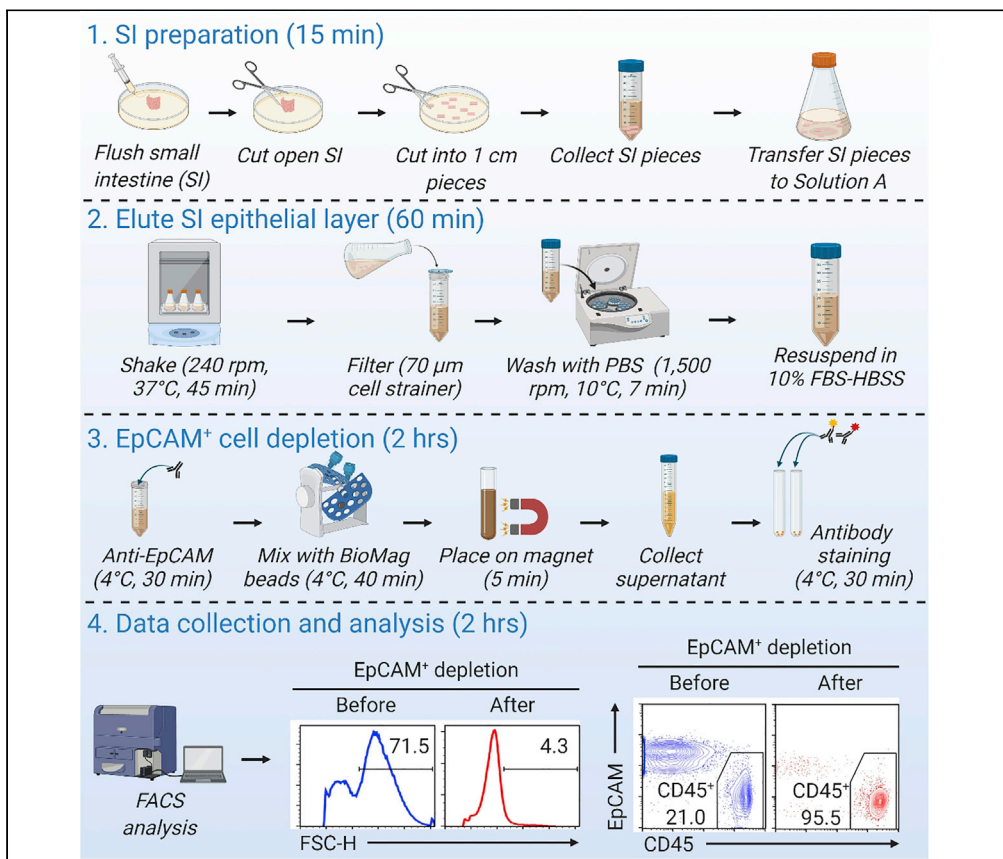


Protocol

High-yield enrichment of mouse small intestine intraepithelial lymphocytes by immunomagnetic depletion of EpCAM⁺ cells



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Highlights

Depletion of EpCAM⁺ epithelial cells to enrich intraepithelial lymphocytes (IELs)

Magnetic bead-based protocol to isolate IELs from the small intestine (SI)

Optimized protocol to achieve high-yield and high-purity isolation of SI IELs

Applicable for isolating rare cell populations, such as iNKT cells, from the SI epithelium

Lymphocytes in the mouse small intestine (SI) epithelium are critical to establish effective barrier immunity. Here, we describe a magnetic cell separation protocol that employs anti-epithelial cellular adhesion molecule (EpCAM) antibodies to deplete epithelial cells and to enrich for intraepithelial lymphocytes (IELs) from SI tissues. The resulting IEL preparation was functionally and phenotypically comparable to IELs isolated using conventional density gradient centrifugation protocols. Moreover, the yield and purity of anti-EpCAM-depleted SI IELs were higher than those enriched by conventional isolation.

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Protocol

High-yield enrichment of mouse small intestine intraepithelial lymphocytes by immunomagnetic depletion of EpCAM⁺ cells

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SUMMARY

Lymphocytes in the mouse small intestine (SI) epithelium are critical to establish effective barrier immunity. Here, we describe a magnetic cell separation protocol that employs anti-epithelial cellular adhesion molecule (EpCAM) antibodies to deplete epithelial cells and to enrich for intraepithelial lymphocytes (IELs) from SI tissues. The resulting IEL preparation was functionally and phenotypically comparable to IELs isolated using conventional density gradient centrifugation protocols. Moreover, the yield and purity of anti-EpCAM-depleted SI IELs were higher than those enriched by conventional isolation.

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

BEFORE YOU BEGIN

⌚ Timing: 30 min

Note: Before starting the cell isolation, design the staining chart for the experiment (see an example in Table 1). The single-color staining tubes are necessary to set up fluorescence color compensation of the flow cytometer. Lymph node (LN) cells can be used for most of the compensation tubes, except for the staining of EpCAM which requires cells from the unfractionated SI epithelial eluate. All animal experiments were reviewed and approved by the National Cancer Institute, Animal Care and Use Committee. All mice were cared for in accordance with NIH guidelines.

1. Switch on and set up the orbital shaker to 240 rpm at 37°C for 45 min.
2. Pre-chill cell centrifuge to 10°C.
3. Pre-chill each 100 mL of 10% Fetal Bovine Serum (FBS)-Hank's Balanced Salt Solution (HBSS), 125 mL of 2% FBS-HBSS, and 25 mL of Phosphate Buffered Saline (PBS) per SI of one mouse to 4°C.
4. Pre-warm 25 mL of Solution A per SI in a 37°C incubator.
5. Label the FACS tubes according to the staining chart.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-mouse CD8 α (Clone: 53-6.7) BV786; Working dilution: 1:10	BD Biosciences	Cat#: 563332 RRID: AB_2721167
Rat anti-mouse EpCAM (Clone: G8.8) eFluor450; Working dilution 1:10	Invitrogen	Cat#: 48-5791-80 RRID: AB_10717391

