PNAS
www.pnas.org

Supplementary Information for

Title: Interleukin-22 promotes phagolysosomal fusion to induce protection against *Salmonella enterica* Typhimurium in human epithelial cells

Authors: Jessica L. Forbester[1,2,*], Emily A. Lees[2,3], David Goulding[2], Sally Forrest[2,3], Amy Yeung[2], Anneliese Speak[2], Simon Clare[2], Eve L. Coomber[2], Subhankar Mukhopadhyay[2], Judith Kraiczy[7], Fernanda Schreiber[2], Trevor D. Lawley[2], Robert E.W. Hancock[9], Holm H. Uhlig[4, 5], Matthias Zilbauer[6,7], Fiona Powrie[4,8] and Gordon Dougan[2,3]

*Jessica L. Forbester
Email: jf8@sanger.ac.uk

[1]Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK

[2] Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK

[3] Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, UK

[4] Translational Gastroenterology Unit, John Radcliffe Hospital, Headley Way, Headington

www.pnas.org/cgi/doi/10.1073/pnas.1811866115

Oxford,OX3 9DU, UK

[5] Department of Paediatrics, John Radcliffe Hospital, Headley Way, Headington Oxford,OX3 9DU, UK

[6] Department of Paediatric Gastroenterology, Hepatology and Nutrition, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK

[7] University of Cambridge Department of Paediatrics, Addenbrooke's Hospital, Hills Road Cambridge, CB2 0QQ, UK

[8] Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Headington, Oxford, OX3 7FY, UK

[9]Centre for Microbial Diseases and Immunity Research, University of British Columbia, 2259 Lower Mall, Vancouver BC, Canada

This PDF file includes:

SI Materials and Methods
References for SI reference citations
Figs. S1 to S7
Captions for datasets S1 to S4

Other supplementary materials for this manuscript include the following:

Datasets S1 to S4

SI Materials and Methods

Bacteria. *S*. Typhimurium SL1344, a well-characterised isolate (1), was used in these studies. *S*. Typhimurium SL1344 *invA* (2) was constructed by moving an *invA*::Km^r deletion from *S*. Typhimurium SL3261 to SL1344 by P22 transduction. A TIMER^{bac}-*Salmonella*, which has been previously described (3),(4), was a kind gift from Dirk Bumann, University of Basel. For stimulation assays, bacteria were grown in Luria-Bertani broth overnight at 37°C with shaking. Before infection for RNA-seq, bacterial cells were re-suspended at 2 x 10^7 CFU mL^{-1} in Dulbecco's phosphate-buffered saline (Sigma-Aldrich), with 1 x 10^6 bacteria added per well of a 24-well plate of iHOs. For

microinjection/viability assays, bacterial cells were suspended at a concentration of 1 x 10^9 CFU mL^{-1} in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) and phenol red (Sigma-Aldrich).

Generation of IL10R2^{comp} iHO. To restore expression of functional IL-10R2 in the IL-10R2^{Pat} hIPSCs, we generated the AAVS1 EF1a-IL10R2-PGK-puro targeting vector by Gibson assembly. The Gibson assembly product was transformed into OneShot TOP10 chemically competent *E. coli* (ThermoFisher) and positive colonies were picked. We isolated plasmids from the positive colonies and confirmed the presence and sequence of EF1α-IL10R2 in the targeting vector by restriction digests, PCR and sequencing. Subsequently, the targeting vector was transformed into competent *E.coli* to isolate endotoxin-free plasmids to transform into the IL10R2^{Pat} hIPSCs. We transfected the mutant human hIPSCs with TALEN-L (CCCCTCCACCCCACAGT), TALEN-R (AGGATTGGTGACAGAAA) and targeting vector via nucleofection (Amaxa Biosystems). The resultant targeted cells were selected on puromycin for 7 days and the surviving colonies were picked and expanded. The positive clones were confirmed by PCR and sequencing.

Ethical approvals. This work involved the use of human cell lines created by the Human Induced Pluripotent Stem Cell Initiative (HIPSCI). Consent was obtained for the use of cell lines for the HIPSCI project from healthy volunteers. A favourable ethical opinion was granted by the National Research Ethics Service (NRES) Research Ethics Committee Yorkshire and The Humber – Leeds West on 10th August 2015, reference number 15/YH/0391. IL10RB^{Pat} hIPSCs were generated at the Wellcome Trust Sanger Institute (WTSI) as part of the Oxford IBD cohort study / COLORS in IBD project with appropriate

ethical approvals (REC: 09/H1204/30; North Staffs LREC / NRES Committee West Midlands – The Black Country (IBD in Oxford).

Culture of primary iHO. Organoids were seeded into Matrigel (Corning) and overlaid with media consisting of 30% base growth medium: Advanced DMEM/F12 (Invitrogen), 2mM GlutaMax (Invitrogen), 10 mM Hepes buffer (Sigma) and 50X B-27 serum-free supplement (Life Technologies); 20% R-Spondin 1 conditioned medium; 50% Wnt3a conditioned medium; with complete medium supplemented with 10mM Nicotinamide, 100 ng mL^{-1} Noggin (Peprotech), 1.25 mM N-acetyl-cysteine (Sigma), 50 ng mL^{-1} EGF (Invitrogen), 10μM SB202190, 500nM A83-01. 1 μM Y-27632 dihydrochloride monohydrate (Sigma) was added when organoids were manually disrupted. Organoids were passaged every 7-10 days by removal from Matrigel using Cell Recovery solution (Corning), centrifugation and removal of supernatant, manual disruption of remaining iHOs in base growth medium using a P200 pipette, centrifugation and re-plating in Matrigel. Medium was changed every 2-3 days.

Culture of murine organoids (iMOs). Mucosal tissue was harvested from wild type and *Il22ra1*-/- mice and seeded for generation of small intestinal organoids according to the protocol described in Sato *et al.* (5). Organoids were seeded into Matrigel (Corning) as described for hIPSC-derived organoids and overlaid with media consisting of 90% base growth media: Advanced DMEM/F12 (Invitrogen), 2mM GlutaMax (Invitrogen), 10 mM Hepes buffer (Sigma), 100x N-2 supplement (Life Technologies) and 50X B-27 serum-free supplement (Life Technologies); 10% R-Spondin 1 conditioned media; with complete medium supplemented with 100 ng mL^{-1} Noggin (Peprotech), 1mM N-acetyl-cysteine (Sigma) and 50 ng mL^{-1} EGF (Invitrogen). 1 μM Y-27632 dihydrochloride monohydrate (Sigma) was added to media for seeding, but not for subsequent passaging. Organoids

were passaged every 3-4 days by removal from Matrigel using Cell Recovery solution (Corning), centrifugation and removal of supernatant, manual disruption of remaining iMOs in base growth media, centrifugation and re-plating in Matrigel. Media was changed every 2-3 days.

