2: Materials and Methods

Collaboration note

Some of the methods described in this chapter have been published as: “Interleukin-22 promotes phagolysosomal fusion to induce protection against *Salmonella enterica* Typhimurium in human epithelial cells” (Forbester et al., 2018) and “Using Human Induced Pluripotent Stem Cell-derived Intestinal Organoids to Study and Modify Epithelial Cell Protection Against Salmonella and Other Pathogens” (Lees et al. 2019).

TEM imaging was performed by David Goulding, and I am grateful for the help of Christine Hale in setting up macrophage cultures, Jessica Forbester for advice on troubleshooting with organoid cultures and Derek Pickard for his assistance in producing the TIMER^{bac} EPEC strain. Bulk RNA-Seq libraries were constructed and sequenced by the DNA pipelines core facility at the Wellcome Trust Sanger Institute, and I thank Artika Nath for her help with the bulk RNA-Seq data analysis and Daniel Kunz for his assistance with the single cell RNA-Seq data analysis. Dr Simon Clare, WTSI, kindly provided the mice used to produce murine organoids.

2.1 Growth and differentiation of hiPSCs into iHO

2.1.1 Culture and passage of induced pluripotent stem cells

Human induced pluripotent stem cells (hiPSCs) were routinely maintained in the Essential 8 Flex™ (Gibco, E8 Flex) medium kit, as per manufacturer’s instructions, to allow weekend-free culture. However, hiPSCs can be adapted from other hiPSC culture systems, such as Essential 8™ (Gibco) and TeSR™-E8™ (Stemcell Technologies) with relative ease. Human intestinal organoids (iHO) were generated using hiPSCs derived from the Kolf2, Rayr2 and Sojd2 lines, as per the protocol published by Forbester et al.[1] These hiPSC were acquired through the Human Induced Pluripotent Stem Cells Initiative Consortium (HipSci; www.hipsci.org), an open-access reference panel of characterised hiPSC lines.[2]

Demographic information for these cell lines is detailed in **Table 2.1**. All lines come from healthy volunteers, with the Kolf2 line having been used extensively in research on host-pathogen interactions by the Dougan group.[3,4] Both this line and the other lines selected as comparators were chosen based on parameters provided during phenotyping by the HipSci consortium; namely high pluritest score, low copy number variations, well characterised assay data and high differentiation potential.

Table 2.1: Demographics of healthy volunteer cell lines used in this study

Cell line:	Donor age (years):	Donor sex:	Donor ethnicity:
Kolf2	55-59	Male	White – British
Sojd2	45-49	Female	White – Other
Rayr2	75-79	Male	White – British

iPSCs were grown until colonies covered approximately 80-90% of the plate surface. Plates for passage were prepared 1 hour prior to use, by adding Vitronectin XF 10 μg/mL (Stemcell Technologies) diluted in Dulbecco's PBS without calcium and magnesium (DPBS; Gibco) to tissue-culture treated plates and E8 Flex medium warmed to room temperature (r.t.). Medium was removed from iPSCs ready for passage and cells washed twice with DPBS (No Ca^{2+} or Mg^{2+}). Versene solution (Life Technologies) was added to plates, and they were incubated at r.t. for 5-8 minutes, until holes started to appear in the centre of the previously confluent iPSC colonies. At this time, Versene was aspirated, discarded, and replaced with E8 Flex. iPSCs were dislodged by washing the plate surface in this medium and cells moved to a 15mL Falcon tube. Vitronectin was aspirated from the pre-coated plates and replaced with E8 Flex, and the appropriate volume of iPSC suspension to give a 1:10 dilution of cells on the new plate. Ratios for splitting can be adjusted dependent on iPSC growth rate of different cell lines. Plates were rocked to disperse the iPSC across their surface, incubated at 37 °C / 5% CO_2 and fed the day following passage.

2.1.2 Differentiation from iPSC to hindgut

The directed differentiation process and cytokines involved are outlined in **Table 2.2.**

Table 2.2: Directed differentiation process for hindgut production from hiPSC

Day:	Activity:
0	iPSCs were split onto a 10cm tissue culture-treated dish, pre-coated with Vitronectin XF as described above, into 10 mL Essential 8 Flex medium supplemented with activin A (10 ng/mL) + basic fibroblast growth factor (bFGF; 12 ng/mL). Growth factors were added to medium directly before use; as was the case in all subsequent steps.
1	Medium was changed to 10 mL Essential 8 Flex medium supplemented with activin A (10 ng/mL) + basic fibroblast growth factor (bFGF; 12 ng/mL).
2	Differentiation was commenced by changing the medium to 10 mL Essential 8 Flex medium supplemented with the growth factors: activin A (100 ng/mL), bFGF (100 ng/mL), Bone morphogenetic protein 4 (BMP-4; 10 ng/mL), phosphoinositol 3-kinase inhibitor LY294002 (10 µM) and GSK3 inhibitor CHIR99021 (3 µM).
3	Medium was changed to 10 mL Essential 8 Flex medium supplemented with activin A (100 ng/mL), bFGF (100 ng/mL), BMP-4 (10 ng/mL) and LY294002 (10 µM). Endoderm specification induced by this medium resulted in visible changes to iPSC colony morphology over the following 24 hours.
4	Medium was changed to 10 mL RPMI/B27 medium supplemented with activin A (100 ng/mL) and bFGF (100 ng/mL). RPMI/B27 medium contains: 500 mL of RPMI Medium 1640 with GlutaMAX supplement, 10 mL B27 Supplement (50X, serum free) and 5 mL non-essential amino acids, with optional addition of 5 mL penicillin–streptomycin (10,000 U/ml).
5	Medium was changed to 10 mL RPMI/B-27 medium supplemented with activin A (50 ng/mL).
6	To begin patterning posterior endoderm to hindgut, medium was changed to 10 mL RPMI/B27 supplemented with CHIR99021 (6 µM) + Retinoic acid (3 µM).
7,8,9	Medium was changed daily with the same composition as for day 6. During these steps visible 3-D structures of hindgut became apparent, covering the surface of the plate.
10	The resulting hindgut was embedded in Matrigel (Corning) (See **Figure 2.1** for outline of process with illustration).

2.1.3 Embedding of hindgut into Matrigel

iHO base growth medium (BGM) was produced (see **Table 2.3** for contents) and filter sterilized before use. Medium was removed from the hindgut plate, which was washed once with DPBS (No Ca^{2+} or Mg^{2+}). 5 mL collagenase solution (see **Table 2.4** for contents) was added to the plate and incubated at 37 °C for 5 minutes. The collagenase was inactivated by addition of 5 mL BGM to plate, hindgut structures were dislodged using a cell scraper, and the resulting

suspension collected into a 15 mL falcon tube. The suspension was centrifuged at 1200 rpm for 1 minute. Supernatant was removed and replaced with 10 mL BGM, and hindgut gently pipetted to break it up into smaller pieces. This solution was centrifuged at 750 rpm for 1 minute and washed twice with BGM by repeating this step. Cells were re-suspended in a small volume of BGM (~300-500 µL) and around 100 µL of this solution was added to 1.5 mL Matrigel. 60 µL of this solution was added to one well of a 24 well plate (set up on a plate heater at 37 °C) and allowed to set. Density was checked under a microscope, and if required, further hindgut solution was added until the desired density of seeding was achieved. Matrigel was then spotted out into the remaining wells on the plate. After incubation of the plate at 37 °C for 10 minutes, 800µL BGM containing growth factors (see **Table 2.5**) was added to each well.

Medium was changed every 2-3 days, or immediately if medium was discoloured; on these occasions, Y-27632 was omitted as it is only required when splitting/seeding. After initial seeding into Matrigel, iHO were allowed around 7 days to develop before splitting. By day 3–4 post-embedding, distinct spheres were visible in the culture.

