DOI:
10.3791/63554(2022)

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

TITLE:

Co-culture of murine small intestine epithelial organoids with innate lymphoid cells

AUTHORS AND AFFILIATIONS:

Emily Read[1,2*], Geraldine M Jowett[1,2,ψ*], Diana Coman[1], Joana F Neves[1†]

1 Centre for Host-Microbiome Interactions, King's College London, London, UK

2 Wellcome Trust Cell Therapies and Regenerative Medicine PhD Programme, King's College London, London, UK

[†]to whom correspondence should be addressed: joana.pereira_das_neves@kcl.ac.uk

*these authors contributed equally to this work.

[ψ] Present address: Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge University, UK

SUMMARY:

This protocol offers detailed instructions for establishing murine small intestine organoids, isolating type-1 innate lymphoid cells from the murine small intestine lamina propria, and establishing 3-dimensional (3D) co-cultures between both cell types to study bi-directional interactions between intestinal epithelial cells and type-1 innate lymphoid cells.

ABSTRACT:

Complex co-cultures of organoids with immune cells provide a versatile tool for interrogating the bi-directional interactions that underpin the delicate balance of mucosal homeostasis. These 3-dimensional, multi-cellular systems offer a reductionist model for addressing multi-factorial diseases and resolve technical difficulties that arise when studying rare cell types such as tissue resident innate lymphoid cells (ILCs). The following article describes a murine system that combines small intestine organoids and small intestine lamina propria derived helper-like type-1 ILCs (ILC1s), which can be readily extended to other ILC or immune populations. ILCs are a tissue resident population that are particularly enriched in mucosa, where they promote homeostasis and rapidly response to damage or infection. Organoid co-cultures with ILCs have already begun shedding light on new epithelial-immune signalling modules in the gut, revealing how different ILC subsets impact intestinal epithelial barrier integrity and regeneration. This protocol will enable further investigations into reciprocal interactions between epithelial and immune cells, which hold the potential to provide new insights into the mechanisms of mucosal homeostasis and inflammation.

INTRODUCTION:

Communication between the intestinal epithelium and gut-resident immune system is central to the maintenance of intestinal homeostasis[1]. Disruptions to these interactions are associated with both local and systemic diseases, including Inflammatory Bowel Disease (IBD) and gastrointestinal cancers[2]. A notable example of one more recently described critical regulator of homeostasis comes from the study of innate lymphoid cells (ILCs), which have emerged as key players in the intestinal immune landscape[3]. ILCs are a group of heterogenous innate immune cells that regulate intestinal homeostasis and orchestrate inflammation largely through cytokine-mediated signalling[4].

Murine ILCs are broadly divided into subtypes based on transcription factor, receptor and cytokine expression profiles[5]. Type-1 ILCs, which include cytotoxic Natural Killer (NK) cells and helper-like type-1 ILCs (ILC1s), are defined by expression of the transcription factor (eomesodermin) Eomes and T-box protein expressed in T cells (T-bet)[6] respectively, and secrete cytokines associated with T helper type-1 (T_H1) immunity: interferon-γ (IFNγ) and tumour necrosis factor (TNF), in response to interleukin (IL)-12, IL-15 and IL-18[7]. During homeostasis, tissue resident ILC1s secrete Transforming Growth Factor ß (TGF-ß) to drive epithelial proliferation and matrix remodelling[8]. Type-2 ILCs (ILC2s) primarily respond to helminth infection via secretion of T helper type-2 (T_H2) associated cytokines: IL-4, IL-5 and IL-13; and are characterized by expression of retinoic acid related orphan receptor (ROR) α (ROR-α)[9] and GATA Binding Protein 3 (GATA-3)[10–12]. In mice, intestinal "inflammatory" ILC2s are further characterized by expression of Killer cell lectin-like receptor (subfamily G member 1, KLRG)[13] where they respond to epithelial tuft-cell derived IL-25[14,15]. Finally, type-3 ILCs, which include lymphoid tissue inducer cells and helper-like type-3 ILCs (ILC3s), are dependent on the transcription factor ROR-γt[16], and cluster into groups that secrete either Granulocyte macrophage colony stimulating factor (GM-CSF), IL-17 or IL-22 in response to local IL-1β and IL-23 signals[17]. Lymphoid tissue inducer cells cluster in Peyer's patches and are crucial for the development of these secondary lymphoid organs during development[18], whereas ILC3s are the most abundant ILC subtype in the adult murine small intestine lamina propria. One of the earliest murine intestinal organoid co-culture systems with ILC3s was harnessed to tease apart the impact of the cytokine IL-22 on Signal Transducer And Activator Of Transcription 3 (STAT-3) mediated Leucine Rich Repeat Containing G Protein Coupled Receptor 5 $(Lgr5)^+$ intestinal stem cell proliferation[19], a powerful example of a regenerative ILC-epithelial interaction. ILCs exhibit imprint-heterogeneity between organs[20,21] and exhibit plasticity between subsets in response to polarising cytokines[22]. What drives these tissue-specific imprints and plasticity differences, and what role they play in chronic diseases like IBD[23], remain exciting topics that could be addressed using organoid co-cultures.

Intestinal organoids have emerged as a successful and reliable model to study the intestinal epithelium[24,25]. These are generated by culturing intestinal epithelial Lgr5^{+} stem cells, or entire isolated crypts which include Paneth cells as an endogenous source of Wnt Family Member 3A (Wnt3a). These 3-dimensional (3D) structures are maintained either in synthetic hydrogels[26] or in biomaterials that mimic the basal lamina propria, for instance thermal-crosslinking basal extracellular matrix (TBEM), and are further supplemented with growth factors that mimic the surrounding niche, most notably Epithelial Growth Factor (EGF), the Bone morphogenetic protein (BMP)-inhibitor Noggin, and an Lgr5-ligand and Wnt-agonist R-Spondin1[27]. Under these conditions, organoids maintain epithelial apico-basal polarity, and recapitulate the crypt-villi structure of the intestinal epithelium with budding stem cell crypts that terminally differentiate into absorptive and secretory cells in the centre of the organoid, which then shed into the internal pseudolumen by anoikis[28]. Although intestinal organoids alone have been hugely advantageous as reductionist models of epithelial development and dynamics in isolation[29,30], they hold tremendous future potential for understanding how these behaviours are regulated, influenced, or even disrupted by the immune compartment.

In the following protocol a method of co-culture between murine small intestinal organoids and lamina propria derived ILC1s is described, which was recently used to identify how this population unexpectedly decreases intestinal signatures of inflammation, and instead contributes to increased epithelial proliferation via TGF-ß in this system[8].

PROTOCOL:

All experiments must be completed in accordance and compliance with all relevant regulatory and institutional guidelines for animal use. Ethical approval for the study described in the following article and video was acquired in accordance and compliance with all relevant regulatory and institutional guidelines for animal use.

All animals were culled by cervical dislocation according to standard ethical procedure, conducted by trained individuals. Slicing of the femoral artery or decapitation was conducted (as appropriate to the protocol in hand) as confirmatory assessments of death, prior to organ and tissue harvest. Animals were housed under specific-pathogen-free conditions (unless stated otherwise) at accredited Charles River and King's College London animal units in accordance with the UK Animals (Scientific Procedures) Act 1986 (UK Home Office Project License (PPL:70/7869 to September 2018; P9720273E from September 2018).

1 Establishing murine small intestinal organoids

This section of the protocol describes the generation of intestinal organoids from the murine small intestine. Crypts are first isolated from tissue, resuspended in TBEM and then incubated with media containing EGF, Noggin and R-Spondin (ENR). Establishing murine small intestinal organoids has also been well described elsewhere[24,25,27].

1.1 Place BME on ice (40 µL/well, 500 µL will thaw in approximately 2-4h, do not leave at room temperature) to thaw and put a standard tissue-culture treated 24-well plate in a 37 °C incubator to pre-warm.

1.2 Prepare ~4 mL or 550 mL per well of ENR medium by adding EGF, Noggin and R-Spondin to basal medium (**Table 1**) and place in a 37 °C incubator.

1.3 Cull a 6-12 week old animal following approved Schedule 1 procedures, and dissect out the small intestine using forceps and microdissection scissors, place in 15 mL phosphate-buffered saline (PBS) on ice.

1.4 Using the stomach and caecum as points of orientation, isolate the desired region of the small intestine (duodenum, jejunum or ileum). In this protocol, the ileum is isolated.

1.5 Submerge the intestine in PBS on a 10cm^2 Petri dish on ice and use forceps to gently, but completely, remove fat from the intestine.