Microinjections and invasion assays. Microinjection was carried out as previously described (6, 7). For intracellular invasion assays, we modified the commonly used gentamicin protection assay (8). The apical side of the iHO IECs is located on the inside of the spherical iHO structure, therefore microinjection provides a solution for introducing stimuli to this luminal surface. To perform microinjections, we used the Eppendorf TransferMan NK2-FemtoJet express system, with injections performed in an environmental chamber, to allow all injections to be carried out at 37°C and 5% CO_2. Bacterial cultures were diluted 1:1 with phenol red so that infected iHOs could be easily identified. After injection, iHOs were incubated for 30 minutes (initial invasion assays) or 90 min (all other invasion assays) at 37°C, 5% CO_2. After 30 or 90 minutes, iHOs were isolated from Matrigel with Cell Recovery Solution (Corning), centrifuged, washed once with PBS, subjected to manual disaggregation of the organoid ultrastructure by vigorous pipetting, resuspended in iHO base growth medium supplemented with 0.1 mg ml^{-1} gentamicin (Sigma) to kill extracellular bacteria, and incubated at 37°C for 1 h. iHO aggregates were then centrifuged and washed once with PBS (Sigma). Cells were lysed with 1% Triton X-100 in PBS. Lysates were serially diluted in PBS, and plated onto pre-warmed LB plates for CFU counting. For intracellular survival assays iHOs were further incubated for 90 min at 37°C post gentamicin incubation, to allow for assessment of intracellular survival without further bacterial invasion. All Gentamicin protection assays were performed with at least three biological replicates. For some experiments iHOs or iMOs were pre-treated with recombinant human or murine IL-22 (100ng mL^{-1}, BioTechne)

for 18 h prior to microinjection. For W7 and Concanamycin A assays, rhIL-22 stimulated iHOs were either treated with 50µM W7 phagolysosomal fusion inhibitor (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, Sigma-Aldrich) for six hours or with 100nM Concanamycin A (Insight biotechnology) for 4 hours prior to injection. Protection assays were performed with three or four biological replicates per condition.

Preparation of RNA, RT-qPCR and RNA-sequencing. iHOs were recovered from Matrigel using Recovery Solution (Corning), washed by centrifugation at 405 g, with RNA purified from iHO cell pellets using the RNeasy minikit (Qiagen). RNA was reverse transcribed with the QuantiTect reverse transcription (RT) kit (Qiagen) according to the manufacturer's protocol. All RT-qPCR experiments were performed with TaqMan gene expression assays and TaqMan gene expression master mix (Applied Biosystems) on the Applied Biosystems StepOne real-time PCR system. RT-qPCR data were analysed via the comparative C_T method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. For RNA sequencing, RNA was prepared from microinjected or rhIL-22 stimulated iHOs, plus corresponding controls for three biological replicates per condition. Samples were prepared for sequencing by the Wellcome Trust Sanger Institute Operations pipeline.

RNA Sequencing. Multiplexed mRNA libraries were prepared by using the Illumina TruSeq protocol. Libraries were analysed on a Bioanalyzer high sensitivity DNA chip (Agilent). Libraries were then sequenced via paired-end sequencing with the Illumina-C HiSeq 2500 platform, producing 100bp paired-end reads. RNA-Sequencing analysis was performed using the Wellcome Trust Sanger Institute Pathogen Informatics RNA-Seq Pipeline for mapping of RNA-Seq data. Each lane of Illumina sequence was assessed for quality on the basis of GC content, average base quality, and adapter contamination.

RNA-Seq reads were aligned with TopHat (9) version 2.0.8 with the human reference version GRCh37 used in the 1000 Genomes project. The read counts per gene were generated with featureCounts version 1.4.5-p1. The annotation for featureCounts came from ENSEMBL 75. Read counts were used to represent gene expression levels. R versions 3.1.2-3.3.1 were used to import count data, and the DESeq2 package was used to normalize the data and detect differentially expressed genes (10). Significantly differentially expressed genes were selected with a cut-off false-discovery rate of less than 0.05 and a fold change of 2.0. Heat maps and principal-component analysis (PCA) plots were constructed from rlog-transformed data with the ggplot2 R library (http://cran.r-project.org /web/packages/ggplot2/index.html). For the most significantly upregulated and downregulated genes, enriched gene ontology terms were identified by using InnateDB with the category Biological Processes selected.

Immunostaining for confocal microscopy. iHOs grown on 4-well glass sterile chamber slides were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature, followed by 3 washes with PBS. Samples were blocked and permeabilised in 2% Triton X-100 (Sigma) in 5% Foetal Bovine Serum (FBS; Sigma-Aldrich) diluted in PBS for 2 h. Primary antibodies were applied overnight at 15°C in 0.25% Triton X-100 in 5% FBS diluted in PBS, and then rinsed 3 times with PBS. Secondary antibodies were applied in the same manner. Nuclei were counterstained with 10nM DAPI dilactate diluted in PBS for 30 min, and samples were rinsed 6 times with PBS, then treated with FocusClear™ (2B Scientific) for 2-4 h at room temperature. Samples were mounted in Prolong-Gold with added DAPI (Invitrogen) and analysed on a Zeiss LSM 510 Meta confocal microscope, or on a Leica SP8 DLS (Digital light sheet) microscope. Human intestinal biopsies were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2h at room temperature, followed by overnight fixation in 1% paraformaldehyde. Fixed

biopsies were then cut into smaller sections and stained similarly to iHOs, but with the following changes: addition of DiD membrane stain applied in conjunction with the secondary antibodies; nuclei counterstaining with 10nM DAPI dilactate diluted in PBS for 3 hours; O/N incubation in FocusClear^{TM}.

Transmission electron microscopy. Infected organoids were fixed at room temperature in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer and then post fixed in 1% osmium tetroxide diluted in sodium cacodylate buffer, mordanted with 1% tannic acid, dehydrated with an ethanol series (contrasting with 2% uranyl acetate at the 30% stage), and embedded and sectioned as previously described (7). FEI 120-kV Spirit BioTWIN transmission electron microscope and F4.15 Tietz charge-coupled device camera were used for imaging.

Flow cytometry analysis of iHOs. iHOs recovered from Matrigel were washed with Dulbecco's Phosphate Buffered Saline (DPBS, no calcium or magnesium, Gibco), re-suspended in 2mL collagenase solution and dissociated into single/small clumps of cells using the gentleMACS Dissociator (GentleMACs C-tubes; program Spleen_01_01, Miltenyl Biotech). Cells were centrifuged at 115 g, and washed with DPBS. Single cells were re-suspended in FACS buffer containing 2mM EDTA (Sigma) in DPBS and blocked with Trustain FcX block (5μl/100μl buffer; BioLegend; 10 min at 4°C). 5μl of directly conjugated anti-human antibody against IL10R2 antigen (BD Pharmingen) or IgG1 κ Isotype matched control (BD Pharmingen) antibodies were added to separate samples and incubated for 30 min, 4°C. Cells were live/dead stained using 0.5μg mL^{-1} calcein blue, AM (GeneCopoeia) and fixable viability dye eFluor 780 (1:2000; eBioscience), or Zombie aqua fixable viability kit (Biolegend). Cells were analysed on a Becton Dickinson

FACsAriaIIIu using FACS Diva software (v8). For analysis of microinjected iHOs forty iHOs per condition were injected with TIMER^{bac}-*Salmonella* and incubated for 2 h at 37°C, 5% CO_2. Injected iHOs were selected for further processing by pipetting, washed in DPBS by centrifugation at 115 g, re-suspended in TrypLE (Gibco) and incubated for 5-10 min at 37°C to dissociate to single cells. Cells were washed with DPBS and live/dead staining and analysis were performed as described above.

