Interaction of *Salmonella enterica* Serovar Typhimurium with Intestinal Organoids Derived from Human Induced Pluripotent Stem Cells

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The intestinal mucosa forms the first line of defense against infections mediated by enteric pathogens such as salmonellae. Here we exploited intestinal “organoids” (iHOs) generated from human induced pluripotent stem cells (hiPSCs) to explore the interaction of *Salmonella enterica* serovar Typhimurium with iHOs. Imaging and RNA sequencing were used to analyze these interactions, and clear changes in transcriptional signatures were detected, including altered patterns of cytokine expression after the exposure of iHOs to bacteria. *S. Typhimurium* microinjected into the lumen of iHOs was able to invade the epithelial barrier, with many bacteria residing within *Salmonella*-containing vacuoles. An *S. Typhimurium* invA mutant defective in the *Salmonella* pathogenicity island 1 invasion apparatus was less capable of invading the iHO epithelium. Hence, we provide evidence that hiPSC-derived organoids are a promising model of the intestinal epithelium for assessing interactions with enteric pathogens.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. Typhimurium* SL1344, a well-characterized isolate used routinely in laboratory experiments (18),...
was used in these studies. In order to examine hIOs during an attenuated bacterial infection, we used an S. Typhimurium SL1344 strain defective in the invA gene of Salmonella pathogenicity island 1 (SPI-1). S. Typhimurium SL1344 carrying an invA deletion was constructed by moving the invA::Kmr deletion from S. Typhimurium SL3261 to SL1344 by P22 transduction.

Microinjection. After at least 6 weeks in culture A1ATD-1 hIOs were challenged with S. Typhimurium SL1344 (1 × 10⁶ CFU ml⁻¹) and left uninfected. Stabilized organoids were incubated for 3 h at 37°C and 5% CO₂, and then RNA was purified with the RNeasy minikit (Qiagen).

Microinjection. After at least 6 weeks in culture, hIOs were prepared for microinjection 4 days prior to infection by passaging as described above. Disaggregated hIOs were resuspended in 200 µl of Matrigel, plated into glass bottom Wilco Wells microinjection dishes (INTRACEL), and covered with hIO base growth medium. The Eppendorf TransferMan NK2-FemtoJet express system was used to inject bacteria into organoids. All injections were carried out in a chamber at 37°C and 5% CO₂. After injection, organoids were incubated for 3 h at 37°C and 5% CO₂ and then fixed for microscopy. For RNA sequencing (RNA-Seq) analysis, 30 organoids per microinjection plate were injected with either PBS or S. Typhimurium SL1344 mixed 1:1 with phenol red dye. hIOs were incubated for 3 h at 37°C and 5% CO₂, Matrigel was dissolved with Cell Recovery Solution (Corning), and phenol red-marked hIOs were isolated. RNA was then purified with the RNeasy minikit (Qiagen).

RT-qPCR. RNA isolated from whole hIOs before or after infection was reverse transcribed with the QuantiTect reverse transcription (RT) kit (Qiagen) according to the manufacturer’s protocol. All RT-quantitative PCR (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 analyzed via the comparative CT method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control.

RNA-Seq and analysis. RNA was prepared from hIOs microinjected with S. Typhimurium SL1344 and hIOs microinjected with PBS (as described above) for three biological replicates per condition. Multiplexed mRNA libraries were prepared by using the Illumina TruSeq protocol and sequenced via paired-end sequencing with the Illumina-C HiSeq 2500 platform. 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 (23) version 2.0.8 with the human reference version GRCh37 used for 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 expression levels. R version 3.1.2 was used to import count data, and the DESeq2 package was used to normalize the data and detect differentially expressed genes (24). Significantly differentially expressed genes were selected with a cutoff false-discovery rate of less than 0.05 and a log change of >2.0-fold. Heat maps and principal-component analysis (PCA) plots were constructed from log-transformed data with the ggplot2 R library (http://cran.r-project.org/web/packages/ggplot2/index.html). For the 25 most significantly upregulated genes, enriched gene ontology terms were identified by using InnateDB with the category Biological Processes selected.