1.6 Cut the tissue longitudinally using microdissection scissors (preferably rounded tip to avoid perforation). Keeping the intestine submerged in PBS, shake to remove faecal matter and maintain the mucous side up to track tissue orientation.

1.7 Transfer tissue to the dry lid of a Petri dish on ice, placing the intestine mucous/villus side up. Holding one end of the intestine with forceps, gently scrape away mucus using the angled edge of a clean glass slide.

1.8 Fill the plate with ice-cold PBS and rinse the tissue.

1.9 Pre-coat a 50 mL tube with PBS2 (PBS + 2% FCS, **Table 1**) to prevent adhesion of the tissue to the plastic and add 10 mL of ice-cold PBS to this tube. Cut the intestine into small (~2 mm) segments into the coated 50 mL tube with PBS.

1.10 Pre-coat a 25 mL pipette with PBS2. Using this pipette, take the segments up and down 5-10 times to clean the tissue fragments.

1.11 Allow the segments to settle for (15-30 seconds), remove and discard the PBS supernatant, and repeat step 1.10 and 1.11 until the solution is clear (approximately 3-4 times).

1.12 Allow the segments to settle and discard PBS supernatant . Add 30 mL ice-cold crypt isolation buffer (**Table 1**).

1.13 Place the tubes on a horizontal roller or rocker at 30-60 revolutions per minute (rpm) (or gentle) at 4 °C for 30 minutes. Do not incubate for faster speed, higher temperature, or longer duration as the crypts will prematurely dislodge and become single cells with lower viability and yield.

NOTE: From this stage onwards, all procedures should be conducted in an aseptic environment using sterile materials and reagents.

1.14 Allow intestinal segments to settle at the base of the 50 mL tube. Carefully remove crypt isolation buffer and place in a 50 mL tube on ice. *[If crypt isolation process was too rigorous, crypts maybe seen in this fraction. It can therefore be kept as a reserve but will optimally be discarded at the end of the protocol.]*

1.15 Add 20 mL of ice-cold PBS to intestinal segments. Pre-coat a 25 mL pipette with PBS2 and use this to pipette intestinal segments up and down 5-8 times.

1.16 Let the segments settle for 30 seconds. Pre-coat a 50 mL tube and 25 mL pipette with PBS2. Using this pipette, collect the supernatant (fraction 1) into the pre-coated 50 mL tube and place on ice. [*This fraction serves to rinse away remaining crypt isolation buffer. However, it also serves as an additional quality control step, if pipetting was too vigorous crypts will be abundant in this rinse fraction, and if it was too gentle there will be very little debris in this fraction. Optimally, fraction 1 is discarded at the end of the protocol, but it can be used to make organoids if issues arise with fractions 2-4].*

1.17 Repeat step 1.15 and 1.16 but adding 10 mL of ice-cold PBS rather than 20 mL and placing the supernatant in a fresh PBS2 pre-coated 50 mL tube (fraction 2).

1.18 Repeat step 1.14 twice more, however pool the resulting supernatant with fraction 2 (into the same 50 mL tube from step 1.14) to give fractions 2-4. This single 50 mL tube should contain the dislodged crypts in 30 mL of ice-cold PBS.

1.19 Switch on an inverted light microscope. Remove a 50 µL aliquot from the pooled 2-4 fractions and place onto a coverslip. Assess for the presence of epithelial crypts. *[If crypts are not present, repeat steps 1.17-1.18 using more force during pipetting until crypts are released. Ensure that crypts were not prematurely dislodged in the crypt isolation buffer from step 1.14 or in fraction 1 from step 1.16.]*

1.20 Pre-coat a 100 µm strainer and 50 mL tube with PBS2. Pass the pooled crypt fractions through this strainer and into the pre-coated 50 mL tube.

1.21 Spin down strained crypt fractions at 300 *g* at 4 °C for 5 minutes.

1.22 Discard the supernatant and resuspend the crypts in 10 mL ice-cold Advanced DMEM/F12. Move the crypts to a 15 mL tube.

1.23 Spin down crypts at 180 *g* at 4 °C for 5 minutes to remove single cells and lymphocytes.

1.24 Resuspend crypts in 1 mL ice-cold Advanced DMEM/F12.

1.25 Remove a 10 µL aliquot and place onto a coverslip. Using an inverted light microscope, count the number of crypts to determine crypt concentration.

1.26 Calculate the volume required for ~400 and ~1500 crypts. Pre-coat a p1000 tip with PBS2 and using this tip pipette the required volumes (containing ~400 and ~1500 crypts) into separate 1.5 mL tubes.

1.27 Spin down crypts at 300 *g* at 4 °C for 5 minutes.

1.28 Remove as much supernatant as possible, then resuspend crypts in 80 µL TBEM on ice (pipette tips can be pre-cooled at 4 °C to prevent matrix gelation, which occurs at 12 °C).

1.29 Remove 24-well plate from step 1.1 from the incubator. Gently pipette 40 µL of crypts into the centre of a well in a slow, circular motion to form a flat but 3D dome structure, or into three separate small domes. *[Viability of the organoids is the lowest in the centre of the dome where nutrients and gases permeate less effectively[31], thus maximising the surface area exposed to media while maintaining a clear 3D structures is critical.]*

1.30 Repeat step 1.29 to create a total of two wells with a density of ~200 crypts per well and two with a density of ~750 crypts per well. Directly place the plate in a 37 °C 5% CO_2 incubator and for 15-20 minutes, taking care to not disrupt the viscous but still liquid matrix domes.

1.31 Add 550 µl of pre-warmed ENR media per well (from step 1.2) and incubate at 37 °C 5% CO_2. At this stage, it is suggested to supplement ENR medium with 5 µM of Wnt agonist (CHIR 99021) and/or 5 µM Rho Kinase inhibitor (Y-27632) for 24-48 hours to improve yield and efficiency of crypt isolation.

1.32 Crypts should close to form rounded structures within 24 hours, and crypt buds should appear within 2-4 days (**Figure 1A**). Replace with fresh ENR medium every ~2 days or when the phenol pH indicator in the culture medium turns pale orange, and before it turns yellow.

2 Maintenance of murine small intestinal organoids

This section of the protocol describes the maintenance and passaging of murine small intestinal organoids. Organoids are first harvested and then mechanically disrupted using a bent p1000 tip. This process breaks large organoids consisting of numerous crypts into multiple smaller fragments for expansion and releases dead cells that have accumulated in the pseudolumen. Murine small intestinal organoid maintenance has also been well described elsewhere[24,25,27].

NOTE: All procedures should be conducted in an aseptic environment using sterile materials and reagents. Passage or expand organoids once every 4-5 days, before bursting of the organoids occurs from substantial accumulation of debris in the organoid lumen. Organoids can be passaged at ratio of 1:2-1:3 depending on organoid density, which will optimally be between 100-200 organoids per well.

2.1 As in steps 1.1 and 1.2, thaw TBEM on ice (40 µL/well). Prepare ENR medium (550 µl per well), place medium and a standard tissue culture treated 24-well plate in a 37 °C incubator.

2.2 Remove the plate containing organoids from the incubator. Discard the media from the well to be passaged.

2.3 Add 500 µl ice-cold Advanced DMEM/F12 to the well and using this p1000 tip (pre-coated in media to avoid organoid sticking to tip interior) harvest the organoids into a 15 mL tube.

2.4 Rinse the bottom of the well with 250-300 µl ice cold Advanced DMEM/F12, ensuring no organoids remain in the well, and pool into the 15 mL tube containing harvested organoids from step 2.3. If passaging multiple wells, repeat steps 2.3 and 2.4, pooling organoids from multiple wells into the same 15 mL tube.

2.5 Spin down organoids at 300 *g* 4 °C for 3 minutes.

2.6 Upon centrifugation, four fractions should become visible: a base fraction with healthy organoids, a central and clear matrix fraction, a matrix fraction containing single and dead cells, and an upper supernatant layer containing single and dead cells. Remove the supernatant, the debris fraction, and as much of the clear matrix fragment as possible without disrupting the pellet of organoids.

2.7 Gently bend the tip of a p1000 pipette tip (approximately 2-5 mm bend). Pre-coat this tip in PBS2 or Advanced DMEM/F12. Using this tip, pipette the organoids up and down 10-20 times to mechanically disrupt the organoids and remaining matrix.

2.8 Spin down at 210 *g* at 4 °C for 3 minutes.

2.9 As in step 2.6, remove the supernatant, the debris fraction, and as much of the clear matrix fragment as possible without disrupting the pellet of dissociated crypts. If healthy crypts or small organoids have not formed a clear pellet, centrifuge again at 300 *g* for 3 minutes.