2.1.4 Maintenance and passage of iHO

iHO require at least 1 month of routine passaging after seeding to facilitate maturation, with splitting required every 4–7 days. Note that there will be some variation in iHO development depending on iPSC line used and the density of the initial culture. During the first few passages there will be visible contaminating cells which are not iHO. These will eventually die, leaving a clean culture of spherical and, after approximately 4 weeks, budded organoids (as demonstrated in **Figure 2.1**). In addition, use of an in-hood imaging system can be used to select and passage only iHO with the desired morphology. As iHO mature, they will require splitting every 6-7 days, dependent on growth rate and density. If any of the following are true, iHO should be split prior to this point:

- The luminal cavities of the iHO start to fill up with dead cells
- The Matrigel starts to disintegrate
- iHO start to grow out of the Matrigel
- The culture is too dense and the medium starts to go yellow rapidly

Splitting process:

Medium was removed from iHO and replaced with 500 µL Cell Recovery Solution (BD) per well. The plate was then incubated at 4 °C for 40-50 minutes, at which point iHO were floating in solution. This solution was then gently pipetted into 15 mL falcon tubes (trying not to break up iHO) and allowed to settle for 3-5 minutes before removing supernatant and single cells. iHO were re-suspended in 5 mL BGM and pipetted gently to wash, then centrifuged at 750 pm for 2 minutes. Supernatant was removed, iHO re-suspended in ~300-500 µL BGM and a P1000 was used to break up iHO into smaller chunks. Around 100 µL of iHO solution was added to 1.5 mL Matrigel and pipetted briefly to mix, then the resulting solution plated out into a 24 well plate and overlaid with BGM containing growth factors as described above. To prepare for an invasion assay experiment, iHO were passaged 4-5 days prior to an experiment as described above, but 120µl droplets of Matrigel/iHO solution were placed into 5mm glass bottomed microinjection dishes. Rather than leaving the iHO suspension in a droplet as with routine passaging, the solution was spread over the bottom of the dish to create a thin layer of Matrigel and overlaid with 2.5 mL base growth medium plus growth factors. If antibiotics had been used in BGM, these were removed and replaced with non-antibiotic supplemented media prior to microinjection experiments.

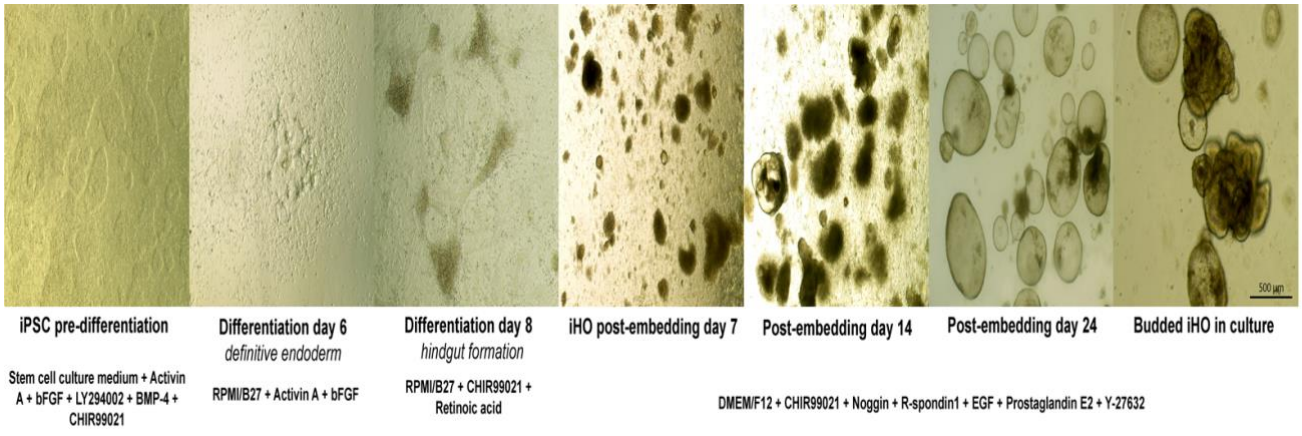

Figure 2.1: Sequential imaging of differentiation process from iPSC to iHO (Images taken on Thermo-Fisher EVOS XL imaging system at 4x / 10x magnification).

Table 2.3: Components of iHO base growth medium

Component:	Manufacturer:	Amount:
Advanced DMEM/F12	Invitrogen	500 mL
B27 serum-free supplement 50x	Life Technologies	10 mL
N2 serum-free supplement 100x	Life Technologies	5 mL
HEPES 1 M	Life Technologies	5 mL
L-glutamine 200 mM	Life Technologies	5 mL
Optional: Penicillin-streptomycin (10,000 U/mL)	Life Technologies	5ml

Table 2.4: Components of collagenase solution

Component:	Manufacturer:	Amount:
Collagenase IV powder	Life Technologies	500 mg
Advanced DMEM/F12	Gibco	400 mL
KnockOut Serum Replacement	Gibco	100 mL
L-glutamine 200 mM	Life Technologies	5 mL
2-Mercaptoethanol	Sigma-Aldrich	3.5 μL

Table 2.5: Growth factors for iHO base growth medium

Component:	Manufacturer:	Amount:
Recombinant human R-spondin 1	R&D	500 ng/mL
Recombinant human Noggin	R&D	100 ng/mL
Epidermal growth factor (EGF)	R&D	100 ng/mL
Prostaglandin E_2	Sigma	2.5 μM
CHIR99021	Abcam	3 μM
Y-27632 dihydrochloride monohydrate	Sigma-Aldrich	10 μM

2.1.5 Phenotyping of iHO

After 4 weeks in culture, iHO underwent phenotyping by means of RT-qPCR, immunostaining for cell types and fixation for transmission electron microscopy (TEM).

RT-qPCR for markers of cell types

RNA was extracted from iHO using RNeasy Mini Kit (Qiagen), and reverse transcribed using Qiagen QuantiTect reverse transcription kit as per the manufacturer's instructions.

Resultant cDNA was used to perform RT-qPCR using TaqMan gene expression assays and gene expression mastermix (Applied Biosystems) on the Applied Biosystems StepOne real-time PCR system. The aim here was to confirm phenotyping by monitoring the upregulation of transcripts for markers of intestinal tissue in comparison to iPSC.

Quantitative PCR (qPCR) was used to perform a comparison of selected gene expression between iPSC and iHO. This approach revealed that markers of pluripotency such as POU5F and NANOG are highly expressed in iPSC, whereas Vil1 (protein coding gene for villin), Lys (lysozyme, secreted by Paneth cells), Muc2 (mucin 2, secreted by goblet cells) and Chga (chromogranin A – produced by enteroendocrine cells) genes were more highly expressed in iHO, indicating differentiation into these cell types (**Figure 2.2**).

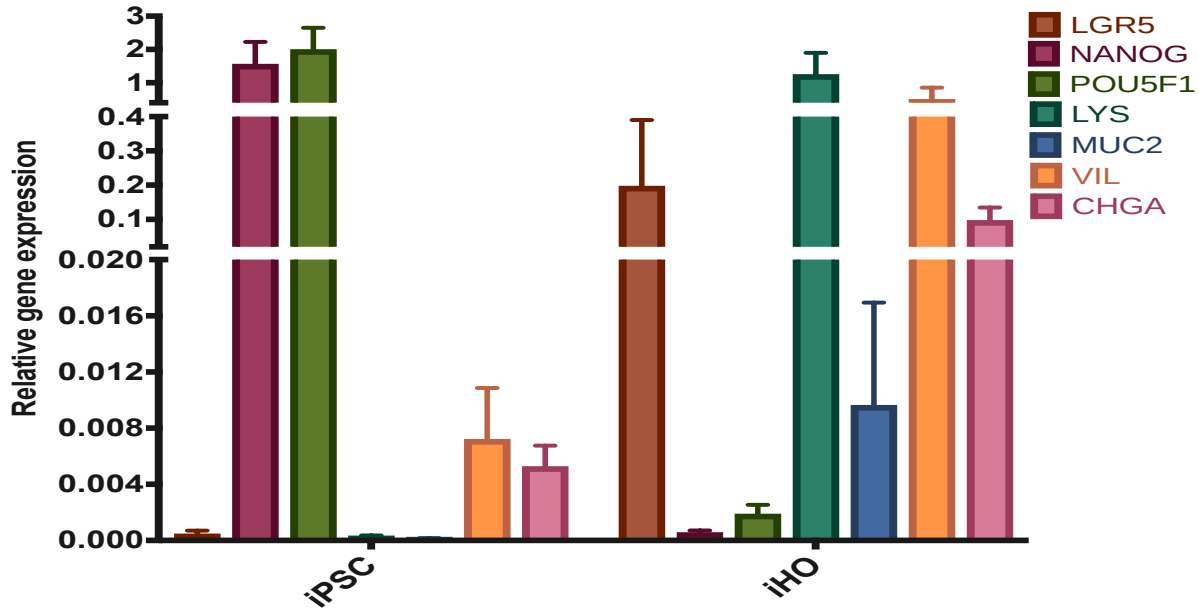

Figure 2.2: Differences in relative gene expression between iPSC and iHO in Kolf2 cell line. Data presented are from 4 technical replicates, with assays repeated 3 times using paired iPSC/iHO of different batches. Data were analysed using the comparative cycle threshold (C_T) method, with GAPDH as an endogenous control.