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rat anti-mouse EpCAM (Clone: G8.8) unconjugated	Invitrogen	Cat#: 14-5791-85 RRID: AB_953626
Rat anti-mouse CD4 (Clone: GK1.5) PE-Cyanine7; Working dilution 1:20	Tonbo Biosciences	Cat#: 60-0041-U100 RRID: AB_2621828
Hamster anti-mouse TCRβ (Clone: H57-597) Alexa Fluor 594; Working dilution 1:40	BioLegend	Cat#: 109238 RRID: AB_2563324
Rat anti-mouse CD45 (Clone: 30-F11) APC-e780; Working dilution: 1:10	Invitrogen	Cat#: 47-0451-82 RRID: AB_1548781
Rat anti-mouse CD8β (Clone: YTS156.7.7) Alexa Fluor 647; Working dilution: 1:10	BioLegend	Cat#: 126612 RRID: AB_2075777
Rat anti-mouse CD16/32 (Clone: 2.4G2) unconjugated; Working dilution 1:50	BD Biosciences	Cat#: 553141 RRID: AB_394656
Hamster anti-mouse TCRγδ (Clone: GL3) FITC; Working dilution 1:20	BD Biosciences	Cat#: 553177 RRID: AB_394688
Rat anti-mouse B220 (Clone: RA3-6B2) FITC; Working dilution 1:20	BD Biosciences	Cat#: 553088 RRID: AB_394618
Rat anti-mouse CD19 (Clone: eBio1D3) eFluor 660; Working dilution 1:10	eBioscience	Cat#: 50-0193-82 RRID: AB_11218286
Mouse anti-mouse CD1d tetramers (PBS-57 loaded) PE; Working dilution 1:200	NIH tetramer facility	Cat#: 55274 RRID: n/a
Goat anti-rat IgG BioMag beads	QIAGEN	Cat#: 310107 RRID: n/a
Goat anti-mouse IgG BioMag beads	QIAGEN	Cat#: 310007 RRID: n/a

Chemicals, peptides, and recombinant proteins

Sodium azide	Sigma-Aldrich	Cat#: S2002-500G
DL-dithiothreitol	Sigma-Aldrich	Cat#: 43815-5G
Modified Cohn fraction V bovine serum albumin (BSA) powder	Equitech-Bio	Cat#: BAC65
Heat-inactivated (56°C for 30 min) standard Fetal Bovine Serum (FBS)	HyClone	Cat#: SH30088.03
0.4% trypan blue	Gibco	Cat#: 15250061
Phosphate Buffered Saline (PBS)	Gibco	Cat#: 10010023
Hank's Balanced Salt Solution (HBSS)	Gibco	Cat#: 14175095

Experimental models: Organisms/strains

Mouse: C57BL/6 (C57BL/6NCR) mice of both sexes and between 6-12 weeks of age	Charles River Laboratories	n/a
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Software and algorithms

FlowJo software	FlowJo LLC	https://www.flowjo.com
GraphPad Prism 7 software	GraphPad	https://www.graphpad.com
Canvas X software	Canvas GFX	https://www.canvasgfx.com

Other

60 μm nylon filters	Merck Millipore	Cat#: NY6000010
100 μm nylon filters	Merck Millipore	Cat#: NY1H00010
70 μm nylon cell strainers	Falcon	Cat#: 352350
5 mL polystyrene round-bottom tube	Falcon	Cat#: 352052
5 mL polypropylene round-bottom tube	Falcon	Cat#: 352063
14 mL polypropylene round-bottom tube	Falcon	Cat#: 352059
50 mL polypropylene conical tube	Falcon	Cat#: 352070
100 mm petri dish	Falcon	Cat#: 351029
Corning® 125 mL polycarbonate Erlenmeyer flask with flat cap	Corning	Cat#: 430421
Monoject blunt cannula 16G	Monoject	Cat#: 8881202322
Monoject 12 mL syringe	Monoject	Cat#: 8881512878
Cell centrifuge	Thermo Fisher Scientific	Model: Legend XTR
Cell culture CO ₂ incubator	Thermo Fisher Scientific	Model: 370
37°C shaker	New Brunswick Scientific	Model: Excella E24
MACSmix™ tube rotator	Miltenyi Biotec	Cat#: 130-090-753

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hemocytometer counting chamber with V-slash	Paul Marienfeld GmbH	Cat#: 0650030
Standard 25 binocular microscope	Zeiss	n/a
Magnetic separator	Polysciences	Cat#: 84102S
BD FACS LSRII flow cytometer	BD Biosciences	n/a

MATERIALS AND EQUIPMENT

Solution A (for the preparation of SI IELs from 4 mice):

Reagent	Final concentration	Amount
10% FBS-HBSS	10% (v/v)	99 mL
DL-dithiothreitol	1 mM	0.0154 g
0.5 M EDTA	5 mM	1 mL
Total	n/a	100 mL

Note: Solution A should always be prepared fresh and kept at 37°C until use.

FACS buffer: store at 4°C.

Reagent	Final concentration	Amount
BSA	0.1% (w/v)	0.5 g
Sodium azide	0.1% (w/v)	0.5 g
HBSS	n/a	500 mL
Total	n/a	500 mL

Note: FACS buffer can be stored at 4°C until used.

2% FBS-HBSS: store at 4°C.

Reagent	Final concentration	Amount
Standard FBS	2% (v/v)	10 mL
HBSS	n/a	490 mL
Total	n/a	500 mL

Note: 2% FBS-HBSS can be stored at 4°C until used.

10% FBS-HBSS: store at 4°C.

Reagent	Final concentration	Amount
Standard FBS	10% (v/v)	50 mL
HBSS	n/a	450 mL
Total	n/a	500 mL

Note: 10% FBS-HBSS can be stored at 4°C until used.

0.08% trypan blue: store at 20°C–25°C.

Reagent	Final concentration	Amount
0.4% trypan blue	0.08% (v/v)	10 mL
PBS	n/a	40 mL
Total	n/a	50 mL

Note: 0.08% trypan blue can be stored at 20°C–25°C until used.

Fc block solution: store at 4°C.

Reagent	Final concentration	Amount
Rat anti-mouse CD16/32 (Clone: 2.4G2)	12.5 µg/mL	25 µL
FACS buffer	n/a	975 µL
Total	n/a	1 mL

Note: Fc block buffer can be stored at 4°C until used.

STEP-BY-STEP METHOD DETAILS

Preparation of small intestine tissues

⌚ **Timing:** 30 min

This section describes how to dissect and process the SI for removal of the epithelial monolayer containing the lymphocytes.

1. Dissection and tissue processing of the SI
 - a. Mice are sacrificed by carbon dioxide asphyxiation. To prevent fur contamination, the carcasses are doused with 70% ethanol. Small dorsal incisions are made in the pelvis region using scissors. The pelt is then removed from the head to the base of the tail by carefully tearing the skin in the transverse plane. See [Figure 1A](#) for an example of dorsal incision, and [Figure 1B](#) for pelt removal.
 - b. Turn over the mice on to its ventral side, and cut open the peritoneal membrane using scissors to expose the intestine. See [Figure 2A](#) for an example of cutting the peritoneal membrane and [Figure 2B](#) for exposing the SI.
 - c. Cut the SI below the pyloric sphincter and above the cecum using scissors. See [Figure 3A](#) for an example of cutting the SI below the pyloric sphincter, and [Figure 3B](#) for cutting the SI above the cecum.
 - d. Pull out the intestine using forceps, while removing the mesenteric fat using another set of forceps. See [Figure 4A](#) for an example of proper mesenteric fat removal during dissection, whereas [Figure 4B](#) shows incomplete fat removal. [Figure 4C](#) is the zoomed-in view of the mesenteric fat.

