aluminum foil and keep at 4°C

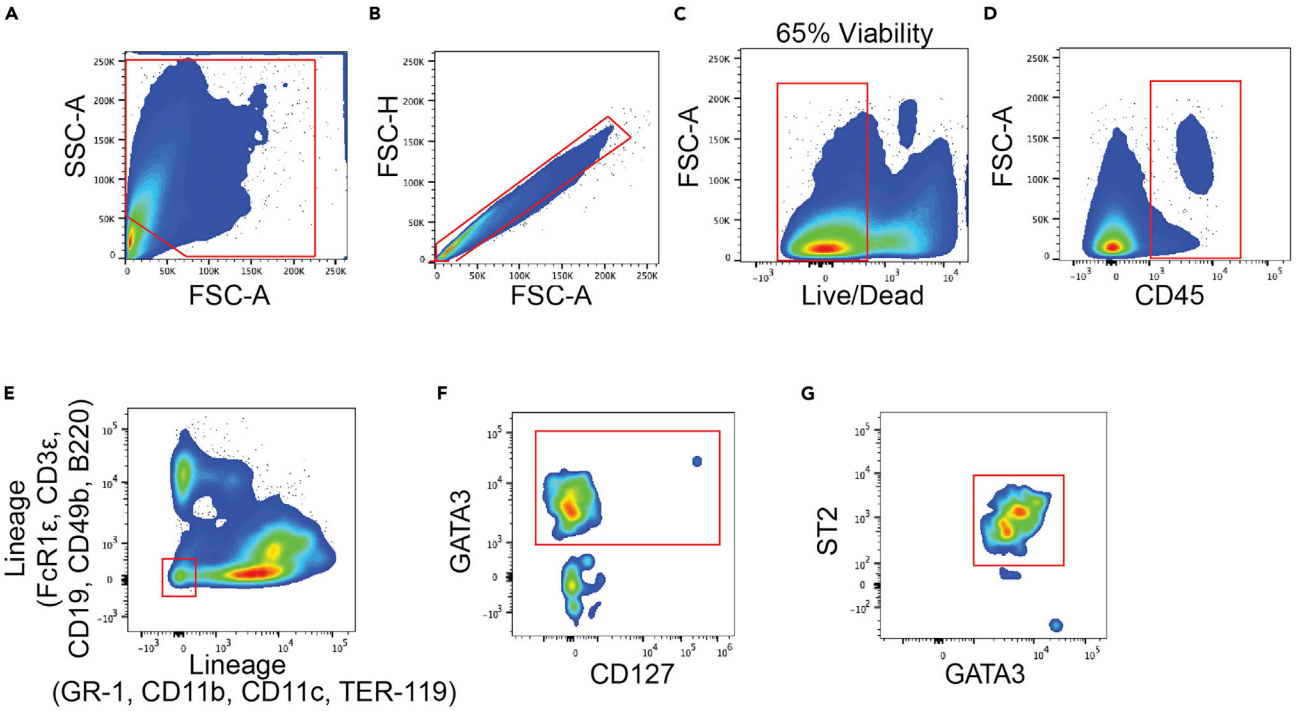

Figure 5. ILC2 gating strategy

(A) Forward and sideward scatter gating to exclude debris.
(B) Singlet cell gating.
(C) Live cell gating.
(D) $CD45^+$ cell gating.
(E) Lineage-negative cell population gating.
(F) $GATA3^+$ cell population gating.
(G) ILC2 were characterized as $GATA3^+ST2^+$ cell population.

Pause point: At this step, the fixed and stained cells can be stored at 4°C for a week prior to moving onto the acquisition of samples by flow cytometry. However, it is preferable to acquire within 24–48 h.

Flow cytometry acquisition

Timing: 2 h (For 5 samples)

This step details the acquisition of our samples using the BD LSRFortessa flow cytometer from our institution. You might have to modify this step to accommodate to your institute's instruments and guidelines.

94. As the samples are fixed, acquisition of the samples can be acquired within 2 days.
95. Take the plate out of the fridge and transfer the samples to individual 5 mL FACS tubes.

Note: If your instrument has a plate reader attached, you may wish to use it and follow your institute's core facility's guidelines.