Immunostaining for markers of constituent cell types in iHO

Organoids were grown in 40µl Matrigel on Millicell EZ slide 4-well slides (Merck) for 5-6 days, then fixed in 4% paraformaldehyde (Sigma) in DPBS (no Ca^{2+} or Mg^{2+}) for 1 hour at r.t. Samples were rinsed x3 in DPBS; then blocked and permeabilised in 2% Triton X-100 (Sigma) with 5% foetal bovine serum (FBS; Sigma-Aldrich) diluted in DPBS for 2 hours. Primary antibodies diluted in 0.25% Triton X-100 / 5% FBS in DPBS were applied overnight at 15°C

(see **Table 2.6** for antibody concentrations and suppliers). Samples were rinsed x3 in DPBS and secondary antibodies applied then incubated for 3-4 hours at r.t. Samples were again rinsed x 3 in DPBS and nuclei counterstained with 10nM DAPI dilactate (Sigma-Aldrich) diluted in DPBS for 30 minutes at r.t. Samples were rinsed x6 in DPBS, immersed in FocusClear™ (2B Scientific) and covered for 2-4 hours to enhance sample transparency. FocusClear™ was removed and the plastic cage taken off samples, which were mounted directly in Prolong Gold with DAPI (Invitrogen), and analysed on the Zeiss LSM 510 Meta or Leica SP8 confocal microscopes. **Figure 2.3** demonstrates representative images of the phenotyping process for iHO from the Kolf2 cell line.

This protocol was also used to investigate protein expression and demonstrate presence of bacteria interacting with the epithelium following microinjection of the organoids with pathogens of interest.

Table 2.6: Antibodies used for immunostaining

Target	Host	Clonality	Isotype	Source	Dilution
Primary antibodies					
MUC2	Mouse	Monoclonal	IgG1	Abcam	1:200
LYZ	Rabbit	Polyclonal	Unknown	Abcam	1:50
VIL1	Mouse	Monoclonal	IgG1	Abcam	1:50
CHGA	Mouse	Monoclonal	IgG2b / IgG1	Abcam / Bio-Techne	1:50 / 1:100
IL-10R2	Rabbit	Polyclonal	IgG	Abcam	1:50
IL-22R1	Rabbit	Polyclonal	IgG	Abcam	1:100
S100A9	Rabbit	Polyclonal	IgG	Abcam	1:200
Rab7	Mouse	Monoclonal	IgG	Abcam	1:50
CARD8	Rabbit	Polyclonal	IgG	Abcam	1:100
Integrin alpha 6	Mouse	Monoclonal	IgG2b	Abcam	1:400
Goat anti-salmonella CSA-1 (FITC)	Goat	Polyclonal	Unknown	Insight Biotechnology	1:20
Secondary antibodies					
Goat anti-rabbit IgG Alexa fluor 456	Goat	Polyclonal	IgG	Life Technologies	1:100
Goat anti-mouse IgG FITC	Goat	Polyclonal	IgG	Abcam	1:100
Goat anti-mouse IgG TRITC	Goat	Polyclonal	IgG	Thermo-Fisher	1:200
Donkey anti-rabbit IgG Alexa fluor 647	Donkey	Polyclonal	IgG	Abcam	1:100
Other compounds used for staining					
Alexa fluor 647 phalloidin	N/A	N/A	N/A	Thermo-Fisher	1:1000
Lectin UEA-I (FITC conjugated)	N/A	N/A	N/A	Sigma-Aldrich	1:100
DiD (4-chlorobenzene sulfonate salt)	N/A	N/A	N/A	Biotium	2µg/ml
DAPI dilactate	N/A	N/A	N/A	Sigma-Aldrich	10nM

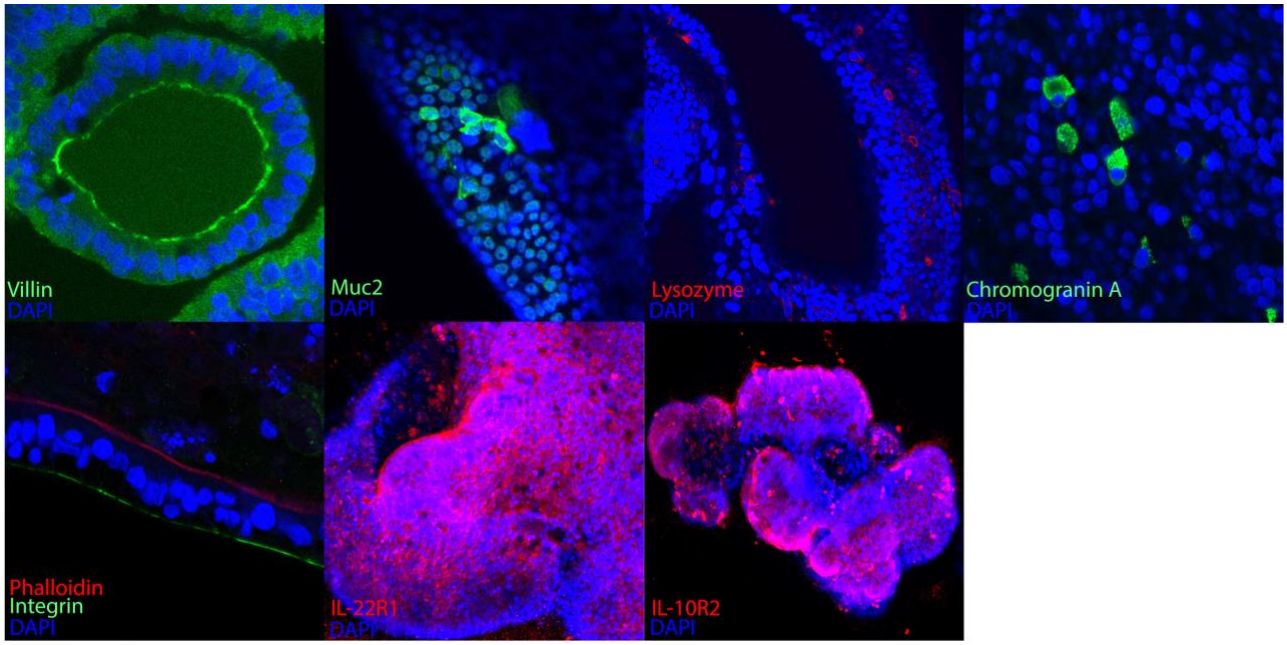

Figure 2.3: Immunostaining with specific antibodies for presence of constituent intestinal cell types in Kolf2 iHO. Imaged are iHO with nuclei stained blue with DAPI. Villin is a cytoskeletal protein of microvilli of the epithelial brush border in the intestine, and therefore a marker of the presence of enterocytes. Secretory cell types are marked by staining of the proteins they produce; lysozyme is an antimicrobial enzyme produced by Paneth cells, chromogranin A is a neuroendocrine secretory protein produced by enteroendocrine cells and mucin 2 is a protein secreted by intestinal goblet cells. Staining for integrin alpha 6 and phalloidin demonstrates an organised, polarised epithelium with an apparently continuous luminal surface. Images taken on Zeiss LSM 510 Meta confocal microscope (villin and phalloidin at 40x magnification, all other markers at 20x magnification).

TEM for constituent cell types in iHO

iHO were grown for 5-6 days on 50mm glass bottomed wells (MatTek corporation). BGM was aspirated and iHO fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (see **Table 2.7** for components) for 1 hour at r.t. Specimens were washed x 3 in sodium cacodylate buffer, then incubated in 1% osmium tetroxide in sodium cacodylate buffer at r.t. for 1 hour. Samples were washed x 3 in sodium cacodylate buffer, transferred to glass vials and stained with 1% tannic acid in sodium cacodylate for 30 minutes at r.t. Samples were then washed with 1% sodium sulphate in distilled water for 10 minutes. Samples were dehydrated through suspension in ethanol in series of concentrations: 30%, 30% with uranyl acetate (also at 30%), 50%, 70%, 90% and then 3 X 100%, for 20 minutes each. A rotator was used to aid infiltration in these and the following steps. Samples were incubated for 2 x 15 minutes in propylene oxide (Sigma-Aldrich), then changed for a 1:1 mix of propylene oxide and Epon resin (Epoxy Embedding Medium kit, Sigma-Aldrich) for 1 hour. This was changed for neat Epon resin overnight, then samples

were embedded in fresh Epon resin in a flat moulded tray and cured at 65°C for 48 hours. 500nm semi-thin sections were cut on a Leica UCT ultramicrotome and stained with toluidine blue on a microscope slide. Images were recorded on the Zeiss Axiovert CCD camera and areas selected for ultrathin 50nm sectioning. Ultrathin sections were collected onto copper grids and contrasted with uranyl acetate and lead citrate before viewing on a FEI 120kV Spirit BioTWIN TEM and recording CCD images on an F4.15 Tietz charge-coupled device camera. **Figure 2.4** displays representative TEM images for iHO from the Kolf2 cell line.