⚠ **CRITICAL:** The complete removal of the visceral and mesenteric fat is necessary to ensure optimal yield and viability of the SI IELs.

- e. Place the SI into a 10 cm petri dish pre-layered with 2% FBS-HBSS and flush out the fecal content with 2% FBS-HBSS using a 12 mL syringe with a 16G blunt needle. Use curved forceps to slide the SI onto the needle as you flush with 2% FBS-HBSS to remove the fecal matter. Transfer the SI to a new dish with 2% FBS-HBSS and repeat the flushing process. See [Figure 5](#) for an example of the SI before ([Figure 5A](#)) and after ([Figure 5B](#)) flushing out of fecal matters.

⚠ **CRITICAL:** If mesenteric fat remains visible on the SI after the first round of flushing, remove the SI from the petri dish and place it on a paper towel, stretching out the SI lengthwise. Be sure to cover the SI with 2% FBS-HBSS to minimize the loss of IELs. Then, starting from one end of the stretched-out SI and moving to the other, peel the SI away from the fat using two curved blunt-edged forceps— the fat will stick to the paper towel. See [Figure 6](#)

Table 1. Staining chart example to check the purity of IELs before and after EpCAM⁺ cell depletion

Tube #	Sample	Antibodies							Live/ dead
		FITC	PE-Cy7	eFluor 450	Alexa fluor 594	Alexa fluor 647	APC-e780	BV 786	Propidium iodide (PI)
1	IELs before depletion	TCR $\gamma\delta$	CD4	EpCAM	TCR β	CD8 β	CD45	CD8 α	Add PI
2	IELs after depletion	TCR $\gamma\delta$	CD4	EpCAM	TCR β	CD8 β	CD45	CD8 α	Add PI
3	LN cells	–	–	–	–	–	–	–	No PI
4	LN cells	TCR $\gamma\delta$	–	–	–	–	–	–	No PI
5	LN cells	–	CD4	–	–	–	–	–	No PI
6	IELs before depletion	–	–	EpCAM	–	–	–	–	No PI
7	LN cells	–	–	–	TCR β	–	–	–	No PI
8	LN cells	–	–	–	–	CD8 β	–	–	No PI
9	LN cells	–	–	–	–	–	CD45	–	No PI
10	LN cells	–	–	–	–	–	–	CD8 α	No PI

Tubes #3 - #10 are compensation tubes that should be used to set up the flow cytometer.

for an example of the post-dissection fat removal process. Once all visible fat is removed, return the SI to the petri dish for a second round of flushing.

- f. Transfer the flushed SI into a new petri dish pre-layered with 2% FBS-HBSS and cut open the SI longitudinally using scissors. See [Figure 7](#) for a visual representation of the cutting process. Shake the SI using forceps to remove mucus and any remaining fecal content. Transfer the SI to a new dish with 2% FBS-HBSS and repeat the shaking process.

△ CRITICAL: Incomplete removal of the mucus leads to decreased IEL viability and yield. Perform visual inspection of the processed SI and confirm that fecal matter and the mucus are completely removed. Ensure to shake the SI gently to avoid stripping off the epithelial layer.

- g. Transfer the cut-open SI into a new petri dish with 2% FBS-HBSS and slice into ~1 cm piece. See [Figure 8A](#) for an example of ~1 cm cut SI pieces.

△ CRITICAL: If the cut SI pieces are longer than 1 cm, IEL recovery can decrease because the subsequent removal of epithelial monolayer will be inefficient.

- h. Transfer the SI pieces into a 50 mL tube and fill up to 25 mL with 2% FBS-HBSS and shake. Pour out the content into a new petri dish. Using forceps, transfer the cut SI pieces into the same 50 mL tube and fill up to 25 mL with 2% FBS-HBSS and shake again.

Note: The washed and processed SI pieces can be kept at this stage on ice when processing more than one SI.

Elution of lymphocytes from the SI epithelial layer

⌚ Timing: 1 h

2. Removal of the epithelial monolayer containing IELs
 - a. Pipette 25 mL of pre-warmed (37°C) solution A into a 125 mL conical flask.
 - b. Pour out the entire contents of the 50 mL tube with SI pieces into a new petri dish. Using forceps, transfer the cut SI pieces from the petri dish into the 125 mL conical flask containing solution A.

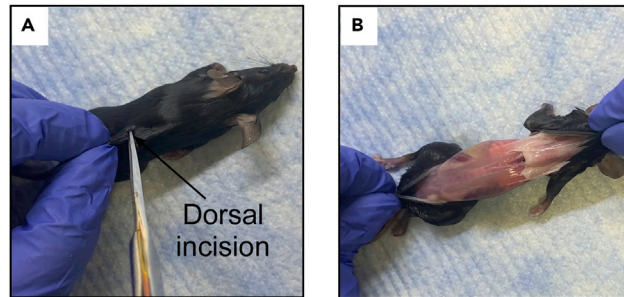


Figure 1. Preparing the mouse for dissection

(A) Example of making a dorsal incision on euthanized mice.
(B) Example of pelt removal by tearing the skin in the transverse plane.

- c. Shake the conical flask at 240 rpm at 37°C for 45 min in a pre-warmed 37°C orbital shaker. See [Figure 8](#) for an example of SI pieces before and after shaking.

Note: After shaking, the solution should be cloudy which indicates effective removal of the SI epithelial layer.

△ **CRITICAL:** Solution A should always be prepared fresh. Solution A and the orbital shaker should be pre-warmed to 37°C for efficient removal of the SI epithelial monolayer.

- d. Transfer the SI eluate, which will be around 25 mL, into a 50 mL tube by passing it through 70 μm cell strainers.
- e. Centrifuge at 1,500 rpm (500 × g) for 7 min at 10°C.
- f. Discard the supernatant and resuspend the pellet in 10 mL cold PBS.

Note: Take an 1 mL aliquot of the cell suspension and set it aside and keep it at 4°C to compare the purity before and after the EpCAM⁺ cell depletion ([Table 1](#)).

- g. Fill up the 50 mL tube with the SI eluate to 25 mL with cold PBS and centrifuge tube at 1,500 rpm (500 × g) for 7 min at 10°C.
- h. Discard the supernatant.

△ **CRITICAL:** Cell suspension should be kept on ice to ensure maximum cell viability.