96. Prepare compensation tubes with each fluorochrome listed above.

Note: For violet laser-excited fluorochromes e.g.,: eFluor450, BV711, BV510, UltraComp compensation beads are recommended due to background autofluorescence. OneComp

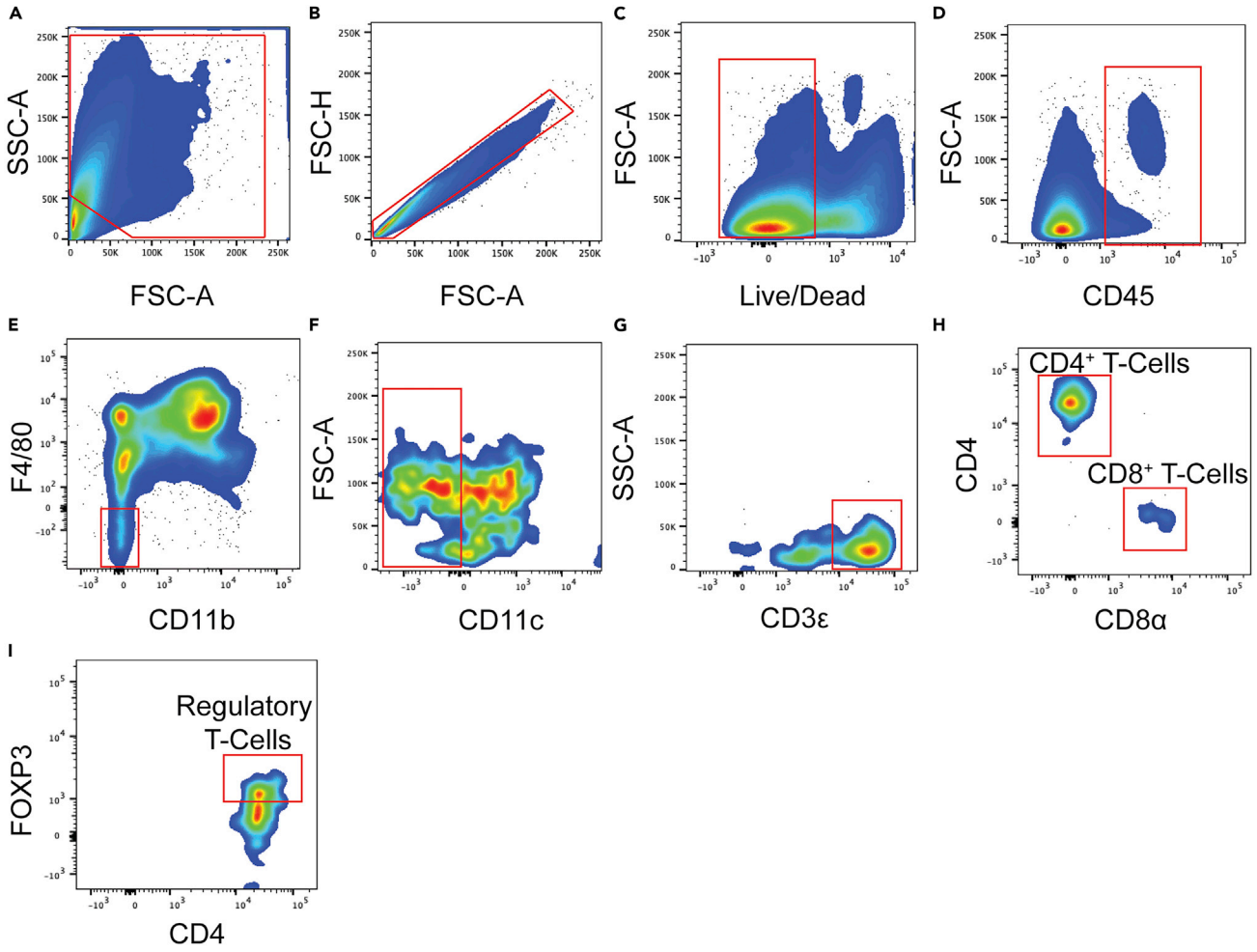

Figure 6. Regulatory T-cells (Tregs) gating strategy

(A) Forward and sideward scatter gating to exclude debris.
(B) Singlet cell gating.
(C) Live cell gating.
(D) $CD45^+$ cell gating.
(E) CD11b and F4/80 double-negative gating.
(F) CD11c negative gating.
(G) $CD3\epsilon^+$ T cell gating.
(H) $CD4^+$ T cell and $CD8^+$ T cell gating.
(I) $CD4^+FOXP3^+$ gating to identify Tregs

compensation beads are typically used for blue, red, yellow-green and UV laser-excited fluorochromes.