Table 2.7: Components of sodium cacodylate buffer

Component:	**Manufacturer:**	**Amount:**
Distilled H_2O	MilliQ	1 litre
Sodium cacodylate trihydrate	Sigma-Aldrich	21.4 g
Magnesium chloride	Sigma-Aldrich	1 g
Calcium chloride	Sigma-Aldrich	0.5g
Hydrochloric acid	Fisher Scientific	Use to adjust to pH 7.42

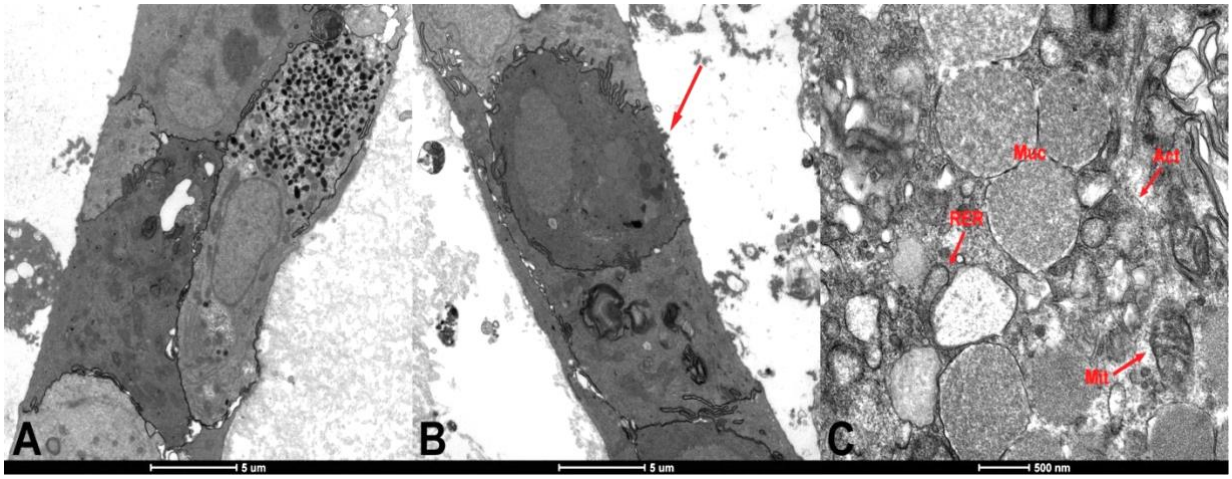

Figure 2.4: TEM images of iHO generated from Kolf2 cell line: (**A**) Enteroendocrine cell (identifiable by presence of secretory products stored in cytoplasmic granules) and (**B**) Goblet cell (recognisable by pale-staining mucin granules visible to right of nucleus). Also visible in this image are microvilli on the luminal aspect of the cell (arrow). (**C**) Magnified goblet cell (labelled are mucin granules (Muc), presence of mitochondria (Mit), actin (Act) and rough endoplasmic reticulum (RER)). Images taken on FEI 120kV Spirit BioTWIN TEM by D Goulding.

2.2 Pre-stimulation of iHO with rhIL-22

18 hours prior to invasion assays, media was aspirated from iHO, replaced with fresh BGM and rhIL-22 (R&D) then added to this culture media to a final concentration of 100 ng/mL.

2.3 Microinjection of iHO and intracellular invasion / intracellular survival / luminal killing assays

Cultures of bacteria of interest in 10 mL Luria-Bertani broth were incubated at 37 °C overnight with shaking, then diluted in DPBS (containing Ca^{2+} and Mg^{2+}) to an optical density of 2 at 600 nm (OD_{600}). Following this, cultures were mixed 1:1 with phenol red. The environmental chamber on the microscope was heated to 37 °C prior to starting the assay. A 6 μm microinjection drill tip (Eppendorf) was loaded with the bacterial culture, attached to the microinjection arm of the Eppendorf TransferMan NK2-FemtoJet express system (see **Figure 2.5**) and injection pressure set to 600kPa, with an injection time of 0.5 secs. The microscope was focused on the iHO plate and a target iHO selected for injection. Each iHO was injected 3 times, and a set number of iHO injected per experiment (30 for invasion assays, 60 for RNA-Seq assays). Due to heterogeneity in iHO size and structure within a culture, it is necessary to inject a large number of iHO to control for variation. The use of phenol red allowed injected iHO to be identified for downstream processing and avoid duplicate injections. Following injections, iHO plates were incubated at 37 °C / 5% CO_2 for 90 minutes (or 3 hours for RNA-Seq / imaging assays), following which, BGM was aspirated, replaced with 3 mL Cell Recovery Solution and incubated at 4°C for 45 minutes. iHO / Cell Recovery Solution was then moved into a 15 mL falcon containing 5 mL DPBS, centrifuged at 1500 rpm for 3 minutes and supernatant removed. For intracellular invasion assays, a modified gentamicin protection assay was performed.[5] iHO were disaggregated by vigorous pipetting and resuspended in 5 mL BGM containing gentamicin (Sigma-Aldrich) at 0.1 mg/mL. This suspension was incubated at 37 °C for 1 hour to kill extracellular bacteria, then centrifuged at 1500 rpm for 3 minutes and washed x 1 with DPBS. iHO were resuspended in 500 μL Triton X-100 1% in DPBS (Sigma-Aldrich) and manually dissociated by pipetting ~50x. This mixture was incubated for 5 minutes at r.t. then serially diluted in DPBS 10-fold to generate 10^{-1}, 10^{-2} and 10^{-3} concentrations. 3 x 20 μL droplets of the neat and diluted solutions were pipetted onto pre-warmed LB agar plates and incubated overnight at 37 °C. Colony counting was performed the following day.

Assays to investigate intracellular invasion versus survival were completed using the technique above, with harvesting at 30 minutes to assay initial invasion or incubation for an

additional 90 minutes following gentamicin treatment, to allow assessment of intracellular survival.

Luminal killing assays were used to assess the survival of *S.* Typhimurium inside the iHO lumen. Here, untreated or rhIL-22 pre-treated iHO were microinjected and incubated for 90 min at 37°C with 5% CO_2. iHO were isolated from Matrigel as described above and care was taken not to disrupt the general structure of phenol red marked organoids. iHO were gently washed in DPBS, centrifuged at 1500 rpm, supernatant removed, re-suspended in 500 μL DPBS and then broken open by vigorous pipetting. The resulting solution containing bacteria, iHO and DPBS was centrifuged at 1500 rpm to pellet cellular material whilst leaving bacteria in suspension. Supernatants were serially diluted and 20 μl spots were plated onto pre-warmed LB agar plates for CFU counting. An alternative method for this assay was used for assays requiring iHO harvest over longer time periods. In these assays, 3 single iHO were isolated from Matrigel at each time point using a disposable scalpel (Swann Morton) and manually disrupted into 100 μL DPBS. This solution was then centrifuged at 1500 rpm, serially diluted and plated as described above. Results for both methods gave comparable bacterial counts when directly compared.

Gentamicin protection assays and luminal killing assays were performed with at least three biological replicates per condition. For statistical comparisons of bacterial counts, unpaired, two-tailed *t* tests or Mann-Whitney tests were carried out, using the Prism 7 software (GraphPad).

Time course assays of both intracellular and luminal contents were performed, with iHO harvested at varying timepoints over the 6 hour period post-injection. Competition assays between different strains of *S*. Typhimurium were carried out by co-infecting iHO with 2 bacterial strains simultaneously, one of which was the TIMER^{bac}-*Salmonella*, as described by Claudi et al,[6] which produces fluorescent orange colonies, thus allowing discrimination between the strains when recovered from the intracellular compartment and plated.

