Enteroviruses infect human enteroids and induce antiviral signaling in a cell lineage-specific manner

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Edited by Peter Palese, Icahn School of Medicine at Mount Sinai, New York, New York, and approved December 23, 2016 (received for review October 19, 2016)

Enteroviruses are among the most common viral infectious agents of humans and are primarily transmitted by the fecal–oral route. However, the events associated with enterovirus infections of the human gastrointestinal tract remain largely unknown. Here, we used stem cell-derived enteroids from human small intestines to study enterovirus infections of the intestinal epithelium. We found that enteroids were susceptible to infection by diverse enteroviruses, including echovirus 11 (E11), coxsackievirus B (CVB), and enterovirus 71 (EV71), and that contrary to an immortalized intestinal cell line, enteroids induced antiviral and inflammatory signaling pathways in response to infection in a virus-specific manner. Furthermore, using the Notch inhibitor dibenzazepine (DBZ) to drive cellular differentiation into secretory cell lineages, we show that although goblet cells resist E11 infection, enteroendocrine cells are permissive, suggesting that enteroviruses infect specific cell populations in the human intestine. Taken together, our studies provide insights into enterovirus infections of the human intestine, which could lead to the identification of novel therapeutic targets and/or strategies to prevent or treat infections by these highly clinically relevant viruses.

enterovirus | enteroid | goblet cells | innate immune | enteroendocrine cells

Enteroviruses are significant sources of human infections worldwide and are primarily transmitted by the fecal–oral route. Nonpoliovirus enteroviruses include coxsackievirus, echovirus, enterovirus 71 (EV71), and enterovirus D68 (EV-D68) and are small (~30 nm) single-stranded RNA viruses belonging to the *Picornaviridae* family. In many cases, enterovirus infections remain asymptomatic, whereas in others, infection is associated with mild flu-like symptoms or much more severe outcomes such as type I diabetes, encephalomyelitis, encephalitis, myocarditis, dilated cardiomyopathy, pleurodynia, acute flaccid paralysis, or even death.

The human gastrointestinal (GI) tract is a complex organ, with an epithelial surface that must provide a protective and immunological barrier in a complex and diverse microbial environment. The epithelium of the small intestine contains at least seven distinct cell subtypes that are responsible for the physiological functions of the intestine, which include nutrient absorption and defense against pathogens. The lack of models that recapitulate the complexity of the GI tract has hindered studies into many aspects of enterovirus infection in this specialized environment. Although murine models have been developed for the study of enterovirus-induced disease (1–4), many of these models require intraperitoneal (i.p.) infection, thereby bypassing the GI tract, or require ablation of the host innate immune system (5, 6). Coupled with species differences between humans and mice, there remains a need to develop human-based platforms to model enterovirus infections of the GI tract.

The full repertoire of mature cells in the small intestine in vivo includes those of absorptive (enterocytes) and secretory (Paneth, goblet, and enteroendocrine) lineages, which are derived from Lgr5⁺ stem cells located at the base of intestinal crypts. Despite

serving as the primary portal for enterovirus entry into the human host, it remains unknown whether enteroviruses target select cell types within the intestine for their initial replication. An ex vivo model of the human intestinal epithelium has been developed, whereby primary intestinal crypts are isolated and cultured into epithelial structures that have been described as “mini-guts,” often termed enteroids (7–9). Primary intestinal crypts are plated onto Matrigel, mimicking the enriched levels of laminin α 1 and α 2 present at crypt bases in vivo (10), and are cultured in the presence of growth factors that induce crucial developmental signaling through the Wnt and Notch pathways. Lgr5⁺ intestinal crypt stem cells differentiate into the various epithelial cell subtypes found in the human small intestine in vivo, resulting in the production of enteroid structures over 4 to 5 days (11). Others have shown that human enteroids can serve as models for the study of enteric infections by human rotavirus (12, 13) and norovirus (14).