97. Perform the acquisition using the BD LSRFortessa

Note: You will require an instrument in your institution's core facility that can accommodate up to a 15-color panel e.g., BD LSRFortessa, BD LSRII.

98. Ensure there is enough sheath fluid in the tank and that the waste tank is not full.
99. Using BD FACSDiva (or the available acquisition software in your institute's core facility) create a new data acquisition matrix

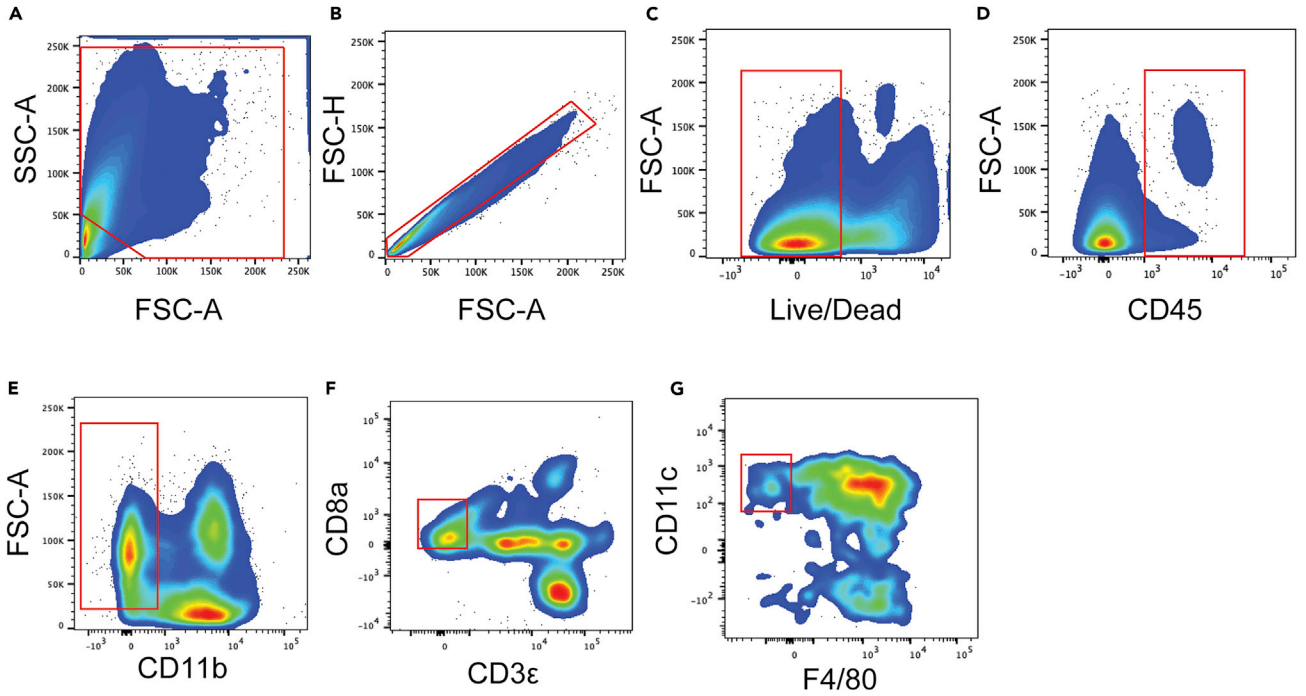

Figure 7. Non-lymphoid tissue dendritic cells (NLT DCs) gating strategy

(A) Forward and sideward scatter gating to exclude debris.

(B) Singlet cell gating.

(C) Live cell gating.

(D) $CD45^{+}$ cell gating.

(E) CD11b negative gating.

(F) CD3ϵ and CD8ϵ double negative gating.

(G) $F4/80^{Low}$, $CD11c^{Mid}$ gating to identify NLT DCs.