3. Counting of cell numbers
 - a. Add 10 μL of the single cell suspension from step 2f to 190 μL 0.08% trypan blue (1:20 dilution).
 - b. Take 10 μL of the cell suspension from step 3a and count the cells using a hemacytometer. ([Troubleshooting 1](#))

Note: We routinely use trypan blue and a hemacytometer to count the number of viable cells. Expect a high rate of cell death (>30%), which will be caused by the mechanical stress during the isolation procedure.

Depletion of EpCAM⁺ SI epithelial cells

⌚ **Timing:** 2 h

This section describes in detail how to deplete SI epithelial cells from the eluate using anti-EpCAM antibodies.

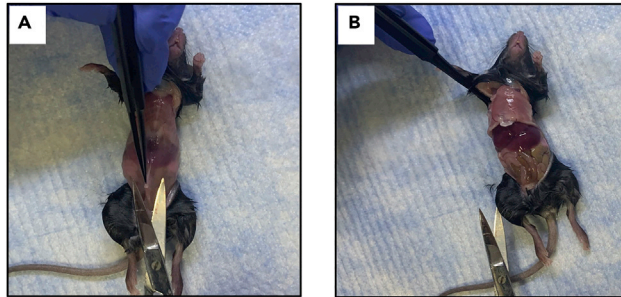


Figure 2. Opening the peritoneal cavity

(A) Example of cutting the peritoneal membrane.
(B) Example of exposing the peritoneal cavity.

4. Incubation with anti-EpCAM antibodies

- a. After determining the cell numbers, resuspend the cells in 10% FBS-HBSS at a concentration of 20 million cells/mL. Empirically, 50–100 million cells are recovered from one SI isolation.
- b. Add 15 μ L/mL of 0.5 mg/mL anti-EpCAM antibody to the resuspended cells and incubate the cell suspension undisturbed on ice for 30 min. ([Troubleshooting 2](#))

Note: During the 30 min incubation, prepare the goat anti-rat IgG-coated and goat anti-mouse IgG-coated BioMag magnetic beads (step 5).

- c. After 30 min of incubation, fill up the tubes up to 25 mL with 10% FBS-HBSS and centrifuge tubes at 1,500 rpm (500 \times g) for 7 min at 10°C to remove excess antibodies.
- d. Discard the supernatant and resuspend the pelleted cells in 10% FBS-HBSS to an estimated concentration of 50 million/mL, based on the cell numbers from step 3b.

5. Washing and preparing BioMag beads

- a. Take each 2 mL of goat anti-rat IgG-coated and goat anti-mouse IgG-coated BioMag magnetic beads for 100 million cells and transfer them into a 14 mL polypropylene round-bottom tube and place the tube on a magnetic stand for 5 min. During the incubation time, the magnetic beads will slowly but firmly adhere to the magnet. ([Troubleshooting 2](#) and [3](#))

Note: Even if the cell numbers are below 100 million, 2 mL of goat anti-rat IgG BioMag beads and goat anti-mouse IgG BioMag should be used. The goat anti-mouse IgG beads are used to deplete B cells from the cell suspension and can be omitted.

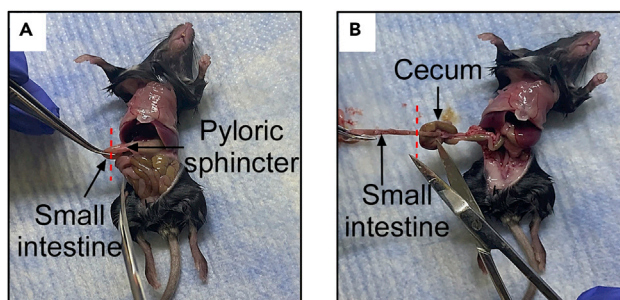


Figure 3. Dissection of SI

(A) An example of cutting the SI below the pyloric sphincter.
(B) An example of cutting the SI above the cecum. The red dashed line represents the line for cutting the SI.

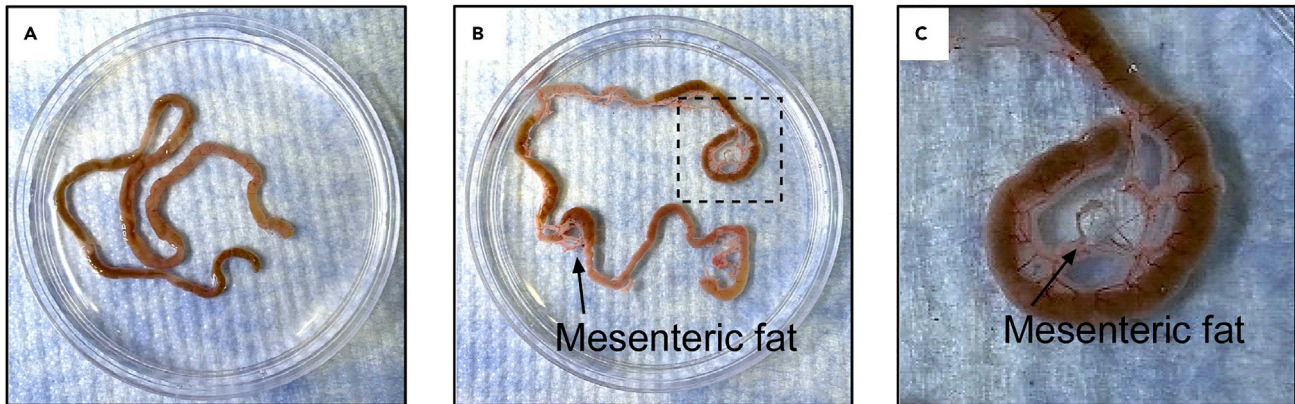


Figure 4. Demonstration of proper SI mesenteric fat removal (step 1d)

- (A) An example of proper SI dissection, free of mesenteric fat.
 (B) An example of improper SI dissection, with incomplete removal of mesenteric.
 (C) Zoomed-in view of the mesenteric fat attached to the SI.

- b. While keeping the tubes attached to the magnet so that the beads remain magnetized, remove the supernatant by aspiration. Resuspend the BioMag beads in 10 mL of 10% FBS-HBSS and place the cell suspension on the magnet for an additional 5 min. Repeat this process one more time.
- c. Discard the supernatant and resuspend the beads in 1 mL of 10% FBS-HBSS. To this suspension, add the cells from step 4d and transfer the suspension into a 5 mL polypropylene tube.
- d. Place the tube on the MACSmix™ tube rotator for 40 min at 4°C. See [Figure 9](#) for an example of before ([Figure 9A](#)) and after ([Figure 9B](#)) rotation of the beads with cells.
- e. After 40 min of rotation, place the tube on a magnet for 5 min and transfer the supernatant into a new 5 mL polypropylene tube. Place the tube on the magnet for another 5 min to remove residual magnetic beads from the cell suspension. See [Figure 10](#) for the depiction of proper removal of magnetic beads. ([Troubleshooting 5](#))

Note: The tube can be placed back on the magnet as many times as necessary to ensure complete removal of magnetic beads.