Anti-human cytokine/chemokine multiplex bead assays. Triplicate 25-µl samples of hIO culture supernatants from unstimulated hIOs and hIOs stimulated with S. Typhimurium SL1344 by addition to the culture medium were analyzed for cytokine/chemokine concentrations. Millipore customized anti-human Milliplex magnetic bead kits were used in accordance with the manufacturer’s instruction, and a multiplex selection of the analytes tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-8 was measured. Briefly, 25-µl aliquots of supernatants or dilutions of a mixed cytokine standard were captured on anti-antibody-coated beads overnight. On the following day, after washing with a 96-well plate-formatted magnet, detection reagents were added and allowed to bind to any captured cytokine present on the beads. Further washes with the 96-well magnet, data were acquired on a Luminex FlexMap3D and analyzed with Luminex X-exponent software by extrap-
Immunohistochemistry. For immunohistochemical analysis, iHOS suspended in Matrigel were fixed in 3% paraformaldehyde and 0.125% glutaraldehyde in PBS for 1 h at room temperature. They were then cooled to 4°C and dehydrated through an ethanol series (25, 50, 70, and 100% for 30 min each), infiltrated with glycol methacrylate (GMA) JB-4 and benzoyl peroxide (JB-4 embedding kit; Sigma-Aldrich), polymerized in gelatin capsules at 4°C for 24 to 48 h, and stored at -20°C. One- to 2-μm sections prepared on a Leica UCT ultramicrotome were mounted on slides precoated with 0.01% (wt/vol) poly-L-lysine hydrobromide. For immunoperoxidase staining, samples were blocked for 30 min with 0.1% sodium azide and 100 μl of 30% hydrogen peroxide and for 30 min with RPMI 1640 culture medium (Invitrogen) with 5% fetal calf serum and 1% BSA and then washed with TBS buffer (0.6 g of Tris, 8 g of sodium chloride, 1 liter of distilled H₂O, 3 to 4 ml of 1 M HCl to pH 7.6) three times for 15 min each. Samples were incubated overnight at 4°C with primary antibodies for mucin 2 (MUC2; ABCAM ab11197), lysozyme (LYZ; ABCAM ab2408), and chromogranin A (CHGA; ABCAM ab36997) in TBS buffer at antibody-dependent dilutions (MUC2, 1:200; LYZ, 1:50; CHGA, 1:50). Bound primary antibodies were detected with the Envision® System-HRP (AEC) (Dako), and nuclei were visualized by countertaining with Mayer’s hematoxylin. Finally, sections were covered with Genex Clear Mount (Sapphire Bioscience) and baked at 80°C for 5 min to set. For immunofluorescence staining for LAMP-1 (ABCAM ab24170), followed by donkey anti-rabbit 647 (ABCAM ab150075) and Salmonella common structural antigen 1 (CSA-1), fluorescein isothiocyanate-labeled (Insight Biotechnology Limited 02-91-99) sections were similarly processed by omitting the first block and diluting all of the antibodies in PBS (LAMP-1, 1:50; donkey anti-rabbit 647, 1:100; CSA-1, 1:20). Sections were mounted in Prolong-Gold with added 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen).

Transmission electron microscopic analysis of infected iHOS. Infected organoids were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde from the standard-curve dilutions. Results are expressed in picograms per milliliter.