In this study, we cultured Lgr5⁺ stem cell-derived enteroids from human fetal small intestines and applied this model to the study of enterovirus infections. We found that human enteroids were susceptible to infection by coxsackievirus B (CVB), echovirus 11 (E11), and EV71 to varying degrees and induced potent antiviral signaling pathways in response to infection in a virus-specific manner. Using the Notch inhibitor dibenzazepine (DBZ) to enrich enteroids with cells of a secretory lineage, we also show that E11 is unable to replicate in MUC2-positive goblet cells. Collectively, these data provide insights into the intestinal cell populations

Significance

Enteroviruses are a leading source of human infections worldwide and are primarily transmitted by the fecal–oral route. However, very little is known regarding the events associated with enterovirus infection of the human gastrointestinal tract. Here, we used a primary stem cell-derived enteroid model to interrogate the susceptibility of human enteroids to a panel of enteroviruses and to determine the antiviral signaling pathways induced in response to infection. Our study provides important insights into the mechanisms associated with enteroviral infections of the human gastrointestinal tract.

Author contributions: C.G.D., M.G., and C.B.C. designed research; C.G.D., A.M.B., C.M., C.J.L., M.G., and C.B.C. performed research; A.M.B., C.M., M.G., and C.B.C. contributed new reagents/analytic tools; C.G.D., M.G., and C.B.C. analyzed data; and C.G.D., M.G., and C.B.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the Sequence Read Archive database (accession no. SRP091501).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1617363114/-DCSupplemental.

Fig. 1. (A) Illustration depicting the strategy for enteroid culturing. Crypts are isolated from whole intestine epithelia and grown in media containing Wnt3a, Noggin, EGF, and R-spondin for 5 days to induce proliferation and differentiation. (Right) Brightfield image of enteroids after 5 days in culture. (Scale bar, 50 μ m.) (B and C) Human epithelial-derived enteroids were immunostained for the goblet cell marker MUC2 (green) and actin (red) (B) or the enterocyte markers E-cadherin (red, Left) or the enteroendocrine marker CHGA (green, Right) and the Paneth cell marker Lysozyme C (red, Right) (C). (Scale bars, 50 μ m.) (D) Hierarchical clustering heat map of differential gene expression profiles [based on log (RPKM) values] between two independent preparations of Caco-2 cells and three independent human enteroid cultures by RNA-seq. (E) Heat map [based on log (RPKM) values] comparing gene expression levels between Caco-2 and enteroid cultures for markers of differentiated small intestinal epithelial cell types: enterocytes (CDX1, SI), goblet cells (MUC2), Paneth cells (Reg3a, DefA5, DefA6), and M-cells (SPIB, GP2). (F) RT-qPCR comparison of expression levels for intestinal genes in Caco-2 cells and human enteroid cultures. Data in F are shown as mean $\pm$ SD and are normalized to Caco-2 cells. *** P < 0.001; ** P < 0.01.

Human Enteroids Are Susceptible to Enterovirus Infection. We next assessed whether human enteroids were susceptible to enterovirus infection and compared their levels of infection to Caco-2 cells, which are permissive to infection (18, 23, 26). Using immunofluorescence microscopy for double-stranded viral RNA (vRNA), which is formed as a replication intermediate, we found that CVB, E11, and EV71 all replicated in human enteroids by 24 hours p.i., although the level of EV71 infection was consistently lower than that of either CVB or E11 (Fig. 2A). Infected cells were positive for the epithelial intermediate filament cytokeratin-19 (Fig. S1A). In addition, we performed RT-qPCR from infected enteroids and compared their levels of vRNA to those in infected Caco-2 cells. Consistent with our immunofluorescence data, we found that enteroids were robustly infected with CVB and E11, with slightly lower efficiency than in Caco-2 cells, but that EV71 replicated to significantly lower levels in enteroids (Fig. 2B). In addition, we found that E11 infection elicited pronounced cytotoxicity, which was associated with loss of crypt morphology and integrity within 24 hours p.i. (Fig. 2C, Left), and the mislocalization of the tight junction protein occludin, which we have shown previously is disrupted by enterovirus infection (24) (Fig. 2C, Right). Consistent with the induction of cell death, we also observed increased levels of released

HMGB1, which is associated with cell necrosis, in medium collected from E11, but not CVB or EV71, infected enteroids (Fig. S1B). To determine the kinetics of E11 replication, we used immunofluorescence and RT-qPCR for vRNA and found that viral dsRNA was generated within 8 hours p.i., with levels increasing until 24 hours p.i. (Fig. 2D), which was confirmed by RT-qPCR for total vRNA (Fig. 2E). Finally, we verified that enteroids produced infectious virus by measuring infectious viral titers and found that in six independent enteroid preparations, E11 titers were similar to those produced from infected Caco-2 cells (Fig. 2F). These data show that human enteroids are permissive to enterovirus infection and are capable of supporting replication and progeny release.

