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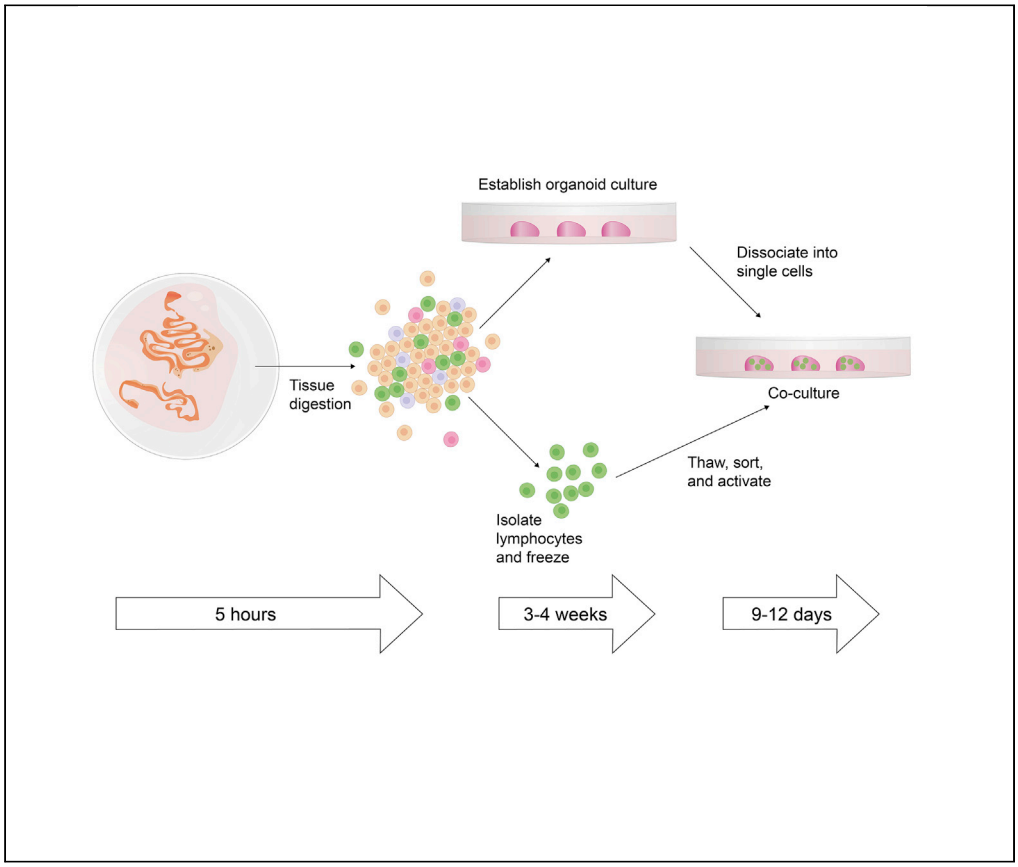
In vitro co-culture of human intestinal organoids and lamina propria-derived CD4$^+$ T cells



Crosstalk between immune cells and intestinal stem cells (ISCs) *in vivo* plays a critical role in tissue homeostasis and inflammation; however, *in vitro* models based on primary cells recapitulating this interaction were lacking. Here, we provide a detailed protocol for an autologous *in vitro* long-term 3D co-culture system of human intestinal CD4$^+$ T cells and ISCs to study T cell-intestinal epithelial cell interactions during tissue development and inflammation.

Renée R.C.E. Schreurs, Martin E. Baumdick, Agata Drewniak, Madeleine J. Bunders

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Highlights

Reproducible generation and culture of intestinal organoids with intestinal CD4$^+$ T cells

Isolation of intestinal crypts and lamina propria-derived lymphocytes

In vitro system to model immune-mediated regulation of intestinal growth and inflammation

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In vitro co-culture of human intestinal organoids and lamina propria-derived CD4$^+$ T cells

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SUMMARY

Crosstalk between immune cells and intestinal stem cells (ISCs) *in vivo* plays a critical role in tissue homeostasis and inflammation; however, *in vitro* models based on primary cells recapitulating this interaction were lacking. Here, we provide a detailed protocol for an autologous *in vitro* long-term 3D co-culture system of human intestinal CD4$^+$ T cells and ISCs to study T cell-intestinal epithelial cell interactions during tissue development and inflammation.

For complete details on the use and execution of this protocol, please refer to Schreurs et al. (2019).

BEFORE YOU BEGIN

This protocol describes the use of primary patient material. Please confirm that you are allowed to process primary patient material with your local Medical Ethics Committee and request informed consent from donors. Tissues are obtained and maintained until processing under sterile conditions. Organoids can be generated from intestinal tissues from various anatomical locations of the intestine as well as different disease modalities and tailored to the research questions.

Prepare buffers and solutions specified in this segment before sample processing. See below for reagents and concentrations. Some reagents and media can be prepared in advance and stored as indicated. A complete list of materials and equipment required can be found in the Key Resources Table (KRT).

> *Alternatives:* In our publication (Schreurs et al., 2019) fetal intestinal organoid cultures were co-cultured with fetal lamina propria-derived CD4$^+$ T cells, however the same protocol was successfully applied to pediatric and adult intestinal organoid cultures and CD4$^+$ T cells from pediatric and adult tissues as well as blood.

> *Note:* Perform the whole protocol under sterile conditions in a cell-culture hood.

Prepare digestion mixes, media and buffers

Timing: 1 h

Prepare the necessary digestion mixes, media and buffers before you begin. Please see recipes and storage conditions in the Materials and Equipment section.

1. EDTA/DTT mix to separate the epithelial cells from the underlying lamina propria.
2. Collagenase D mix to digest the lamina propria after epithelial detachment.
3. FACS buffer for cell staining and sorting
4. T cell medium
5. Advanced DMEM/F12 (AD+) medium
6. Human intestinal stem cell (HISC) medium
7. Antibody mix to stain cells for sorting