100. Ensure that all the forward scatter and side scatter parameters are selected (e.g., Area (A), Height (H), and Width (W))
101. Assign each channel to your fluorochromes and delete the rest
102. Prime the instrument twice to remove any debris that may block and clog the instrument during acquisition
103. Perform (Cytometer Setup and Tracking) CST if your institute core facility requires the first user of the day to do so
104. Use your unstained control to set the voltages of your FSC-A, SSC-A and fluorochrome by right-clicking on Cytometer Settings → Application Settings → Create Worksheet, which creates a new global worksheet
105. In the SSC-A and FSC-A plot, draw a gate around the lymphocyte population. In the other plots showing the fluorochromes, make sure the negative population is on the "+" sign
106. Perform compensation using your compensation tubes and set an interval gate for the negative population peak and move your P2 gate on the positive population peak
107. Calculate compensation.

Note: In BD FACSDiva it is Instrument → Instrument Setup → Calculate Compensation. Click "Link & Save".

108. Click on the little sheet icon to get back to the analysis schematic and set up your gating strategy
109. Acquire all samples, including FMO and unstained controls and ensure that the threshold rate is at 10,000 events/second

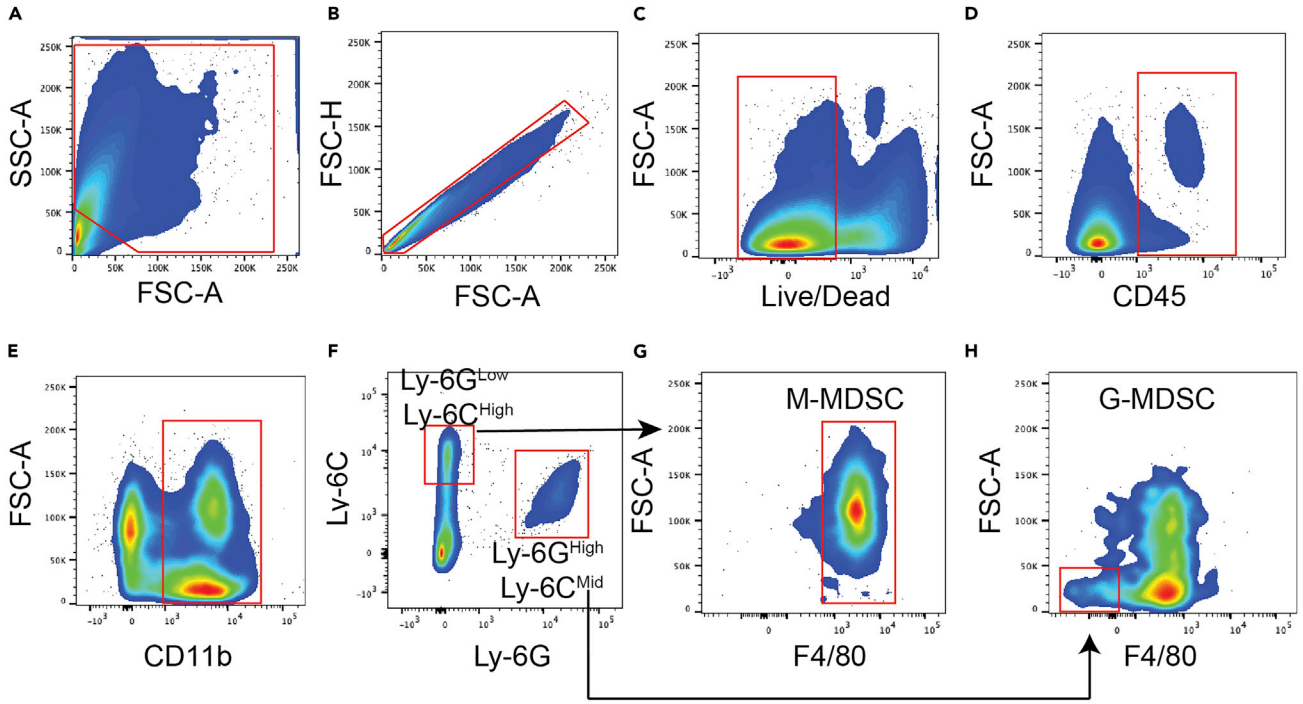

Figure 8. Myeloid-derived suppressor cells (MDSC) gating strategy

(A) Forward and sideward scatter gating to exclude debris.

(B) Singlet cell gating.

(C) Live cell gating.

(D) CD45^{+} cell gating.

(E) CD11b positive gating.

(F) Ly-6G^{Low} and Ly-6C^{High} gating for M-MDSCs and Ly-6G^{High} and Ly-6C^{Mid} for G-MDSCs.

(G) F4/80^{High} gating to identify M-MDSCs.

