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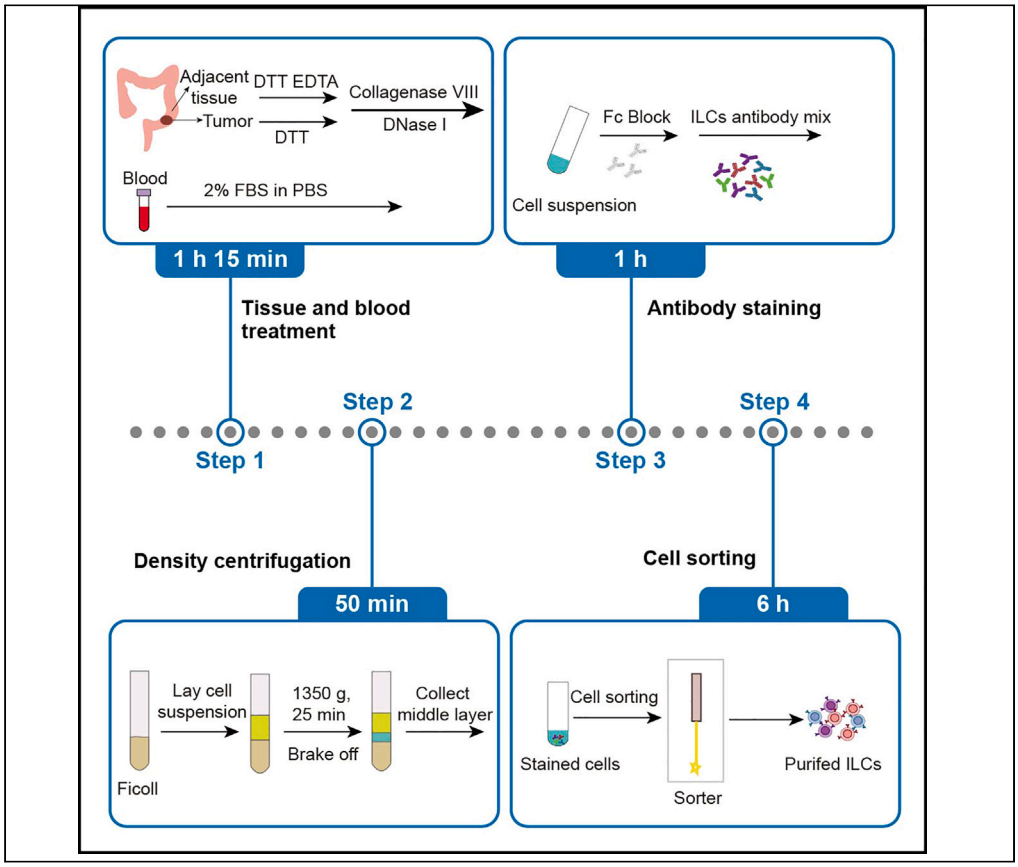
Analysis and purification of innate lymphoid cells in human intestine and blood by flow cytometry



The role of innate lymphoid cells (ILCs)—including natural killer cells, helper-like ILC1s, ILC2s, ILC3s and lymphoid tissue inducers—in human cancer is still poorly understood due to the scarcity of cell number. To address this, we present a protocol to analyze or purify ILCs from human blood, adjacent intestine, and colorectal tumor tissue. We describe steps for tissue and blood treatment, density centrifugation, antibody staining and cell sorting.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Tissue preparation of human intestine and tumor

Enrichment of lymphoid cells by density centrifugation

Gating strategy and sorting of human innate lymphoid cells

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Analysis and purification of innate lymphoid cells in human intestine and blood by flow cytometry

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SUMMARY

The role of innate lymphoid cells (ILCs)—including natural killer cells, helper-like ILC1s, ILC2s, ILC3s, and lymphoid tissue inducers—in human cancer is still poorly understood due to the scarcity of cell number. To address this, we present a protocol to analyze or purify ILCs from human blood, adjacent intestine, and colorectal tumor tissue. We describe steps for tissue and blood treatment, density centrifugation, antibody staining, and cell sorting.