For assays investigating inhibition of phagolysosomal fusion, iHO that had been pre-treated with rhIL-22 were either treated with 50μM W7 phagolysosomal fusion inhibitor (*N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, Sigma-Aldrich) for 6 hours

prior to injection, 100nM Concanamycin A (Insight Biotechnology) for 4 hours prior to injection, or left untreated. Microinjections and gentamicin protection assays were then completed as described above. **Table 2.8** lists all bacterial strains used for infection assays.

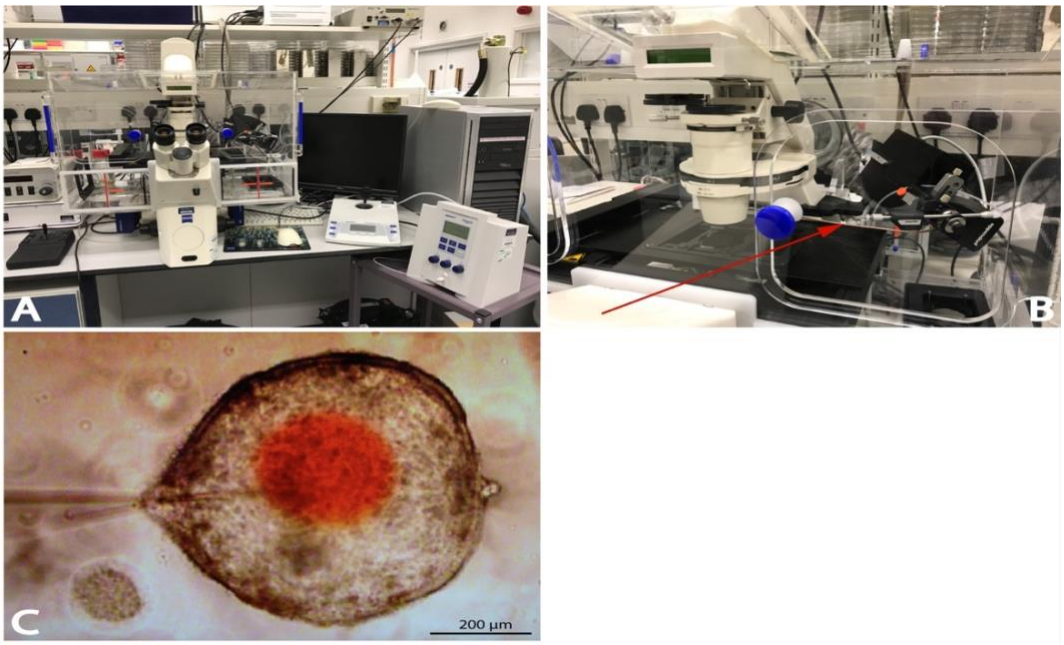

Figure 2.5 Microinjection of iHO with *S*. Typhimurium SL1344. Image (**A)** shows the setup of the microinjection system with environmental chamber, allowing for injection of the iHO at 37 °C. The Eppendorf TransferMan NK2-FemtoJet express system fitted with microcapillary (**B**) allows bacterial inoculum to be delivered directly into the luminal cavity of the iHO. By mixing bacterial inoculums with phenol red, infected iHO can be marked for downstream processing (**C**). Images were taken at ×100 magnification.

Table 2.8: Bacterial strains used for infection assays

Strain name and number:	**Source:**
S. Typhimurium SL1344 WT	Derek Pickard, WTSI
S. Typhimurium ST4/74 WT	Derek Pickard, WTSI
S. Typhimurium ST4/74 Δ*PhoPQ*	Derek Pickard, WTSI
S. Typhimurium SL1344 Δ*invA*	Derek Pickard, WTSI
S. Typhimurium TIMER^{bac}-*Salmonella* SL1344	Dirk Bumann, University of Basel
Enteropathogenic *Escherichia Coli* (EPEC) E2348/69	Derek Pickard, WTSI
TIMER^{bac}- EPEC E2348/69	Produced in collaboration with Derek Pickard
S. Enteritidis 6206	Rafal Kolenda, University of Wroklaw
S. Enteritidis 6174	Rafal Kolenda, University of Wroklaw
S. Enteritidis P125109 (PT4)	Derek Pickard, WTSI
S. Typhi Ty2 BRD948 Δ*aroC, aroD, htrA*	Derek Pickard, WTSI
S. Typhimurium ST313 D23580	Derek Pickard, WTSI
S. I:4,[5],12:i:− *(S*. Typhimurium variant) ST34 VNB1779	Stephen Baker, OUCRU (clinical isolate, Vietnam)
S. I:4,[5],12:i:− *(S*. Typhimurium variant) ST34 VNB2140	Stephen Baker, OUCRU (clinical isolate, Vietnam)
S. I:4,[5],12:i:− *(S*. Typhimurium variant) ST34 VNB2315	Stephen Baker, OUCRU (clinical isolate, Vietnam)
S. I:4,[5],12:i:− *(S*. Typhimurium variant) ST34 VNS20081	Stephen Baker, OUCRU (clinical isolate, Vietnam)
S. I:4,[5],12:i:− *(S*. Typhimurium variant) ST34 VNS20101	Stephen Baker, OUCRU (clinical isolate, Vietnam)
S. Typhi Quailes strain	Oxford Vaccine Group (challenge strain)
S. Paratyphi A NVGH308	Oxford Vaccine Group (challenge strain)
S. Typhi E02-1180 SGB90	Derek Pickard, WTSI (clinical isolate, India)
S. Typhi 2010K-0515 101TY	Derek Pickard, WTSI (clinical isolate, Kenya)
S. Typhi 2010K-0517 116TY	Derek Pickard, WTSI (clinical isolate, Kenya)

2.4 Electroporation of TIMER^{bac} plasmid into alternative bacteria

The TIMER^{bac} plasmid was electroporated into Enteropathogenic *Escherichia Coli* (EPEC) to provide fluorescent bacteria for imaging of bacterial-epithelial interactions in the iHO lumen. To obtain plasmid DNA, TIMER^{bac} Salmonella were incubated overnight at 37°C in 10 mL LB broth with shaking, then a Qiagen plasmid midi prep kit was used as per manufacturer's instructions to extract the TIMER^{bac} plasmid, which was stored at 4°C until use. Cultures of EPEC strain E2348/69 (serotype O127:H6) in 10 mL LB broth were incubated at 37°C with shaking. 1 mL of overnight culture was added to a conical flask containing 100 mL of LB broth and re-incubated at 37°C with shaking for 2-3 hours until an OD_{600} of 0.3 was reached. This solution was split into 4 x 25 mL aliquots and centrifuged at 4000 rpm at 4°C for 10 mins. Supernatant was discarded and bacteria resuspended in 30 mL ice-cold glycerol 10% (Sigma-Aldrich) before again being centrifuged at 4000 rpm at 4°C for 10 mins. This glycerol washing step was repeated, supernatant discarded and bacterial pellet re-suspended in 130 µL glycerol. 60 µL of this suspension and 3-5 µg of PCR product was added to pre-cooled 2mm electroporation cuvettes (Cell Projects Ltd). The extracted plasmid RNA was electroporated into bacterial cells at 2.4 kV using the Bio-Rad Micropulser. 500 µL of S.O.C. medium (Invitrogen) was added to the cuvette, which was incubated at 37°C for 2 hours to allow cells to recover. 3 x 100 µL bacterial solution was plated onto LB plates containing ampicillin (for maintenance of plasmid) and incubated overnight at 37°C. 20 colonies from these plates were placed onto fresh agar plates and again incubated overnight at 37 °C. Colonies fluoresced orange, indicating the successful transfer of the TIMER^{bac} plasmid.

2.5 Production and culture of murine organoids (iMO)

Mucosal tissue was harvested from 2 wild type C57/B6 mice and 2 equivalent mice harbouring the IL-22RA1$^{-/-}$ mutation and seeded into Matrigel in order to generate small intestinal iMO as described by Sato *et al*.[7] Organoids were overlaid with media comprising 90% base growth media (see **Table 2.9** for composition) and 10% R-Spondin 1 conditioned media. Growth factors were added to media in the concentrations described in **Table 2.10**.

In addition, 1 µM Y-27632 was added to media for initial seeding, but was not required for subsequent passages. iMO were passaged every 3-4 days by dissolving Matrigel using Cell Recovery Solution, then centrifugation at 2000 rpm for 2 minutes. Supernatant was removed, iMO were resuspended in BGM and manually disrupted via vigorous pipetting. Disrupted iMO were resuspended in Matrigel at a ratio of 1:3 – 1:5 depending on desired density, re-plated into 6 well plates and overlaid with BGM, which was changed every 2-3 days.