(H) F4/80^{Low} gating to identify G-MDSCs

110. Once acquisition is completed, export your data according to your institute's core facility's guidelines.

Note: Ensure that it is FCS3.0 to allow analysis on the BD FlowJo program.

111. Perform cleaning and shutdown (if last person of the day) procedures according to your institute's core facility guidelines.

⚠ CRITICAL: As ILC2s are a rare cell population, we acquire all the stained cells in the tube and do not set a pre-determined number of events to acquire. We then normalize to CD45+ cells, or as an absolute number of cells from the total cell count, tissue weight, and total cells acquired via FACSDiva.

⚠ CRITICAL: Depending on the timepoint following 4-HT induction of melanomagenesis, we have a variable range of ILC2s acquired. For Day 7, our range is approximately 10,000–40,000 ILC2s per 1 million CD45+ cells, while at Day 14 it is approximately 3,000–60,000 ILC2s per 1 million CD45+ cells, and at Day 40 it is from 4,000–15,000 ILC2s per 1 million CD45+ cells.

EXPECTED OUTCOMES

From the digestion of the late-stage endpoint tumors, the number of cells will range from 8 million to 10 million cells. However, when working with skin samples from Day 7 and 14 mice (following the last

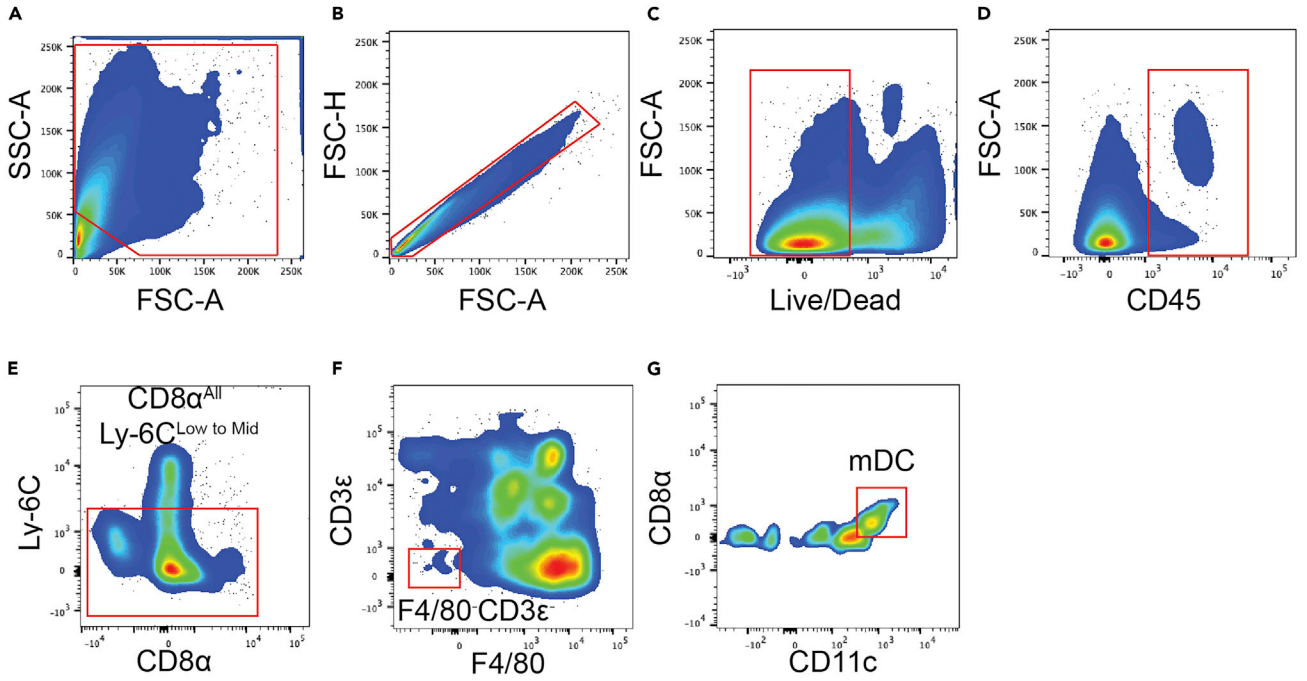

Figure 9. Monocytic tissue dendritic cells (mDCs) gating strategy

(A) Forward and sideward scatter gating to exclude debris.

(B) Singlet cell gating.