For complete details on the use and execution of this protocol, please refer to Qi et al. (2021).[1]

BEFORE YOU BEGIN

Institutional permissions

Clinical samples were collected from colorectal cancer patients after informed consent. Approval had been obtained by the local ethnics committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Preparation of wash buffer

Timing: 15 min

1. Wash buffer 1 is also called intra-epithelial lymphocyte (IEL) buffer.[2] 10 or 20 mL of Wash buffer 1 (depending on tissue size) is used to isolate the intra-epithelial layer. Usually, 10 mL for tissue of weight less than 0.5 g is used, 20 mL for larger tissue.

Wash buffer 1 (Stock solution)	Final concentration	Amount
HEPES (1 M)	15 mM	300 μL
EDTA (0.5 M)	5 mM	200 μL
DTT (2 M)	1 mM	10 μL
FBS	10%	2 mL
PBS		17.49 mL
Total		**20 mL**

2. Wash buffer 2 should be made for tumor tissue as follows. The volume of each tumor tissue is 10–20 mL depending on tissue size.

Wash buffer 2 (Stock solution)	Final concentration	Amount
DTT (2 M)	6.5 mM	65 μL
FBS	10%	2 mL
PBS		17.935 mL
Total		**20 mL**

⚠ CRITICAL: The Wash buffer should be freshly prepared before application, and PBS should be prewarmed at 37°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Fc receptor blocking solution (1:50 dilution)	BioLegend	Cat#422302; RRID: AB_2818986
Anti-human TCR γδ antibody, clone B1, FITC (1:100 dilution)	BD Biosciences	Cat# 559878; RRID: AB_397353
Anti-human TCR αβ antibody, clone T10B9.1A-31, FITC (1:100 dilution)	BD Biosciences	Cat# 555547; RRID: AB_395931
Anti-human CD3 antibody, clone UCHT1, FITC (1:100 dilution)	BD Biosciences	Cat# 555916; RRID: AB_396217
Anti-human CD19 antibody, clone HIB19, FITC (1:100 dilution)	BD Biosciences	Cat# 555412; RRID: AB_395812
Anti-human CD14 antibody, clone M5E2, FITC (1:100 dilution)	BD Biosciences	Cat# 555397; RRID: AB_395798
Anti-human CD16 antibody, clone 3G8, FITC (1:100 dilution)	BD Biosciences	Cat# 555406; RRID: AB_395806
Anti-human CD94 antibody, clone HP-3D9, FITC (1:100 dilution)	BD Biosciences	Cat# 555888; RRID: AB_396200
Anti-human CD123 antibody, clone 7G3, FITC (1:100 dilution)	BD Biosciences	Cat# 558663; RRID: AB_1645485
Anti-human CD34 antibody, clone 581, FITC (1:100 dilution)	BD Biosciences	Cat# 555821; RRID: AB_396150
Anti-human CD303 (BDCA-2) antibody, clone AC144, FITC (1:100 dilution)	Miltenyi Biotec	Cat# 130-090-510; RRID: AB_244167
Anti-human FceR1 alpha antibody, clone (AER-37 (CRA1)), FITC (1:100 dilution)	Thermo Fisher Scientific	Cat# 11-5899-42; RRID: AB_10732835
Anti-human CD127 antibody, clone eBioRDR5, PE (1:100 dilution)	Thermo Fisher Scientific	Cat# 12-1278-42; RRID: AB_10717663
Anti-human CD45 antibody, clone 2D, Alexa Fluor 700 (1:100 dilution)	Thermo Fisher Scientific	Cat# 56-9459-42; RRID: AB_2574511
Anti-human CD117 (c-kit) antibody, clone 104D2, BV421 (1:100 dilution)	BioLegend	Cat# 313216; RRID: AB_11148721
Anti-human CD294 (CRTH2) antibody, clone BM16, PE-CF594 (1:100 dilution)	BD Biosciences	Cat# 563501; RRID: AB_2738244
Chemicals, peptides, and recombinant proteins		
Collagenase type VIII	Sigma-Aldrich	Cat#C2139
DNase I	Sigma-Aldrich	Cat#DN25
DL-Dithiothreitol	Sigma-Aldrich	Cat#D9779
Lymphoprep	STEMCELL	Cat#07851
Fetal bovine serum	Thermo Fisher Scientific	Cat#10437028
Penicillin-Streptomycin	GIBCO	Cat#5140122
RPMI 1640	Corning	Cat#10-041-CV
Phosphate buffered saline	Corning	Cat#21-040-CMR
Normal mouse serum	Jackson ImmunoResearch Laboratories	Cat#015-000-120
Brilliant stain buffer	BD Horizon	Cat#563794
EDTA	Invitrogen	Cat#AM9260G
Software and algorithms		
FlowJo version 10	Treestar	https://www.flowjo.com/; RRID: SCR_008520