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD45-FITC (HI30)	eBioscience	Cat# 11-0459; RRID: AB_10852703
Anti-human CD3-PE-CF594 (SP34-2)	BD Horizon	Cat# 562406; RRID: AB_11154406
Anti-human CD45RA-BV650 (HI100)	BD Horizon	Cat# 563963; RRID: AB_2738514
Anti-human CCR7-PE-Cy7 (3D12)	BD Pharmingen	Cat# 560922; RRID: AB_10561680
Anti-human CD4-BV570 (RPA-T4)	BioLegend	Cat# 300533; RRID: AB_10896788
Live-Dead Fixable Red Dead Cell Stain Kit	Invitrogen	Cat# L23102
Biological samples		
Fetal intestinal tissues	HIS Mouse Facility, AUMC Amsterdam	n/a
Chemicals, peptides, and recombinant proteins		
HEPES	Sigma-Aldrich	Cat# H3375; CAS: 7365-45-9
n-Acetyl-L-cysteine	Sigma-Aldrich	Cat# A9165; CAS: 616-91-1
[Leu^{15}]-Gastrin	Sigma-Aldrich	Cat# G9145; CAS: 39024-57-2
Nicotinamide	Sigma-Aldrich	Cat# N0636; CAS: 98-92-0
SB202190	Sigma-Aldrich	Cat# S7067; CAS: 152121-30-7
2-Mercaptoethanol	Sigma-Aldrich	Cat# 516732; CAS: 60-24-2
0.4% Trypan Blue	Sigma-Aldrich	Cat# T8154; CAS: 72-57-1
(Heat-inactivated) Fetal Bovine Serum (FBS)	Biological Industries	Cat# 04-007
Human IL-2 IS, research grade	Miltenyi	Cat# 130-097-742
Anti-CD3 (1XE)	Sanquin	Cat# M1654
Anti-CD28 (15E8)	Sanquin	Cat# M1650
Matrigel, phenol red-free, LDEV-free	Corning	Cat# 356231
Noggin conditioned medium (NCM)	AUMC	Home-made
R-spondin conditioned medium (RCM)	AUMC	Home-made
Wnt3a conditioned medium (WCM)	AUMC	Home-made
TrypLE express	Invitrogen	Cat# 12605-036
Advanced Dulbecco's Modified Eagle's Medium (DMEM)/F12	Invitrogen	Cat# 12634-028
GlutaMAX Supplement	Invitrogen	Cat# 35050061
B27 Supplement	Invitrogen	Cat# 17504-044
N2 Supplement	Invitrogen	Cat# 17502-048
Recombinant Mouse Epidermal Growth Factor (mEGF)	Invitrogen	Cat# PMG8045
Penicillin/Streptomycin (P/S)	Invitrogen	Cat# 15140-122
Gentamicin	Gibco	Cat# 15710-049
A83-01	Tocris	Cat# 2939; CAS: 909910-43-6
RHO/ROCK pathway inhibitor (Y)	STEMCELL Technologies	Cat# Y-27632; CAS: 129830-38-2
Collagenase D	Roche	Cat# 11088866001; EC:3.4.24.3
DNase I	Worthington Biochemical Corporation	Cat# LS002007
Percoll (GE Healthcare)	Sigma-Aldrich	Cat# GE17-0891-01
1,4-Dithiothreitol (DTT)	Sigma-Aldrich	Cat# D8255; CAS: 6892-68-8
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Cat# 03690; CAS: 60-00-4
Dimethyl sulfoxide (DMSO) EMSURE ® ACS	Supelco	Cat# 102952; CAS: 67-68-5

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Iscove's Modified Dulbecco's Medium (IMDM) with HEPES and L-glutamine	Lonza	Cat# BE12-722F
Software and algorithms		
FACSDiva	BD Biosciences	Version 8; RRID: SCR_001456
ImageJ	NIH	Version 1.50i; RRID: SCR_003070
FlowJo	Treestar	Version V10.5.0; RRID: SCR_008520
Other		
Cell strainer, 70 μm	Falcon, Corning	Cat# 352350
UltraComp eBeads	eBioscience	Cat# 01-2222-42
Culture dishes 145 × 20 mm	Cellstar	Cat# 639160
Centrifuge tubes, canonical, 50 mL	Cellstar	Cat# 227261
Centrifuge tubes, canonical, 15 mL	Cellstar	Cat# 188271
Microcentrifuge tubes, 1.7 mL	Corning	Cat# CLS3620
Sterile polystyrene culture plates, 6 wells	Corning	Cat# CLS3516
Sterile polystyrene culture plates, 24 wells	Corning	Cat# CLS3524
Cryogenic vials	Corning	Cat# CLS8672
Puradisc syringe filters, 0.2 μm	Whatman	Cat# WHA10462200
DeBakey forceps (for tissue dissection)	n/a	n/a
Adson forceps (for tissue dissection)	n/a	n/a
Metzenbaum scissors (for tissue dissection)	n/a	n/a
Bürker chamber	n/a	n/a
Incubator, 37°C, 5% CO_2	n/a	n/a
StrataCooler	n/a	n/a
Freezer, −80°C	n/a	n/a
Liquid nitrogen freezer	n/a	n/a
Plate shaker	n/a	n/a
Fluorescence-activated cell sorter (FACS)	n/a	n/a
Bright-field microscope with camera	n/a	n/a

MATERIALS AND EQUIPMENT

EDTA/DTT mix		
Reagent	Final concentration	Amount
IMDM	n/a	24.2 mL
EDTA (0.5 M)	5 mM	250 μL
DTT (1 M)	2 mM	50 μL
FBS	1%	250 μL
P/S (100×)	1×	250 μL
Total	**n/a**	**25 mL**

Note: Prepare and use on day of tissue processing.

Collagenase D mix		
Reagent	Final concentration	Amount
IMDM	n/a	29.4 mL
Collagenase D	1 mg/mL	30 mg
FBS	1%	300 μL
DNase I	1000 U/mL	300 μL
Total	**n/a**	**30 mL**

Note: Prepare and use on day of tissue processing.

FACS buffer		
Reagent	Final concentration	Amount
PBS	n/a	500 mL
FBS	1%	5 mL
EDTA (0.5 M)	2 mM	2 mL
Total	**n/a**	**507 mL**

Note: Can be prepared in advance and stored at 4°C for at least 2 months under sterile conditions.

T cell medium		
Reagent	Final concentration	Amount
IMDM	n/a	450 mL
FBS	10%	50 mL
Gentamycin (10 mg/mL)	6 μg/mL	300 μL
2-mercaptoethanol (1:280 pre-diluted 14.3 M pure liquid)	50 μM	0.5 mL
Total	**n/a**	**500.8 mL**

Note: Can be prepared in advance and stored at 4°C for up to 4 weeks under sterile conditions.

Advanced DMEM/F12 (AD+) medium		
Reagent	Final concentration	Amount
Advanced DMEM/F12	n/a	500 mL
GlutaMAX (100×)	1×	5 mL
P/S (100×)	1×	5 mL
HEPES (1 M)	10 mM	5 mL
Total	**n/a**	**515 mL**

Note: Can be prepared in advance and stored at 4°C for up to 4 weeks under sterile conditions.

Human intestinal stem cell (HISC) medium		
Reagent	Final concentration	Amount
First mix the following components for 2× EGF/Noggin/Rspondin (ENR) medium:		
AD+ medium	n/a	4 mL
B27 supplement (50×)	2×	400 μL
N2 supplement (100×)	2×	200 μL
n-Acetylcysteine (500 mM)	2.5 mM	50 μL
Noggin conditioned medium (NCM, see notes)	20%	2 mL
Rspondin conditioned medium (RCM, see notes)	40%	4 mL
mEGF (50 μg/mL)	0.1 μg/mL	20 μL
Total	**n/a**	**10.7 mL**
Then, to make 21 mL HISC, add the following components to 10.7 mL 2× ENR medium:		
[Leu^{15}]-Gastrin (10 μM)	10 nM	20 μL
Nicotinamide (1 M)	10 mM	200 μL
A83-01 (500 μM)	0.5 μM	20 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Wnt3a conditioned medium (WCM, see notes)	50%	10 mL
SB202190 (20 mM; optional, see notes)	10 μM	10 μL
Human IL-2 (500 000 U/mL; optional, see notes)	50 U/mL	2 μL
Y (1 mM; optional, see KRT and notes)	10 μM	20 μL
Total	n/a	21 mL

Note: When prepared, store 2× ENR at 4°C for up to maximally 3 weeks and HISC at 4°C for up to 1 week.