(C) Live cell gating.

(D) $CD45^{+}$ cell gating.

(E) $CD8\alpha^{All}$ and Ly-$6C^{Low}$ gating.

(F) $F4/80^{-}CD3\epsilon^{-}$ for mDCs.

(G) $CD8\alpha^{Low}$ and $CD11c^{Mid\ to\ High}$ to identify mDCs

day of 4-HT treatment), we typically count less than 2 million cells. We have included the gating strategies for ILC2s, T-Cells, dendritic cells, MDSCs, and macrophages (Figures 5, 6, 7, 8, 9, and 10 respectively). We have also included the levels of ILC2s, which is a rare population at 3 different timepoints during the course of melanoma development (Days 7, 14 and 40) (Figure 11).

LIMITATIONS

A limitation of the $BRAF^{V600E}/PTEN^{null/null}$ melanoma mouse model is the development of spontaneous tumors in approximately 10%–15% of male mice per cohort in various anatomical locations. $BRAF^{V600E}/PTEN^{null/null}$ female mice are not used for this protocol, as approximately 40% per cohort will develop spontaneous tumors. While the cell viability of digested late-stage endpoint tumors (i.e., Day 40 following the last day of 4-HT treatment) remained consistently high, one may encounter issues with viability when working with Day 24 tumors. While this protocol shows the markers used to identify skin and tumor ILC2s, care must be taken when attempting to identify ILC2s in the lung, gut, or other parts of the body. While ILC2s express the above-mentioned markers, ILC2s at various sites express different levels of markers (Ricardo-Gonzalez et al., 2018, Meininger et al., 2020, Simoni et al., 2017). Having said that, using a "base" of lineage negative, CD127 and GATA3 to identify total ILC2s can be used and then the other markers can be gated. An alternative is to use Mean Fluorescence Intensity to determine the levels of activation and/or inhibition markers at different timepoints.

TROUBLESHOOTING

Problem 1

Pigmented lesions and tumors not formed after 4-Hydroxytamoxifen treatment.