FIG 1 A1ATD-1 hIPSC-derived intestinal organoids show increased expression of genes for markers of adult intestinal tissue. (A) At differentiation day 30, the expression of adult intestinal markers CHGA (enteroendocrine cells), LYZ (Paneth cells), MUC2 (goblet cells), and VIL1 (enterocytes) was significantly greater in A1ATD-1 iHOS than in A1ATD-1 hIPSCs (P = 0.0023, P = 8.41 × 10⁻⁵, P = 0.002, and P = 4.4 × 10⁻⁵, respectively). (B) Primers were validated by measuring the expression of the same markers in RNA isolated from human ileum. (C) Log fold change in the expression of pluripotency markers. The expression of NANOG and POUF51 was significantly lower in A1ATD-1 iHOS on both days 25 (25d) (P = 4.64 × 10⁻³ and P = 1.99 × 10⁻³) and 55 (55d) (P = 3.55 × 10⁻⁵ and P = 3.63 × 10⁻⁵) than in their A1ATD-1 hIPSC progenitors. Each RT-qPCR was performed with a TaqMan gene expression assay specific for each gene and analyzed via the comparative C_T method with GAPDH as an endogenous control. *, P < 0.05; **, P < 0.001; ***, P < 0.0001.
dehydrate in 0.1 M sodium cacodylate buffer (1 liter of dH2O, 21.4 g of sodium cacodylate, 1 g of MgCl$_2$, 0.5 g of CaCl$_2$, adjusted to pH 7.42 with HCl), postfixed in 1% osmium tetroxide diluted in sodium cacodylate buffer, dehydrated with an ethanol series, and then embedded with the Epoxy Embedding Medium kit (Sigma-Aldrich). After embedding, samples were cured at 65°C for 48 h. Semithin (0.5-μm) sections were cut on a Leica UCT ultramicrotome and stained with toluidine blue on a microscope slide with suitable areas selected for ultrathin 50-nm sectioning. Ultrathin sections were collected on copper grids and contrasted with uranyl acetate and lead citrate before viewing on an FEI 120-kV Spirit BioTWIN transmission electron microscope. Images were taken on an F4.15 Tietz charge-coupled device camera.

**Invasion assays.** Microinjection was carried out as described above. To assess the invasion of iHO epithelial cells by bacteria, we modified the commonly used gentamicin protection assay (25) for use in iHOS. Forty iHOS per microinjection dish were injected with either wild-type or invA mutant S. Typhimurium SL1344. Injected iHOS were incubated for 90 min at 37°C and 5% CO$_2$. iHOS were isolated from Matrigel with Cell Recovery Solution (Corning), centrifuged, washed once with PBS, subjected to manual disaggregation of the organoid ultrastructure, resuspended in iHO base growth medium supplemented with 0.1 ml$^{-1}$ gentamicin (Sigma), 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 20-μl spots were plated onto prewarmed LB plates for CFU counting. Protections assays were performed with three biological replicates.

**Statistical analysis.** For statistical comparisons, we used unpaired, two-tailed t tests done with the Prism 6.0b software (GraphPad).

**Nucleotide sequence accession numbers.** RNA-Seq data are stored in the European Genome-Phenome Archive under study accession number EGA00001001253. Data will be made available to all researchers upon request to the Data Access Committee (DAC) for the Wellcome Trust Sanger Institute, accession number EGAC00001000205. The named person of contact for the DAC for the Wellcome Trust Sanger Institute is Giselle Kerry (gh2@sanger.ac.uk). The restriction on data access is required for human donor protection.

**RESULTS**

IHOs generated from A1ATD-1 hIPSCs recapitulate features of the human intestine in vitro. Human intestinal organoids were generated from A1ATD-1 hIPSCs, which were initially reprogrammed from dermal fibroblasts (21), in accordance with a previously published differentiation protocol (22). After 30 days in culture, there were significantly higher levels of the mRNAs for MUC2 (mucin 2, goblet cells), VIL1 (Villin 1, enterocytes), CHGA (chromogranin A, enteroendocrine cells), and LYZ (lysozyme, Paneth cells) in A1ATD-1 iHOS than in A1ATD-1 hIPSCs (Fig. 1A). These four markers were also highly expressed in control human ileal tissue (Fig. 1B). In contrast, 25 days after resuspension in Matrigel and the addition of iHO base growth medium, we observed dramatically lower mRNA levels of the pluripotency markers NANOG and POUF51 in iHOS than in A1ATD-1 hIPSCs (Fig. 1C). We also observed distinct morphological changes in our iHOS cultures marking the differentiation process to endoderm, hindgut, and finally iHOS (Fig. 2A). After 1 to 2 weeks in Matrigel, the iHOS formed spheroid structures of cells enclosing a hollow lumen, with some crypt-like structures evident in the iHO ultrastructure. To further dissect the morphology of the iHO wall, we
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A