- f. Transfer the supernatant into a new 14 mL tube and keep on ice until further use.

Note: Measure the volume of the supernatant to determine recovered cell numbers.

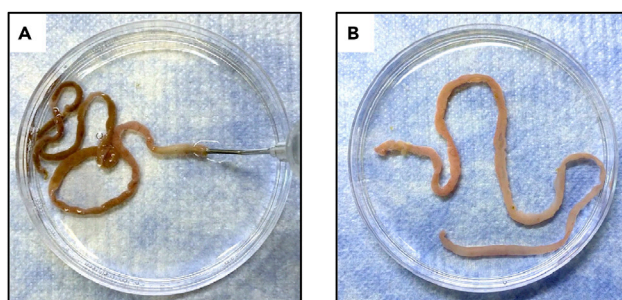


Figure 5. Flushing out fecal matters from the SI (step 1e)

- (A) An example of the SI before starting the flushing process, with the end of a blunt needle inserted into the lumen of SI.
 (B) An example of the SI after both flushes, with all fecal matter removed.

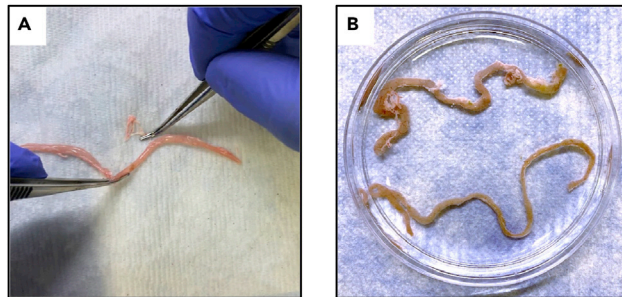


Figure 6. Post-dissection mesenteric fat removal (step 1e, if necessary)

(A) Demonstration of mesenteric fat removal from SI after dissection.

(B) Comparison of SI attached to mesenteric fat (top) with the same SI after post-dissection fat removal (bottom). This SI has been cut in half for the purpose of comparison.

6. Counting of cell numbers

- Add 10 μL of the single cell suspension from step 5f to 190 μL 0.08% trypan blue (1:20 dilution).
- Take 10 μL of the cell suspension from step 6a and count the cells using a hemacytometer. ([Troubleshooting 4](#))

Antibody staining

⌚ Timing: 1 h

This section describes how to stain cells for purity check.

7. Surface marker staining

- Take 5 million IELs and transfer them into 5 mL polystyrene round-bottom tubes. Add 2 mL of cold FACS buffer to each tube and centrifuge the tubes at 1,500 rpm (500 \times g) for 7 min at 10°C.
- Discard the supernatant and stain with antibodies to surface markers in FACS buffer, according to the dilution factors as indicated below.

Antibody	Dilution for working concentration
Hamster anti-mouse TCR $\gamma\delta$ FITC	1:20 v/v
Rat anti-mouse CD4 PE-Cy7	1:20 v/v
Hamster anti-mouse TCR β Alexa Fluor 594	1:40 v/v
Rat anti-mouse EpCAM eFluor450	1:10 v/v
Rat anti-mouse CD8 β Alexa Fluor 647	1:10 v/v
Rat anti-mouse CD8 α BV786	1:10 v/v
Rat anti-mouse CD45 APC-e780	1:10 v/v

- Use 10 μL each of the diluted antibodies to make a master mix for antibodies, according to the number of samples. Make extra amounts of the master mix (calculate for 2 or 3 more samples than necessary) to secure enough reagents and to compensate for pipetting errors.

Note: We recommend making a master mix, which includes all the corresponding fluorescence-conjugated antibodies and the anti-mouse CD16/32 blocking antibody, when staining with multiple antibodies to reduce variability between samples by pipetting.

- Add 70 μL of the antibody master mix to each tube. For the single-color compensation tubes, add 10 μL of the diluted antibody according to the staining chart. Add 5 μL anti-mouse CD16/32 (Clone: 2.4G2) to all tubes.

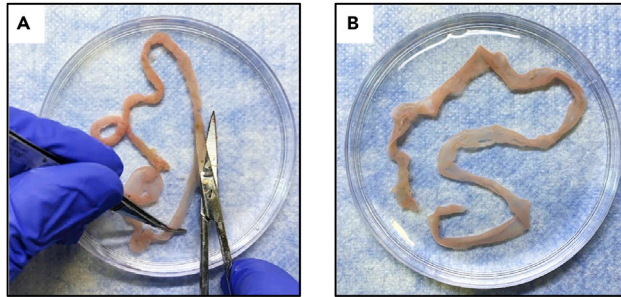


Figure 7. Longitudinal cutting of the SI (step 1f)

(A) Depiction of the longitudinal cutting process.

(B) An example of the SI after opening it up lengthwise.

- e. Incubate tubes at 4°C for 30 min.
- f. Add 2 mL of cold FACS buffer to each tube, and then spin down cells at 1,500 rpm (500 × g) for 7 min at 10°C.
- g. Discard the supernatant and repeat step 7f to wash the cells again.
- h. Discard the supernatant and resuspend pellet with 200 μL cold FACS buffer.
- i. Filter the cells into new FACS tubes by passing through 60 μm nylon filters and place the tubes on ice until flow cytometric analysis.

Note: Filter the cell suspension through 60 μm nylon filters to remove cellular aggregates. To prevent reaggregation, cells should be not filtered earlier than 30 min before the start of data acquisition.

Sample collection and data analysis

⌚ Timing: 2 h

This section describes how to collect samples using a flow cytometer and which software to use for data analysis.

8. Use unstained cells and single-color control staining to set appropriate photomultiplier tube (PMT) voltages and compensations for each parameter.
9. At least 0.5 million PI-negative live-gated cells should be collected per tube at a flow rate of 6,000 events/second or less. A representative voltage setting for acquisition would be as follows: FCS:

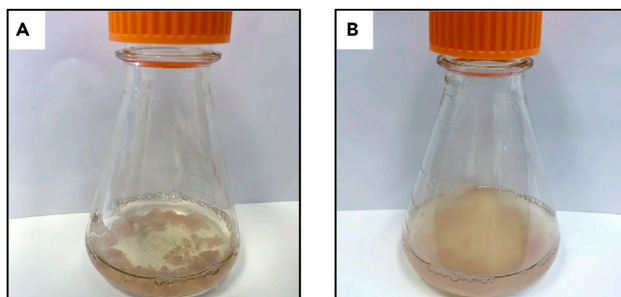


Figure 8. Release of the SI epithelial layer by orbital shaking (step 2c)

(A) SI fragments that were cut into ~1 cm piece in solution A before shaking.