Table 2.9: Base growth media for iMO

Component:	**Manufacturer:**	**Amount:**
Advanced DMEM/F12	Invitrogen	500 mL
GlutaMax	Life Technologies	5 mL
HEPES 1M	Life Technologies	5 mL
N2 serum-free supplement 100x	Life Technologies	5 mL
B27 serum-free supplement 50x	Life Technologies	10 mL

Table 2.10: Growth factors for iMO media

Component:	**Manufacturer:**	**Amount:**
Recombinant murine Noggin	Peprotech	100 ng/mL
N-acetyl-cysteine	Sigma	1 mM
Murine epidermal growth factor (EGF)	Invitrogen	50 ng/mL

iMO produced in this way underwent phenotyping via light microscopy and immunostaining for cell-specific markers, with methods as for these assays done in iHO (**Figure 2.6 & 2.7**).

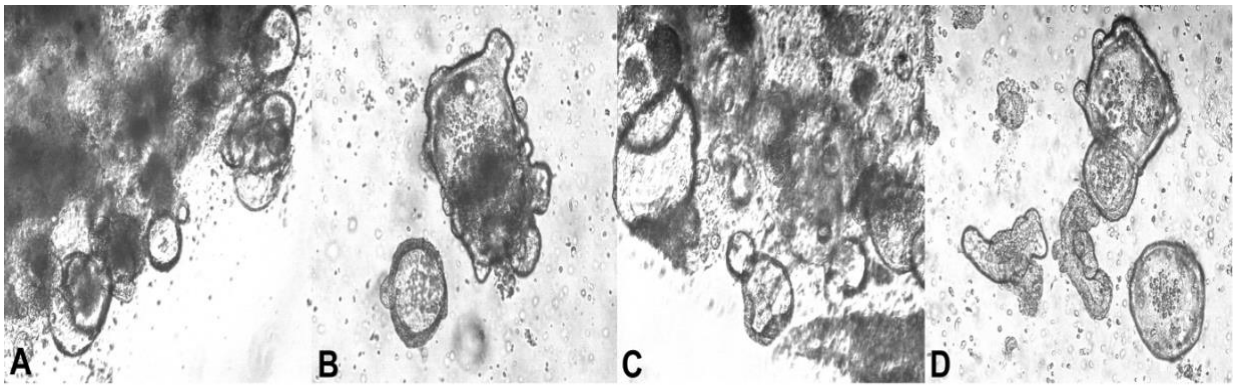

Figure 2.6: Light microscopy of iMO development. Panels A & C show WT and IL-22RA1$^{-/-}$ iMO respectively at day 3 post-embedding, and panels B & D at passage 13. Clearing of non-organoid tissue and development of budded structure is noted in both WT and IL-22RA1$^{-/-}$ lines.

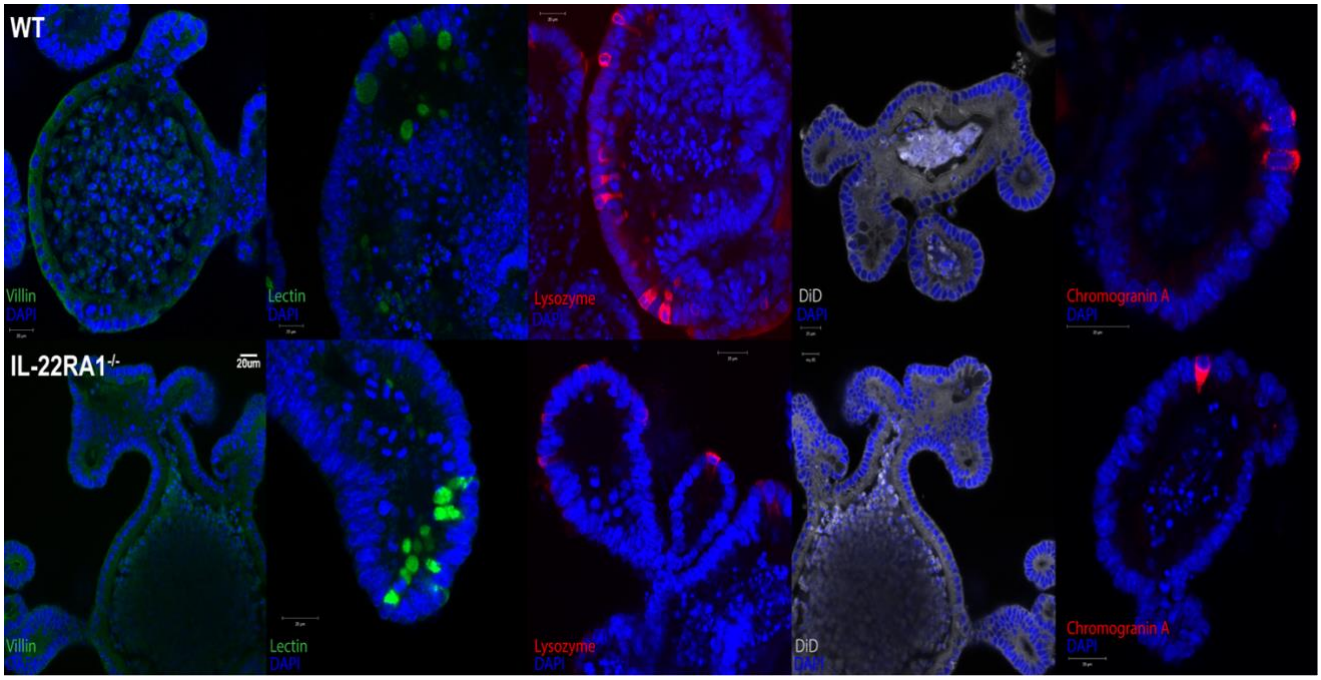

Figure 2.7: Immunostaining of iMO with specific antibodies for presence of constituent intestinal cell types. Imaged are iMO from WT and IL-22RA1$^{-/-}$ mice with nuclei stained blue with DAPI. iMO epithelial cells are stained with villin, Paneth cells with lysozyme and enteroendocrine cells with Chromogranin A. Goblet cells are stained with Lectin, and membrane staining is done with DiD . Images taken on Zeiss LSM 510 Meta confocal microscope at 20x magnification (scale bars indicate 20μm in each panel).

2.6 Microinjection of iMO and intracellular invasion assays

The set up for these assays was similar to that outlined for microinjection of iHO in 2.3. Differences with the iMO injection protocol were that iMO were microinjected at a pressure of 400 kPa and injection time 0.1 secs, with one injection per iMO, reflecting the smaller internal volume of these organoids. Because of difficulties manually disaggregating iMO related to their small size; following injection, incubation and washing in DPBS, iMO were re-suspended in 1 mL TrypLE (Gibco), incubated at 37°C for 3 minutes, then gently pipetted to dissociate cells to a single cell solution. 2 mL BGM was added to the solution to stop the dissociation reaction, samples were centrifuged at 2000 rpm for 3 minutes and re-suspended in BGM containing gentamicin at 0.1 mg/mL. The remainder of the protocol was identical to that described in 2.3.

2.7 Single cell sequencing of iHO following rhIL-22 stimulation

The detailed single cell sequencing and data analysis protocol is provided in **Appendix 1.** Briefly, iHO were grown for 5 days, stimulated with rhIL-22 100ng / mL for 18 hours or left unstimulated. iHO were dissociated to single cells and FACS sorted onto 96 well plates. Cells were lysed and mRNA extracted and polyadenylated then reverse transcribed with SmartSeq-2 PCR. Nextera libraries were produced from resultant cDNA and were further amplified, pooled and submitted to the WTSI sequencing pipeline.

2.8 Western blotting for proteins of interest in iHO

Western blots were used to demonstrate the presence / absence of caspase-1 in iHO produced from hiPSC lines with a mutation in the CARD8 gene versus a healthy control. iHO were harvested using Cell Recovery Solution and washed once with ice-cold DPBS (No Ca^{2+} or Mg^{2+}). This was replaced with 200-300 μL chilled Radio Immunoprecipitation Assay (RIPA) buffer (see **Table 2.11** for contents) and samples were agitated for 30 minutes at 4°C, followed by centrifuging at 4°C for 20 minutes at 12,000 rpm. Supernatants were aspirated, placed into chilled microcentrifuge tubes and underwent bicinchoninic acid assays (Pierce BCA Protein Assay Kit, Thermo-Fisher), run on the FLUOstar Omega microplate reader (BMG Labtech) to determine protein concentration. Samples were diluted in RIPA buffer and denatured by addition of Laemmli buffer (see **Table 2.12** for composition) and heating to 95°C for 5 mins.