2.10 Calculate the required volume of TBEM, 80-120 µL (depending on if a 1:2 or 1:3 passage ratio is used) per well of harvested organoids.

2.11 Resuspend the pellet in the calculated volume of TBEM and apply 40 µl per well to the pre-warmed 24-well plate to form a dome. Directly place the plate in a 37 °C 5% CO_2 incubator and for 15-20 minutes.

2.12 Add 550 µl of ENR medium per well and incubate at 37 °C 5% CO_2. Replace with fresh ENR medium every 2-3 days.

3 Isolation of small intestinal lamina propria innate lymphoid cells

This section of the protocol describes the isolation of ILC1 from the murine small intestine lamina propria of *RORγt*^{GFP} reporter mice. This involves extracting the intestine, removing epithelial cells, and then digesting tissue. Lymphocytes are then separated by density gradient separation and ILC1 isolated via FACS. FACS isolation following the gating strategy in **Figure 2** requires the extracellular staining mastermix described in **Table 4,** with the additional staining controls described in **Tables 2-3** for machine (**Table 2**) and gating (**Table 3**) set up. For the isolation of a live, pure ILC1 population *RORγt*^{GFP} reporter mice are required to gate out *RORγt*$^{GFP+}$ ILC3. Tissue processing for isolation of lamina propria lymphocytes has also been well described elsewhere[32].

NOTE: Tissue from individual biological animal replicates is kept separate in this protocol. These samples should be appropriately labelled and kept on ice whenever possible.

3.1 Tissue processing

NOTE: All reagents except for digestion enzymes should be allowed to reach room temperature to ensure rapid tissue digestion.

3.1.1 Place TBEM on ice (40 µL/well, 500 µL will thaw in approximately 2-4 hours, do not leave at room temperature) to thaw and put a standard tissue-culture treated 24-well plate in a 37 °C incubator to pre-warm.

3.1.2 Prepare fresh epithelium removal and digestion buffer (**Table 1**). Prepare isotonic low viscosity density gradient medium (LVDGM) (90% LVDGM, 10% 10X PBS), and prepare 40% isotonic LVDGM and 80% isotonic LVDGM in neutralising buffer (**Table 1**).

3.1.3 Cull a 6-12 week old animal following approved Schedule 1 procedures, dissect out the small intestine using forceps and microdissection scissors and place in 15 mL PBS on ice.

3.1.4 Submerge the intestine in PBS on a 10cm^2 Petri dish on ice and use forceps to gently but completely remove fat from the intestine.

3.1.5 Remove Peyer's patches (~ 5-10, running in a line opposite the line of fat tissue) using microdissection scissors to deplete Lymphoid tissue inducer cells and B-cells. *[Peyer's patches are absent in Rag$^{-/-}$ and other Lineage-depleted animals].* Unlike air bubbles, Peyer's patches will remain in place if nudged.

3.1.6 Cut the tissue longitudinally using microdissection scissors (preferably rounded tip to avoid perforation). Keeping the intestine submerged in PBS, shake to remove faecal matter.

3.1.7 Cut the tissue into 2-4 cm length pieces and transfer into a 50 mL tube.

3.1.8 Add approximately 20-40 mL of ice-cold PBS and shake rigorously for 5-15 seconds to remove mucous and debris.

3.1.9 Discard the contents of the tube onto a fresh Petri dish, and replace intestinal fragments into the same 50 mL tube using forceps.

3.1.10 Repeat steps 3.1.8 and 3.1.9 3-4 times, or until the PBS is clear.

3.2 Epithelium removal

3.2.1 Place intestinal fragments into a fresh Petri dish on ice. Using forceps, pick up intestinal segments and remove excess liquid by tapping on a dry surface.

3.2.2 Cut the tissue into ~0.75-1 cm pieces into a fresh 50 mL tube. Add 5-7 mL of epithelium removal buffer (**Table 1**). Vortex the tube and ensure all intestinal segments are submerged in the buffer.

3.2.3 Incubate the samples at 37°C, with medium rocking (100-150 rpm) for 12-15 minutes.

3.2.4 Vortex the tube for 20-30 seconds.

3.2.5 Repeat steps 3.2.1-3.2.4 once more, discarding the cloudy supernatant (fraction contains epithelial and intra-epithelial cells) and replacing with fresh epithelial removal buffer.

3.3 Digestion of the tissue

3.3.1 Tip contents on to a fresh Petri dish. Using forceps, pick up intestinal segments and tap on a dry surface to discard excess liquid. Place segments into a fresh Petri dish on ice.

3.3.2 Cluster segments into the centre of the Petri dish into a dense mass. Using either 2 scalpels or sharp scissors, finely shred the tissue until it reaches a viscous consistency that could pass through a p1000 pipette tip.

3.3.3 Using tweezers, place the minced tissue into a clean 50 mL tube. Rinse the Petri dish with 1-2 mL of digestion buffer (**Table 1**) to collect any remaining tissue and pool into the 50 mL tube with the shredded tissue.

3.3.4 Add 5-7 mL of digestion buffer to shredded tissue and ensure that all tissue is collected at the bottom of the tube.

3.3.5 Incubate the samples at 37°C, with medium rocking (~100-150 rpm) for 15 minutes. Vortex the tube for 20-30 seconds every 5 minutes to aid the digestion.

3.3.6 Whilst samples are incubating, place a 40 µm cell strainer on top of a fresh 50 mL tube for each sample. Coat the filters with 1-2 mL neutralizing buffer (**Table 1**).

3.3.7 Vortex the samples for 20-30 seconds after the incubation has finished.

3.3.8 Filter the partially digested tissue through the coated 40 µm filter into the 50 mL tube with neutralising buffer.

3.3.9 Use tweezers to collect undigested tissue from the 40 µm filter and place back into the 50 mL tube that the digestion was performed in, for a second round of digestion. Filters can be removed and rinsed with digest buffer to dislodge any undigested tissue, which may adhere to the filter.

3.3.10 Once as much undigested tissue as possible has been removed from the filter and placed back into the 50 mL tube that digestion was performed in, the filter can be rinsed with 1-2 mL neutralizing buffer over the 50 mL tube containing the filtered supernatant.

3.3.11 Add 20-25 mL neutralising buffer to the filtered supernatant and place on ice to maintain the viability of isolated cells during the second round of tissue digestion.

3.3.12 Add another 5-10 mL of digestion buffer to the undigested tissue and repeat steps 3.3.5-3.3.10, ensuring to filter the digested tissue into the same tube as used in 3.3.8. (the same filter can also be reused). Rinse the filter with neutralising buffer until the 50 mL line is reached, then discard any remaining undigested tissue.

3.4 Lymphocyte isolation by density gradient

3.4.1 Spin the collected supernatants for at 500 *g* for 10 minutes.

3.4.2 During step 3.4.1, add 5 mL of 80% isotonic LVDGM to a 15 mL tube for each sample.

3.4.3 Following centrifugation, discard the supernatant from the filtered, digested tissue. Resuspend the pellet in 10 mL of 40% isotonic LVDGM, ensure the pellet is well homogenised and no large chunks remain.

3.4.4 Setting the pipette aid to its slowest setting, tilt the 15 mL tube of 80% isotonic LVDGM, and very gently overlay the 10 mL of cell suspension in 40% isotonic LVDGM. It is critical that the cell suspension does not mix with the 80% fraction. *[If the fractions should ever accidentally mix,*

rapidly distribute lymphocytes that reached the 80% fraction between two 50 mL tubes filled with PBS, and centrifuge for 10 min at 500 g. Then, discard supernatant, and repeat step 3.4.3-3.4.4.]

3.4.5 Spin the tubes at 900 *g*, 20°C, with the acceleration and deacceleration set to the lowest setting to ensure the fractions are not disrupted. Centrifuge for 20 minutes (not including break time).

NOTE: All procedures from this step onwards should be conducted in an aseptic tissue culture hood using sterile materials and reagents.

3.4.6 Pre-coat a 50 mL tube for each sample with PBS2 and add 45 mL of ice-cold PBS.

3.4.7 After centrifugation, gently remove the upper debris layer from the 15 mL tube. Coat a p1000 tip with PBS2 and use to collect the lymphocyte-laden interphase between the 40-80% gradients into the 50 mL tube containing ice-cold PBS.

