University of Dundee

Isolation, Characterization, and Culture of Intestinal Intraepithelial Lymphocytes

James, Olivia Jane; Vandereyken, Maud; Swamy, Mahima

Published in:
Innate Lymphoid Cells

DOI:
[10.1007/978-1-0716-0338-3_13](https://doi.org/10.1007/978-1-0716-0338-3_13)

Publication date:
2020

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

James, O. J., Vandereyken, M., & Swamy, M. (2020). Isolation, Characterization, and Culture of Intestinal Intraepithelial Lymphocytes. In S. Amarnath (Ed.), *Innate Lymphoid Cells: Methods and Protocols* (pp. 141-152). (Methods in Molecular Biology; Vol. 2121). Humana Press. https://doi.org/10.1007/978-1-0716-0338-3_13

General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Isolation, characterisation and culture of intestinal intraepithelial lymphocytes

Olivia Jane James¹, Maud Vandereyken¹, Mahima Swamy¹

¹ Medical Research Council Protein Phosphorylation and Ubiquitylation Unit (MRC-PPU), School of Life Sciences, University of Dundee, Dundee, DD1 5EH, UK

Corresponding author: Dr. Mahima Swamy, MRC-PPU, Wellcome Trust Biocentre 3, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK. Email: m.swamy@dundee.ac.uk

Running title: Isolation and characterization of intestinal IEL

Abstract

Intestinal intraepithelial lymphocytes (IEL) comprise distinct groups of innate-like and memory T cells that collectively form one of the largest T cell compartments in the body. IEL are located within the intestinal epithelium and are the first immune cells in the gut to interact with the food, microbiota, and pathogens that the gut is continually exposed to. IEL can respond rapidly to external insults to protect the small intestinal epithelium but are also considered regulatory cells that are important to maintain the homeostasis of the gut. However, the mechanisms of IEL activation and their interactions with the epithelium remain largely elusive. Indeed, IEL are not commonly evaluated even in studies of gut immunology, potentially because they are perceived as being difficult to isolate and study. In this protocol, we present a simplified method to isolate IEL from the murine small intestine and provide representative data for flow cytometric analyses of the different IEL subsets. We also outline two procedures for culturing IEL, which can permit functional studies and coculture with epithelial cells. These strategies should make studies of this large but enigmatic T cell compartment more accessible and open up understanding of homeostatic mechanisms in the intestine, and tissue-associated immunity.

Key words: intraepithelial lymphocytes, IEL, innate-like lymphocytes, small intestine, $\gamma\delta$ T cells, unconventional T cells

1. Introduction

The intestinal lumen is home to the diverse network of commensal bacteria that are crucial for intestinal homeostasis, but also to many potentially pathogenic stimuli that could cause the body harm. The main role of the epithelium is nutrient absorption, but it also acts as an important physical barrier between the external environment (intestinal lumen) and the host (lamina propria). This physical barrier is reinforced by resident immune cells, such as intraepithelial lymphocytes (IEL), which are a specialised and extremely heterogeneous population of T lymphocytes that sit between the single-cell layer of epithelial cells. It is estimated that for every 10 epithelial cells, there is one IEL, and considering the surface area of the small intestine, IEL account for a substantial population of the body's lymphocytes [1]. Thus, understanding how these cells function in both health and disease is fundamental to our understanding of intestinal homeostasis.

IEL are T cells and therefore express a T cell antigen receptor (TCR) consisting of either $\alpha\beta$ or $\gamma\delta$ chains, alongside TCR co-receptors CD8 $\alpha\beta$ or CD8 $\alpha\alpha$ and to a lesser extent CD4(+/-). In mice, the most prevalent IEL subsets within the epithelium of the small intestine are those expressing TCR $\gamma\delta$ alongside CD8 $\alpha\alpha$ (TCR $\gamma\delta$ CD8 $\alpha\alpha$), which account for half the total IEL pool. The high percentage of cells expressing TCR $\gamma\delta$ is one of the main phenotypes distinguishing IEL from conventional peripheral blood T cells, where TCR $\gamma\delta$ -expressing T cells only account for a small percentage. The remaining IEL subsets are those expressing TCR $\alpha\beta$ in addition to either CD8 $\alpha\beta$ or CD8 $\alpha\alpha$ (TCR $\alpha\beta$ CD8 $\alpha\beta$ and TCR $\alpha\beta$ CD8 $\alpha\alpha$, respectively). IEL differ in many ways from conventional peripheral T cells, not only in the conformation of the TCR and CD8 coreceptors but also in their constitutive expression of activation markers such as CD44 and CD69. In addition, they express a range of other activating and inhibitory receptors, such as NK receptors, allowing them to rapidly respond to danger and stress signals in their microenvironment [1]. Further, as resident T cells, IEL do not recirculate and are retained in the epithelium by interactions such as $\alpha E\beta 7$ (also known as CD103) on their surface binding to E-cadherin on epithelial cells [2].