MATERIALS AND EQUIPMENT

Collagenase VIII stock

Dissolve 1 g of Collagenase VIII in serum-free RPMI 1640 medium to make a stock of 333 mg/mL within 15 min. Aliquot in 50 μL per vial, and store at −80°C.

DNase I

Dissolve the DNase I in 10% glycerol to make a stock solution of 150 mg/mL. Aliquot in 100 μL per vial, and store at −80°C.

Complete RPMI 1640 medium

Complete RPMI 1640 medium is prepared using 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin.

Flow buffer

Flow buffer is prepared by mixing 2 mL of 0.5 M EDTA stock (2 mM) and 10 mL FBS, and adding PBS to adjust the volume to 500 mL. Flow buffer should be prepared in advance and store at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

Tissue pretreatment

Timing: 1 h 15 min

This step details how to wash the samples, remove the epithelial cell layer in adjacent tissue, and tumor.

1. Collect and record basic information of the tissue, e.g., weight, length.
2. Remove visible blood vessels and adipose tissue from the samples with forceps.
3. Wash each tissue with 10 mL ice-cold PBS in 10 cm cell-culture dish.
4. Cut the adjacent tissue into small pieces about 0.3 cm^2 in area by scissors.
5. Put the adjacent tissue into 50 mL tube containing 20 mL Wash buffer 1 and shake at 200 rpm for 1 h at 37°C.
6. Cut the tumor tissue into big pieces (0.2 cm in length), wash in 50 mL tube containing 20 mL Wash buffer 2, and shake at 200 rpm for 15 min at 37°C.
7. After the washing step, shake normal tissues powerfully (4–5 cycles per second) for 5 min to dissociate the epithelial fraction, while shake the tube with tumor tissue gently (1 cycle per second) for 5 min.
8. Discard the supernatant directly and wash the tissue twice with 10 mL of PBS at about 25°C.

Tissue digestion

Timing: 1 h 30 min

This step details how to digest the adjacent gut tissue and CRC tumor into single cell suspensions.

9. Preparation of digestion buffer. Digestion buffer should be prepared before digestion using pre-warmed complete 1640 medium.

Digestion buffer	Final concentration	Amount
DNase I	0.1 mg/mL	26.8 μL
Collagenase Type VIII	0.38 mg/mL	45.7 μL
Complete 1640 medium		39.92 mL
Total		**40 mL**

10. After washing, dry the tissue with dust-free paper, and place into 1.5 mL Eppendorf tube.
11. Cut tissues into very small pieces (about 1 mm in length) with scissors and transferred into 50 mL tube containing 10–20 mL digestion buffer depending on the tissue weight.
12. Place the tubes at a slope of about 45° to maximize contact with the liquid. Shake the tubes at 200 rpm for 60 min at 37°C.
13. The digested tissues are shaken vigorously with hand to dissociate thoroughly for 5 min.
14. Add 10 mL complete RPMI 1640 medium to slow down the digestion. In order to increase single-cell yield, use 21-gauge syringe to aspirate back and forth to mechanically dissociate the residual tissue at about 25°C.
15. Filter cell suspension by 100 μm cell strainer into a new canonical 50 mL tube.
16. Centrifuge at 700 g for 5 min at about 25°C. Discard the supernatant, and scatter the cell pellet gently with 12.5 mL complete 1640 medium.
17. Add 12 mL of Ficoll in a 50 mL tube and carefully lay the above cell suspension at the top of Ficoll.
18. Centrifuge at 1,350 g for 25 min, at about 25°C. Set the acceleration at 1 grade, and deceleration at 0 grade.
19. Carefully collect the medium layer into new 50 mL tubes and adjust the volume into 10 mL with ice-cold PBS.
20. Centrifuge at 700 g for 5 min at 4°C, and resuspend cells with 5 mL flow buffer. Pass through 70 μm cell strainer into a 15 mL tube.
21. Calculate the cell number by mixing 20 μL of cell suspension with 20 μL trypan blue using a hemocytomter. Record cell number and viability.