PC1: 75% variance
PC2: 11% variance

Condition
SL1344
Unstim

B

C

cytokine-mediated signaling pathway
positive regulation of NF-kappaB import into nucleus
positive regulation of nitric oxide biosynthetic process
positive regulation of gene expression
extracellular matrix organization
positive regulation of transcription from RNA polymerase II promoter
positive regulation of angiogenesis
chondrosis
response to lipopolysaccharide
negative regulation of fat cell differentiation
mammary gland epithelium development
positive regulation of cell proliferation
positive regulation of NF-kappaB transcription factor activity
cardiac muscle cell contraction
signal transduction
intercellular adhesion
positive regulation of inflammatory response
immune response
innate immune response
inflammatory response

D

Relative gene expression

A1ATD-1 IHOs
A1ATD-1 IHOs + SL1344

IL1B
IL23A
TNF
IL8
CXCL2

E

IL8 (pg/mL)

A1ATD-1 IHOs
A1ATD-1 IHOs + SL1344

IL6 (pg/mL)

A1ATD-1 IHOs
A1ATD-1 IHOs + SL1344

TNF (pg/mL)

A1ATD-1 IHOs
A1ATD-1 IHOs + SL1344
examined iHO cellular structure in more detail by transmission electron microscopy (Fig. 2B). We were able to visualize a polarized monolayer of epithelial cells, with a clear distinction between the apical and basal sides, and microvilli present on the apical surface. Structures resembling tight junctions between epithelial cells were also evident (Fig. 2C). Finally, we detected the expression and localization of MUC2, LYZ, and CHGA in iHOS with marker-specific antibodies and immunohistochemistry within subsets of cells in the iHO wall (Fig. 2D). The cells expressing these markers were distributed around the iHO and were not solely congregated in one region, illustrating that A1ATD-1 iHOS comprise enterocytes interspersed with cells harboring genetic signatures typical of Paneth, goblet, and enteroendocrine cells.