Echovirus 11, but Not CVB, Infection of Human Enteroids Induces Antiviral Signaling. In cell lines, including Caco-2 cells, enteroviruses robustly attenuate the host innate immune system, and infection is not

Fig. 2. (A) Enteroids infected with CVB, E11, or EV71 for 24 hours, or mock-infected controls, were immunostained for vRNA (in green) using an antibody against dsRNA. DAPI-stained nuclei are shown in blue. (Scale bar, 50 μ m.) (B) RT-qPCR for CVB, E11, or EV71 vRNA from Caco-2 cells (gray) or three independent enteroid preparations (blue). Data are shown as Δ Ct relative to actin. (C, Left) Images depicting the cytopathic effect in an E11-infected enteroid compared with a mock-infected control. Images are merged composites of differential interference contrast (DIC), DAPI-stained nuclei (in blue), and vRNA (in red). (C, Middle) Confocal micrographs of mock- or E11-infected enteroids immunostained for vRNA (green) and occludin (red) 24 hours following infection. Zoomed image of the white box shown in Middle at Right. (Scale bars, Left and Middle, 50 μ m; Right, 15 μ m.) (D) Immunofluorescent staining for vRNA (red, vRNA) and DAPI-stained nuclei over a time course of E11 infection in enteroids ranging from early (8 hours) to advanced (24 hours) stages of infection. (Scale bar, 50 μ m.) (E) E11 RNA levels as determined by RT-qPCR throughout a time course of infection (at the indicated hours p.i.) in an enteroid culture. (F) E11 titers (shown as plaque-forming units per milliliter) in supernatants of Caco-2 or enteroid cultures 24 hours p.i. Data in B, E, and F are shown as mean $\pm$ SD and are normalized to vRNA levels at 4 hours p.i. (E) * P < 0.05; ** P < 0.01; *** P < 0.001.

usually accompanied by the induction of significant antiviral signaling (27). To assess whether human enteroids respond to enterovirus infection by inducing antiviral signaling, we performed RNA-seq analyses from enteroids infected with CVB, E11, or EV71. Consistent with our immunofluorescence and RT-qPCR studies, we found that both E11 and CVB robustly infected enteroids, whereas the levels of EV71 infection were significantly lower, as assessed by FPKM (fragments per kilobase of transcript per million mapped reads) values (Fig. 3A). For consistency, independent enteroid preparations (six total) were assigned numerical identifiers to compare infections and transcriptional changes in matched preparations. Surprisingly, we found that whereas E11 infection induced the differential expression of 350 transcripts, CVB infection only induced changes in 13 transcripts, with only one shared transcript between viruses (Fig. 3B and Fig. S2A and B). Interestingly, we found that enteroids potently induced cytokines, chemokines, and IFN-stimulated genes (ISGs) in response to E11 infection; however, these same pathways were not induced by CVB or EV71 infection (Fig. 3C). Gene Set Enrichment Analysis (GSEA) (20) revealed the enrichment of transcripts associated with NF- κ B signaling [false discovery rate (FDR), 0] and IFN signaling (FDR, 0.02) in E11-infected enteroids (Fig. S3A). The induction of antiviral and inflammatory signaling in E11-infected enteroids was confirmed by RT-qPCR in additional enteroid preparations, which all exhibited pronounced induction of

these pathways in response to infection (Fig. 3D and Fig. S3B). In addition, we confirmed that these pathways were induced specifically in E11-infected enteroids and not in Caco-2 cells, or in response to CVB, using RT-qPCR for HERC5 (an IFN-inducible E3 ligase) (28) and CXCL11 (Fig. 3F). We also found that enteroids treated with the synthetic dsRNA ligand polyinosinic-polycytidylic acid [poly(I:C)] or infected with E11 induced the expression of an inflammatory mediator, whereas infection with CVB did not, suggesting that stimulation of toll-like receptor (TLR3) might be involved in this induction (Fig. S3C). Finally, using an ELISA for CXCL10, we confirmed that the observed transcriptional changes correlated with the production of high levels of protein only in E11-infected enteroids and not in CVB- or EV71-infected enteroids, or in Caco-2 cells (Fig. 3F). Collectively, these data show that human enteroids respond to E11, but not CVB, infection by inducing antiviral signaling pathways, suggesting that these signals are induced in a virus-specific manner.