Preparation of peripheral blood mononuclear cells (PBMCs)

Timing: 1 h

This step details how to isolate PBMCs in whole blood.[3]

22. Blood is collected with anticoagulant-treated tubes.
23. Dilute blood samples with equal volume of 2% FBS in PBS (1:1 dilution) at about 25°C.
24. Pipet up and down to mix the blood and buffer.
25. Add 12 mL of Ficoll to a 50 mL conical tube.
26. Gently transfer about 16 mL of diluted blood over the Ficoll layer.
27. Centrifuge the tubes at 1,350 g for 25 min, at about 25°C. Set the acceleration at 1 grade, and deceleration at 0 grade, to avoid disturbing the PBMCs interface.
28. Remove the diluted plasma layer at top with Pasteur pipette.
29. Collect the PBMCs layer, the middle white layer, into a new 15 mL conical tube.
30. Wash the PBMCs once with 8 mL ice-cold PBS.
31. Centrifuge at 700 g for 5 min at 4°C.
32. Carefully remove the supernatant.
33. Add 2 mL RBC lysing buffer and incubate the mixtures on ice for 5 min.
34. Add 5 mL PBS to the tube, and centrifuge at 700 g for 5 min.
35. Remove the supernatant and resuspend the PBMCs with 2 mL flow buffer and kept on ice for staining.

Antibody mix preparation

Timing: 15 min

This step details antibody mixes for staining ILCs.

36. Make antibody mix for staining ILCs.
    a. Staining buffer: 20 μL of mouse serum and 50 μL of Brilliant Strain Buffer is added into 130 μL of flow buffer. Usually, 200 μL staining buffer per sample.
    b. Block Solution: 2 μL of Fc block in 98 μL staining buffer/sample.
    c. Antibody Mixture: 2 μL of FITC-conjugated against each lineage markers (TCR γδ, TCR αβ, CD3, CD19, CD14, CD16, CD94, CD123, CD34, CD303 and FcεRI),[4] 2 μL of Alexa Fluor 700-CD45, 2 μL of PE-CD127, 2 μL of BV421-CD117 and 2 μL of PE-CF594-CD294 in 70 μL staining buffer/sample. This is a 2-fold antibody mixture. Final antibody dilution is 1:100.

Staining

Timing: 60 min

This step details for staining of ILCs for each sample.

37. Cells prepared from both tissue and blood are then washed with 1 mL of flow buffer and centrifuged at 700 g for 5 min at 4°C.
38. Pellets are blocked with 100 μL of Block Solution for 10 min at 4°C.
39. Add 100 μL of Antibody Mixture directly to the cells, vortex gently. Incubate for 30 min in dark at about 25°C.
40. Wash with 1 mL of flow buffer, vortex briefly and centrifuge at 700 g for 5 min at 4°C.
41. Discard the supernatant, resuspend the pellet with 500 μL flow buffer and filter through 40 μm nylon strainer.

Sorting

Timing: 6 h

This step details how to purify ILCs on a BD FACSAria III cell sorter.