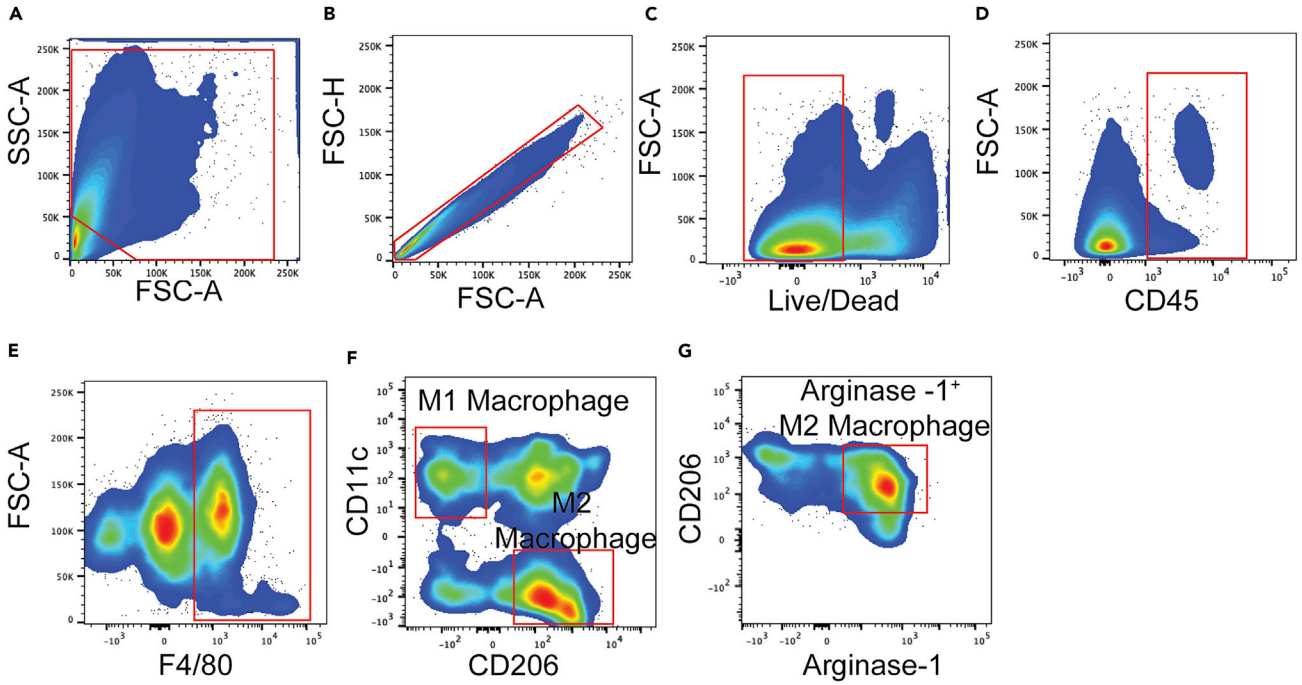

Figure 10. Macrophage gating strategy

(A) Forward and sideward scatter gating to exclude debris.

(B) Singlet cell gating.

(C) Live cell gating.

(D) $CD45^{+}$ cell gating.

(E) $F4/80^{High}$ population gating.

(F) $CD11c^{High}$ and $CD206^{Low}$ gating for M1 Macrophages, and $CD11c^{Low}$ and $CD206^{Mid\ to\ High}$ gating for M2 Macrophages.

(G) M2 Macrophages express high levels of Arginase-1.

Potential solution 1

Backcrossing of mice with previous generations may be warranted due to genetic drift.

Re-genotype mice to ensure the presence of *Tyr::CreER* transgene, remove the breeder mice immediately if pubs were detected negative for *Tyr::CreER*. In addition, all mice used in this study were tested $PTEN^{flox/flox}$ and $BRAF^{CA/+}$. Primer sequences can be found at (Huang et al., 2021).

Problem 2

Low cell count after tumor digestion and single cell isolation.

Potential solution 2

This may be possible especially with early-timepoint tissues such as non-pigmented skin and pigmented skin. Pooling of cells may be warranted.

Problem 3

Low viability after tumor digestion and single cell isolation.

Potential solution 3

Reduce incubation in digestion media for 8–12 h.

Problem 4

No positive staining of markers by flow cytometry.

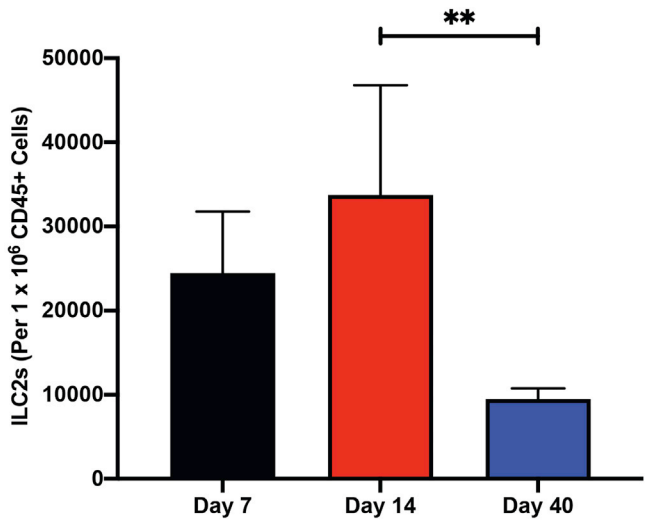

Figure 11. Detection of ILC2s at 3 different timepoints during melanoma progression (mouse model shown in Figure 2)

ILC2s reduce in number in melanomas characterized on Day 40, compared to Days 7 and 14. One-way ANOVA. All values are represented as mean ± SD. $^{**}P \leq 0.01$.

Potential solution 4

Repeat compensation with new set of single-stained, and unstained controls and adjust PMT voltages.

You may need to use a different fluorophore that might work on your institute's flow cytometer.

Re-titration of antibodies may be warranted to suit your institute's flow cytometer.

Problem 5

Intracellular stain cannot be detected.

Potential solution 5

Increase eBioscience FOXP3 Fixation/Permeabilization solution incubation to 1 h and at room temperature instead.

It is possible to use the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Catalog #554714) and to follow the manufacturer's instructions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sonia del Rincon PhD, soniavictoria.delrincon@mcgill.ca

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

J.H.F. and S.V.d.R. designed the study and edited the manuscript. S.S.K. developed the staining panels. S.S.K. and C.G. developed the skin digestion protocol. N.G., F.H., and D.P. maintained the mouse colony and breeding. S.S.K. performed the sample collection, processing, and staining and flow cytometry analysis, drafted the manuscript, and designed the figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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