Goblet Cells Are Not Infected by Echovirus 11. Next, we took advantage of the ability of primary human enteroids to develop into multiple cell types, such as those of absorptive (enterocytes) and secretory (goblet, enteroendocrine, and Paneth) lineages to determine whether enteroviruses target specific cell types in the human intestine. By immunofluorescence microscopy, we observed what appeared to be the lack of infection of MUC2⁺ goblet cells in enteroids infected with E11 (Fig. S4A). Goblet cells are characterized by high levels of cytoplasmic MUC2, which is localized at the basal region of the cell body (Fig. S4B). However, the low abundance of total MUC2⁺ cells in enteroids (~10% of total cells) (Fig. 4C), coupled with the lack of infection in 100% of cells, complicated an assessment of whether MUC2⁺ cells resisted E11 infection. Thus, we sought to alter the ratio of cells of absorptive and secretory lineages using the Notch inhibitor DBZ to increase secretory cell differentiation (29). To do this, enteroids were grown in the presence of DBZ (or DMSO control), beginning 48 hours following the initiation of culturing. We found that DBZ treatment induced a dramatic increase in the numbers of MUC2⁺ cells, with an increase from ~10% to ~50% (Fig. 4A and C). In addition, we also observed a significant enhancement in the numbers of CHGA-positive enteroendocrine cells following DBZ treatment (Fig. 4B and C). Consistent with the enhancement in the total numbers of secretory cells, DBZ-treated enteroids exhibited significant increases in the expression of goblet (MUC2) and Paneth (Reg3A and DefA6) markers and modest decreases in an enterocyte marker (CDX1) compared with DMSO-treated controls as assessed by RT-qPCR (Fig. S4C). As expected, this effect was specific for human enteroids, as DBZ had no effect on the expression of secretory cell markers in Caco-2 cells (Fig. S4D). To further profile the changes induced by DBZ treatment, we compared the transcriptional profiles of DMSO- and DBZ-treated enteroids by RNA-seq. These studies revealed the up-regulation of transcripts associated with enteroendocrine (Neurog3, INSM1, Pax4) and cells of secretory lineage (MUC2, GFI1, FOXA2, Reg3A, DefA5-6, and SPINK4) with a corresponding down-regulation of transcripts associated with M-cells (SPIB), cells of absorptive lineage (BEST4), stem cell markers (OLFM4 and LGR5), and Notch signaling-associated factors (HES1) (Fig. 4D).

We next performed immunofluorescence microscopy for vRNA and either CHGA or MUC2 in E11-infected enteroids that had been treated with DMSO or DBZ. We observed an association between CHGA-positive cells and vRNA (Fig. 4F and H) but found that vRNA was rarely associated with MUC2-positive cells (Fig. 4G and H), suggesting that E11 is unable to replicate in goblet cells. We next determined whether the reduced infection of MUC2⁺ cells resulted from their unique induction of antiviral signaling by performing RNA-seq in DMSO- or DBZ-treated enteroids infected with E11. FPKM values indicated that DBZ treatment had little impact on the total levels of vRNA (Fig. 4I). Differential expression

Fig. 3. (A) Table of FPKM values for CVB, E11, or EV71 in three (CVB, EV71) or five (E11) independent enteroid preparations as determined by RNA-seq. In A and C, six independent enteroid preparations are assigned numerical identifiers (1–6) to facilitate direct comparison of transcript changes between matched preparations. (B) Venn diagram of transcripts induced by E11 or CVB infection, with only one transcript shared between viruses. (C) Heat map [based on log(RPKM) values] of highly up-regulated antiviral and proinflammatory transcripts in E11-, CVB-, or EV71-infected enteroids compared with mock-infected enteroids. (D) RT-qPCR for the indicated genes in three additional enteroid preparations (labeled 1–3) infected with E11. (E) HERC5 and CXCL11 mRNA levels as determined by RT-qPCR in Caco-2 cells and two independent enteroid preparations infected with CVB or E11 (left y axis). vRNA levels are also shown for each sample, relative to CVB levels in matched infections (right y axis). (F) ELISA for CXCL10 (shown as picograms per milliliter) from four independent enteroid preparations infected with CVB, E11, or EV71 for 24 hours compared with mock-infected controls. Data in D–F are shown as mean $\pm$ SD and are normalized to mock-infected controls (D and E left y axis). ** $P < 0.01$; *** $P < 0.001$. In C, gray denotes transcripts with zero mapped reads.

which to model enterovirus–GI tract interactions that may recapitulate events associated with virus infections in vivo.