3.4.8 Spin the tubes at 300 *g*, 4°C for 5 minutes.

3.4.9 Discard the supernatant and resuspend the pellet in 500 µL FACS buffer (PBS + 2% FCS + 0.5 mM EDTA + 10 mM HEPES, **Table 1**). Pre-coat a 40 µm filter with FACS buffer and use to filter the cell suspension into a flow tube. Rinse the 50 mL tube with an additional 500 µL FACS buffer and filter into the same flow tube.

3.4.10 Remove a 10 µl aliquot from the resulting 1 mL cell suspension. Count the lymphocyte yield using a cell counter or hemocytometer and calculate the cell concentration for each sample.

3.5 Sample preparation for sorting – extracellular staining

NOTE: The antibodies used in the extracellular staining mastermix, as well as the reagents to prepare the fixable viability dye and Fc block solution, are used in concentrations enough for up to 5 x 10^6 cells per 100 µL. Volumes should be adjusted accordingly.

NOTE: For FACS machine compensation to sort ILC1, single colour controls for each of the fluorophores in the extracellular staining mastermix are required. For the antibodies, compensation beads that contain a positive antibody binding bead population and a negative antibody non-binding bead population can be used. For the ultraviolet (UV) fixable viability dye single colour control, compensation beads that contain amine reactive (for positive signal) and non-reactive (for negative signal) populations can be used. It is not recommended to use cells

from the *RORγt*^{GFP} reporter mice for the UV fixable viability dye single colour control, as these cells will contain a GFP^{+} signal.

3.5.1 Remove a small aliquot of ~10 µL from each sample, pool into a separate flow tube containing 250 µl of FACS buffer and place on ice. This will be used for the unstained control.

3.5.2 Add 1-2 mL of PBS to the remaining sample tubes. Centrifuge at 300-400 *g* for 3-5 minutes.

3.5.3 Prepare 200 µL of UV fixable viability dye solution per sample. Dilute UV fixable viability dye (resuspended in DMSO following manufacturer's instructions) 1:500 in PBS (or following manufacturer's instructions).

3.5.4 Discard the supernatant and resuspend the samples in 200 µL of UV fixable viability dye solution. Vortex the tubes and incubate in the dark at 4°C for 10-15 minutes.

3.5.5 Add 1-2 mL of FACS buffer to the tubes to quench the UV fixable viability dye , vortex for 10 seconds and centrifuge the tubes at 300-400 *g*, 4°C for 3-5 minutes.

3.5.6 Prepare 200 µL of Fc block solution per sample, 0.25 mg/mL Fc block in FACS buffer. Discard the supernatant from the centrifuged tubes.

3.5.7 Add 200 µL of Fc block solution to each of the samples, vortex for 10 seconds and incubate in the dark at 4°C for 10 minutes.

3.5.8 Add 500 µL of FACS buffer to a fresh flow tube. Remove a 2-5 µL aliquot from each sample and pool into the tube for the fluorescence minus one (FMO) controls.

3.5.9 Vortex the FMO tube for 10 seconds and distribute evenly (100 µl per tube) into fresh flow tubes labelled for each of the FMO controls (Lineage cocktail FMO, CD127 FMO, KLRG1 FMO, NKp46 FMO and NK1.1 FMO, **Table 3**). Add all the antibodies minus the antibody of interest to each tube (as described in **Table 3**) and vortex for 10 seconds. Place the FMO controls aside in the dark at 4°C.

3.5.10 Centrifuge the samples (not FMO controls) at 300-400 *g*, 4°C for 3-5 minutes.

3.5.11 Prepare extracellular staining mastermix, 200 µL per sample with the final antibody dilutions described in **Table 4.** Staining volume may have to be adjusted for the appropriate cell concentration (up to 5 x 10^6 cells per 100 µL) depending on cell counts from step 3.4.10.

3.5.12 Add extracellular staining mastermix to samples, vortex for 10 seconds and place aside in the dark at 4°C.

3.5.13 Prepare a fresh single colour control for each antibody intended to be used in the gating strategy (**Figure 2**). Vortex antibody compensation beads for 20 seconds, add 1 drop of beads to a flow tube and stain with 0.5 µL of antibody (as shown in **Table 2,** or following manufacturer's instructions) and vortex for 10 seconds.

3.5.14 Prepare a UV fixable viability dye single colour control using an amine reactive compensation bead kit. Vortex amine reactive compensation beads for 20 seconds, add 1µL of Live/Dead UV dye (as shown in **Table 2,** or following manufacturer's instructions) and vortex for 10 seconds.

3.5.15 Incubate samples, FMOs and single colour controls in the dark, at 4°C, for 30 minutes.

3.5.16 During this time, prepare tubes to collect sorted cells. Add 250-300 µL 10% FBS in Advanced DMEM/F12 to 1.5 mL tubes for each sample.

3.5.17 After the incubation add 2 mL of PBS2 to each of the samples, FMO controls and single colour controls.

3.5.18 Centrifuge the samples, FMO controls and single colour controls at 300-400 *g*, 4°C for 3-5 minutes.

3.5.19 Discard the supernatant from all the centrifuged tubes. Resuspend the samples and FMO controls in 250 µL of FACS buffer and vortex for 10 seconds.

3.5.20 Resuspend the single colour controls in 250 µL of PBS2. Add 1 drop of amine non-reactive beads to UV fixable viability dye single colour control only. Vortex all controls for 10 seconds.

3.5.21 Cells are now ready to be sorted. Keep samples, FMO controls and single colour controls on ice in the dark whenever possible to improve cell viability and prevent loss of signal from photobleaching.

4 Co-culture of small intestinal organoids with innate lymphoid cells

This section describes the co-culture of sorted murine small intestinal ILC1 (isolated following the protocol in section 3) with murine small intestinal organoids (described in sections 1 and 2). Organoids should optimally be used 1-2 days following passage. Co-culture involves harvesting the organoids, adding the appropriate number of ILC1, centrifuging to pellet organoids and ILC1 together, and resuspending in BME. Complete this section as soon as possible once ILC1 have been isolated.

NOTE: All procedures should be conducted in an aseptic environment using sterile materials and reagents.

4.1 Ensure TBEM from step 3.1.1 has thawed.

4.2 Harvest 1-2 day old organoids as described in section 2.3-2.4.

4.3 Resuspend the organoid pellet in 1 mL of cold ice-cold Advanced DMEM/F12. Do not bend the p1000 tip. If required, organoids can be placed in separate PBS2 coated 1.5 ml tubes on ice for a short period of time to be distributed between different co-culture conditions.

NOTE: Only begin preparation of organoids once ILC samples are ready for co-culture, and work on ice and rapidly as soon as organoids have been harvested to minimise epithelial cell death.

4.4 Pre-coat one 1.5 mL tube in PBS2 per ILC replicate sample. Distribute ~100-200 organoids (approximately 1 well of a 24 well plate) into each tube.

4.5 Pre-coat a p1000 tip in PBS2 and use to assess the volume of ILC1s after FACS purification (250-300 µL + sorted volume). Determine the concentration of ILC1 per mL using number of recorded cells from the sort and determined volume.

4.6 Using the same PBS2 coated p1000 tip, add no fewer than 500 ILC1s to the PBS2 coated 1.5ml tube containing the organoids. If the number of ILC1s yielded from the sort is less than 500, we recommend adding the organoid directly to the original sort tube to minimise loss of cells from transfer.

4.7 Spin down ILC1 and organoids at 300 *g* at 4 °C for 5 minutes. If possible, avoid small diameter tabletop centrifuges, as these will pool the cells along the edge of the tube interior as opposed to creating a pellet at the tip of the tube. Since cell numbers are very low, it is imperative to minimise loss of cells during these steps.

4.8 Slowly and gently remove as much of the supernatant as possible without disturbing the pellet (which may not be visible by eye, especially in no-organoid ILC-only controls). Place the sample on ice.

4.9 Resuspend the cold pellet in 30 µL per well of TBEM. While holding the tube on a cold surface (e.g., a small box of ice placed in the tissue culture hood) mix the organoids and ILC at least 10-15 times to ensure even distribution. Triturate frequently but gently, to avoid damaging the organoids and the formation of bubbles.

4.10 Apply 30 µL per well of ILC-organoids in TBEM to a pre-warmed 24 or 48 well plate to form a single dome. Directly place the plate in a 37 °C 5% CO_2 incubator and for 10-20 minutes.

NOTE: It strongly recommended that ILC-only and organoid-only controls are set up for downstream analysis. ILC1 are rare, but GFP^- ILC2 can be substituted for immunofluorescence or FSC/SSC flow cytometry controls where scientifically appropriate.