**Stimulation of A1ATD-1 iHOS with S. Typhimurium SL1344 results in altered patterns of gene expression.** To probe the changes in the transcriptome of A1ATD-1 iHOS induced after infection of iHOS with salmonellae, we microinjected iHOS with S. Typhimurium SL1344 or PBS as a control and performed a global RNA-Seq analysis. After differential expression analysis, we observed 1,448 genes significantly upregulated in iHOS infected with S. Typhimurium SL1344 (cutoff, log change of >2-fold, adjusted \( P \) value, <0.01) and 577 genes significantly downregulated (cutoff, log change of <−2-fold; adjusted \( P \) value, <0.01) compared to controls. PCA (Fig. 3A) demonstrated clustering of the samples dependent on treatment, with those samples infected with SL1344 forming a group distinct from the uninjected iHOS, suggesting different patterns of gene expression in A1ATD-1 iHOS injected with SL1344. A heat map of the 30 most highly upregulated genes in iHOS after infection with S. Typhimurium SL1344 consists of a high proportion of genes associated with response to infection (Fig. 3B). ILs, essential mediators of the interactions between immune cells and nonhematopoietic cells (26), comprise 6 of the 30 most highly upregulated genes after infection of iHOS with SL1344. One of the highly expressed ILs was IL-23A, which has recently been linked with protection of the intestinal barrier through interaction with the IL-22 signaling pathway (27). IL-20 is also highly expressed after infection with SL1344, and this IL is associated with epidermal function and psoriasis (28); however, its role in intestinal infection is undetermined (29). After differential-expression analysis, we detected significant upregulation of goblet cell-associated genes such as GCNT3 and MUC2 (30) (log2 change, 1.5022-fold; adjusted \( P \) value, 1.68 \( \times \) 10−46; log2 change, 1.3399-fold; adjusted \( P \) value, 0.0059, respectively), suggesting that multiple cell types that make up the iHO can respond to SL1344 stimulation. Interestingly, one of the highly upregulated genes was BIRC3, which is also upregulated in enteroendocrine cells infected with *Chlamydia trachomatis* (31). Many genes encoding proinflammatory cytokines, including CCL20, IL1B, IL23A, CSF1, CXC10, IRAK2, TL17, TNF, TNFIP1, TNFAIP6, and CCL2, were also significantly upregulated. Factors involved in the innate immune response, inflammation, cytokine-mediated signaling, and the response to lipopolysaccharide were all enriched in iHOS after infection with *S. Typhimurium SL1344* (Fig. 3C; see Data Sets S1 and S2 in the supplemental material). To validate transcriptional changes in iHOS after infection with *S. Typhimurium SL1344*, we carried out further microinjections with PBS or SL1344 and measured the mRNAs for IL-8, IL1B, IL23A, TNF, and CXC12 with quantitative assays (Fig. 3D). The relative log fold expression of the mRNAs for these five genes increased significantly in iHOS infected with SL1344, in comparison with that in organsoids infected with PBS. Because of the morphology of organsoids in culture, it is possible to stimulate them apically via microinjection or basally through addition to the culture medium. We produced a global RNA-Seq data set by using basally stimulated iHOS. To this end, we left clusters of iHOS untreated or stimulated with *S. Typhimurium SL1344* by independent addition to the iHO base growth medium and performed an RNA-Seq analysis complementary to that carried out for the microinjected samples. Forty-nine of the 100 most highly upregulated genes were replicated in both data sets (see Data Set S1 and S3 in the supplemental material), and we observed enrichment in similar pathways after a gene ontology term analysis (see Data Sets S3 and S4 in the supplemental material). After stimulation with *S. Typhimurium SL1344*, we measured the expression of the cytokines TNF-α, IL-6, and IL-8 with Luminescent cytokine assays to confirm their production and secretion by iHOS (Fig. 3E). We detected significantly greater expression of all three cytokines in the supernatants of SL1344-stimulated iHOS than in unstimulated iHOS (\( P = 3.34 \times 10^{-4} \), \( P = 6.33 \times 10^{-7} \), and \( P = 3.89 \times 10^{-4} \), respectively).

**Microinjection of iHOS allows direct modeling of S. Typhimurium interaction with intestinal cells.** Our RNA-Seq analysis highlighted changes in the global transcriptional landscapes that are induced after pathogen sensing by the intestinal epithelial cells that make up the iHO structure. To further model bacterial invasion, we wanted to determine if the iHO system recapitulated hallmarks of *Salmonella* invasion such as the formation of the *Salmonella*-containing vacuole (SCV). Previous studies have used organoid dissection to introduce pathogens into the luminal cavity (12); however, we found that a microinjection system was a robust method (16). We could inject iHOS with a mixture of a bacterial suspension and phenol red, which made it easy to distinguish injected and noninjected iHOS in a single culture dish (Fig. 4A). After injection, iHOS retained their inoculum for the 3-h