However, there is still relatively limited research into the functionality of these unconventional T cells, compared to T cell found in the spleen and blood.

Germane to our understanding of IEL is a good protocol to process and isolate these cells. Multiple protocols exist for IEL isolation [3, 4], and various technical improvements have been suggested, including the use of multiple Nylon wool columns [5], multiple Percoll gradients [6], elutriation centrifugation [7], mostly designed to improve the purity of IEL preparations from intestinal epithelial cells. However, we find that for routine analyses, such as evaluating the subsets and numbers of IEL in various relevant knockout mouse models, the simplified protocol presented here is sufficient to get large numbers of IEL (up to 10 million per murine small intestine), within 2 hours of euthanizing a mouse. This protocol enriches murine small intestinal IEL to about 50-60%; if purer populations are needed, this can be achieved by using a magnetic cell separation technique to quickly enrich for a specific population, for example, CD8 IEL. We have described a straightforward staining panel that allows for not only the identification of total IEL but also distinction between the various subsets. Finally, we describe short-term culture of IEL supported by IL-15 [8], and a longer term culture protocol for intestinal IEL that involves stimulating IEL through their TCR in the presence of a cocktail of cytokines [9, 10].

2. Materials

2.1 Dissection of Murine Small Intestine

Dissection instruments (Fine Science Tools) consisting of:

Fine scissors (Toughcut, straight)

Reusable Feeding Needle 20 Gauge Straight (referred to as gavage needle in methods)

Bonn artery scissors with Ball tip

Graefe Extra Fine Forceps Curved Serrated (2x)

10-20ml disposable syringe

1X Dulbecco's Phosphate Buffered Saline (PBS), store at 4°C.

Supplemented RPMI media: RPMI 1640 media, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic mix, 1% L-glutamine (all from Thermo Fisher Scientific). Incubate at 37°C in a water bath prior to IEL isolation.

2.2 Isolation of IEL from Small Intestine

100µm cell strainers (1x per gut)

DL-Dithiothreitol (DTT): 1M stock solution in H₂O, sterile filter and store at -20°C in 500µl aliquots. Use for IEL isolations at 1Mm in supplemented media

10X PBS

Percoll: Prepare a 90% isotonic stock by adding 110ml of 10x PBS to 1L Percoll, this stock is kept at 4°C and used to make fresh working stocks (36/67%) of Percoll solutions for IEL isolations

36% Percoll: prepare 10ml by adding 4ml of 90% Percoll stock to 6ml 1x PBS

67% Percoll: prepare 10ml by adding 7.5ml of 90% Percoll stock to 2.5ml 1x PBS

2.3 Characterization of IEL Subsets by Flow Cytometry

FACS buffer: 1x PBS, 1% FBS, 0.05% sodium azide

Fc block (Purified anti-mouse CD16/32 Antibody, clone: 93): 1:200 in FACS buffer

4',6-diamidino-2-phenylindole (DAPI): 1:20,000 in FACS buffer

Antibodies for identification of IEL subsets:

TCRβ-APC (clone: H57-597): 1:200 in FACS buffer

TCRγδ-PerCP-eFluor710 (clone: GL3): 1:200 in FACS buffer

CD8 β -FITC (clone: H35-17.2): 1:500 in FACS buffer

CD8 α -PE (clone: 53-6.7): 1:400 in FACS buffer

CD4-PE/Cy7 (clone: RM4-5): 1:400 in FACS buffer

2.4 Culture of Intestinal IEL

Culture medium: RPMI 1640 media, supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM β -mercaptoethanol, 2.5 mM HEPES, and non-essential amino acids.

anti-CD3 ϵ antibody (clone: 2C11).