Preparation of protein and western blotting. iHOs for protein preparation were harvested by removal from Matrigel by incubation with Cell Recovery Solution. Cells were washed in ice cold PBS, then chilled Radio Immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris pH 8, cOmplete protease inhibitor cocktail (Roche), in ddH_2O) was added to the cell pellets. Lysate was maintained under constant agitation for 30 min at 4°C followed by centrifugation at 16,000 g for 20 min. Supernatant was transferred to chilled microcentrifuge tubes for further analysis. Protein concentration of plasma and protein extracts was determined by bicinchoninic acid (BCA) assay (Pierce), as per manufacturer's instructions. Protein extracts were diluted for blotting in RIPA buffer and prepared for gel electrophoresis by addition of 4X laemmli buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue in 0.125 M Tris HCl pH 6.8), and heating to 95 °C for 5 min. As a control for STAT3 activation blots we also used lysate from Hela cells stimulated for 15 min with IFN-α (Millipore, 47-226). Samples were cooled to room temperature before electrophoresis by SDS-PAGE. 1μg mL^{-1} of each sample was loaded onto 12% Mini-PROTEAN TGX precast gels (Biorad) and run at 175 V for 45 min with Tris/glycine-running buffer. Proteins were blotted onto ethanol-activated polyvinylidene difluoride (PDVF) membrane using a semi-dry transfer system (70 mA, 75 min) in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol in ddH_2O).

Membranes were blocked by incubation in 5% milk in PBS-T (0.1% Tween-20 in PBS) for 1 h. Primary antibody in 2% milk in PBS-T was added for 1 h. Membranes were then washed three times for 5 min in PBS-T. Appropriate HRP- conjugated secondary antibody was added for 45 min in 2% milk in PBS-T, and membranes were washed three times for 15 min in PBS-T. Amersham Enhanced Chemiluminescence (ECL) Western Blotting Detection reagent was used for detection of polypeptides with Amersham Hyperfilm ECL (both GE Healthcare) as per the manufacturer's instructions. All membrane incubations were carried out at room temperature on a rocking platform.

Generation of S100A9$^{-/-}$ iPSC line. The knockout of S100A9 was generated by a single T base insertion in the third exon containing the EF-hand motif at cDNA position 228 using CRISPR/Cas9 in the Kolf2 human iPSC line. This was achieved by nucleofection of 10^6 cells with Cas9-crRNA-tracrRNA ribonucleoprotein (RNP) complexes. Synthetic RNA oligonucleotides (target site: 5'- AGACAAGCAGCTGAGCTTCG -3', WGE CRISPR ID: 915082321, 225 pmol crRNA/tracrRNA) were annealed by heating to 95°C for 2 min in duplex buffer (IDT) and cooling slowly, followed by addition of 122 pmol recombinant eSpCas9_1.1 protein (in 10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT). Complexes were incubated at room temperature for 20 minutes before electroporation. After recovery, cells were plated at single cell density and colony were picked into 96 well plates. 96 clones were screened for heterozygous and homozygous mutations by high throughput sequencing of amplicons spanning the target site using an Illumina MiSeq instrument. Final cell lines were further validated by Illumina MiSeq. Homozygous targeted clone was used in downstream differentiation assays.

Additional supporting data:

S100A9 WT sequence: GCAGCTGAGCTTCGAGGAGTTCA

S100A9 MUT sequence: GCAGCTGAGCTTTCGAGGAGTTCA

Insertion of T at position 228

PCR amplification primers:

F: TTTGGTATGTGCTCAGTGTCTG

R: GAAGAGGTGGAAGAAGCACAC

WT Protein

MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVIE

HIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP

MUT Protein

MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVIE

HIMEDLDTNADKQLSFRGVHHADGEANLGLPREDARG.RGPWPPP.ARPRGGHPL

Antibodies for flow cytometry. IL-10R2 primary antibody and isotype control were purchased from BD biosciences. S100A9 antibody was purchased from Biolegend.

Antibodies for western blotting. STAT3 primary antibody was purchased from Cell Signalling Technologies and pSTAT3 from Abcam. Goat anti-rabbit and rabbit anti-mouse HRP/immunoglobulins were from Dako.

Antibodies for immunostaining. MUC2, LYZ, VIL1, RAB7, CHGA, IL10R2, IL22R1, S100A9 and MUC4 primary antibodies and donkey anti-rabbit 647 and Goat pAb to Ms IgG FITC were purchased from Abcam. CSA-1 primary antibody was from Insight Biotechnologies and Alexa fluor 456 goat anti-rabbit IgG from Life Technologies.

Statistical analyses. For statistical comparisons, we used unpaired, two-tailed *t* tests for comparing gene expression analyses and unpaired, two-tailed Mann Whitney *U* tests for

comparing bacterial counts data. For FACS analyses with iHOs infected with TIMER^{bac}-*Salmonella* we used Kruskal-Wallis tests with Dunn's multiple comparision test to compare the number of infected IECs in each condition. To compare proportions, we used Fisher's exact tests. All statistical analyses were done with the Prism 6.0b software (GraphPad). A p value of ≤ 0.05 was considered to be significant. Pathway analysis for RNA-Sequencing data was performed using the analysis tools available on the InnateDB website (http://www.innatedb.com/index.jsp). The hypergeometric analysis algorithm and Benjamini Hochberg p-value correction methods were used in all pathway analysis.