Note: NCM and RCM are home-made, employing Rspo-I-Fc or Noggin-Fc-producing HEK293T cells, which are cultured over 2–3 weeks to obtain NCM and RCM, this has been previously described in detail by Broutier et al. (2016), Vonk et al. (2020), Pleguezuelos-Manzano et al. (2020). Stably transfected Wnt3a-producing L-cells are available to produce Wnt3a, and supernatant is harvested to obtain WCM. The generation of WCM has also been described in detail by Broutier et al. (2016), Vonk et al. (2020), and Pleguezuelos-Manzano et al. (2020). Quality control upon usage of a new batch of RCM, NCM and WCM is recommended by testing the efficacy of each new batch in established organoid cultures individually. Alternatively, recombinant R-Spondin, recombinant Noggin, recombinant Wnt3a or Wnt surrogates are commercially available. Suggested concentrations for human recombinant R-Spondin (1 μg/mL), human recombinant Noggin (100 ng/mL), and human recombinant Wnt3a (100 ng/mL) (Sato et al., 2011) as well as Wnt surrogates (concentrations in the range of 0.5–10 nM) (Janda et al., 2017; Miao et al., 2020) have been published. However, titration of these proteins is recommended to optimize efficacy.

Note: Prepare HISC *with* SB202190 and *without* IL-2 when culturing intestinal organoids alone. However, when co-culturing intestinal organoids with $CD4^+$ T cells prepare HISC *without* SB202190 and *with* IL-2.

Note: Prepare HISC *with* Y only if using the HISC to culture organoids from single cells, either immediately after seeding the single cells harvested from tissue or after cultured organoids have been turned into single cells using TrypLE (see 'Generation of organoid-T cell co-cultures; Produce a single-cell suspension from intestinal organoid cultures'). Otherwise, prepare HISC *without* Y.

Antibody mix to obtain viable $CD45^+CD3^+CD4^+CCR7^-CD45RA^-$ Tem cells		
Reagent	Final concentration	Amount
FACS buffer	n/a	89.25 μL
Live-Dead Fixable Red (1:50 pre-diluted in FACS buffer)	1:5000	1 μL
CD45-FITC	1:100	1 μL
CD3-V500	1:25	4 μL
CD4-APC	1:80	1.25 μL
CCR7-PE	1:40	2.5 μL
CD45RA-BV650	1:100	1 μL
Total	n/a	100 μL

Note: Prepare and use on the day of cell sorting, keep at 4°C and in the dark until use.

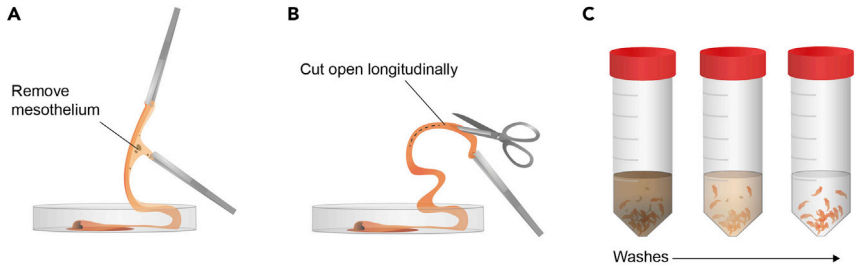

Figure 1. Preparation of the intestine

(A) Remove mesothelium from the intestine.

(B) Cut the intestine open longitudinally.

(C) Intestinal tissue cut into 0.5×0.5 cm segments and washed by shaking in PBS.

STEP-BY-STEP METHOD DETAILS

Establishing organoid cultures from intestinal tissue samples

Timing: 2–3 h

This section describes the generation of stable organoid cultures from fetal intestinal tissues and adaptations for the generation of organoids from pediatric or adult tissues. Organoids can be generated from a tissue piece as small as regular biopsies (1-2 mm in size) obtained at endoscopy or larger tissue sections obtained at surgery. The latter is described here as it requires more steps before isolating the stem cell containing crypts. Typically, around 2 cm^2 tissue is sufficient for both organoid generation and subsequent lamina propria lymphocyte isolation. Biopsies, although sufficient for organoid generation, are insufficient starting material to retrieve enough lamina propria lymphocytes for co-culture in most cases (see also 'isolation of lymphocytes from intestinal lamina propria tissue samples').

1. Remove the mesothelium surrounding the intestine (Figure 1A) and remove the muscular layer in case of pediatric and adult tissues (see also Schreurs et al. [2017]).
2. Cut the intestine open longitudinally (Figure 1B).
3. Cut the intestinal tissue into small pieces (~0.5×0.5 cm) (Figure 1C).
4. Wash tissue pieces 3–4× in 30 mL PBS in a 50 mL centrifuge tube by shaking vigorously until the faeces is removed and the supernatant is clear (see note) (Figure 1C).
5. Remove PBS and distribute tissue pieces in wells of a 6-well plate.
6. Add 5 mL EDTA/DTT mix per well.
7. Incubate for 1 hr in EDTA/DTT mix at 4°C on a shaker (400–600 rpm).
8. Vigorously pipet tissue pieces in EDTA/DTT mix up-and-down 20× with a 25 mL serological pipet.
9. Filter the collected supernatant through a 70 μm cell strainer in a 50 mL centrifuge tube, *do not discard the tissue pieces but place them on ice in a 50 mL centrifuge tube and proceed with the isolation of lymphocytes from lamina propria.*
10. Spin the filtered cells 10 mins at 500 *g* and 4°C.
11. Discard the supernatant and resuspend the pellet in 10 mL AD+, transfer to a 15 mL centrifuge tube.
12. Spin tube 5 mins at 500 *g* and 4°C.
13. Resuspend the pellet in the appropriate volume of AD+ and transfer to a pre-cooled 1.7 mL microcentrifuge tube. Per 1/24 well use 10 μL AD+ (see note).
14. Mix the cell suspension with Matrigel (see note) at a ratio of 1:2 (add 20 μL Matrigel per 1/24 well). Avoid creating bubbles in the Matrigel; slowly pipet the Matrigel on top of the pellet by ejecting the Matrigel against the wall of the tube above the cell pellet, do not go past the first stop on the pipet. Turn down the volume of the pipet to 50% of the Matrigel-cell suspension volume and gently mix by slowly pipetting up-and-down without going past the first stop on the pipet.
15. Seed 3 drops of 10 μL per well in a pre-warmed 24-well plate. See also troubleshooting problem 2.
16. Incubate the plate for 10 mins at 37°C.

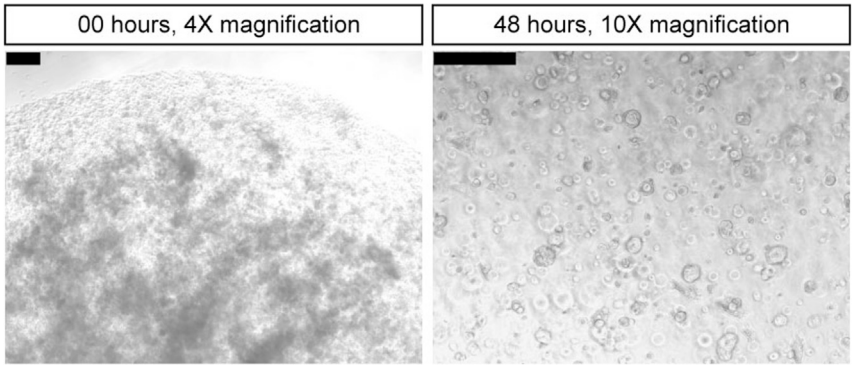

Figure 2. Generating organoids from fetal intestinal epithelium

The image on the left represents fetal intestinal epithelium seeded after detachment at a high density. The image on the right displays a magnified image of the same culture after 48 h where the formation of organoids can be observed. Images were acquired on the Leica DM IRB Inverted microscope (Leica). The black scale bar represents 150 μm.