Table 2.11: Components of RIPA buffer

Component:	**Manufacturer:**	**Amount:**
Distilled H_2O	MilliQ	9 mL
Sodium chloride	VWR	150 mM
Triton X-100	Sigma-Aldrich	100 μL
Sodium deoxycholate	Sigma-Aldrich	0.05 g
Sodium dodecyl sulphate 10%	Sigma-Aldrich	100 μL
TRIS hydrochloride (pH 8)	Invitrogen	50nM
cOmplete Protease Inhibitor Cocktail	Roche	1 tablet

Table 2.12: Components of Laemmli buffer

Component:	Manufacturer:	Amount:
TRIS hydrochloride (pH 6.8)	Invitrogen	3.6 mL
Sodium dodecyl sulphate 20%	Invitrogen	4.5 mL
Glycerol 100%	Invitrogen	4.5 mL
β-mercaptoethanol	Sigma-Aldrich	2.4 mL
Bromophenol blue	Honeywell Fluka	0.74 μg

Samples were cooled to room temperature and electrophoresed by SDS-PAGE. 1 μL /mL of each sample was loaded onto Mini-PROTEAN TGX 12% gels (Bio-Rad), with All Blue Precision Plus Protein Standards (Bio-Rad) for comparison, and run at 175 V for 45 mins, with TRIS/glycine running buffer (National Diagnostics). Semi-dry transfer was completed onto ethanol-activated polyvinylidene difluoride (PDVF) membranes (Thermo-Fisher) at 70 mA for 75 mins in transfer buffer (see **Table 2.13** for components). Membranes were blocked in 5% milk in DPBS and 0.1% Tween-20 (PBS-T) (Bio-Rad), before incubating in primary antibody in 2% milk in PBS-T. Membranes were washed x 3 in PBS-T and incubated for 45 minutes with HRP-conjugated secondary antibody. Membranes were washed x 3 in PBS-T, developed using Clarity ECL blotting substrates (Bio-Rad) and imaged on the ImageQuant LAS 4000 (GE Healthcare). All membrane incubations were completed on a rocking platform at r.t. β-actin was used as an internal control for protein loading (see **Table 2.14** for antibodies used for Western blotting).

Table 2.13: Components of transfer buffer (1 L)

Component:	Manufacturer:	Amount:
TRIS hydrochloride	Invitrogen	5.62 g
Sodium dodecyl sulphate 10%	Invitrogen	3.7 mL
Glycine	Sigma-Aldrich	2.93 g
Methanol	Fisher Scientific	200 mL
Distilled H_2O	MilliQ	800 mL

Table 2.14: Antibodies used for Western blotting

Target	Host	Clonality	Isotype	Source	Dilution
Primary antibodies					
β-actin	Rabbit	Polyclonal	IgG	Abcam	1:2500
Caspase-1	Mouse	Monoclonal	IgG2a	R&D	1:1000
Secondary antibodies					
Goat anti-rabbit immunoglobulins/HRP	Goat	Polyclonal	IgG	Dako	1:2000
Goat anti-mouse immunoglobulins/HRP	Goat	Polyclonal	IgG	Dako	1:2000

2.9 FACS for expression of proteins in iHO after stimulation / infection

iHO were stimulated with rhIL-22, and/or microinjected with bacteria of interest and incubated for 90 minutes. iHO were recovered from Matrigel using Cell Recovery Solution, washed in DPBS and incubated with TrypLE for 5-10 minutes at 37°C until they had dissociated into a single cell solution. Samples were washed with BGM to de-activate TrypLE and then in DPBS, before splitting into the requisite amount of tubes to stain for proteins of interest. Samples were incubated with viability staining compounds for 15 mins at r.t. in the dark. Cells were fixed in 4% paraformaldehyde at 4°C for 20 mins (optional). Cells were blocked with 3% BSA in PBS for 30min at 4°C, centrifuged at 4000 rpm and re-suspended in Perm/Wash buffer (BD) for 15 mins at r.t. in the dark. Samples were then stained with required antibodies diluted in Perm/Wash, washed x 2, re-suspended in FACS buffer and transferred to Fortessa FACS tubes, and run on the Becton Dickinson 8 FACSAria IIIu using FACS Diva software (v8). **Table 2.15** lists antibodies used for FACS assays.

Table 2.15: Antibodies / stains used for FACS assays

Target	Host	Clonality	Isotype	Source	Dilution
Primary antibodies					
S100A9	Rabbit	Polyclonal	IgG	Abcam	1:1000
S100A9 (isotype control)	Rabbit	Polyclonal	IgG	Abcam	1:1000
Other compounds used for staining					
DAPI dilactate	N/A	N/A	N/A	Sigma-Aldrich	10 µM
Zombie aqua fixable viability kit	N/A	N/A	N/A	BioLegend	1:100
Fixable viability dye eFluor 780	N/A	N/A	N/A	eBioscience	1:2000
Calcein blue, AM	N/A	N/A	N/A	GeneCopoeia	0.5 µg/mL

2.10 Production of hiPSC lines with isogenic mutations

For studies on the mechanism of rhIL-22-related enhancement of phagolysosomal fusion, an S100A9 knockout hiPSC line was produced by the Cellular Generation and Phenotyping (CGaP) facility at the WTSI as outlined in **Appendix 2**.

2.11 Growth and differentiation of hiPSC into macrophages

Embryoid body formation

hiPSCs from the Kolf2 cell line were cultured on mouse embryonic feeder (MEF) plates (gelatinised plates coated with mouse embryonic fibroblasts (Amsbio)), in Hu iPS base medium (BM) (see **Table 2.16** for components) containing hFGF basic (R&D) at a concentration of 4 ng/mL, as per the method by Hale et al.[8]

Table 2.16: Components of Hu iPS base medium (BM)

Component:	**Manufacturer:**	**Amount:**
Advanced DMEM F12	Invitrogen	400 mL
Knockout serum (KOSR)	Invitrogen	100 mL
L-glutamine 200 mM	Invitrogen	5.5 mL
Mercaptoethanol	Sigma-Aldrich	3.5 µL
Optional: Penicillin-streptomycin (10,000 U/mL)	Invitrogen	5 mL

Cells were harvested from plates using collagenase / dispase mix (Becton Dickinson), and the suspension centrifuged at 1200 rpm for 7 mins. Supernatant was aspirated and replaced with 5 mL BM, cells counted and re-suspended at 1 x 10^6 cells/ mL. 2 mL of this suspension was aliquoted into tissue culture treated 10 cm plates (Corning) with 8 mL BM. Plates were incubated at 37 °C / 5% CO_2 for 3-4 days to allow formation of embryoid bodies (EBs).

Embryoid bodies to monocytes

EBs were harvested into a 50 mL Falcon tube and allowed to settle, before removal of supernatant and resuspension in monocyte differentiation base medium (see **Table 2.17** for composition) with rhM-CSF at 50 ng/mL (R&D) and rhIL-3 at 25 ng/mL. 10 mL of this suspension was plated onto gelatinised tissue culture treated 10 cm plates and incubated at 37 °C / 5% CO_2. Medium was changed every 7-8 days, until day 21, at which point it was possible to harvest floating monocyte precursors and refill plates still containing adherent EBs with fresh medium, in order to repeat this harvest on a weekly basis.