42. Label collection tubes with indicated sample name, and add 1 mL of RPMI 1640 medium containing 20% FBS.
43. Gently vortex the tubes to rinse the tube before collection.
44. Set up the cell sorter with a 70 μm nozzle.
45. Set PMT voltages based on clear separation of positive and negative single staining.[5]
46. Auto-compensation is calculated with compensation beads stained with each dye. Unstained beads are used as background.
47. ILCs are gated on $CD45^+$ $Lineage^-$ $CD127^+$ after the lymphocyte, and singlets gates.
48. The ILCs are sorted using the 4-well purity mode.
49. The flow rate is set under 4 grade, and cell rates are set under 12,000 events per second.
50. After the sorting procedure, tubes are unloaded, and adjusted to 2 mL volume with RPMI 1640 medium containing 20% FBS.
51. Cells are centrifuged at 700 g for 5 min at 4°C. And supernatants are removed with pipette with about 200 μL liquid left in the tube.
52. The above cell suspension is transferred into a new 1.5 mL Eppendorf tube.
53. The cells are centrifuged at 1,500 g for 5 min at 4°C.
54. After centrifugation, the liquid is gently removed with a pipette.
55. The cell pellets might be visible under the light.

⚠ CRITICAL: It is critical to leave about 200 μL liquid in the tube to avoid discarding the cells. The liquid volume of 1.5 mL Eppendorf tube is better than 1 mL to avoid cells sticking on the wall of tubes.

Optional: Cells could be centrifuged at 700 g for 5 min and resuspended in PBS containing Fixable Viability stain 520 (1:1000) for 10 min at 4°C before staining for dead cell removal.

Note: The digestion buffer volume is tissue weight dependent. 5 mL or less volume could be added when the tissue is less than 0.3 g. 10 mL for 0.3–0.5 g of tissue. 20 mL digestion buffer could be used when the weight is more than 0.5 g.

EXPECTED OUTCOMES

Using the above method, we could get cell suspensions of viability larger than 90%. In addition, we develop gating strategies for innate lymphoid cells in adjacent intestinal tissue, CRC tissue and peripheral blood. The key to this gating strategy is to use enough negative markers to exclude unwanted cells and use CD127 to define total ILCs.

Helper-like ILCs are gated as by first gating on the lymphocytes gate based on forward scatter and side scatter. Then singlets are gated. After gating the CD45-positive gate, the ILCs are gated based on lineage negative and CD127 positive. Lineage negative (Lin^-) ($TCR\gamma\delta^-$ $TCR\alpha\beta^-$ $CD3^-$ $CD19^-$ $CD14^-$ $CD16^-$ $CD94^-$ $CD123^-$ $CD34^-$ $CD303^-$ $Fc\varepsilon RI^-$) $CD127^+$ helper-like ILCs (Figure 1). Lineage markers are used to exclude a group of cell mixtures. CD3, TCR αβ, and TCR γδ are used to exclude T cells, CD19 for B cells, CD16 and CD94 for NK cells, CD14 and CD123 for myeloid cells, FcεRI and CD123 for granulocytes, CD34 for hematopoietic stem cells, and CD303 and CD123 for plasmacytoid DCs.[6] Due to the tissue residence properties of ILCs,[7] the abundance of ILCs in adjacent tissue is higher than in blood (Figures 1A and 1B). It is easier to gate ILCs in peripheral blood (Figure 1A) and adjacent tissue (Figure 1B) than in tumor tissue (Figure 1C), due to the downregulation of lineage markers in tumor tissue.

LIMITATIONS

In this study, we mainly focus on processing blood, adjacent and tumor tissue of CRC patients. Although it works well for lymphocytes, it should be tested to get other cell fractions from these tissues. To process for other tissues than the intestine, it should be tested. In this study, we focus on gating the helper-like ILCs, and exclude NK cells from the gating. To study the NK cells, CD94, and CD16 should be different colors from the lineage gate.

TROUBLESHOOTING

Problem 1

Low cellular viability (related to steps 10–21).

Potential solution

This happens when the samples are delayed from the hospital to the laboratory. To avoid this, try to protect the human sample in ice-cold complete RPMI1640 medium, and minimize the transportation time. The muscle, adipose tissue layer in the adjacent gut tissue will also result in decreased viability. Try to remove this as clearly as possible. Over-digestion will also reduce cell viability. The enzyme should be tested in different batches.

Problem 2

Inadequate cell number (related to steps 1 and 22).

Potential solution

The frequency of ILCs varies across patients. In addition, tissue weight and blood volume will also affect the cell number. Usually, the ILCs number in the adjacent tissue is enough for downstream scRNA-seq. Additionally, the ILCs number in peripheral blood and tissue varies a lot. Increasing the tissue weight and blood volume will help.