Cytokines: Human IL-2 (Proleukin, stock 10,000U/ml), murine soluble IL-15 (Immunotools, stock 100 μ g/ml), murine IL-3 (R&D Systems, stock 1x10⁴U/ml), murine IL-4 (R&D Systems, stock 2x10⁴U/ml), murine IL-15/IL-15R Complex Recombinant Protein (eBioscience, stock 100 μ g/ml). Prepare stock concentrations in PBS containing 1% BSA, after dissolving lyophilized cytokines as per the manufacturer's instructions.

3. Methods

3.1 Dissection of Murine Small Intestine

1. Cut intestine from ~1cm below stomach (proximal duodenum), pulling out the entire length of the small intestine and removing the fat and mesentery using tweezers as you do so. Make the final cut to release the small intestine ~1cm before caecum (terminal ileum) (*See Fig. 1; pictures 1-3, also see Note 1 and 2*).
2. Flush the intestine with 10-20ml ice-cold PBS using a syringe with a gavage needle attached. Put the gavage needle into the widest opening of the intestine (the end cut from nearest the stomach)

and slowly infuse PBS until all the luminal content is removed. Push the gut onto the gavage needle as you flush until it is empty (*see Fig. 1; pictures 4 and 5, also see Note 2*).

3. [OPTIONAL] Peyer's patches may be removed at this point using curved dissection scissors (*see Note 3*).
4. Using ball tip scissors cut the intestine longitudinally to expose the lumen. Insert the ball tip of the scissors into the widest opening of the intestine and continue to push the rest of the intestine onto the scissors while simultaneously cutting until the intestine is cut open along its length (*see Fig. 1; picture 6*).
5. Using forceps, pick up the intestine midway along its length so that it is folded in half, and hold it over a 50ml falcon tube containing 25ml pre-warmed supplemented RPMI media (*see Fig. 1; picture 7, see Note 4*).
6. Cut the small intestine transversely into small 5-10mm pieces into the media (*see Fig. 1; picture 7*).

3.2 Isolation of IEL from the Small Intestine

1. Add DTT to the media containing the intestinal pieces to a final concentration of 1mM.
2. Incubate for 40 minutes with constant shaking at room temperature (*see Note 5*).
3. Centrifuge for 5 minutes at 500 g at room temperature.
4. Discard supernatant and resuspend the pieces in 10ml warm supplemented media.
5. Vortex at medium speed for 3 minutes (*see Note 6*).
6. Pass the resuspension through a 100µm cell strainer and collect the flow-through into a new 50ml Falcon tube (this flow-through contains the IEL).
7. Put the intestinal pieces back into the Falcon tube and resuspend in 10ml of fresh media.
8. Repeat steps 9 & 10 for a total of 2 vortex steps.
9. Use a further 30ml to wash the cell strainer completely and top up the flow-through to 50ml.
10. Discard the intestinal pieces.

11. Centrifuge the resulting flow-through for 5 minutes at 500 g at room temperature.
12. The cells will be in a dense pellet with a red tint, resuspend the pellet in 8ml 36% Percoll and pipette up and down to thoroughly mix.
13. For good separation of IEL from epithelial cells, we set up two Percoll gradients per gut.
Take 2 fresh 15ml falcon tubes and add 4ml of 67% Percoll into each.
14. Create a Percoll density gradient by overlaying 4ml of 36% Percoll (mixed with the cells) on top of the 67% Percoll (*see Note 7*).
15. Centrifuge for 30 minutes at 700 g at room temperature without brake (*see Note 8*).
16. Following centrifugation, the cells of interest appear at the interface between the 36% Percoll top layer and the 67% Percoll bottom layer (*see Fig. 2*).
17. Pipette or aspirate off the epithelial cells and top layer of Percoll containing epithelial cells.
18. Using a p1000 pipette, collect the IEL from the interface, dispensing them into a new 15ml Falcon tube.
19. Top up to 15ml with warm media and centrifuge at 700 g for 5 minutes to wash cells out of Percoll.
20. Repeat the wash step in medium of choice depending on what the cells are going to be used for, for example; if the cells are to be put into culture, do a second wash in culture medium, if the cells are to be used for flow cytometry do a second wash in FACS buffer.
21. Isolated IEL are now ready to use, expected yield is 4×10^6 - 1×10^7 total live cells per intestine (*see Note 9*).
22. If higher purity of IEL is required, it is possible to perform a second Percoll step after the 36/67% density gradient by removing the IEL layer, spinning down the cells and resuspending the cell pellet in 5ml of 40% Percoll to be centrifuged for 20 minutes at 500 g. This will further separate some of the remaining epithelial cells from the IEL, giving a purer population. However, for the purest IEL populations, it is advisable that immunomagnetic

isolation kits are used, which will incur a decrease in cell numbers but will improve purity (see **Note 10, Fig. 3**).