4.11 Add 550 µL of complete ILC1 medium (ENR + IL-2 + IL-7 + IL-15 + 2-Mercaptoethanol, **Table 1**) with any desired experimental cytokines or blocking antibodies, per well and incubate at 37 °C 5% CO_2.

4.12 Gently remove plate from incubator every 1-2 days, allow the plate to sit in tissue culture hood for 1 minute to ensure lymphocytes are settled.

4.13 Remove 200-250 µl media, and place into an empty well of a 24-well plate. Check this supernatant well with an inverted microscope to ensure no lymphocytes were removed. If lymphocytes are present, centrifuge these, and resuspend in 300 µl fresh ILC1 medium, and add to remaining 200-250 µl media in the original well. If supernatant is clear, simply add 300 µl fresh ILC1 medium to the co-cultures. Ensure media is replenished every 1-2 days or when media becomes pale-orange/yellow.

NOTE: Do not allow media to evaporate sufficiently that the tip of the matrix dome breaks the surface of the media. Always ensure that matrix is completely submerged. Excess evaporation can be avoided by using central wells in the tissue culture plates and adding ~600 µL PBS to the surrounding wells.

NOTE: Co-cultures with adult ILC are stable for 1-4 days, after which point the organoids that were seeded without major disruption will rupture and reseed as new crypts. If analysing the epithelium, it is recommended that cultures are analysed within 1-4 days of establishing co-cultures.

NOTE: Downstream analysis can be performed using immunofluorescence, flow cytometry, or FACS purification of target populations into lysis buffer for gene expression analysis by single cell or bulk RNA-seq or RT-qPCR. ILC1 viability is well maintained in culture, and the population will

undergo mild expansion, with ~500 ILC1 with 100 SIO undergoing 2-3 fold expansion on average. However, this yield will be impacted by additional treatments, with TGF-neutralisation improving and p38-inhibition decreasing the absolute number of ILC1 after co-culture[8]. Any unanticipated loss of more than 50% of seeded ILC1 numbers may be the result of either an imbalanced ratio of ILC1 to SIO (increase number of seeded crypts), SIO contamination (ensure antibiotic cocktail is functioning and test supernatant for mycoplasma), or of quality issues in the cytokine stocks, with ILC1 being particularly sensitive to a lack of IL-2 or IL-15. Co-culture supernatants from concentrated 96 or 48-well plates have been successfully used for ELISAs. When dissociating co-cultures, we recommend incubating cells with DNAse after a 20 minute gentle trypsin replacement or EDTA-based dissociation to single cells to prevent cell clumping from damaged epithelial cells.

REPRESENTATIVE RESULTS:

When successfully completed, freshly isolated crypts should form budding crypt structures within 2-4 days (**Figure 1A**). Healthy and robust organoid cultures should be actively growing and can be passaged and expanded as detailed in the protocol.

This protocol describes the isolation of small intestinal ILC1 from the RORγt^{GFP} murine transgenic reporter line, which allows isolation of live ILC1 by FACS (**Figure 2**). Using the protocol outlined here, the expected ILC1 count range is 350-3500 isolated cells.

After being seeded with organoids, co-cultures can be visualized by immunocytochemistry (**Figure 3A-B**). ILCs and epithelial cells can also be analysed by flow cytometry, as demonstrated in **Figure 3C**. ILC1 upregulate epithelial CD44, characterised by flow cytometry (**Figure 4A-B**) and immunocytochemistry (**Figure 4C**). Specifically, ILC1 induce expression of the CD44 v6 splice variant in organoids, as demonstrated by RT-qPCR (**Figure 4D**).

FIGURE AND TABLE LEGENDS:

Table 1: Media and buffer compositions.

Table 2: Single colour controls. *Composition of single colour controls to isolate small intestine lamina propria ILC1 using the gating strategy defined in Figure 2.*

Table 3: Fluorescence minus one (FMO) mastermixes. *Composition of FMO mastermixes for Lineage cocktail FMO, CD127 FMO, KLRG1 FMO, NKp46 FMO and NK1.1 FMO. FMO mastermixes contain all of the antibodies used with the exception of one and are used to stain a sample aliquot. Lineage cocktail is defined as CD19, CD3e, CD5, Ly-6G/Ly-6C.*

Table 4: Extracellular staining mastermix. *The concentrations are adjusted for staining up to 5 x 10^6 cells in 200 µL FACS buffer. Details of antibodies used can be found in the Materials section.*

Figure 1. Murine small intestinal organoids. *Representative image of successfully generated small intestinal organoids 2-3 days post passage (A). Unsuccessful culture demonstrated in (B). Scale bar 100 µm.*

Figure 2. Gating strategy to isolate ILC1s from the small intestine lamina propria of transgenic RORγt^{GFP} reporter mice. *Representative flow cytometric plot of ILC1 isolation from the small intestine lamina propria of transgenic RORγt^{GFP} reporter mice by FACS. ILC1s are defined as live, CD45$^+$, Lin$^-$ (CD3, CD5, CD19, Ly6C), CD127$^+$, KLRG1$^-$, RORγt$^-$, NKp46$^+$ and NK1.1$^+$). Representative from N = >50 mice.*

Figure 3. Organoid and ILC1 co-cultures. *Bright-field images (A), confocal microscopy images (B) and FACS plots (C) of small intestinal organoids (SIO) cultured alone (top) or with ILC1s (bottom) (representative of experiments with ILC1s from N = 3 mice). (B) Staining with CD45 illustrates ILC1s and Zonula occludens protein 1 (ZO-1) marks epithelial cells in organoids. Scale bars 50 µm. (C) previously gated on single, live cells. Epithelial cellular adhesion molecule (EpCAM) marks intestinal epithelial cells (IEC) in organoids, CD45 marks ILC1s. Figure adapted from*[8].

Figure 4. ILC1s in co-culture with small intestinal organoids drive upregulation of CD44 in intestinal epithelial cells. (A) *Representative cytometric plot of CD44 expression in epithelial cellular adhesion molecule (EpCAM) positive epithelial cells (live, CD45$^-$, EpCAM$^+$) from small intestinal organoids (SIO) cultured alone (left) or with ILC1s for 4 days (right). (B) Flow quantification of CD44 expression in intestinal epithelial cells (IEC) (ILC1s from N = 5 mice). (C) Representative confocal microscopy image of CD44 localization in d4 SIO alone (left) or co-cultured with ILC1s (right) (representative of N = 3 mice). (D) RT-qPCR with exon-specific primers for CD44 splice variants s, v4, and v6 (N=3). Scale bars 50 µm. Figure adapted from*[8].

DISCUSSION:

This protocol describes the methods for establishing murine small intestine organoids, isolating rare ILC1 by minimizing the loss of lymphocytes during the intestinal dissociation protocol, and establishing co-cultures between these two compartments. There are many steps to this protocol, and while some are specific to ILC1s, this approach can be applied to other intestinal immune cell types, and co-culture setups can be modularly adapted to suit individual research questions. However, here we will highlight a number of critical steps that we would not recommend deviating from and offer troubleshooting guidelines for the more technically challenging elements of this protocol.

The use of murine small intestinal organoids from single Lgr5^{+}-eGFP intestinal stem cells is becoming increasingly well established[33,34]; however, in this protocol we suggest isolating intact, entire crypts of Lieberkuehn from CD45.1 animals. Not only do intact crypts recover more rapidly than single Lgr5^{+} cells, but the use of CD45.1 animals without a GFP reporter ensures that no cross-contaminating CD45.2^{+} ILC are analysed from the organoid co-cultures, and is compatible with the use of immune cells containing a GFP-based reporter. In our experience, no mesenchymal or immune cells carry over after 1-3 passages of the organoids. The use of CD45.2 or other animals for establishing organoids is therefore entirely acceptable, however the use of CD45.1 adds a layer of certainty for those new to organoid-immune co-cultures. During organoid establishment, if crypts are not present at step 1.1.19, more rigorous manual shaking may be necessary to dislodge the intact crypts. Environmental factors such as ambient room temperature (e.g., whether the procedure is carried out in the summer or the winter) may add some variability to incubation timings during dissociation. The seeding density of crypts will impact initial organoid formation yield, we therefore recommended seeding a minimum of two different densities to ensure success (e.g. 200-750 suggested here, but this range can be adapted based on an individual needs).