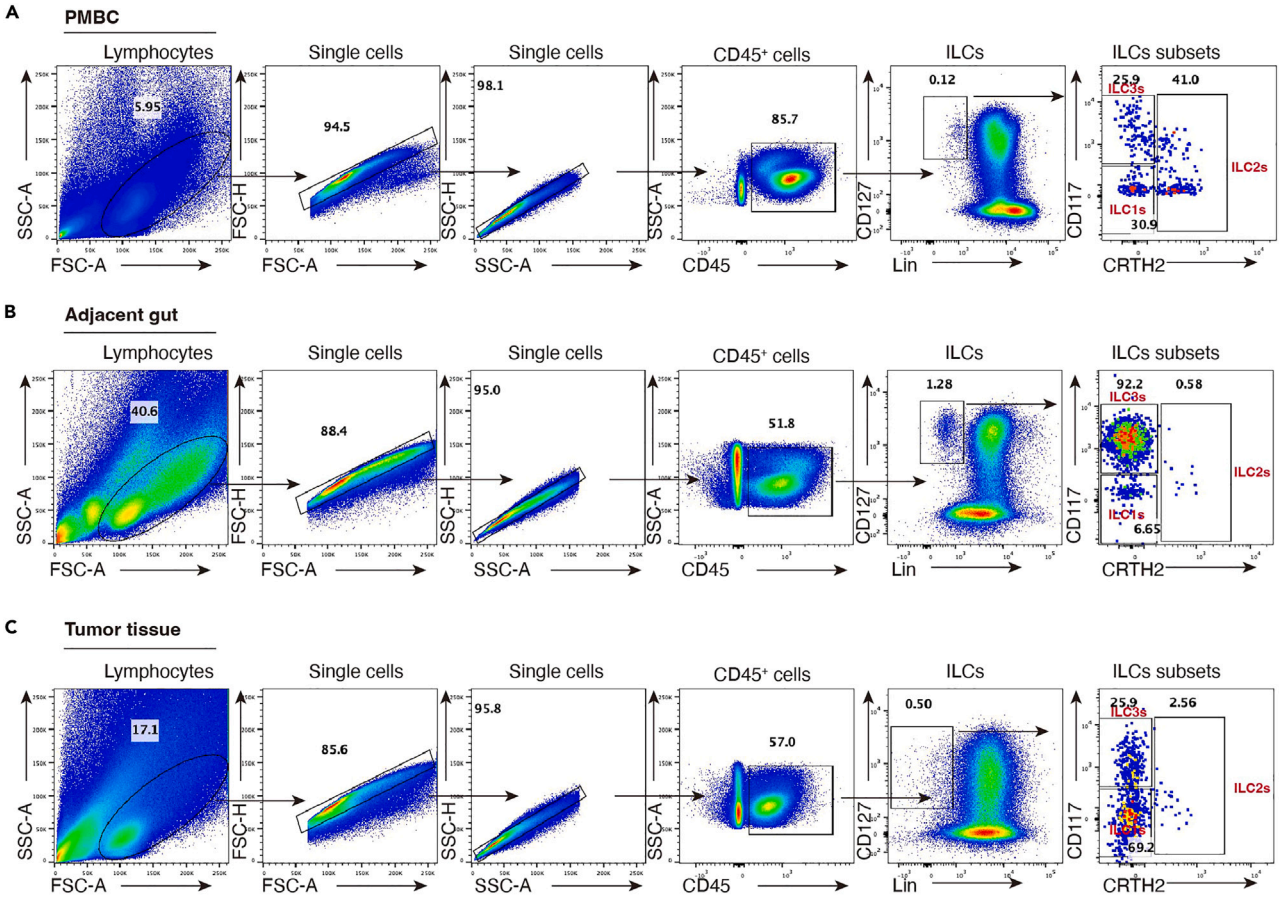

Figure 1. Gating strategy for helper-like ILCs in peripheral blood, adjacent gut, and tumor tissue

(A) Gating strategy for human ILCs in peripheral blood. ILCs are defined as $CD45^+$, lineage-negative (Lin^-) and CD127-positive ($CD127^+$) singlets. ILC1s are defined as $CD45^+$ Lin^- $CD127^+$ $CD117^-$ $CRTH2^-$, ILC2s as $CD45^+$ Lin^- $CD127^+$ $CRTH2^+$; ILC3s as $CD45^+$ Lin^- $CD127^+$ $CD117^+$ $CRTH2^-$. Lineage markers include TCRγδ, TCRαβ, CD3, CD19, CD14, CD16, CD94, CD123, CD34, CD303 and FcεRI.