17. Add 500 μL HISC+Y per well and culture at 37°C and 5% CO_2.
18. Change the medium every 2–3 days. Switch to Y-free medium the first time the culture is refreshed, see 'culturing of intestinal organoids'.
19. Passage organoids after 5–10 days, depending on growth rate, see 'culturing of intestinal organoids; passaging organoid cultures'.

Note: In our experience, organoid cultures generated from intestinal tissues are rarely infected. Therefore, we do not recommend washing the tissue with antibiotics or adding extra antibiotics to the cultures generated. However, when infections frequently occur, these can be prevented by culturing organoids in media containing the antimicrobial agent Primocin (InvivoGen; working concentration 100 μg/mL).

Note: The number of wells that can be seeded from one intestine depends on the size of the tissue. As a rule of thumb, resuspend the pellet in just enough AD+ until the pellet is no longer too viscous to pipet with a P20 tip. From one 1 fetal intestinal tissue piece (which equates to between 10–15 cm^2 tissue) usually around 4–5 wells can be seeded (or 12–15 densely packed Matrigel drops of 10 μL each). This will result in a highly densely seeded cell suspension, dilute with AD+ if necessary (Figure 2).

Note: 24-well plates should be pre-warmed for at least 1 h in the incubator at 37°C. Additionally, Matrigel should be thawed on ice for at least 1 h before it is liquid enough to pipet, always keep Matrigel at 4°C during this protocol and store at −20°C when not in use. Do not let the Matrigel warm above 8°C until culturing organoids in the incubator (see also troubleshooting problem 4). If Matrigel drops are too fluid and do not adhere into a small dome extend pre-warming of the plates up to 1 week before seeding.

Isolation of lymphocytes from intestinal lamina propria tissue samples

Timing: minimum 4 h depending on size of tissue

This section describes how to isolate lymphocytes from intestinal lamina propria tissue after epithelial detachment and organoid culture generation. To retrieve sufficient lamina propria lymphocytes for co-culture experiments, typically a minimum of 2 cm^2 intestinal tissue is preferred. Although there is a lot of variability between donors and tissue origin, 2 cm^2 intestinal tissue should yield at least 1–5 million mononuclear cells.

Lamina propria lymphocyte (LPL) isolation

20. Combine all tissue pieces left over after detachment of the epithelial cells (see 'establishing organoid cultures from intestinal tissue samples' step 9) in 1 well of a 6-well plate and mince with scissors.
21. Transfer the minced tissue to a 50 mL centrifuge tube and add 15 mL Collagenase D mix.
22. Incubate for 30 mins at 37°C on a shaker at 100 strokes/min.
23. Collect the supernatant and filter through a 70 µm cell strainer.
24. Spin the tube with the cell suspension at 10 mins at 500 *g* and 4°C.
25. Discard the supernatant and wash the cells with PBS and place on ice.
26. To increase cell yield, resuspend any tissue pieces that may remain after the first digestion in 15 mL fresh Collagenase D mix.
27. Incubate for 30 mins at 37°C on a shaker at 100 strokes/min.
28. Collect the supernatant and filter through a 70 µm cell strainer.
29. Pool the cells and spin 10 mins at 500 *g* and 4°C.
30. Discard the supernatant and wash the cells with PBS.

Lymphocyte isolation and freezing

31. Resuspend the cells in 10 mL HBSS, then layer the cells on 4 mL standard isotonic Percoll solution (SIP; 0,24 mL 10× PBS + 2,16 mL Percoll + 1,6 mL PBS).
32. Gradient centrifuge the cells for 20 mins at 18°C–22°C and 1000 *g* (acceleration in 120 sec; 0 break).
33. Collect the lymphocyte ring.
34. Wash 2× with PBS (see steps 24 and 25).
35. Count the cells with a Bürker chamber, exclude dead cells with 0.4% Trypan Blue.
36. Resuspend the cell pellet in ice-cold FBS and dropwise add an equal volume of ice-cold FBS + 20% DMSO (reaching an end concentration of 10% DMSO), transfer cells to a cryovial and freeze in a StrataCooler at −80°C (see note).
37. On the next day, transfer the frozen cryovials to a liquid nitrogen tank.

Note: This protocol describes the isolation of LPL from fetal intestinal tissue, however it can also be successfully applied to pediatric or adult intestinal tissues. Please see Schreurs et al. (2017) for a detailed protocol on epithelial detachment and subsequent harvest of LPL from pediatric and adult tissues.

Note: It is recommended to freeze maximum 20 million/mL lamina-propria derived lymphocytes with a recovery of around 70% after thawing.

Pause point: After establishment of the organoid culture and freezing of the lamina propria lymphocytes, as it takes some time for the organoid cultures to grow, the protocol has a natural pause point here.

Culturing of intestinal organoids

Timing: ~5 h/week

This section details the maintenance of long-term organoid cultures by weekly passaging the cultures.

Long term organoid cultures

38. Change the HISC medium of the organoid cultures every 2–3 days. As the organoid cultures are embedded in Matrigel on the plate, medium can be easily removed without disturbing the

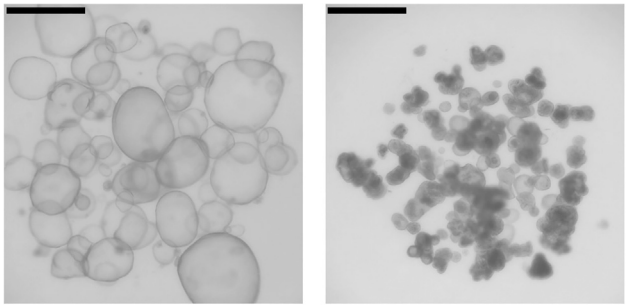

Figure 3. Organoid culture of epithelial cells from fetal intestines

The image on the left represents an organoid culture from fetal intestinal epithelial cells in stem cell medium (HISC), this culture is ready for passing. The image on the right represents differentiated organoids from the same passage cultured in differentiation medium. Images were acquired on the AxioZoom V16 (Zeiss). The black scale bar represents 1 mm.

Matrigel droplets. Make sure the fresh medium is at 18–22°C when added to the cultures as colder medium will liquefy the Matrigel droplets. See also troubleshooting problems 1 and 5.

39. Immediately after seeding the organoids from fresh intestine or after turning organoid cultures into single cells using TrypLE (see also the section 'produce a single cell suspension from intestinal organoid cultures'), add Y to HISC, then switch to HISC without Y with the next refreshment of medium.
40. Passage organoid cultures every 5–7 days, depending on growth (Figure 3). If not passaged frequently enough, the organoids will differentiate and lose their stem cell qualities (Figure 3). Split the cell cultures depending on density, usually 1 well can be split into 4 new wells each week. Monitor the growth rate and density of the organoid cultures closely as there is donor-to-donor variation in growth rates. Ideal density during normal culture is best judged by microscopy; per Matrigel droplet organoids are ideally close together and touching but not overlapping between 30–40 organoids per 10 μL Matrigel droplet at passaging 1:4. Too dense seeding of organoids will lead to faster differentiation represented by darkening and loss of round cystic appearance of the organoids (Figure 3).
41. Organoid cultures stabilize around passage (P) 3, therefore we recommend to not use organoid cultures for experiments prior to P3. Organoid cultures prior to P3 can have remaining debris from the isolation and slightly more tubular shape before stable cystic cultures are obtained.