Once established, intestinal organoid cultures are heterogenous both between lines, along the gastrointestinal tract (e.g. duodenum vs. ileum), and even within the same well of organoid cultures[35,36]. Although we have found this protocol to be robust over many different batches of organoids, it is possible that this heterogeneity could contribute to data variability. It is good practice to be consistent with organoid maintenance (passaging and media changes) to attempt to reduce technical noise from phenotypically irrelevant data. This includes being consistent with the pre-seeding passaging timeline and with the force used to dissociate organoids. We also recommend using the same basal matrix (and lot number of matrix) for experiments being compared, and for experiments to be performed using biological replicates of organoids derived from different animals - when financially and technically feasible – to ensure that results are robustly reproducible.

In establishing co-cultures, the ratio of immune to epithelial cells is a critical consideration that will require optimization based on the research questions. If the impact of epithelial cells on ILC is being interrogated, the number of organoids seeded will need to be sufficient to saturate all ILC. Conversely, when assessing the impact of ILC on the epithelium, it is possible that different ILC/epithelial ratios will result in different phenotypic outputs, reflecting differential states of ILC subset enrichment in mucosa.

A strength of this protocol is that it balances reductionist culture conditions with complex cell types. However, the behaviours of other ILC subsets in these cultures may be dependent on factors not present in this particular protocol. For example, in the Lindemans' protocol used for ILC3 co-cultures, IL-23 was additionally supplemented into co-culture media to support ILC3 maintenance and activation[8]. We found IL-15 to be particularly important in the maintenance of ILC1 in the co-culture system described in this protocol, which was congruent with previous reports of ILC1 requiring this cytokine for homeostasis, though not development[6]. To activate ILC, or to maintain ILC2s, the growth medium may require further optimisation. Moreover, other cellular compartments in the intestine, aside from the epithelium, regulate ILCs. For example, intestinal neurons are known to modulate ILC2s partly through the activity of secreted neuropeptides[37]. Microbial factors also influence ILC phenotype[38]. This limitation could be overcome through the addition of these elements, e.g., cytokines, peptides, or microbial factors, into the co-culture system. This could even allow for the interrogation of the interaction between ILCs and multiple cellular compartments in a reductionist setting. Following this logic, it is absolutely critical that anti-biotic/anti-mycotic reagents are added and frequently replenished to organoid media prior to establishing co-cultures, and that all cultures are performed in aseptic environments, as any culture contamination (e.g., fungal growth or mycoplasma) would likely activate the antigen non-specific ILCs, creating significant phenotypes that may not be present in non-contaminated cultures. For this reason, we do not recommend withdrawal of anti-biotic/-mycotic reagents, even in the co-cultures, as we have not observed that these have any adverse impact on the epithelium or ILCs.

This method provides a unique way to characterize signalling modules between ILCs and the intestinal epithelium, allowing for the biology of both compartments to be investigated. In comparison to other *in vitro* methods consisting of a single cell type, the system presented here is more comparable to *in vivo* physiology and enables multiple potential signalling mechanisms between epithelial cells and ILCs to be interrogated. Other methods of *in vitro* ILC culture predominantly rely on stromal feeder cell lines, such as OP9 or OP9-DL1[39]. This line is derived from newborn mouse calvaria, which are not representative of the intestinal environment. While these have provided the gold standard for maintaining ILCs *in vitro* to date, they suffer substantial limitations in their application to understanding the impact of ILC on the epithelium.

The co-culture protocol we describe between murine small intestinal organoids and lamina propria derived ILCs has significant research applications. Research from our own lab has used this system of co-culture to determine the role of ILC1 derived TGFß in expansion of $CD44^{+}$ epithelial crypts[8], which contributes to our understanding of epithelial dynamics in inflammatory bowel disease. These studies contribute to an increasing body of literature that underpin the critical importance of epithelial-ILC signalling in intestinal homeostasis and inflammation[3].

ACKNOWLEDGMENTS:

E.R. acknowledges a PhD fellowship from the Wellcome Trust (215027/Z/18/Z). G.M.J. acknowledges a PhD fellowship from the Wellcome Trust (203757/Z/16/A) and a NIHR Guy's and St Thomas' (GSTT) Biomedical Research Centre **(**BRC) Bright Sparks Precision Medicine Early Career Research Award. D.C. acknowledges a PhD studentship from the NIHR GSTT BRC. J.F.N. acknowledges a Marie Skłodowska-Curie Fellowship, a King's Prize fellowship, an RCUK/UKRI Rutherford Fund fellowship (MR/R024812/1) and a Seed Award in Science from the Wellcome Trust (204394/Z/16/Z). We also thank the BRC flow cytometry core team based at Guy's Hospital. Rorc(γt)-GfpTG C57BL/6 reporter mice were a generous gift from G. Eberl (Institut Pasteur, Paris, France). CD45.1 C57BL/6 mice were kindly given by T. Lawrence (King's College London, London) and P. Barral (King's College London, London).

DISCLOSURES:

The authors do not declare any conflicts of interest.

REFERENCES:

1. Martini, E., Krug, S. M., Siegmund, B., Neurath, M. F. & Becker, C. Mend Your Fences. *Cellular and Molecular Gastroenterology and Hepatology* **4**, 33–46 (2017).
2. Peterson, L. W. & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature reviews. Immunology* **14**, 141–53 (2014).
3. Diefenbach, A., Gnafakis, S. & Shomrat, O. Innate Lymphoid Cell-Epithelial Cell Modules Sustain Intestinal Homeostasis. *Immunity* **52**, 452–463 (2020).
4. Ebbo, M., Crinier, A., Vély, F. & Vivier, E. Innate lymphoid cells: major players in inflammatory diseases. *Nature Reviews Immunology* **17**, 665–678 (2017).
5. E, V. *et al.* Innate Lymphoid Cells: 10 Years On. *Cell* **174**, 1054–1066 (2018).
6. Klose, C. S. N. *et al.* Differentiation of Type 1 ILCs from a Common Progenitor to All Helper-like Innate Lymphoid Cell Lineages. *Cell* **157**, 340–356 (2014).
7. Bernink, J. H. *et al.* Interleukin-12 and -23 Control Plasticity of CD127+ Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. *Immunity* **43**, 146–160 (2015).
8. Jowett, G. M. *et al.* ILC1 drive intestinal epithelial and matrix remodelling. *Nature Materials 2020 20:2* **20**, 250–259 (2020).
9. Wong, S. H. *et al.* Transcription factor RORα is critical for nuocyte development. *Nature Immunology 2012 13:3* **13**, 229–236 (2012).
10. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–1370 (2010).
11. Mjösberg, J. *et al.* The Transcription Factor GATA3 Is Essential for the Function of Human Type 2 Innate Lymphoid Cells. *Immunity* **37**, 649–659 (2012).
12. Hoyler, T. *et al.* The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of

Type 2 Innate Lymphoid Cells. *Immunity* **37**, 634–648 (2012).
13. Huang, Y. *et al.* IL-25-responsive, lineage-negative KLRG1hi cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nature Immunology 2014 16:2* **16**, 161–169 (2014).
14. J, von M., M, J., HE, L. & RM, L. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature* **529**, 221–225 (2016).
15. Gerbe, F. *et al.* Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature 2016 529:7585* **529**, 226–230 (2016).
16. G, E. *et al.* An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nature immunology* **5**, 64–73 (2004).
17. Spits, H. *et al.* Innate lymphoid cells--a proposal for uniform nomenclature. *Nature reviews. Immunology* **13**, 145–9 (2013).
18. RE, M., P, R. & IL, W. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* **7**, 493–504 (1997).
19. Lindemans, C. A. *et al.* Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature* **528**, 560–564 (2015).
20. Meininger, I. *et al.* Tissue-Specific Features of Innate Lymphoid Cells. *Trends in Immunology* **41**, 902–917 (2020).
21. Dutton, E. E. *et al.* Characterisation of innate lymphoid cell populations at different sites in mice with defective T cell immunity. *Wellcome Open Research 2018 2:117* **2**, 117 (2018).
22. Bal, S. M., Golebski, K. & Spits, H. Plasticity of innate lymphoid cell subsets. *Nature Reviews Immunology* **20***,* 552-565 (2020). doi:10.1038/s41577-020-0282-9
23. Bernink, J. H. *et al.* Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nature immunology* **14**, 221–9 (2013).
24. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–5 (2009).
25. A, O. *et al.* Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nature medicine* **15**, 701–706 (2009).
26. Gjorevski, N. *et al.* Designer matrices for intestinal stem cell and organoid culture. *Nature* **539**, 560–564 (2016).
27. Sato, T. & Clevers, H. Primary Mouse Small Intestinal Epithelial Cell Cultures. in *Methods in molecular biology (Clifton, N.J.)* **945**, 319–328 (2012).
28. Date, S. & Sato, T. Mini-gut organoids: reconstitution of the stem cell niche. *Annual review of cell and developmental biology* **31**, 269–89 (2015).
29. Bartfeld, S. Modeling infectious diseases and host-microbe interactions in gastrointestinal organoids. *Developmental Biology* **420**, 262–270 (2016).
30. Dutta, D., Heo, I. & Clevers, H. Disease Modeling in Stem Cell-Derived 3D Organoid Systems. *Trends in Molecular Medicine* **23**, 393–410 (2017).
31. Tallapragada, N. P. *et al.* Inflation-collapse dynamics drive patterning and morphogenesis in intestinal organoids. *Cell Stem Cell* **28**, 1516-1532.e14 (2021).
32. Qiu, Z. & Sheridan, B. S. Isolating Lymphocytes from the Mouse Small Intestinal Immune System. *Journal of Visualized Experiments : JoVE* **132**, e57281 (2018). doi:10.3791/57281