3.3 Characterization of IEL Subsets by Flow Cytometry

The major IEL subsets routinely retrievable from the murine small intestine are: $\text{TCR}\gamma\delta^+ \text{CD4}^- \text{CD8}\alpha\alpha^+$ ($\text{TCR}\gamma\delta \text{CD8}\alpha\alpha$), $\text{TCR}\alpha\beta^+ \text{CD4}^- \text{CD8}\alpha\alpha^+$ ($\text{TCR}\alpha\beta \text{CD8}\alpha\alpha$), $\text{TCR}\alpha\beta^+ \text{CD4}^- \text{CD8}\alpha\beta^+$ ($\text{TCR}\alpha\beta \text{CD8}\alpha\beta$), $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD8}\alpha^-$ ($\text{TCR}\alpha\beta \text{CD4}$) and $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD8}\alpha\alpha^+$ ($\text{TCR}\alpha\beta \text{DP}$). $\text{TCR}\alpha\beta \text{CD8}\alpha\beta$ and $\text{TCR}\alpha\beta \text{CD4}$ IEL are commonly viewed as tissue-resident memory T cells that have migrated into the epithelium after antigen exposure [11, 12]. Conversely, the former two subtypes are commonly viewed as unconventional or innate-like T cells that constitutively populate the intestinal epithelium after migrating there directly from the thymus or having undergone selective expansion *in situ* [13]. A basic staining protocol to characterize the presence of these four subsets by flow cytometry is presented below.

1. Count cells and resuspend to a final concentration of 1 million cells/ml in FACS buffer.
2. Plate cells out at 200 μ l per stain (per well) in a round-bottom 96-well plate (see **Note 11**).
3. Centrifuge the plate at 500g for 2 minutes at 10°C.
4. Flick off supernatant and vortex plate to resuspend cells.
5. Add 25 μ l Fc block (1:200 in FACS buffer) and incubate for 5 minutes on ice.
6. Make a 2x concentrated multistain mastermix of all fluorophore-conjugated antibodies to be used in FACS buffer (see **Table 1** for antibodies we use to identify IEL subsets).
7. Prepare appropriate compensation controls.
8. Add 25 μ l of fluorophore-antibody mastermix to the cells containing the Fc block.
9. Incubate for 15 minutes on ice and protect from light.
10. Add 150 μ l FACS buffer and centrifuge at 500g for 2 minutes at 10°C.
11. Flick off supernatant and vortex the plate.
12. Add 200 μ l FACS buffer + live/dead dye such as DAPI to acquire samples on a flow cytometer (see **Note 12**).

13. Transfer the stained samples and compensation controls to suitable flow cytometry tubes and acquire on a flow cytometer, such as the BD LSR Fortessa.
14. After acquisition and appropriate compensation, the basic gating strategy and expected distributions of the different IEL subsets are shown in **Fig. 4**.

3.4 Culture of Murine Intestinal IEL

IEL can be sorted or plated out immediately after Percoll isolation for further culture. IEL are prone to cell death *ex vivo* [14], however IL-15 maintains their survival in the short term [8], and we find that IL-15 complexed to the IL-15 receptor α subunit is more efficient than IL-15 on its own. For longer term cultures, and to expand IEL numbers for downstream analyses, TCR stimulation in the presence of a cocktail of cytokines is required.

For short-term culture:

1. Count IEL and resuspend to a final concentration of 1 million cells/ml in culture medium containing 20ng/ml murine IL-15/IL-15R Complex.
2. Plate cells in a 96-well round-bottom plate at 200 μ l per well.
3. Incubate at 37°C, 10% CO₂. Cells can be maintained in IL-15 for 1-3 days.

For longer term cultures:

4. Coat a 96-well round bottom plate with anti-CD3 ϵ antibody (1 μ g/ml in PBS, 50 μ l per well) for 2 hours at 37°C.
5. Wash with 1xPBS.
6. Count IEL and resuspend 0.5 million cells/ml in culture medium. Add a cytokine cocktail containing IL-2 (10 IU/ml), IL-3(100 IU/ml), IL-4 (100 IU/ml) and soluble IL-15 (100 ng/ml).
7. Plate cells in the anti-CD3 ϵ coated 96-well round bottom plate at 200 μ l per well.
8. Incubate at 37°C, 10% CO₂ for 48 hours.