**FIG 3** Microinjection of A1ATD-1 iHOS with *S. Typhimurium SL1344* results in upregulation of genes associated with infection and inflammation. After at least 6 weeks in culture, A1ATD-1 iHOS were microinjected with a mixture of phenol red dye and *S. Typhimurium SL1344* with the Eppendorf TransferMan NK2-FemtoJet express system. iHOS were incubated at 37°C and 5% CO\(_2\) for 3 h, phenol red-marked organoids were isolated, and RNA was prepared. (A) PCA of RNA-Seq expression data for three biological replicates per condition illustrates distinct differences in gene expression patterns between iHOS stimulated with SL1344 and unstimulated iHOS. (B) Heat map of the RNA-Seq expression data calculated with DESeq2 for the 30 most significantly upregulated genes after the addition of SL1344 to A1ATD-1 iHOS. (C) Enriched biological processes upregulated after stimulation of A1ATD-1 iHOS with *S. Typhimurium SL1344* (for the genes associated with each pathway and \( P \) values, see Data Set S1 in the supplemental material). (D) RT-qPCR showing that transcripts for the cytokines IL-8, CXC12, IL23A, TNF-α, and IL1B are significantly upregulated in iHOS injected with SL1344 in comparison to those in iHOS injected with PBS (\( P = 1.56 \times 10^{-4} \), \( P = 2.35 \times 10^{-9} \), \( P = 4.21 \times 10^{-8} \), \( P = 1.28 \times 10^{-4} \), and \( P = 6.42 \times 10^{-4} \), respectively). RT-qPCR data were analyzed via the comparative \( C_{\text{t}} \) method with GAPDH as an endogenous control. (E) After stimulation of iHOS with *S. Typhimurium SL1344*, induction of TNF-α, IL-6, and IL-8 production was shown to be significantly upregulated with Luminescent cytokine assays of supernatants collected from stimulated and unstimulated iHOS (\( P = 3.34 \times 10^{-5} \), \( P = 6.33 \times 10^{-7} \), and \( P = 3.89 \times 10^{-4} \), respectively). Assays were performed with supernatants from three biological replicates.
incubation time. We observed *S. Typhimurium* in the iHO lumen, close to the microvilli (Fig. 4B), and also inside the epithelial cells, residing in structures that resemble SCVs (Fig. 4C).

An *S. Typhimurium* invA mutant is deficient in invasion of iHOs. To further confirm the potential of the iHO system in studying *Salmonella* infection, we investigated the ability of an *S. Typhimurium* SL1344 invA mutant to infect following microinjection into the iHO lumen. InvA is an important component of the SPI-1 invasion system, and this derivative is attenuated in terms of the ability to enter epithelial monolayers (32). To address how this mutant behaved in the iHO system, we modified the gentamicin protection assay (25) regularly used to determine the percentage of bacterial invasion of epithelial monolayers. We found that after the microinjection of either wild-type or invA mutant *S. Typhimurium* SL1344, we recovered significantly different numbers of CFU after plating epithelial cell lysates (P = 0.0092), with SL1344 invA showing 30-fold less invasiveness (Fig. 5A). To confirm our finding, we fixed injected organoids

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FIG 4 Microinjection of organoids derived from hIPSCs provides a model of *S. Typhimurium* SL1344 infection. Overnight cultures of *S. Typhimurium* SL1344 were diluted 1:1 with phenol red and injected into the lumen (L) of A1ATD-1 iHOs with the Eppendorf TransferMan NK2-Femtojet express system. (A) iHOs retained the inoculum for 3 h subsequent to injection. Arrowheads mark the interface between the bacterial inoculum and human cells. (B and C) Transmission electron micrographs 3 h after injection showing SL1344 populating the lumen of the iHO and residing inside the epithelial cells in SCVs after invasion (arrow) (B; enlarged in panel C).

FIG 5 *S. Typhimurium* SL1344 invA mutant is deficient in invasion of iHOs. A1ATD-1 iHOs were microinjected with wild-type (WT) or invA mutant SL1344 and incubated at 37°C and 5% CO₂ for 90 min. (A) Log numbers of CFU per milliliter recovered from cells of iHOs after microinjection and modified gentamicin protection assay, showing that WT SL1344 is significantly (~30×) more invasive than the invA mutant (P = 0.0092). (B) Fluorescence staining of GMA JB-4- and benzoyl peroxide-fixed sections for CSA-1 (green) and LAMP-1 (red) and an overlay of the two images showing the colocalization (yellow) of bacteria and host protein after invasion by WT SL1344. invA mutant SL1344 was distinguishable from WT SL1344 by its lack of induction of LAMP-1 expression.
after 2 h of incubation and stained them for host protein LAMP-1 and Salmonella CSA-1 (Fig. 5B). In iHOs infected with the S. Typhimurium invA mutant, we detected no salmonellae inside cells and an almost undetectable positive signal for LAMP-1, suggesting that the invA mutant was not being internalized and the host cell autophagy pathways were not being significantly triggered. In contrast, wild-type SL344 could be found localized within vacuoles inside epithelial cells, frequently colocalized with LAMP-1, in a similar manner to that observed in other in vivo and in vitro systems (33).

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