SI Figures

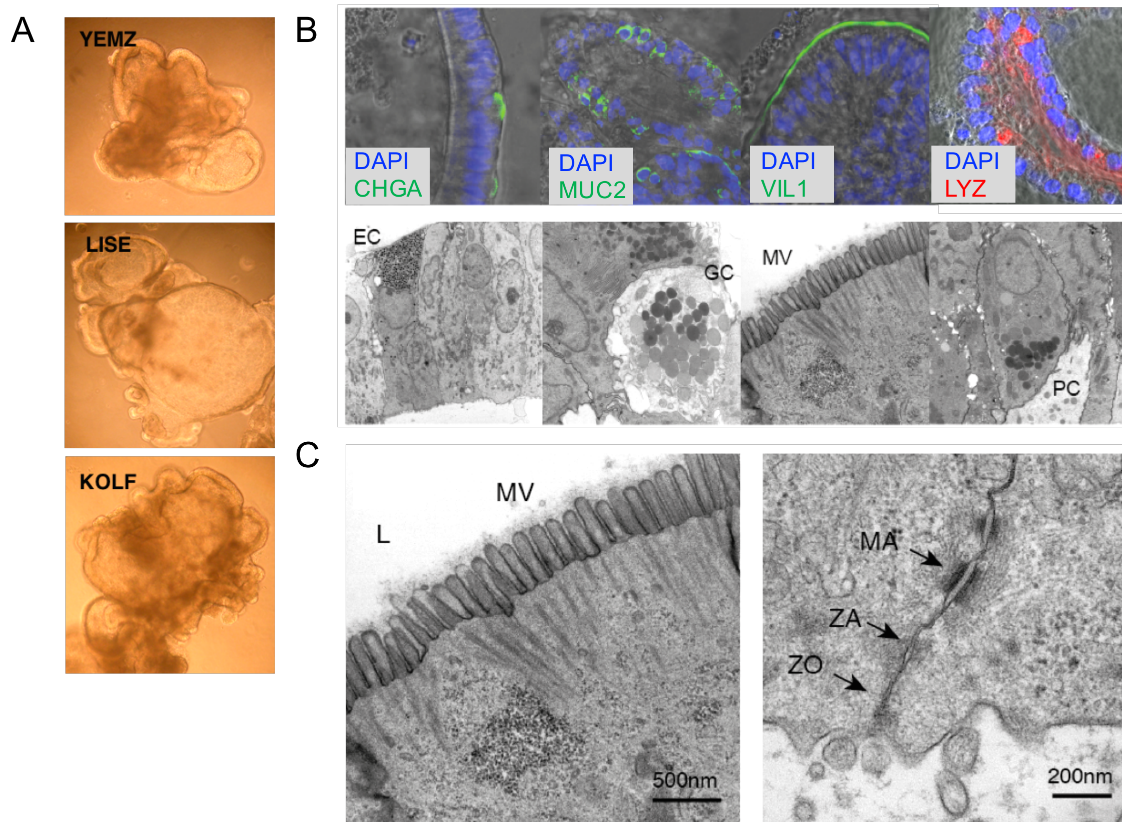

Figure S1. iHOs generated from healthy control Kolf2, Lise1 and Yemz1 hIPSC lines display characteristics of mature intestinal epithelium. (**A**) iHO morphology in culture. (**B**) After at least 6 weeks in culture iHOs were examined for markers of matured intestinal cell types, CHGA (Chromogranin A, enteroendocrine cells; green), MUC2 (Mucin 2, goblet cells; green), VIL1 (Villin 1, enterocytes; green), LYZ (Lysozyme, Paneth cells; red) and DAPI (nuclei, blue), by immunofluorescence, with corresponding TEM micrographs of these cell types depicted below (EC = enteroendocrine cell; GC = Goblet cell; MV = Microvilli; PC = Paneth cell). (**C**) TEM micrographs demonstrating features of the intestinal epithelium (MV = microvilli; L = lumen), with tight junctions visible between epithelial cells (ZO = zonula occludens; ZA = zonula adherens; MA = macula adhaerens). Images were selected as representative for features from images taken from multiple iHOs generated from all three healthy control lines (6).

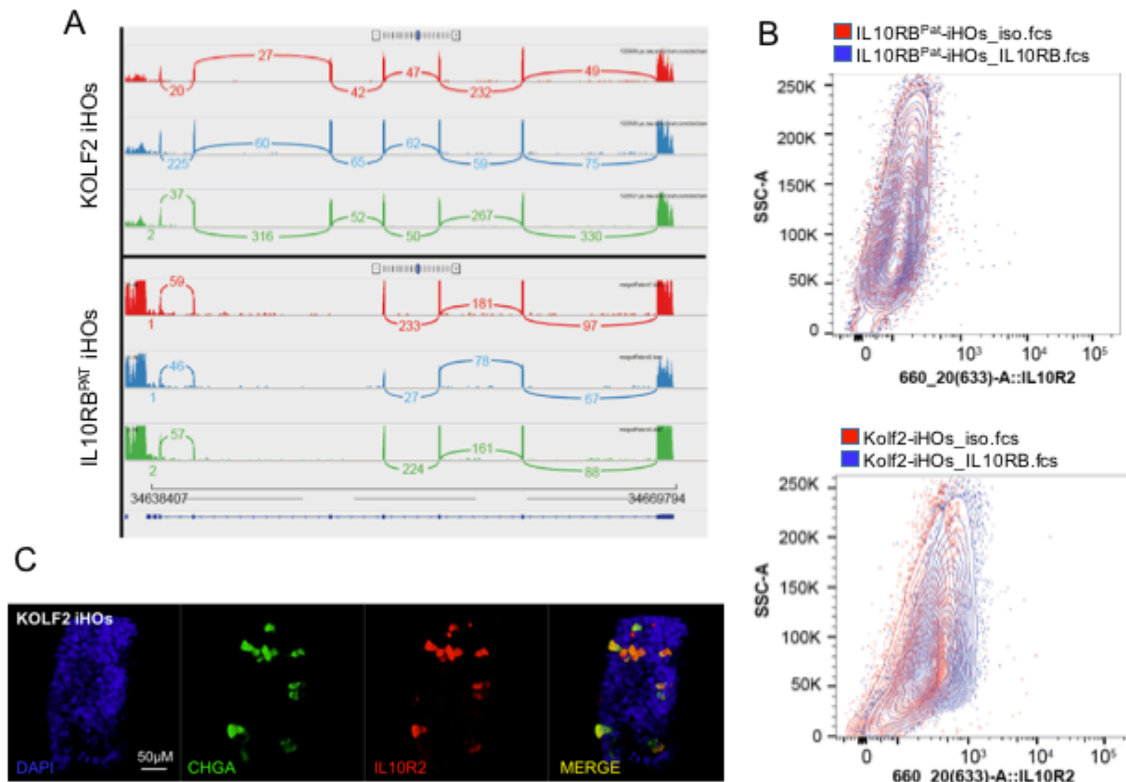

Figure S2. IL10RB iHOs do not express IL10RB, in contrast to healthy control Kolf2 iHOs. (**A**) Representative sashimi coverage plots for IL10RB generated in IGV from RNA-Seq datasets for untreated iHOs generated from Kolf2 and IL10RB^{Pat} hIPSCs, demonstrating loss of coverage of exon 3 in IL10RB^{Pat} iHO samples. RNA-Seq data from three biological replicates is presented for each iHO line (highlighted in different colours). (**B**) To further validate the immunostaining data presented in **Figure 1**, expression of IL10R2 positive cells was examined using flow cytometry. Data presented from sorted live IECs from Kolf2 iHOs and IL10RB^{Pat} iHOs, stained with IL-10R2 antibody (blue) or an isotype control (red), confirming loss of receptor expression in IL10RB^{Pat} iHOs. (**C**) Kolf2 iHOs were examined for CHGA (green) and IL10R2 (red) expression by immunofluorescence, with visible colocalisation present (yellow).