Table 2.17: Components of monocyte differentiation base medium

Component:	**Manufacturer:**	**Amount:**
X-Vivo 15 with gentamicin	Lonza	500 mL
L-glutamine 200 mM	Invitrogen	5.5 mL
Mercapto-ethanol	Sigma-Aldrich	3.5 µL
Optional: Penicillin-streptomycin (10,000 U/mL)	Invitrogen	5 mL

Monocytes to macrophages

Monocyte precursors were counted and resuspended at the concentration required for each assay in macrophage differentiation base medium (MDBM; see **Table 2.18** for composition) containing hM-CSF (R&D) at 100 ng/mL, then plated onto appropriately-sized tissue culture treated dishes. Cells were incubated at 37 °C / 5% CO_2 for 6-7 days prior to use in assays. The first batch of macrophages from a differentiation were checked for human CD14 and CD34 expression by flow cytometry. Phenotyping of these macrophages via TEM has been previously reported.[8]

Table 2.18: Components of macrophage differentiation base medium (MDBM)

Component:	**Manufacturer:**	**Amount:**
RPMI 1640	Sigma	500 mL
Foetal bovine serum (heat-inactivated)	Sigma	50 mL
L-glutamine 200 mM	Invitrogen	5.5 mL
Optional: Penicillin-streptomycin (10,000 U/mL)	Invitrogen	5 mL

2.12 Intracellular infection assays using hiPSC-derived macrophages

Macrophages were plated at a density of $1x10^5$ cells / well and incubated for 7 days prior to infection. Cultures of bacteria of interest were set up in 10 mL LB broth and incubated overnight at 37°C with shaking. Medium overlaying cells was replaced with fresh MDBM immediately prior to assay. Bacterial cultures were diluted to an OD_{600} of 1 and appropriate volume of bacteria added to each well to give a multiplicity of infection (MOI) of 10:1. Cells were incubated for 1 hour at 37 °C / 5% CO_2, infection medium removed and cells washed with DPBS. This was replaced with MDBM containing gentamicin at 0.1 mg/mL for 1 hour to kill extracellular bacteria. Cells were washed again in DPBS and incubated in MDBM for a further 4 hours.

To establish intracellular bacterial counts at 6 hours, macrophages were washed with DPBS and lysed with 500 µL 1% Triton X-100 in DPBS. This mixture was serially diluted in DPBS 10-fold to generate 10^{-1}, 10^{-2} and 10^{-3} concentrations. 3 x 10 µL droplets of the neat and diluted solutions were pipetted onto pre-warmed LB agar plates and incubated overnight at 37 °C. Colony counting was performed the following day.

2.13 Immunostaining of infected hiPSC-derived macrophages

Macrophages were grown on glass coverslips inside tissue culture treated 24-well plates (Corning) at a concentration of $1x10^5$ cells / well. Gentamicin protection assay was carried out as described above following infection of macrophages with *S*. Typhimurium SL1344, *S*. Typhi (Quailes strain) or *S*. Paratyphi A (NVGH308). At the 6 hour timepoint, macrophages were fixed with 4% paraformaldehyde in DPBS (no Ca^{2+} or Mg^{2+}) for 20 minutes, then washed in DPBS and covered in 0.1% Triton X-100 in DPBS for 10 mins at r.t. to permeabilise cells. Cells were washed with DPBS and covered with 1% BSA (Fisher Scientific) in DPBS for 15 mins at r.t. (blocking buffer). Antibodies were diluted in 1% BSA, and CSA-1 applied at 1:50 for 1 hour at r.t. in the dark. Cells were washed x 3 in DPBS and incubated with Alexa fluor 647 Phalloidin (Thermo-Fisher) at 1:1000 for 1 hour at r.t. in the dark. Cells were again washed x 3 with DPBS and coverslips mounted onto 20 µL aliquots of Prolong Gold with

DAPI placed onto Superfrost glass slides (VWR). Slides were stored overnight at 4 °C in the dark and images taken on the Leica SP8 confocal microscope. Number of bacteria per macrophage were recorded for the first 150 macrophages imaged from each condition, using fields chosen at random and compared across groups. Three biological replicates were completed for this assay. Antibodies used for this assay are listed in **Table 2.6**.

2.14 TEM of infected hiPSC-derived macrophages

Macrophages were grown on tissue culture treated 6-well plates (Corning) at a concentration of $2.5x10^5$ cells / well. Gentamicin protection assay was carried out as described above following infection of macrophages with *S*. Typhimurium SL1344, *S*. Typhi (Quailes strain) or *S*. Paratyphi A (NVGH308). MDBM was aspirated and macrophages fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (see **Table 2.7** for components) for 1 hour at r.t. Specimens were washed x 3 in sodium cacodylate buffer, then incubated in 1% osmium tetroxide in sodium cacodylate buffer at r.t. for 1 hour. Samples were washed x 3 in sodium cacodylate buffer. Adherent macrophages were then shaved off the plate using a Teflon strip and transferred to a 1.5 mL Eppendorf tube, centrifuged and the supernatant removed. Macrophages were dehydrated by suspension in ethanol in the series of concentrations: 30%, 30% with uranyl acetate (also at 30%), 50%, 70%, 90% and then 3 X 100%. Samples were incubated for 2 x 15 minutes in propylene oxide and embedded in araldite resin (Sigma-Aldrich). Ultrathin sectioning was then completed, with sections collected onto copper grids and contrasted with uranyl acetate and lead citrate before viewing on a FEI 120kV Spirit BioTWIN TEM and recording CCD images on an F4.15 Tietz charge-coupled device camera.

2.15 Luminex assays for cytokines post-infection in iHO and macrophages

These assays were completed on iHO from Kolf2, Sojd2 and Rayr2 cell lines and macrophages from Kolf2 line infected with CL3 pathogens (*S*. Typhi, *S*. Paratyphi A), therefore supernatants were filtered prior to removal from the CL3 facility and analysis. 200 µL supernatant was removed from all iHO and macrophage samples prior to infection, then at 6 hours post-infection for macrophages, or 1.5 hours and 3 hours post-infection for iHO.

Supernatants were passed through Costar Spin-X centrifuge tube 0.22 μm filters (Corning) and stored in sterile Eppendorf tubes at -80°C prior to analysis.

Once defrosted, a custom Luminex panel (MILLIPLEX MAP kit, Millipore) was used as per manufacturer's instructions to assay levels of: EGF, TGF-α, GROa, CD40L, IL-1RA, IL-1, IL-6, IL-8 and TNF-α in each sample. Samples were run in duplicate on the Luminex MAGPIX instrument and results collected using the xPONENT software. Data analysis was performed in R Version 3.6.0 and Prism version 7.0 software (GraphPad). LOG_2 fold change in median fluorescence intensity versus baseline was calculated for each sample and results compared using unpaired Student's t-test.

2.16 Bulk RNA-Seq for infected iHO and macrophages

Macrophages were cultured at concentration of $2.5x10^5$ cells/well and gentamicin protection assays were performed as described in 2.12. Cells were harvested at the 6 hour time point by washing in DPBS (no Ca^{2+} or Mg^{2+}), followed by addition of 250 μL RLT buffer (RNeasy mini kit, Qiagen), agitation of cells and transfer into 15 mL Falcon tube.

60 iHO per plate were microinjected with bacteria of interest and incubated for 3 hours. iHO were harvested using Cell Recovery Solution, placed into 15 mL Falcon tubes, washed in DPBS (no Ca^{2+} or Mg^{2+}) and 350 μL RLT buffer added to samples.

RNA extractions were performed using the RNeasy mini kit, as per manufacturer's instructions, and RNA was eluted into 40 μL RNase-free water and frozen at -80°C prior to submission to the WTSI's RNA sequencing pipeline. Samples were sequenced on the HiSeq 4000 in dual indexed pools, generating 75bp paired-end reads for both iHO and macrophage infection assays. Subsequent QC and alignment steps are detailed separately for iHO and macrophage samples in **Appendix 3**.

References:

1. Forbester JL, Hannan N, Vallier L, Dougan G. Derivation of Intestinal Organoids from Human Induced Pluripotent Stem Cells for Use as an Infection System. *Methods Mol Biol.* 2016.
2. Leha A, Moens N, Meleckyte R, et al. A high-content platform to characterise human induced pluripotent stem cell lines. *Methods.* 2016;96:85-96.
3. Forbester JL, Lees EA, Goulding D, et al. Interleukin-22 promotes phagolysosomal fusion to induce protection against Salmonella enterica Typhimurium in human epithelial cells. *Proc Natl Acad Sci U S A.* 2018;115(40):10118-10123.
4. Yeung ATY, Hale C, Lee AH, et al. Exploiting induced pluripotent stem cell-derived macrophages to unravel host factors influencing Chlamydia trachomatis pathogenesis. *Nat Commun.* 2017;8:15013.
5. Raffatellu M, Wilson RP, Chessa D, et al. SipA, SopA, SopB, SopD, and SopE2 contribute to Salmonella enterica serotype typhimurium invasion of epithelial cells. *Infect Immun.* 2005;73(1):146-154.
6. Claudi B, Sprote P, Chirkova A, et al. Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. *Cell.* 2014;158(4):722-733.
7. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology.* 2011;141(5):1762-1772.
8. Hale C, Yeung A, Goulding D, et al. Induced pluripotent stem cell derived macrophages as a cellular system to study salmonella and other pathogens. *PLoS One.* 2015;10(5):e0124307.