Passaging organoid cultures

42. Pipet 1 mL ice-cold AD+ in a 15 mL centrifuge tube per culture and keep on ice.
43. Remove the medium from each well.
44. Harvest the organoids by liquefying the Matrigel with 1 mL ice-cold AD+ per well and transfer into a 15 mL centrifuge tube (maximum of 8/24 wells per 15 mL centrifuge tube). See also troubleshooting problem 3.
45. Fill the tube up to 14 mL with ice-cold AD+.
46. Centrifuge for 5 mins at 200 *g* and 4°C.
47. Remove the supernatant with a 5 mL serological pipet and P200 tip, remove as much medium as possible including any Matrigel on top of the pellet (Figure 4).
48. Resuspend the pellet in 1 mL AD+.
49. Mechanically disrupt the organoids by pipetting multiple times (20 repetitions) with a P1000, followed by disruption with a P200, and then a P20 tip.
50. Check organoid fragments under a microscope to confirm they are disrupted.
51. Repeat mechanical disruption until organoids no longer appear round and cystic, but rather like shards (Figure 5).
52. Top up to 14 mL with ice-cold AD+.

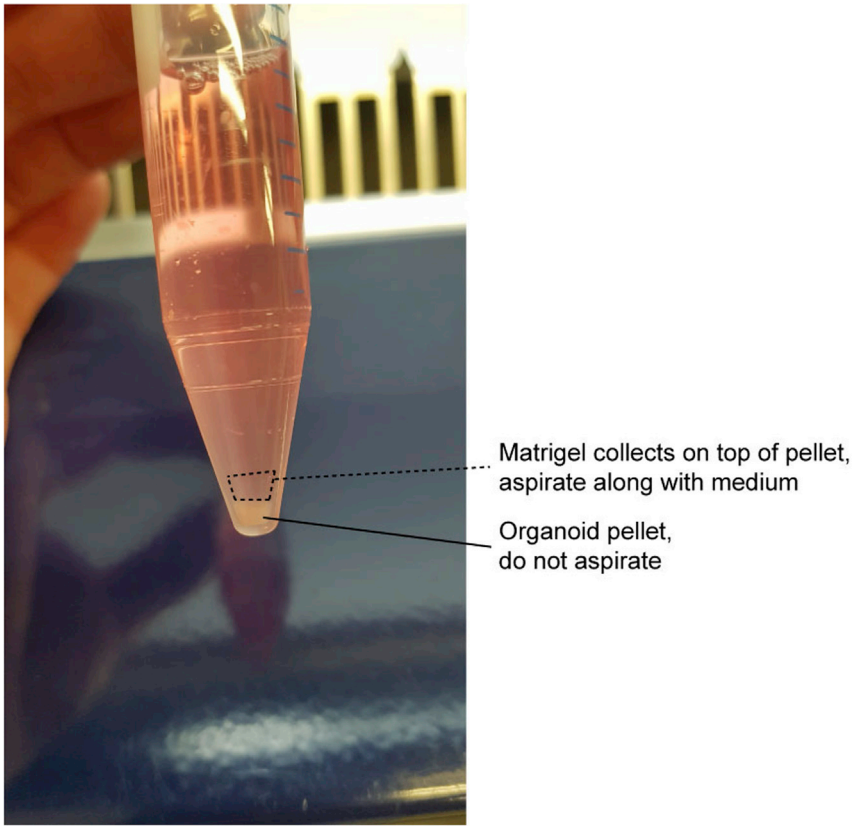

Figure 4. Pelleted organoids

When pelleting organoids there will be a white organoid pellet visible in the bottom of the 15 mL tube. Additionally, Matrigel may be observed floating atop the pellet, which is here indicated by the dashed box in the image. When aspirating the medium, remove the Matrigel atop the pellet but do not remove the white pellet at the bottom of the tube containing the organoids.

53. Centrifuge for 5 mins at 200 *g* and 4°C.
54. Remove the supernatant with a 5 mL serological pipet and any remaining medium with a P200 tip.
55. Resuspend the pellet in the appropriate volume of AD+ and transfer to a pre-cooled 1.7 mL microcentrifuge tube. Per 1/24 well use 10 µL AD+.
56. Mix the cell suspension with Matrigel at a ratio of 1:2 (add 20 µL Matrigel per 1/24 well).
57. Seed 3 drops of 10 µL per well in a pre-warmed 24-well plate.
58. Incubate the plate for 10 mins at 37°C.
59. Add 500 µL HISC per well and culture at 37°C and 5% CO_2.
60. Change medium every 2–3 days.
61. Passage organoids every 5–7 days.

Preparation of lamina propria lymphocytes for co-culture with intestinal organoids

Timing: minimum 5 h depending on cell yield

Once stable organoid cultures have been established, lamina propria lymphocytes can be added to create co-cultures. This section describes how to prepare the lamina propria lymphocytes isolated and frozen in section 'isolation of lymphocytes from intestinal lamina propria tissue samples' for co-culture with intestinal organoids.

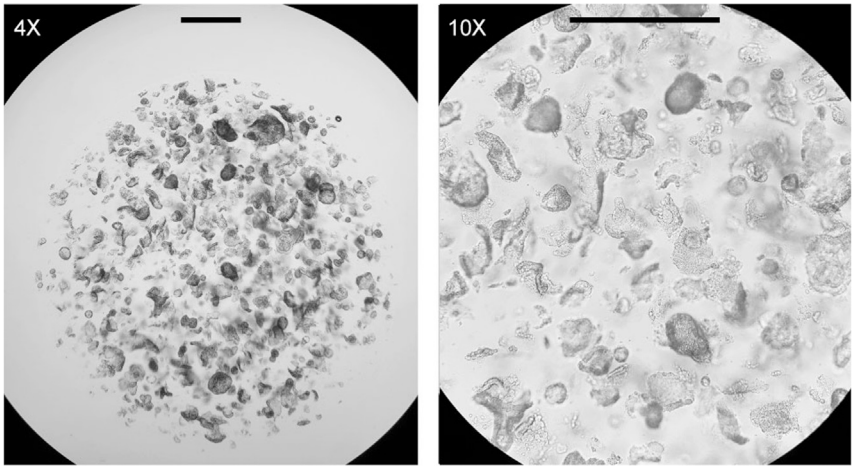

Figure 5. Organoid appearance after passaging

Both images represent an organoid culture that was disrupted by pipetting in order to passage the culture. After disruption of the culture, organoids appear like 'shards'. Image on the left 4× magnification, image on the right of the same droplet at 10× magnification. Images were acquired on a Leica DM IRB Inverted microscope (Leica). The black scale bar represents 0.5 mm.

Thawing of lamina-propria derived lymphocytes

62. Thaw the cells at 37°C in a water bath until a pea-sized clump of ice is left.
63. Transfer the cell suspension to a 50 mL centrifuge tube.
64. Add dropwise 20 mL ice-cold IMDM with 30% FBS while shaking the tube. Mix well by pipetting.
65. Centrifuge for 10 mins at 500 *g* and 4°C.
66. Remove the supernatant and wash the pellet 2× with PBS, while keeping on ice.
67. Count the live cells using a Bürker chamber and 0.4% Trypan Blue solution.