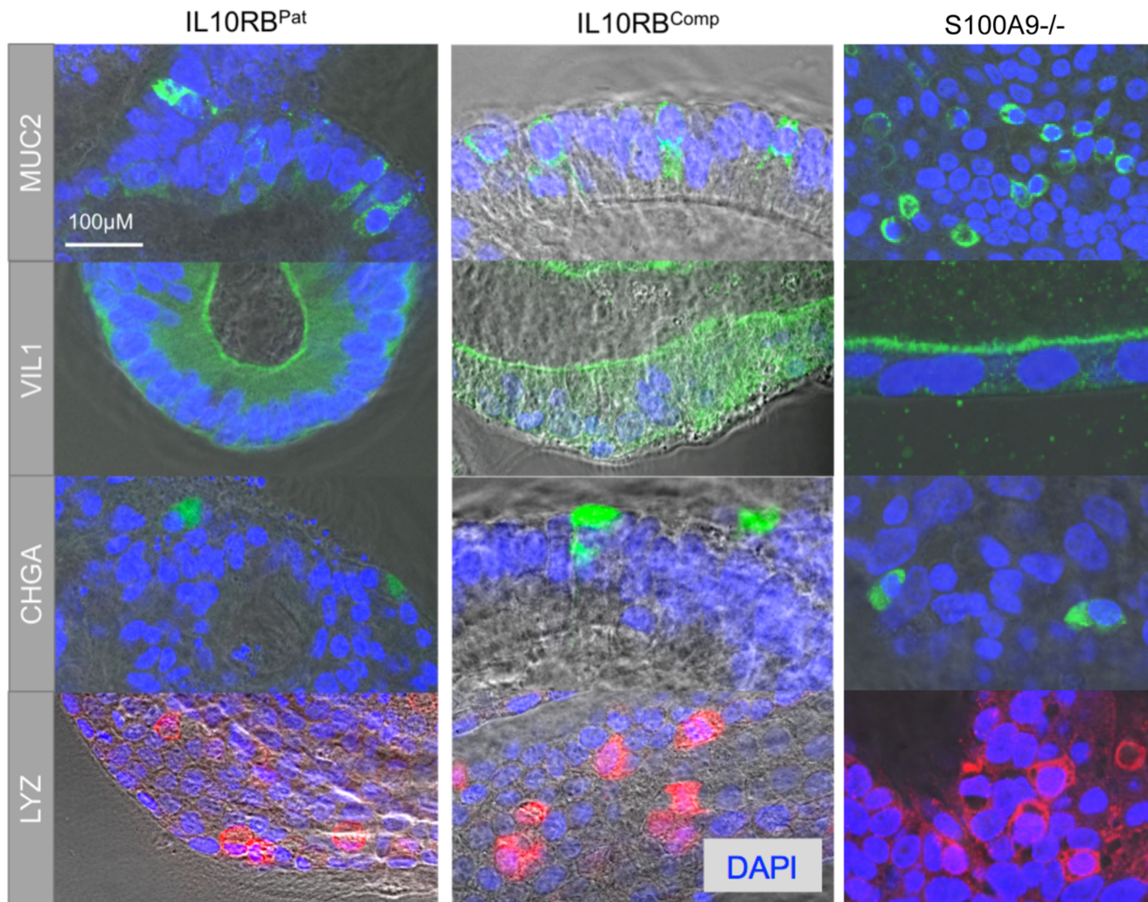

Figure S3. IL10RB^{Pat}, IL10RB^{Comp} and S100A9$^{-/-}$ iHOs display markers of differentiated intestinal cell types. After at least 6 weeks in culture iHOs were examined for markers of matured intestinal cell types, CHGA (green), MUC2 (green), VIL1(green), LYZ (red) and DAPI (blue), by immunofluorescence.

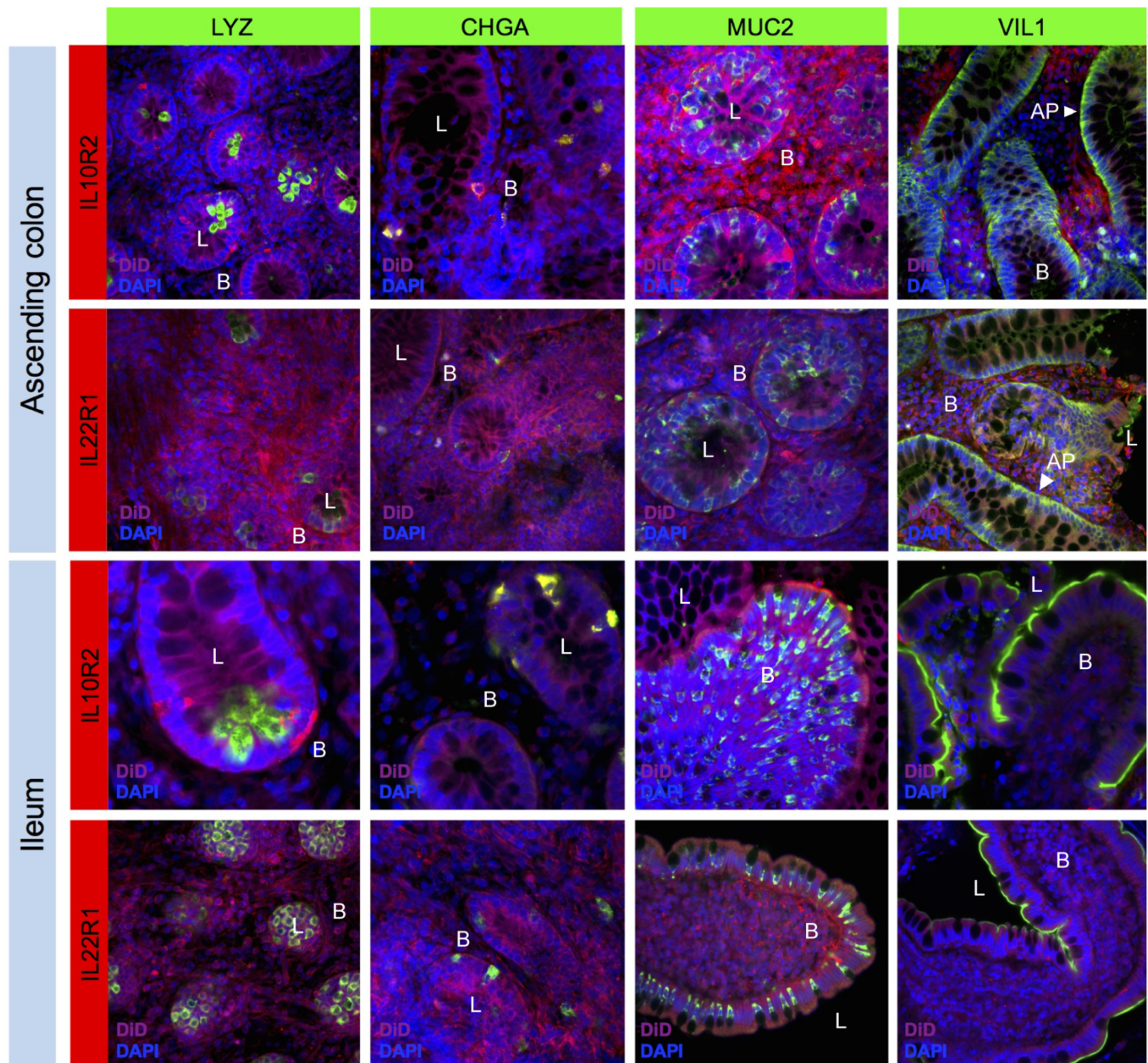

Figure S4. Primary human intestinal tissue was isolated by gastrointestinal biopsy from ileum and ascending colon and examined for expression of IL10R2 (red), IL22R1 (red), LYZ (green), CHGA (green), MUC2 (green), VIL1 (green), DAPI (blue) and DiD (purple; cell membrane stain) by immunofluorescence. L = Lumen; B = Basal; AP = apical surface