(B) Cloudy solution A after the orbital shaking and incubation is completed.

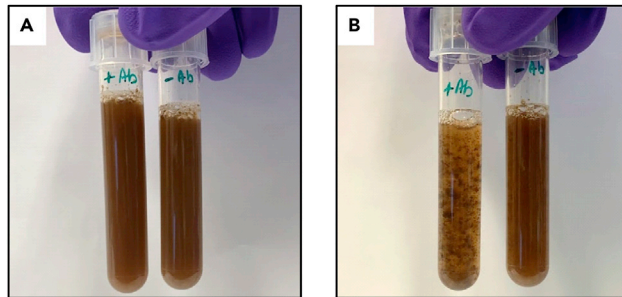


Figure 9. Mixing of magnetic beads and cells (step 5d)

(A) An example of bead and cell mixture before rotation. Anti-EpCAM antibodies were added to the cells in the tube on the left, while the tube on the right contains no anti-EpCAM antibodies.

(B) Image of the same tubes after rotation. The clumping of epithelial cells to the BioMag beads, which is expected, is only seen in the tube with anti-EpCAM antibodies.

565, SSC: 330, FITC: 500, PE-Cy7: 630, eFluor 450: 390, Alexa Fluor 647: 460, Alexa Fluor 594: 510, APC-e780: 580, BV786: 580, PI: 375.

Note: The voltage of each parameter needs to be adjusted using unstained compensation tubes and single color-stained compensation tubes for each experiment (Table 1).

- The .FCS format flow cytometry files were analyzed using the software FlowJo with linear scales for Forward Scatter Height (FSC-H) and Forward Scatter Width (FCS-W), and log scales for fluorochrome parameters. Statistical analyses were performed using the software GraphPad Prism 7, and the figures for publication were created with the graphical software Canvas X.

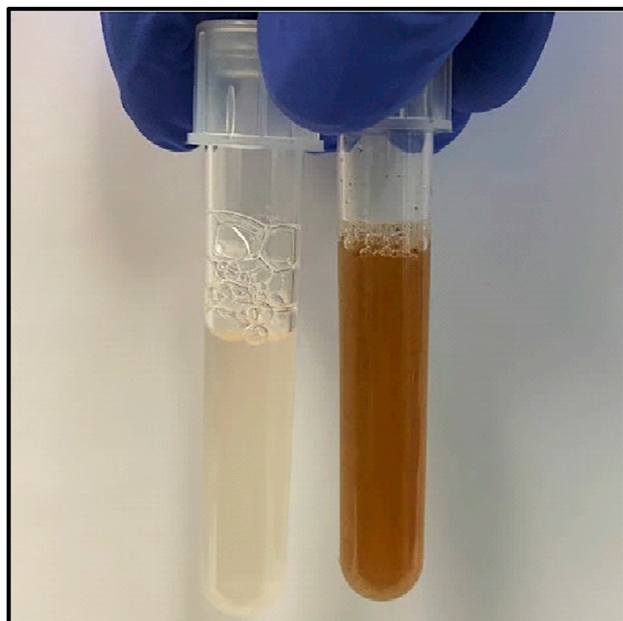


Figure 10. Magnetic beads removal (step 5e)

Comparison of a cell suspension with proper removal of magnetic beads (left) to a cell suspension that failed to completely remove BioMag beads (right).

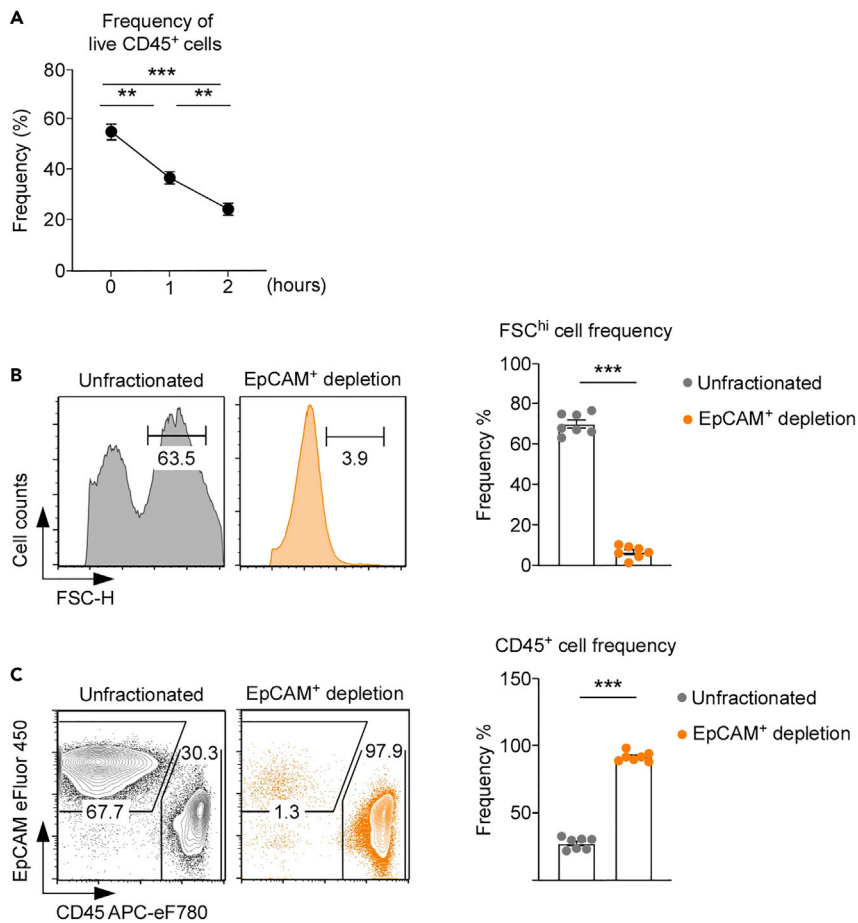


Figure 11. EpCAM⁺ cell depletion of SI epithelial eluate

(A) The graph shows the frequency of live (Ghost dye-negative) CD45⁺ cells in SI eluates incubated for the indicated times *in vitro*. Results are summarized from 3 independent experiments with a total of 7 WT mice.

(B) Forward scatter (FSC-H) analyses of SI IELs before (left) and after EpCAM⁺ depletion (right), gated on live cells. The bar graphs show the summary of FSC^{hi} cell frequency before and after EpCAM⁺ depletion from 3 independent experiments with a total of 7 WT mice.

(C) EpCAM versus CD45 profiles of SI IELs before (left) and after EpCAM⁺ depletion (right) gated on live cells. The bar graphs show the summary of CD45⁺ cell frequency before and after EpCAM⁺ depletion from 3 independent experiments with a total of 7 WT mice. The data are shown as the mean \pm SEM. p values were determined by one-way ANOVA with Tukey's multiple comparison test (A) or two-tailed paired Student's t test (B and C). ** p < 0.01, *** p < 0.001.