Isolate lamina propria-derived effector memory $CD4^+$ T cells using flow-cytometry-based sorting

68. Resuspend the LPL in FACS buffer.
69. Use a Bürker chamber to determine the cell count.
70. Pellet the cells at 400 *g* at 4°C for 5 mins, then remove the supernatant.
71. Prepare the antibody-mix to stain $CD4^+$ T surface molecules (for the recipe please see 'materials and equipment'; see also troubleshooting problem 7) and keep on ice and in the dark until further usage. Use 100 µL antibody mix per 10 million mononuclear cells per donor. The antibody amount can be adjusted dependent on the cell count.
72. Resuspend the LPL in the antibody mix and incubate for 30 mins at 4°C, in the dark and shaking at 600 strokes/min.
73. After incubation, wash the cells by adding 1 mL (per 100 µL antibody mix) FACS buffer to the suspension; pellet the cells at 400 *g* at 4°C for 5 mins, then remove the supernatant.
74. Repeat step 73 to wash the cells again, reducing background fluorescence.
75. Resuspend the cells at maximum 25 million cells/mL in FACS buffer.
76. Acquire the labeled mononuclear cells on a FACS-sorter and use the following gating strategy (Figure 6) to sort viable $CD45^+CD3^+CD4^+CCR7^-CD45RA^-$ Tem cells into tubes filled to at least 10% with T cell medium.
77. Pellet the cells at 400 *g* and 4°C for 5 mins, remove the supernatant and pool the tubes if required. Use T cell medium.

Note: In step 75 a maximum suspension of 25 million cells/mL is best suited for the 4-laser FACS Aria IIu SORP (BD Biosciences) used in Schreurs et al. (2019). However, you may wish

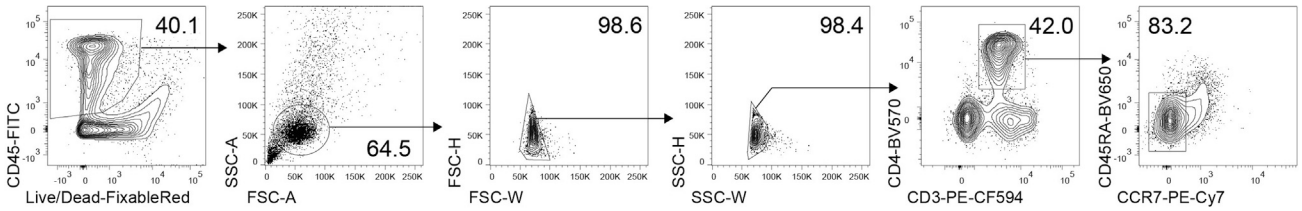

Figure 6. Gating strategy

Flow cytometric gating strategy to sort viable CD45$^+$CD3$^+$CD4$^+$CCR7$^-$CD45RA$^-$ cells from fetal intestinal lamina propria.

to resuspend the cells at a different concentration. Please consult the local FACS operator or aim for a suspension that yields maximum 12 000 events/s.

Note: Please make sure that all antibodies have been properly titrated for your cell type and that a compensation matrix is set-up on the FACS-sorter. Here, UltraComp eBeads were used.

Alternatives: In this publication, CD4$^+$ effector-memory T (Tem; viable CD45$^+$CD3$^+$CD4$^+$-CCR7$^-$CD45RA$^-$) cells were isolated, however, the antibody mix can be adjusted to isolate other immune cell populations residing in the lamina propria or from blood or other adult tissues.

Alternatives: As an alternative to FACS-sorting CD4$^+$ T cells, magnetic-activated cell isolation techniques can be used to isolate CD4$^+$ T cells according to the manufacturer's instructions.

TCR activation of lamina propria-derived CD4$^+$ T cells

78. Resuspend the sorted cells in T cell medium at 4 million cells/mL.
79. Prepare 2× stimulation medium by adding the following components per 1 mL T cell medium.
    a. 0.4 µL 1.5 mg/mL anti-CD3 (end concentration 0.6 µg/mL)
    b. 2 µL 2 mg/mL anti-CD28 (end concentration 4 µg/mL)
80. Combine the cells suspended in T cell medium (part 1) and 2× stimulation medium (part 2) in equal volumes to achieve an end concentration of 2 million cells/mL in 1× stimulation medium.
81. Incubate the cell suspension for 2 h at 37°C and 5% CO_2.

Alternatives: As an alternative, other TCR stimulation reagents can be used. However, the required concentration for efficient T cell activation should be titrated beforehand, as the strength of the anti-CD3 can also vary batch-to-batch.

Generation of organoid-T cell co-cultures

Timing: 2–3 h

This section of the protocol details seeding of CD4$^+$ T cells and epithelial stem cells to generate organoid-T cell co-cultures and their maintenance.

Produce a single-cell suspension from intestinal organoid cultures

82. Pre-warm (37°C) TrypLE and prepare 10 mL AD+ with 10% FBS (18°C–22°C).
83. Remove the supernatant from organoid cultures.
84. Harvest the organoids by liquefying the Matrigel using 800 µL ice-cold AD+ per well. Transfer the organoids in suspension to a 15 mL tube. Do not exceed 6 wells per 15 mL tube.
85. Fill the tubes containing the organoids in suspension up to 14 mL with ice-cold AD+ and pellet the organoids at 200 *g* and 4°C for 5 mins. Remove the supernatant.

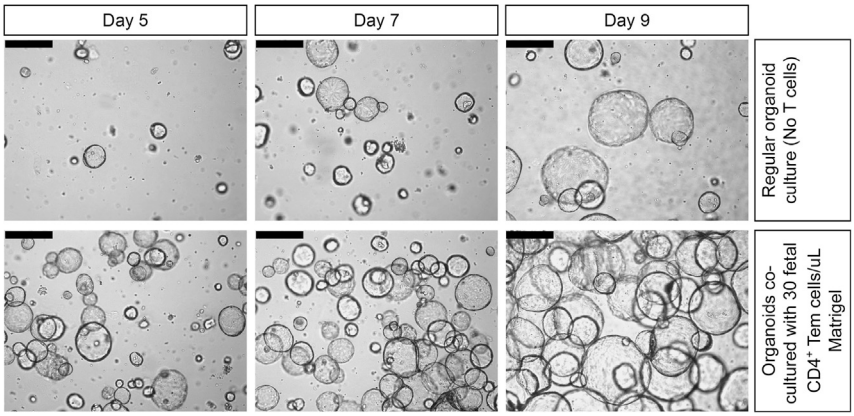

Figure 7. Fetal intestinal organoids and CD4$^+$ Tem cells in co-culture

The top row represents organoids cultured under normal conditions in HISC-SB+IL-2, without the addition of T cells. The bottom row represents organoids from the same fetal intestine, cultured under similar conditions but with the addition of activated viable CD4$^+$ Tem cells (30 cells/μL Matrigel). Images were acquired on the Leica DM IRB Inverted microscope (Leica) on days 5, 7, and 9 post initiation of single cell culture or co-culture. The black scale bar represents 150 μm.

86. Per 6 Matrigel drops (10 μL each) of harvested organoids, add 200 μL TrypLE to the pellet and resuspend. Manually shake in a water bath (37°C) for 2 min, then pipet up-and-down 20 times (with force) using first a P200 and then a P20 tip. Inspect the digestion by holding the tube under the microscope.
87. Repeat step 86, including adding fresh TrypLE to the suspension, until >95% single cells are observed and for a maximum of 4 rounds.
88. Add 10 mL AD+ with 10% FBS per tube containing organoids to inactivate the TrypLE. Incubate at 18°C–22°C for 5 mins.
89. Pellet the cells at 400 *g* and 4°C for 5 mins, then remove the supernatant.
90. Add up to 14 mL ice-cold AD+ to each tube and repeat step 89.
91. Resuspend the cells in ice-cold AD+ and count the total number of single cells in the organoid cell suspension with a Bürker chamber.
92. Per 1/24 well, take 10 000 single organoid cells (3333 cells/droplet) and keep in AD+ on ice.