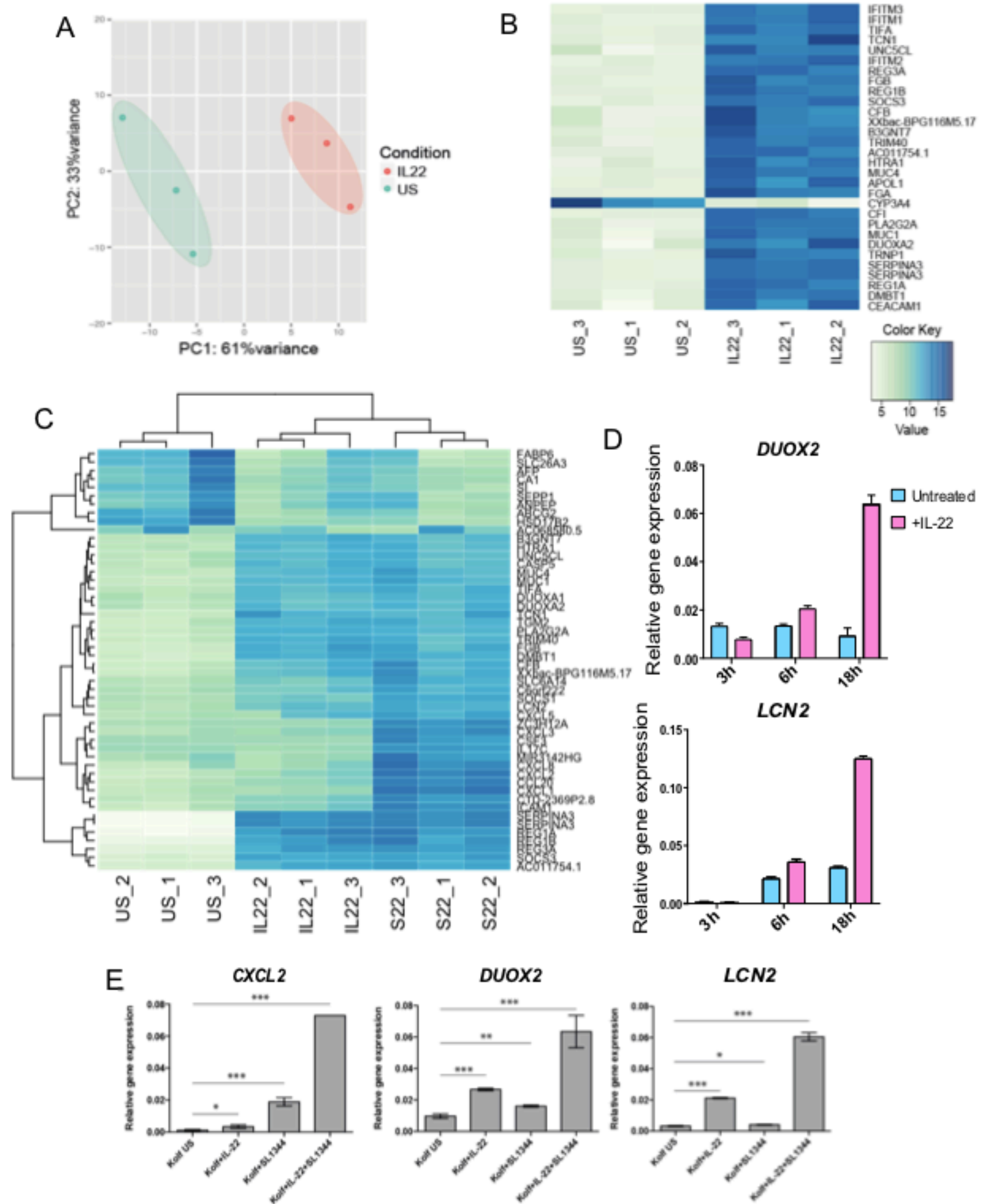

Figure S5. iHOs display changes in transcriptional responses when challenged with IL-22 or IL-22 and *S*. Typhimurium SL1344. (**A**) PCA of RNA-Seq expression data from iHOs treated for 18 hours with 100ng mL^{-1} IL-22 or left untreated for three biological replicates

per condition, demonstrating distinct differences in gene expression patterns between the treated and untreated iHOs. (**B**) Heat map of the RNA-Seq expression data calculated with DESeq2 for the 30 most significantly differentially expressed genes after addition of IL-22 to Kolf2 iHOs, with data presented from three biological replicates per condition. (**C**) Heat map of the RNA-Seq expression data calculated with DESeq2 for the 50 most significantly differentially expressed genes after addition of IL-22 and *S*. Typhimurium SL1344 to Kolf2 iHOs, with data presented from three biological replicates per condition. (**D**) RT-qPCR demonstrates that transcripts for Lipocalin-2 (LCN2) and Dual Oxidase 2 (DUOX2) are not upregulated in Kolf2 iHOs 3 hours and 6 hours after IL-22 stimulation (**E**) RT-qPCR for selected IL-22 regulated genes demonstrates that when IL-22 pre-stimulated iHOs are subsequently challenged with *S*. Typhimurium SL1344, gene expression of the IL-22 regulated genes *CXCL2*, *DUOX2* and *LCN2* is more highly upregulated than with IL-22 alone (Unpaired, two-tailed, Student's *t* tests). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