9. Wash the cells twice and resuspend in IL-2 (10 IU/ml) containing culture medium at a concentration of 100,000 cells per well in a fresh noncoated 96-well plate (*see Note 13*).
10. Replate the cells every 3-4 days in fresh IL-2-containing medium. Cells can be utilized for further downstream analyses after a further 10-12 days in culture.

4. Notes

1. Remove the small intestine as quickly as possible after culling the mouse as this has a significant influence on the number of IEL isolated. It is also important to keep the gut in ice-cold PBS until it is processed into the RPMI, to minimize cell death and losses in cell numbers.
2. There are on average 6-10 Peyer's patches in a healthy adult mouse small intestine, and they tend to be concentrated at the ileal end. We do not routinely remove Peyer's patches, as with the mild isolation protocol above, there is minimal contamination of IEL with Peyer's patch lymphocytes, and these can normally be gated out as they are mainly CD4⁺ CD8 α ^{-ve} CD103^{-ve} T cells.
3. Do this within a high-walled beaker as luminal content tends to spray through leaks under tension.
4. A minimum of 10ml media per gut can be used.
5. This depends on what is available. We use a rotating shaker at room temperature; however 37°C is optimal.
6. As a guide, a medium speed on a Vortex Genie is achieved by setting the speed between 7 & 8.
7. Take your time when creating the Percoll density gradients as the layers can easily mix if you are not careful, a good gradient is best achieved using a 3ml Pastette or a Pipette Boy set at the lowest ejection speed.
8. To achieve a low brake force in a Beckman Coulter Allegra X-12R centrifuge, set acceleration to 6 and deceleration to 3.

9. The yield tends to increase with age and varies dependent on the facility in which the mice are bred. Numbers indicated here are from C57BL6/J mice obtained from Charles River and analyzed between 8 and 12 weeks of age.
10. More than 90% of live cells isolated via the IEL isolation methods described here are CD45+, >80% express CD3 ϵ and >60% are CD8 α + (*see Fig. 3*), making these surface receptors ideal for enrichment of IEL following a Percoll density gradient. We routinely use a CD8 α positive selection kit to further purify IEL, such as the EasySep™ CD8 α positive selection kit provided by StemCell Technologies.
11. Staining in a 96-well plate allows you to use less cells and a smaller amount of antibody, it can also be faster when washing and more accurate due to similar dead volume in each sample after every wash.
12. There are many dead epithelial cells remaining in the stained cell samples, so adding a live/dead discrimination dye is vital for exclusion of these cells and hence for cleaner gating of IEL subpopulations.
13. During culture of IEL, there is always an initial loss in cell numbers after 1 day, but numbers then stay stable (in short-term cultures) or recover and increase (in long-term cultures).

Acknowledgments

MV and MS are supported by the Wellcome Trust and Royal Society (Sir Henry Dale Fellowship to MS, 206246/Z/17/Z), and OJJ is supported by a Wellcome Trust PhD studentship (215309/Z/19/Z). We are grateful to the members of Prof. Adrian Hayday's lab, King's College London, from whom these protocols were modified, particularly Dr. Sarah Clarke, who developed the protocol for long term culture of IEL

References

1. Bas A, Swamy M, Abeler-Dörner L et al. (2011) Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 108:4376-4381
2. Binda E, Erhart D, Schenk M et al. (2009) Quantitative isolation of mouse and human intestinal intraepithelial lymphocytes by elutriation centrifugation. *Journal of immunological methods* 344:26-34
3. Lai YG, Gelfanov V, Gelfanova V et al. (1999) IL-15 promotes survival but not effector function differentiation of CD8⁺ TCR α beta⁺ intestinal intraepithelial lymphocytes. *Journal of immunology* 163:5843-5850
4. Lee Mosley R, Klein JR (1992) A rapid method for isolating murine intestine intraepithelial lymphocytes with high yield and purity. *Journal of immunological methods* 156:19-26
5. Lefrancois L, Lycke N (2001) Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer's patch, and lamina propria cells. *Current protocols in immunology* / edited by John E Coligan [et al] Chapter 3:Unit 3.19
6. Lundqvist C, Hammarström ML, Athlin L et al. (1992) Isolation of functionally active intraepithelial lymphocytes and enterocytes from human small and large intestine. *Journal of immunological methods* 152:253-263