⚠ CRITICAL: Perform each step at the suggested temperature as Matrigel only liquefies at 4°C.

Alternatives: Specific research questions may require different concentrations of single epithelial cells and immune cells seeded together, these ratios should be tested in preliminary experiments to assess the impact of altering the cell input. For example, including a higher concentration of intestinal epithelial cells in the 10 μL drops will increase organoid development however can also promote differentiation and then impair proliferation. Similarly, increased numbers of CD4$^+$ T cells can also impact the results of the cultures as illustrated in our related publication (Schreurs et al., 2019). The optimal organoid-to-immune cell ratio and numbers for the specific tissue or disease setting should be tested and for example immune histochemical assessment of the tissues can inform on physiological and pathophysiological ratios of epithelial cells and immune cells.

Generation and maintenance of organoid-T cell co-cultures

93. Resuspend sorted activated CD4$^+$ Tem cells in AD+ at a concentration of 90 cells/μL (3× the desired end concentration).

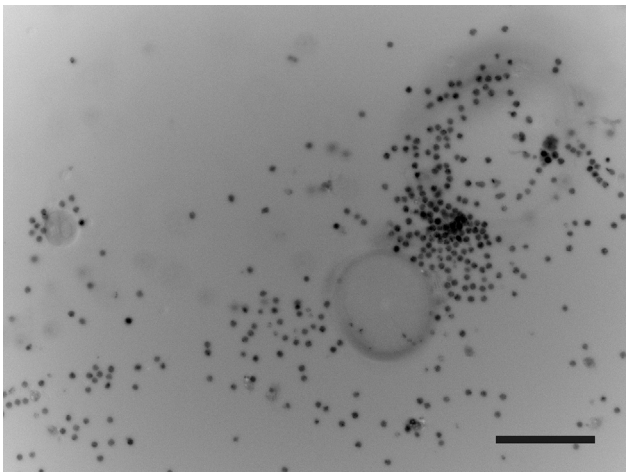

Figure 8. CellTrace Violet (CTV)-stained T-cells present in co-cultures

The image shows T cell-organoid co-cultures with T cells stained by CTV. Images were acquired on an EVOS M5000 Imaging System (Thermo Fisher). The black scale bar represents 100 μm.

94. Pellet the appropriate number of intestinal organoid single cells suspension (10,000 single cells per 1/24 well) at 400 *g* and 4°C for 5 mins, then remove the supernatant.
95. Resuspend the single organoid cell pellet (step 94) in the appropriate volume of AD+-harboring 90 cells/μL $CD4^+$ Tem and transfer to a pre-cooled 1.7 mL microcentrifuge tube. Per 1/24 well use 10 μL AD+ with resuspended T cells at 90 cells/μL and resuspended single organoid cells at 1000 cells/ul.
96. Mix the cell suspension with Matrigel at a ratio of 1:2 (add 20 μL Matrigel per 1/24 well) for a final concentration of 30 $CD4^+$ Tem cells/μL (300 cells/droplet) and 333 single organoid cells/uL (3333 cells/droplet).
97. Seed 3 drops of 10 μL (step 96) per well in a pre-warmed 24-well plate. See troubleshooting problem 6.
98. Incubate plate for 10 mins at 37°C.
99. Add 500 μL HISC+Y-SB+IL-2 per well and culture at 37°C and 5% CO_2.
100. Change the medium every 2–3 days. Switch to Y-free medium the first time the culture is refreshed.
101. Track organoid development with a microscope (Figures 7 and 9). See the next section ('imaging and quantification of organoid-T cell co-cultures') for suggestions on tracking organoid development and quantification thereof. Briefly, organoid growth should be visible by day 5 and the culture can be maintained for 10–15 days depending on the growth speed of the organoids.

⚠ CRITICAL: Double check your calculations to achieve the desired end concentration of the co-cultured cells.

Note: To determine the presence and location of T cells within the organoid cultures, T cells can be incubated with reagents such as CellTrace Violet (Invitrogen) or DAPI prior to sorting and can thereby be visualized by microscopy (Figure 8).

Imaging and quantification of organoid-T cell co-cultures

Timing: 1–2 h on each microscopy day

This section of the protocol offers examples on imaging and analyses of organoid-T cell co-cultures.

102. Measure organoid development by imaging co-cultures every 2–3 days with a light microscope capable of acquiring brightfield images.

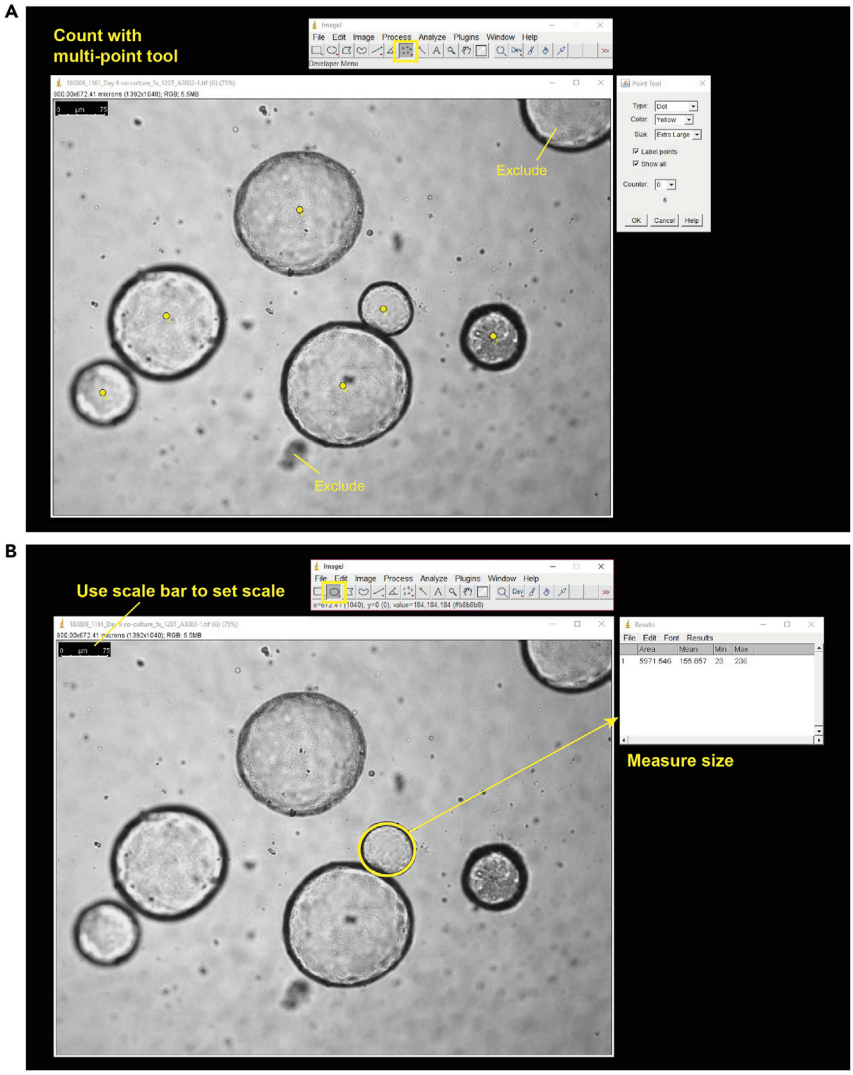

Figure 9. Analyzing organoid growth

(A) Organoids can be counted using the ImageJ built in 'Multi-point tool' (highlighted in yellow). Because organoids are grown in a dome-shaped Matrigel droplet, some organoids might be blurry or only partially within the field of view. Therefore, it is recommended to define a set of rules prior to counting and apply those rules to all images. For example, we exclude i) any organoids that do not have clearly defined edges/appear blurry as interpretation may vary between observer and ii) organoids that are >50% outside the field of view.