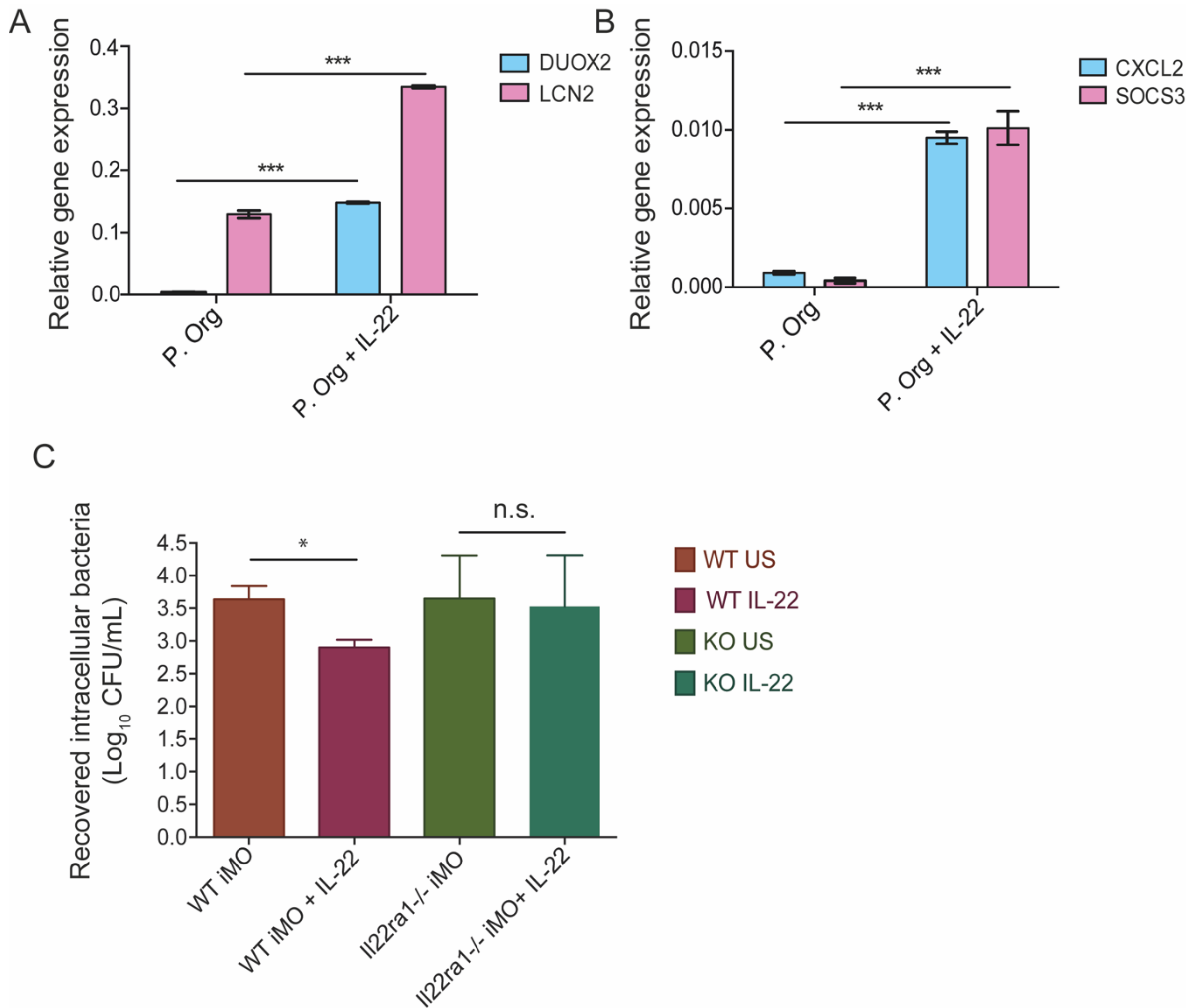

Figure S6. Primary duodenal organoids (P. Org) are responsive to rhIL-22. (**A** & **B**) Transcripts for IL-22 regulated genes Lipocalin 2 (*LCN2*), Dual Oxidase 2 (*DUOX2*), Suppressor of cytokine signalling 3 (*SOCS3*) and *CXCL2* are significantly upregulated after addition of 100ng mL^{-1} IL-22 for 18 hours, in comparison to unstimulated primary organoids (Unpaired, two-tailed Mann Whitney *U* tests). Data presented shows the mean and s.d. from three biological replicates per condition. ***, $P < 0.0001$. (**C**) Modified gentamicin protection assays in iMOs generated from wild type mice show that pre-treatment with IL-22 results in significantly less invasion after microinjection of *S.* Typhimurium SL1344 (p = 0.002; unpaired, two-tailed Mann Whitney *U* test), but this phenotype is not observed in iMOs generated from *Il22ra1*-/- mice (p = 0.7338; unpaired,

two-tailed Mann Whitney *U* test). Assays were performed on three batches of iMOs per mouse, with data presented showing the mean from two mice per genotype +SEM. 25 iMOs were injected per replicate.

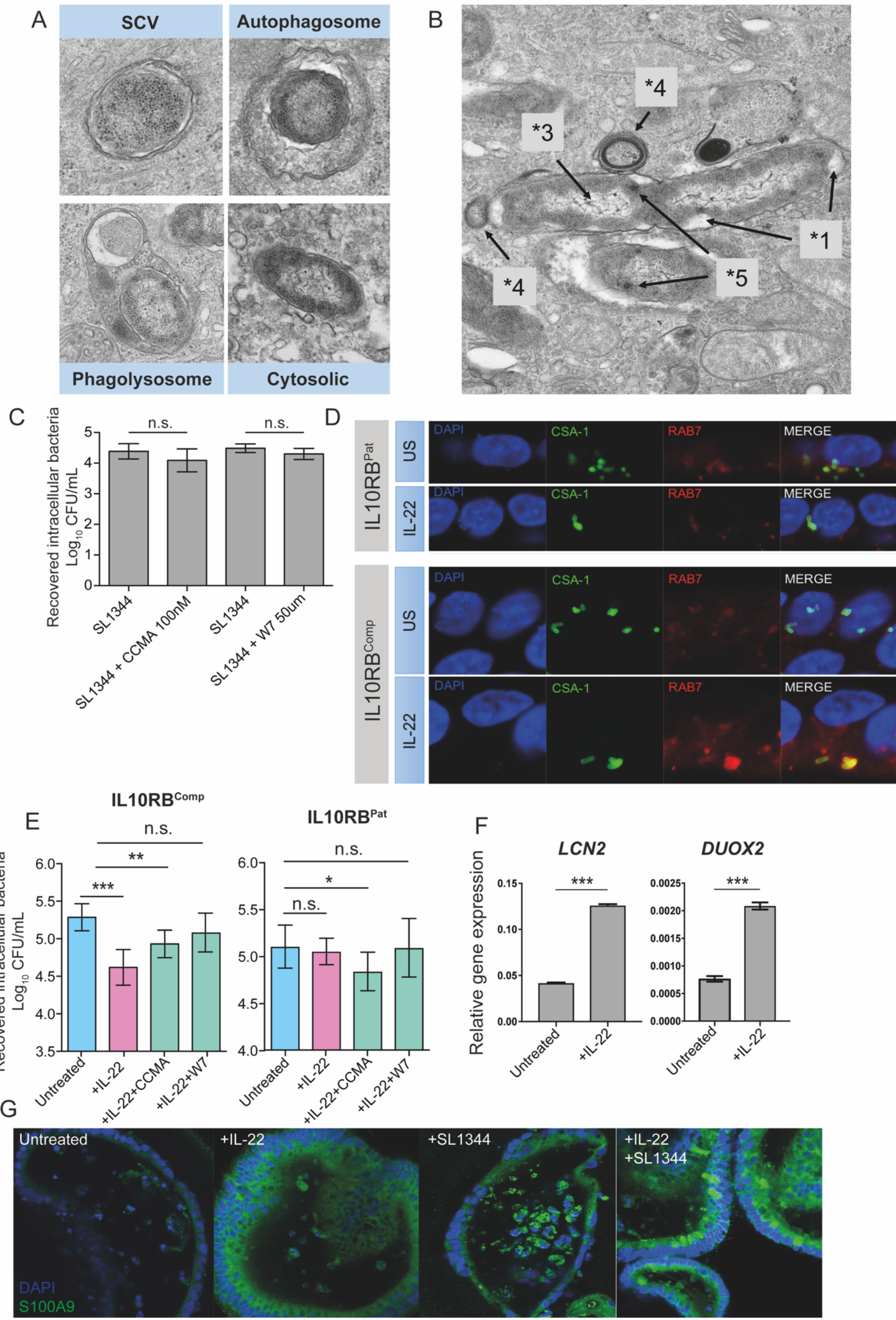
A
SCV
Autophagosome
Phagolysosome
Cytosolic
B
*1
*3
*4
*5
C
Recovered intracellular bacteria Log10 CFU/mL
n.s.
SL1344
SL1344 + CCMA 100nM
SL1344 + W7 50um
D
IL10RBPat
IL10RBComp
US
IL-22
DAPI
CSA-1
RAB7
MERGE
E
IL10RBComp
IL10RBPat
n.s.
***
**
*
Untreated
+IL-22
+IL-22+CCMA
+IL-22+W7
F
LCN2
DUOX2
Relative gene expression
***
Untreated
+IL-22
G
Untreated
+IL-22
+SL1344
+IL-22
+SL1344
DAPI
S100A9