EXPECTED OUTCOMES

The SI eluate comprises intestinal epithelial cells and IELs (Prakhar et al., 2021). The physical separation of IELs from SI epithelial cells is necessary to preserve IEL viability because unfractionated IELs undergo rapid cell death (Figure 11A). Upon extraction, SI epithelial cells release copious amounts of ATP into the environment. Such extracellular ATP binds to the purinergic receptor P2RX7 that are abundantly expressed on IELs and trigger apoptosis (Stark et al., 2018). Hence, it is important to remove intestinal epithelial cells from the SI eluate to ensure the survival of IELs.

The current protocol allows for the isolation of SI IELs at higher purity and greater yield than the conventional methods which are mostly based on density gradients (Montufar-Solis and Klein, 2006; Prakhar et al., 2021; Reissig et al., 2014). Anti-EpCAM⁺ depletion effectively removes the larger-sized cells from the SI epithelial eluate which correspond to intestinal epithelial cells (Figure 11B,

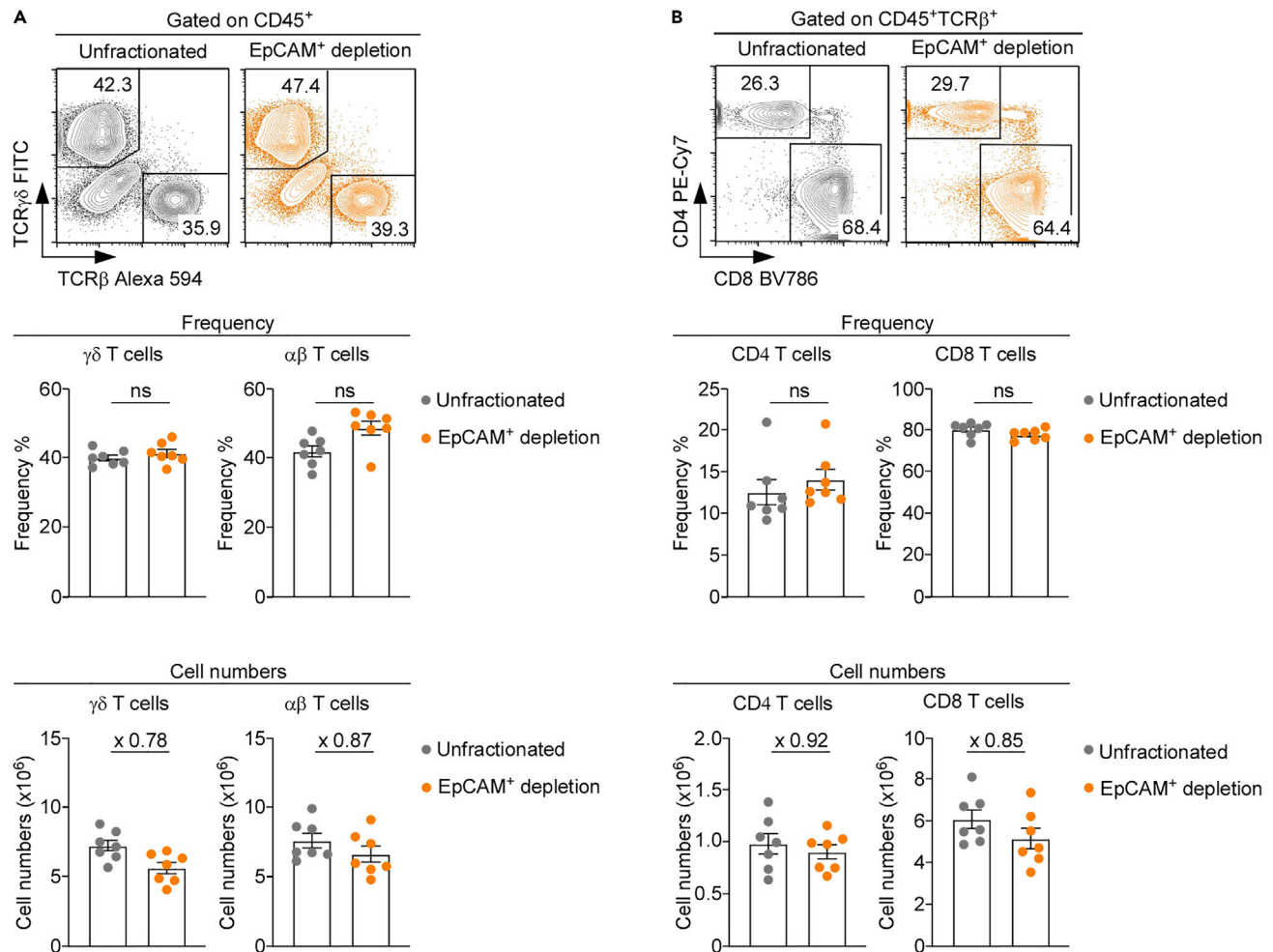


Figure 12. Efficiency of SI IEL T cell recovery by EpCAM⁺ depletion

(A) TCR $\gamma\delta$ versus TCR β profiles of CD45⁺-gated SI IELs before and after EpCAM⁺ depletion (top). The graphs show the frequency (middle) and numbers (bottom) of recovered $\gamma\delta$ and $\alpha\beta$ T cells per one mouse before and after EpCAM⁺ depletion. The results show the summary of 3 independent experiments with a total of 7 WT mice.

(B) CD4 versus CD8 profiles of CD45⁺TCR β ⁺-gated SI IELs isolated from WT mice before and after EpCAM⁺ depletion (top). The graphs show the frequency (middle) and numbers (bottom) of recovered CD4⁺ and CD8⁺ T cells per one mouse before and after EpCAM⁺ depletion. The results show the summary of 3 independent experiments with a total of 7 WT mice. The data are shown as the mean \pm SEM. p values were determined by two-tailed paired Student's t test (A and B). ns, non-significant.

left), and it also enriches for smaller-sized cells that are mostly IELs (Figure 11B, right). Indeed, EpCAM⁺ depletion routinely enriched CD45⁺ hematopoietic origin cells with \sim 90% purity from SI epithelial lavage (Figure 11C). The cellular composition of IELs showed similar frequencies of both $\gamma\delta$ and $\alpha\beta$ T cells before and after anti-EpCAM depletion (Figure 12A, top and middle), and resulted in minimal cell loss during purification (Figure 12A, bottom). Furthermore, the frequency of CD4⁺ and CD8⁺ IEL T cells also remained comparable before and after anti-EpCAM⁺ depletion (Figure 12B, top and middle), and we were able to recover SI IELs with minimal cell loss during purification (Figure 12B, bottom). Consequently, anti-EpCAM depletion permits the effective and high-yield enrichment of SI IELs.