(B) Organoid size can be measured using the ImageJ "Measure" tool (Analyze > Measure). First, set the correct scale (Analyze > Set Scale) to convert pixels to known size i.e., using the scale bar in the top left corner. Secondly, use the "Oval" tool (highlighted in yellow) to outline the edges of individual organoids and acquire their measurement with the "Measure" tool. The black scale bar represents 75 μm.

103. Image each Matrigel droplet.

104. Count organoid number per field of view and determine size of individual organoids by masking the organoids in ImageJ software, using the 'Multi-point tool' and 'Measure' features (Figure 9).

Alternatives: Organoids can also be quantified by analyzing the cell count by flow cytometry. Next to providing the cell count this type of measurement can be combined with epithelial cell markers such as LRG5, Ki-67, EPCAM, MUC2, sucrose isomaltase or villin to assess epithelial cell differentiation. Similarly, the T cells can be assessed by flow cytometry (for example via addition of CD45, CD3, IL-22 antibodies) to assess differentiation or cytokine production.

For flow cytometric analyses of organoids and T cells, a single cell suspension is needed which can be obtained as described in the section 'Produce a single cell suspension from intestinal organoid cultures' and incubated with antibodies of choice. Additionally, once single cell suspensions are obtained, RNA can be isolated for RNA-Seq. Furthermore, protein secretion can be measured in the culture supernatant by ELISA or multiplex bead-assay.

EXPECTED OUTCOMES

This protocol describes the establishment of human intestinal organoid-$CD4^+$ Tem co-cultures for *in vitro* modeling of T cell-intestinal stem cell interactions, building on previously developed intestinal organoid models (Sato et al., 2009). We have generated intestinal organoid cultures derived from several types of intestinal tissues (e.g., fetal, pediatric or adult) as well as isolated lymphocytes from these samples. Therefore, our protocol serves as a basis to investigate crosstalk between immune cells and intestinal epithelial cells for studies investigating immune and intestinal ontogeny and inflammation. Furthermore, this protocol provides a framework to examine the crosstalk of intestinal epithelial cells in organoids with other immune cell subsets and can identify soluble and membrane bound factors, which are key players in the communication between immune cells and intestinal stem cells. While our work so far focused on the interaction of immune cells with intestinal stem cells, this protocol can also be extended to crosstalk of immune cells with differentiated intestinal epithelial cells such as goblet cells, enterocytes or enteroendocrine cells to identify other mechanisms involved in epithelial regeneration as well as immune responses against pathogens infecting epithelial cells.

LIMITATIONS

This protocol describes the successful establishment of a long-term co-culture system of human intestinal organoids and immune cells, allowing the study of specific interactions between intestinal epithelial and immune cells (Bar-Ephraim et al., 2020). However, several limitations are implied; functional behavior of intestinal epithelial and $CD4^+$ T cells might be affected by the culture conditions requiring further investigation of the co-culture medium composition as well as the influence of the extracellular matrix gel to optimize the system. The recently developed completely synthetically produced hydrogels could serve as an alternative with likely less influence on the behavior of immune cell (Gjorevski and Lutolf, 2017). Furthermore, this system is lacking other cell types such as stromal cells and other immune cells. In addition, the microbiota, which emerges as an important parameter in intestinal diseases and homeostasis, is not included in the described protocols, however others have advanced these models (Min et al., 2020). The long-term co-culture system of intestinal organoids and T cells provides a system for basic and translational research questions for the human setting which can be transferred to other tissues and immune cell types for future applications.

TROUBLESHOOTING

Problem 1

No or low efficiency of organoid formation after seeding from tissue.

Potential solution

Cells are seeded too sparsely or too densely. Both can impede organoid formation. As a rule of thumb, upon seeding the cells should be relatively dense in the center of the Matrigel droplet, while still allowing distinguishing individual cells in the periphery of the droplet (Figure 2), cells will naturally collect in the center of the droplet. Ensure the addition of ROCK inhibitor Y to the organoid medium when seeding primary epithelial cells from tissue

Problem 2

Organoid density is not optimal while seeding and is either i) too low or ii) too high.

Potential solution

i) When organoid density is too low, try to concentrate the organoids by centrifugation and resuspension in a smaller volume of medium and Matrigel (1:2 ratio). ii) When organoid density is too high, add additional medium and Matrigel (1:2 ratio) to dilute the organoid suspension.

Problem 3

Organoid loss during passaging.

Potential solution

Rather pipet manually than using an aspirator to remove the last volume of supernatant. Furthermore, collect the supernatant in a separate tube, which can be centrifuged again to not loose organoids in case you accidentally aspirated part of the pellet.

Problem 4

Matrigel solidifies while seeding.

Potential solution

This can happen while seeding many wells. Avoid the Matrigel solidifying by using a cold block for storage of the Matrigel-organoid cell mix while seeding, or make multiple smaller aliquots of the Matrigel-organoid cell mix rather than having all the volume in one tube that warms up during seeding.

Problem 5

Organoids differentiate while in HISC.

Potential solution

The quality of the Wnt3a conditioned medium is critical to maintain the 'stemness' of the stem cells. In case organoids differentiate while in HISC, which can be observed by darkening of organoids and losing their characteristic round and cystic shape (Figure 3), we recommend the preparation of a fresh batch of Wnt3A conditioned medium followed by quality control experiment before using this batch. Recently developed Wnt-surrogate is an alternative to Wnt3a conditioned medium (Janda et al., 2017). Alternatively, when the organoids increasingly differentiate with each passage, they need to be more vigorously disrupted during passage by pipetting or treatment with TrypLE.

Problem 6

Inconsistent density of cells between droplets/wells.

Potential solution

Cells quickly sink to bottom of the tube containing Matrigel if stored too long on ice. Seed immediately after mixing the cell suspension with Matrigel and mix properly before seeding. When many wells are seeded, mix the suspension by regularly pipetting up and down.

Problem 7

No or poor antibody-fluorophore signal.

Potential solution

Double check that all antibodies were added to the mastermix. Optimize laser settings at the flow cytometer. Use single-stained cells for compensation instead of beads.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Madeleine J. Bunders (madeleine.bunders@leibniz-hpi.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data supporting the current study are subject to the rules of regulations of the ethical committee of the Amsterdam University Medical Center. Requests for data should be directed to the lead contact, Madeleine J. Bunders (madeleine.bunders@leibniz-hpi.de).

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AUTHOR CONTRIBUTIONS

R.R.C.E.S., M.E.B., A.D., and M.J.B. designed and performed the experiments. R.R.C.E.S., M.E.B., and A.D. collected samples and analyzed the data. R.R.C.E.S., M.E.B., and M.J.B. wrote the protocol. M.J.B. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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