Figure S7. Enhanced lysosomal fusion with SCVs is observed in IL-22 treated iHOs**.** (**A**) Features used for scoring presented in **Table 1** and **Fig. 4**. Visual characterisation of SCV used for scoring presented in **Table 1** (**B**) Visual characterisation of bacterial cell damage/stress used for scoring: Widening of periplasmic space (1); Membrane damage and ragged appearance (2); Decrease in cytosol density (3); Direct contact with lysosomes (4); Volutin granules present (5) (**C**) Modified gentamicin protection assays in Kolf2 iHOs show that pre-treatment with W7 or Concanamycin A does not result in significantly different recovery of intracellular *S*. Typhimurium SL1344 in comparison to untreated iHOs (W7, p = 0.2; CCMA, p = 0.4; unpaired, two-tailed Mann Whitney *U* tests) (**D**) $IL10RB^{Pat}$ and $IL10RB^{Comp}$ iHOs challenged with IL-22 for 18 hours, or left untreated, and subsequently microinjected with *S*. Typhimurium SL1344, were examined for RAB7 (red), CSA-1 (green) and DAPI (blue) by immunofluorescence (Original magnification: 63x), with colocalisation between RAB7 and CSA-1 (yellow) visible in IL-22 pre-treated $IL10RB^{Comp}$ samples. (**E**) $IL10RB^{Pat}$ or $IL10RB^{Comp}$ iHOs were pre-treated with 100ng mL^{-1} IL-22 for 18 hours, then treated with the phagolysosomal inhibitors W7 at 50μM for 6 hours or Concanamycin A (CCMA) at 100nM for 4 hours, or left untreated, after which modified gentamicin protection assays were performed. There was no significant difference in recovered intracellular bacteria from W7 treated $IL10RB^{Comp}$ iHOs and $IL10RB^{Comp}$ untreated iHOs (p = 0.1135; unpaired, two-tailed Mann Whitney *U* tests). Data presented shows the mean from three biological replicates with three technical replicates per assay, +/- SEM (**F**) Transcripts for IL-22 regulated genes Lipocalin 2 (*LCN2*) and Dual Oxidase 2 (*DUOX2*) are significantly upregulated after addition of 100ng mL^{-1} rhIL-22 for 18 hours, in comparison to unstimulated iHOs (Unpaired, two-tailed, Student's *t* tests). *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; n.s., not significant. Data presented from four technical replicates. RT-qPCR was performed with a TaqMan gene expression assay specific for

LCN2 or *DUOX2* and analysed via the comparative C_T method with GAPDH as an endogenous control. (**G**) Kolf2 iHOs challenged with IL-22 for 18 hours, or left untreated, and subsequently microinjected with *S*. Typhimurium SL1344, were examined for S100A9 (green) and DAPI (blue) by immunofluorescence (Original magnification: 20x).

Supplementary Table S1. Effect of IL-22 treatment and IL10RB expression on *Salmonella* infections.

	Histopathological scores after *Salmonella* treatment					
Pathology	**Kolf2**	**Kolf2 + IL-22**	**IL10RB^{Pat}**	**IL10RB^{Pat} + IL-22**	**IL10RB^{Comp}**	**IL10RB^{Comp} + IL-22**
Salmonella containing vacuole	65	65	49	29	59	64
Autophagosome	1	2	0	0	0	0
Phagolysosome	6	30	27	20	21	53
Cytosolic	1	5	2	0	3	0
Fisher's exact test		p=0.0003		p=0.5751 n.s.		p=0.0072

Histopathological scores from 1mm iHO mucosa, taken in 100μM sections, using scoring features presented in *SI Appendix*, Fig. S7 A & B, demonstrating a significant increase in phagolysosomes in IL-22 treated IL10R2 expressing iHOs injected with *S*. *enterica* Typhimurium SL1344, in comparison to untreated iHOs injected with *S*. *enterica* Typhimurium SL1344 (Fisher's exact tests). n.s., not significant.

SI Data sets

Additional data table S1 (separate file)

Top 50 genes significantly upregulated after treatment of Kolf2 iHOs with 100ng/ml rhIL-22 ($p < 0.05$), accompanied by gene symbols, gene names, significance p-values and raw counts in treated and untreated iHOs

Additional data table S2 (separate file)

Top 25 biological processes identified by InnateDB enriched in Kolf2 iHOs after treatment with 100ng/ml rhIL-22 for 18h

Additional data table S3 (separate file)

Top 100 genes significantly upregulated after treatment of Kolf2 iHOs with 100ng/ml rhIL-22 followed by treatment with S. Typhimurium SL1344 for 3h ($p < 0.05$), accompanied by gene symbols, gene names, significance p-values and raw counts in treated and untreated iHOs

Additional data table S4 (separate file)

Top 25 biological processes identified by InnateDB enriched in Kolf2 iHOs after treatment with 100ng/ml rhIL-22 for 18h, followed by treatment with *S.* Typhimurium SL1344 for 3h

1. Siggins MK*, et al.* (2011) Absent bactericidal activity of mouse serum against invasive African nontyphoidal Salmonella results from impaired complement function but not a lack of antibody. *Journal of immunology* 186(4):2365-2371.
2. Everest P*, et al.* (1999) Evaluation of Salmonella typhimurium mutants in a model of experimental gastroenteritis. *Infection and immunity* 67(6):2815-2821.
3. Claudi B*, et al.* (2014) Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. *Cell* 158(4):722-733.
4. Sorensen M*, et al.* (2003) Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Lett* 552(2-3):110-114.
5. Jung P*, et al.* (2011) Isolation and in vitro expansion of human colonic stem cells. *Nature medicine* 17(10):1225-1227.
6. Forbester JL, Hannan N, Vallier L, & Dougan G (2016) Derivation of Intestinal Organoids from Human Induced Pluripotent Stem Cells for Use as an Infection System. *Methods in molecular biology*.
7. Forbester JL*, et al.* (2015) Interaction of Salmonella enterica Serovar Typhimurium with Intestinal Organoids Derived from Human Induced Pluripotent Stem Cells. *Infection and immunity* 83(7):2926-2934.
8. Raffatellu M*, et al.* (2005) SipA, SopA, SopB, SopD, and SopE2 contribute to Salmonella enterica serotype typhimurium invasion of epithelial cells. *Infection and immunity* 73(1):146-154.
9. Kim D*, et al.* (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology* 14(4):R36.
10. Love MI, Huber W, & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15(12):550.