(B) Gating strategy of human ILCs in adjacent gut.

(C) Gating strategy of human ILCs in tumor tissue. SSC, side scatter; FSC: forward scatter. PBMC, peripheral blood mononuclear cells. Arrows indicating that the gating is always from the drawn gate on the subsequent plot.

Problem 3

Too many debris in PBMCs (related to steps 22–25).

Potential solution

The platelets in PBMCs will increase the debris events during cell sorting. To reduce this, centrifuge the PBMCs at 200 g for 10 min, and collect the supernatants for use.

Problem 4

Difficulty in gating ILCs (related to step 47).

Potential solution

ILCs population is rare. The ILCs in adjacent intestine tissue are easy to gate than in the other two tissues. We recommend to gate ILCs in adjacent tissue, and refer this to slightly modify in blood and tumor. If there are adequate cells, fluorescence minus one (FMO) could also work.

Problem 5

Application of this protocol to non-immune cell analysis in intestine (related to steps 6, 17–19).

Potential solution

If the target cells are epithelial or fibroblasts, we suggest use Wash buffer 2 to pretreat both tumor and adjacent tissue as in step 6. Skip the Ficoll centrifugation steps from steps 17–19.

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. Bing Su (bingsu@sjtu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate specific datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, J.Q. and B.S.; investigation, J.Q. and L.H.; formal analysis, J.Q.; resources, L.H. and Z.W.; writing – original draft, J.Q.; writing – review & editing, J.Q. and B.S.; funding acquisition, L.S. and B.S.; supervision, B.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Qi, J., Crinier, A., Escalière, B., Ye, Y., Wang, Z., Zhang, T., Batista, L., Liu, H., Hong, L., Wu, N., et al. (2021). Single-cell transcriptomic landscape reveals tumor specific innate lymphoid cells associated with colorectal cancer progression. Cell Rep. Med. *2*, 100353. https://doi.org/10.1016/j.xcrm.2021.100353.

2. Qiu, J., Heller, J.J., Guo, X., Chen, Z.M.E., Fish, K., Fu, Y.X., and Zhou, L. (2012). The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity *36*, 92–104. https://doi.org/10.1016/j.immuni.2011.11.011.

3. Dutertre, C.A., Becht, E., Irac, S.E., Khalilnezhad, A., Narang, V., Khalilnezhad, S., Ng, P.Y., van den Hoogen, L.L., Leong, J.Y., Lee, B., et al. (2019). Single-cell analysis of human mononuclear phagocytes reveals subset-defining markers and identifies circulating inflammatory dendritic cells. Immunity *51*, 573–589.e8. https://doi.org/10.1016/j.immuni.2019.08.008.

4. Björklund, Å.K., Forkel, M., Picelli, S., Konya, V., Theorell, J., Friberg, D., Sandberg, R., and Mjösberg, J. (2016). The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. Nat. Immunol. *17*, 451–460. https://doi.org/10.1038/ni.3368.

5. Cossarizza, A., Chang, H.D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., Andrä, I., Andreata, F., Annunziato, F., Arranz, E., et al. (2021). Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). Eur. J. Immunol. *51*, 2708–3145. https://doi.org/10.1002/eji.202170126.

6. Bernink, J.H., Mjösberg, J., and Spits, H. (2017). Human ILC1: to be or not to be. Immunity *46*, 756–757. https://doi.org/10.1016/j.immuni.2017.05.001.

7. Gasteiger, G., Fan, X., Dikiy, S., Lee, S.Y., and Rudensky, A.Y. (2015). Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. Science *350*, 981–985. https://doi.org/10.1126/science.aac9593.