Advantages

The anti-EpCAM⁺ method also allows for the improved recovery of minor cell populations, such as invariant natural killer T (iNKT) cells, when compared to the conventional density gradient method

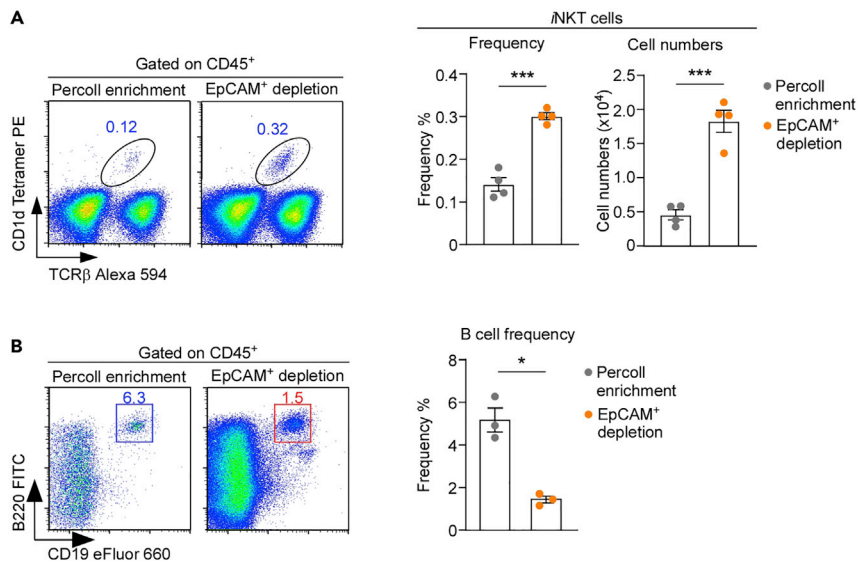


Figure 13. Advantages and limitations of EpCAM⁺ cell depletion

(A) CD1d Tetramer versus TCRβ profiles of CD45⁺ SI IELs isolated by density gradient (Percoll) enrichment or EpCAM⁺ depletion (left). The graphs (right) show the frequency and numbers of recovered CD1d Tet⁺ iNKT cells isolated by Percoll enrichment or EpCAM⁺ depletion. Results show the summary of 2 independent experiments with a total of 4 WT mice.

(B) B220 versus CD19 profiles of CD45⁺ SI IELs isolated by density gradient (Percoll) enrichment or EpCAM⁺ cell depletion (left). The frequencies of CD19⁺B220⁺ mature B cells (right) show the summary of 2 independent experiments with a total of 3 WT mice. The data are shown as the mean ± SEM. p values were determined by two-tailed paired Student's t test (A and B). * p < 0.05, *** p < 0.001.

(Figure 13A left). In fact, when using the anti-EpCAM⁺ method, the frequency of recovered iNKT cells is approximately 2 × greater and the number of cells yielded is around 4 × greater than the respective iNKT cell frequencies and numbers recovered using the density gradient method (Figure 13A right).

LIMITATIONS

EpCAM⁺ cell depletion requires the use of rat anti-EpCAM IgG antibodies (clone G8.8, rat IgG) and magnetic beads that are conjugated with anti-rat IgG antibodies. Importantly, the anti-rat IgG antibodies cross-react to mouse immunoglobulin, thus binding to mouse B cells. Therefore, cell depletion with rat IgG BioMag beads removes B cells from the SI epithelial cell eluates. Such a loss of B cells does not occur in SI IELs isolated using conventional density gradient methods (Figure 13B) (Velazquez et al., 2008). Consequently, the EpCAM⁺ depletion method is not recommended when aiming to isolate B cells from SI IELs.

TROUBLESHOOTING

Problem 1

Low cell counts (less than 50 million cells per mouse) in the SI eluates.

Potential solution

The Solution A should be always freshly prepared with EDTA and DL-dithiothreitol and kept at 37°C prior to SI tissue processing. Cells may stick to plasticware. Consider using plasticwares made from polypropylene which would minimize cell binding to the tube walls.

Problem 2

Incomplete removal of EpCAM⁺ cells from the SI epithelial eluate, which results in low purity of SI IELs and that can be caused by the following two reasons:

Insufficient amounts of anti-EpCAM antibodies.

Insufficient amounts of BioMag anti-rat-IgG beads.

Potential solution

Solution 1: Titrate the amount of anti-EpCAM antibodies to find the optimal amount or concentration of antibodies for epithelial cell depletion.

Solution 2: Because there can be differences between batches or lots of BioMag beads, the optimal amount of magnetic anti-rat-IgG beads should be re-titrated by performing small scale pilot experiments where increasing amounts of magnetic anti-rat-IgG beads are added to the isolation processes. Optimal amounts of magnetic beads can be determined by identifying the beads concentration that achieves the most effective removal of epithelial cells with the lowest amounts of beads.

Problem 3

Anti-rat IgG magnetic beads are not commercially available.

Potential solution

Protein A-conjugated magnetic beads would also work because the anti-EpCAM antibody (Clone: G8.8) is of the rat-IgG2a isotype which binds to protein A with high-affinity. Alternatively, biotin-conjugation of G8.8 antibodies and streptavidin-conjugated magnetic beads could be used.

Problem 4

Low cell viability of isolated SI IELs. The purified IELs are usually used for other downstream analyses, such as *in vitro* activation, differentiation, or functional assays. A low survival rate of the SI IELs is detrimental for the correct analyses of these cells.

Potential solution

Complete the tissue processing procedure as soon as possible following extraction of the SI. Always keep the buffers ice-cold, and prevent the cell suspension to warm up to 20°C–25°C before they are mixed with magnetic beads.

Problem 5

Magnetic separation of BioMag beads from cell suspension is inefficient.

Potential solution

Optimally, all the magnetic beads in the cell suspension should be captured by the magnetic separator. We routinely employ the 15 mL/50 mL magnetic tube separator from Polysciences (Cat# 84102S) to remove the beads, and we found this device highly effective. Nonetheless, inserting the tubes into the separator can sometimes result in creating some space between the magnet and the tube. Taping or clamping the tubes directly to the magnetic separator removes the space in-between, thus greatly increasing the effect of the magnetic field and improving the magnetic separation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jung-Hyun Park (Parkhy@mail.nih.gov).

Materials availability

No new materials were generated.

Data and code availability

All flow cytometry data acquired during this study are available upon request. No new dataset was generated.

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AUTHOR CONTRIBUTIONS

P.P. and V.G. performed the experiments, analyzed the data, and wrote the manuscript. J.-H.P. supervised the project and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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