33. Sato, T. & Clevers, H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science (New York, N.Y.)* **340**, 1190–4 (2013).
34. O'Rourke, K. P., Ackerman, S., Dow, L. E. & Lowe, S. W. Isolation, Culture, and Maintenance of Mouse Intestinal Stem Cells. *Bio-protocol* **6**, (2016).
35. Serra, D. *et al.* Self-organization and symmetry breaking in intestinal organoid development. *Nature* **569**, 66-72 (2019). doi:10.1038/s41586-019-1146-y
36. Lukonin, I. *et al.* Phenotypic landscape of intestinal organoid regeneration. *Nature* **586**, 275–280 (2020).
37. Cardoso, V. *et al.* Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature 2017 549:7671* **549**, 277–281 (2017).
38. Gury-BenAri, M. *et al.* The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell* **166**, 1231-1246.e13 (2016).
39. Seehus, C. & Kaye, J. In vitro Differentiation of Murine Innate Lymphoid Cells from Common Lymphoid Progenitor Cells. *Bio-protocol* **6**, (2016).

Figure 1

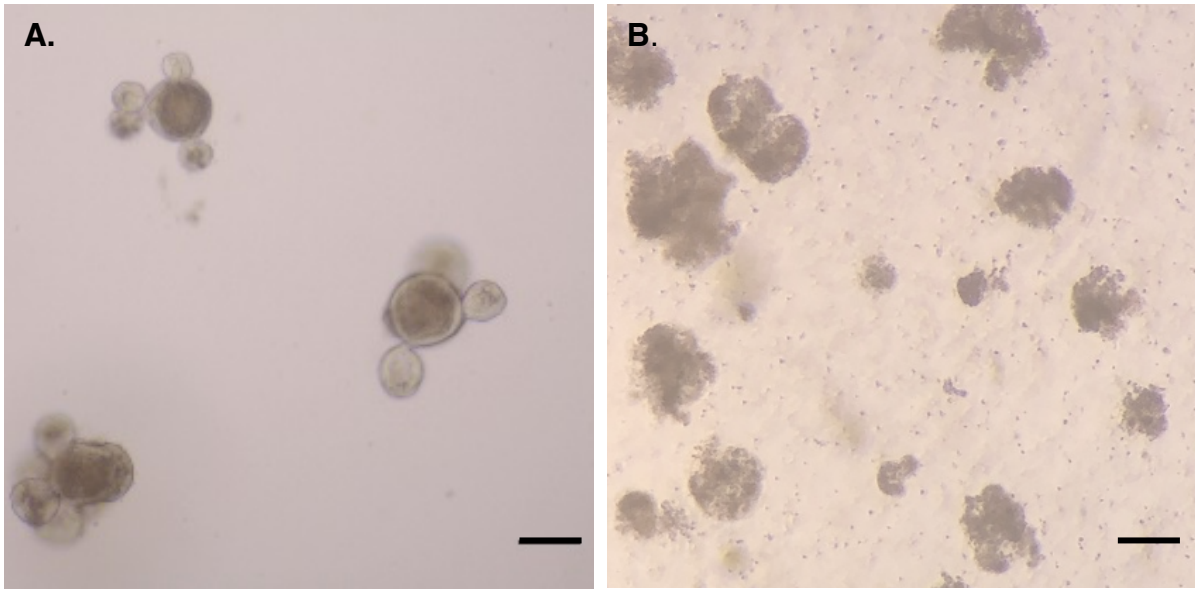


Figure 2

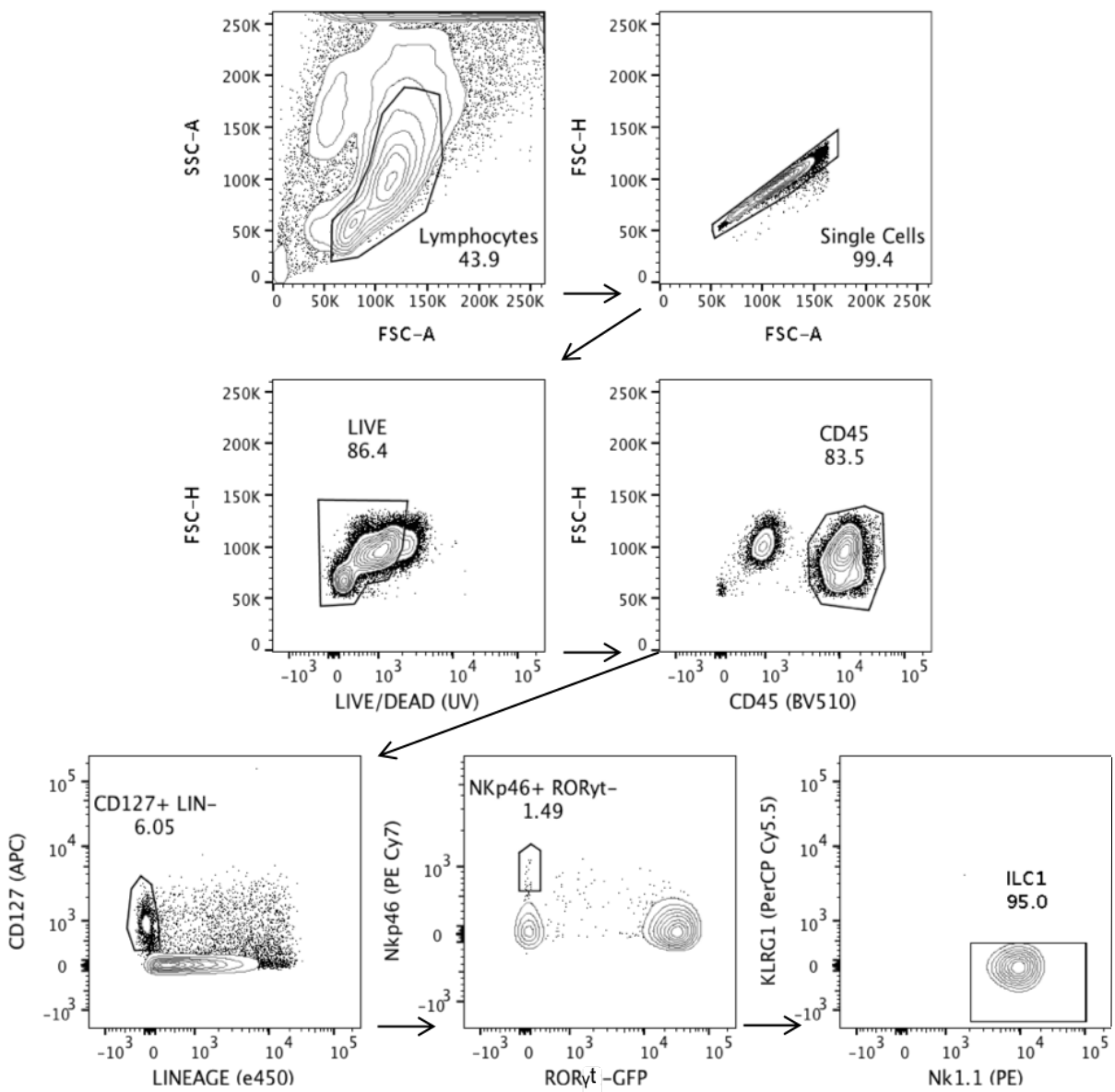

SSC-A
FSC-A
Lymphocytes
43.9
FSC-H
FSC-A
Single Cells
99.4
FSC-H
LIVE/DEAD (UV)
LIVE
86.4
FSC-H
CD45 (BV510)
CD45
83.5
CD127 (APC)
LINEAGE (e450)
CD127+ LIN-
6.05
Nkp46 (PE Cy7)
RORγt -GFP
NKp46+ RORyt-
1.49
KLRG1 (PerCP Cy5.5)
Nk1.1 (PE)
ILC1
95.0