7. McDonald BD, Jabri B, Bendelac A (2018) Diverse developmental pathways of intestinal intraepithelial lymphocytes. *Nature Reviews Immunology* 18:514-525
8. Montufar-Solis D, Klein JR (2006) An improved method for isolating intraepithelial lymphocytes (IELs) from the murine small intestine with consistently high purity. *Journal of immunological methods* 308:251-254
9. Mucida D, Husain MM, Muroi S et al. (2013) Transcriptional reprogramming of mature CD4⁺ helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes. *Nature Immunology* 14:281-289
10. Olivares-Villagómez D, Van Kaer L (2018) Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. *Trends in immunology* 39:264-275
11. Schenkel JM, Masopust D (2014) Tissue-Resident Memory T Cells. *Immunity* 41:886-897
12. Schön MP, Arya A, Murphy EA et al. (1999) Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *Journal of immunology* 162:6641-6649
13. Swamy M, Abeler-Dörner L, Chettle J et al. (2015) Intestinal intraepithelial lymphocyte activation promotes innate antiviral resistance. *Nature Communications* 6:7090
14. Viney JL, Macdonald TT (1990) Selective death of T cell receptor gamma/delta⁺ intraepithelial lymphocytes by apoptosis. *European Journal of Immunology* 20:2809-2812

Table 1. Staining panel (example) and antibody dilutions.

Sample	Antibody-Fluorophore (2x concentrated dilution)
Sample 1: Multistain mastermix for identifying IEL (example)	CD4-PE Cy7 (1:200)
	TCR β -APC (1:100)
	TCR $\gamma\delta$ -Percp ef710 (1:100)
	CD8 α -PE (1:200)
	CD8 β -FITC (1:250)

This table shows a basic staining panel used to identify IEL isolated via the methods described in this chapter. The fluorophores used are for demonstration purposes and can be substituted as appropriate.

Fig. 1 Dissection of murine small intestine – step by step representative images for protocol**Fig. 2 Diagram of 36/67% Percoll density gradient before and after centrifugation**

Before centrifugation, 4ml of resuspended cells in 36% Percoll is overlaid onto 4ml 67% Percoll. After centrifugation, the epithelial cells appear as a dense layer at the very top with more epithelial cells diffused amongst the 36% Percoll layer. The IEL are found at the interface between the 36% and 67% Percoll layers as a cloud-like band. Beneath the 67% Percoll layer, at the base of the 15mL centrifugation tube, a small pellet of red blood cells and dead cells are seen.

Fig. 3 Flow cytometric data showing the expression of various surface markers on cells isolated via Percoll density gradient.

Small lymphocytes account for about half of all the cells isolated using the methods described in this manuscript, and most (>90%) are live as judged by lack of expression of live/dead dye such as DAPI. The percentage of live lymphocytes will vary from mouse to mouse and will also depend on quality of the isolation. Generally, >90% of all live lymphocytes isolated via this method are CD45+, >70% are CD3ε+ and >60% are CD8α+, making these surface receptors useful markers to use for magnetic- or fluorescence-based cell separation techniques to gain purer populations of IEL following a Percoll separation.

Fig. 4 Identification of IEL subpopulations

A) Gating strategy used to identify distinct IEL subpopulations using flow cytometry. Representative plots show IEL isolated using a 36/67% Percoll density gradient. Cells were gated on size using forward scatter (FSC) vs side scatter (SSC) to separate out cells of interest from debris/non-lymphoid cells. Live cells (>90%) were gated on as those that did not express DAPI, as DAPI only penetrates cells with permeabilized cell membranes such as those of dead cells. Live cells were then separated into the various IEL subpopulations based on cell surface marker expression of T cell receptors and T cell receptor-associated proteins; TCRγδ, TCRβ, CD4, CD8α and CD8β. **B)** Expected frequencies of IEL subpopulations using the suggested gating strategy, shown as a percentage of live single cells. TCRαβ DP are not depicted in the figure as they typically account for <1% of the total live cells. Each dot represents 1 murine IEL isolation; n=8.

Before centrifugation

After centrifugation

% Small Lymphocytes

% Live

% CD45+

% CD3+

% CD8α+

FSC-A

A **B**