Figure 3

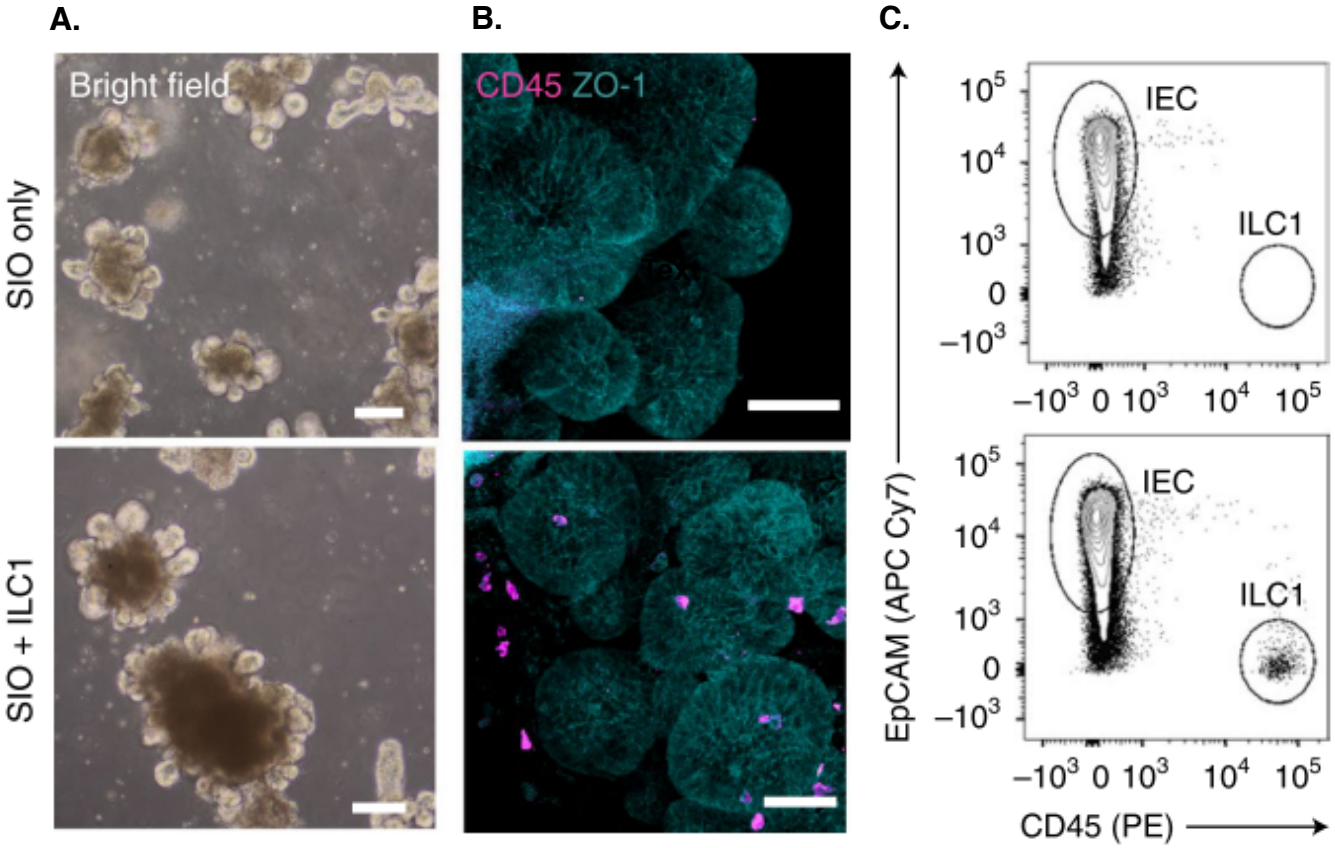
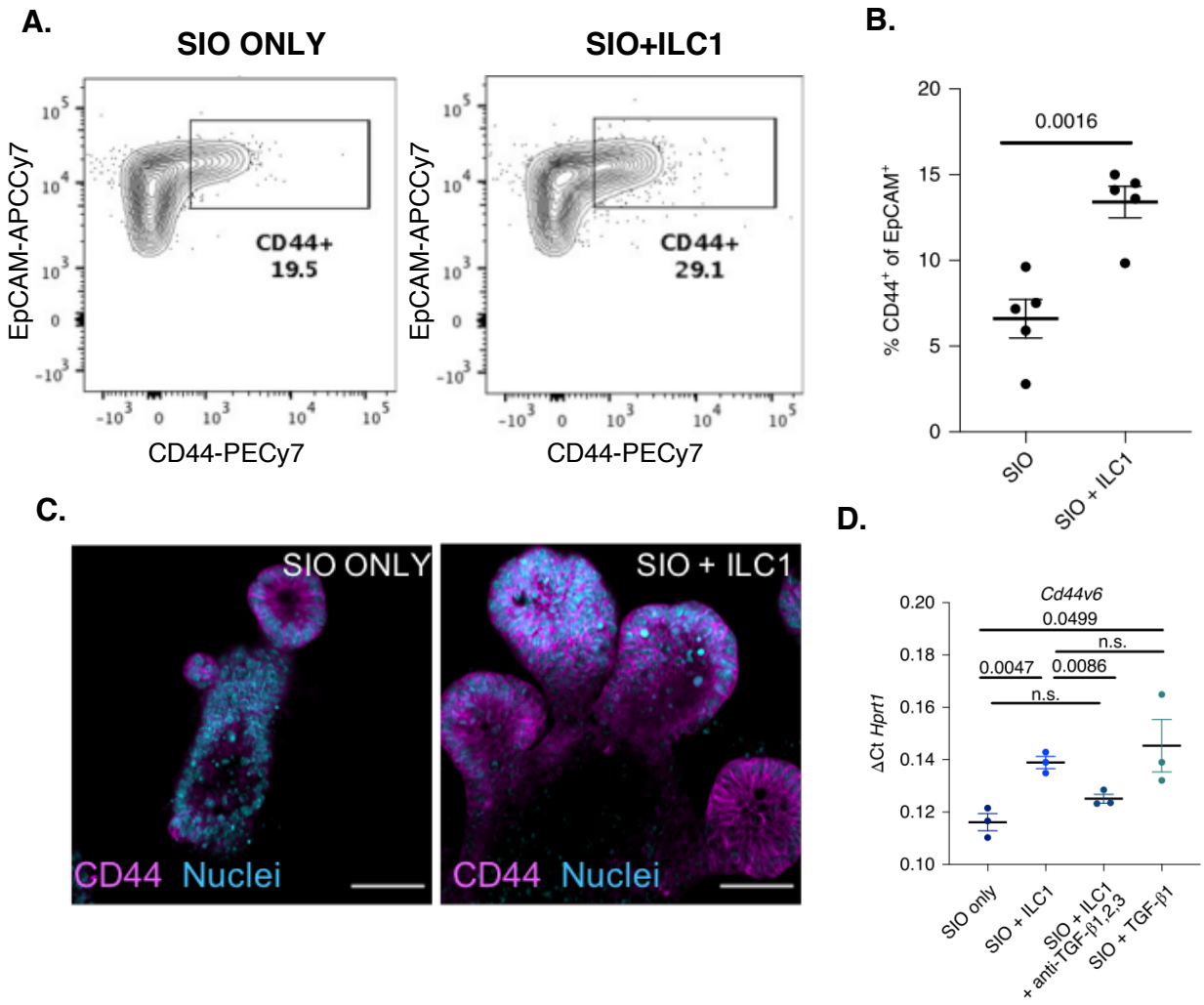

A.
B.
C.
Bright field
CD45 ZO-1
SIO only
SIO + ILC1
IEC
ILC1
EpCAM (APC Cy7)
CD45 (PE)

Figure 4


A.
SIO ONLY
SIO+ILC1
EpCAM-APCCy7
CD44-PECy7
CD44+
19.5
CD44+
29.1
B.
% CD44+ of EpCAM+
0.0016
SIO
SIO + ILC1
C.
SIO ONLY
SIO + ILC1
CD44 Nuclei
CD44 Nuclei
D.
Cd44v6
ΔCt Hprt1
0.0499
n.s.
0.0047
0.0086
n.s.
SIO only
SIO + ILC1
SIO + ILC1 + anti-TGF-β1,2,3
SIO + TGF-β1