Although our data indicated that CVB, E11, and EV71 replicated in enteroids, there were differences in the relative permissiveness of enteroids to enterovirus infections, with EV71 infecting enteroids with low efficiency by comparison with CVB and E11. It is not clear why EV71 failed to infect enteroids to significant levels even when infected with the same inoculum as CVB and E11, whereas Caco-2 cells were readily infected. Based upon transcriptional profiling, the levels of known enterovirus receptors were similar between enteroids and Caco-2 cells (Fig. S5A). It is therefore possible that in enteroids, there are differences in the accessibility of viral receptors, which may result from differences in receptor localization between Caco-2 cells and fully differentiated enteroids, which have a more complex 3D structure that alters viral access to the apical surface. Although it is possible that innate immune detection and signaling in enteroids may be more effective at controlling EV71 infection, we did not detect the induction of any significant antiviral signaling pathways in EV71 enteroids by RNA-seq, suggesting that the restriction of EV71 infection occurs earlier in the viral life cycle.

We detected the robust induction of transcripts associated with antiviral signaling in response to E11 infection of enteroids, including a number of ISGs, cytokines, and chemokines. Interestingly, this induction was specific for E11, as CVB infection did not elicit these same pathways. In cell culture models, enteroviruses use virally encoded proteases to cleave host signaling molecules to diminish antiviral signaling (reviewed in ref. 27). It is unknown whether E11 or other echoviruses are less efficient at suppressing these pathways or whether mechanisms of detection differ between enteroviruses. However, our data suggest that there may be important differences between how the human intestine responds to enteroviral infections in a virus-specific manner, which could impact a variety of aspects of viral pathogenesis. However, despite the robust induction of antiviral signals, E11 replicated efficiently in enteroids, with similar efficiency as in Caco-2 cells, which do not induce similar antiviral pathways, suggesting that the virus has mechanisms to evade this response.

Of particular importance, we found that E11 was unable to replicate in goblet cells but that enteroendocrine cells supported

replication. Little is known about the susceptibility of various intestinal epithelial subtypes to enterovirus infections. Previous studies using M-like cells derived by the coculturing of Caco-2 cells with lymphocytes showed that poliovirus readily adhered to the apical surfaces of these cells and was rapidly transcytosed to the basolateral space (within 1–2 hours p.i.) (30). These findings led to speculation that enteroviruses might bypass the intestinal epithelium entirely and would instead transmigrate across the intestinal barrier through noninfectious transcytosis across M cells. However, our studies indicate that enteroviruses (including poliovirus) (Fig. S5B) robustly infect enteroids and may specifically target cells of specific lineages or subtypes to cross the intestinal barrier. It is unclear why MUC2-expressing goblet cells are less permissive to E11 infection, although our RNA-seq findings from DBZ-treated enteroids do not indicate that unique antiviral innate pathways are induced in MUC2-enriched enteroids. Thus, it seems likely that other properties of these cells, such as their ability to secrete mucins or the presence of mucin-enriched cytoplasmic secretory granules, may limit enterovirus replication.

Taken together, we show that human enteroids can be used to model the multicellular environment of the GI epithelium, which serves as a key cellular portal by which enteroviruses enter their human hosts. Our findings provide important insights into events associated with the earliest stages of enterovirus infection and demonstrate that human enteroids can be used as platforms to define the complex dialogue that exists between enteroviruses and the intestinal epithelium, which undoubtedly have profound impacts on enterovirus pathogenesis.

ACKNOWLEDGMENTS. We thank William Horne (Children's Hospital of Pittsburgh) for assistance with RNA-seq, Abraham Brass (University of Massachusetts) for providing anti-dsRNA antibody, Jian Yu (University of Pittsburgh Cancer Institute) for helpful suggestions related to DBZ experiments, and Jeffrey Bergelson (Children's Hospital of Philadelphia) for careful review of the manuscript. This project was supported by NIH Grants T32 AI-060525 (to C.G.D.) and R01-AI081759 (to C.B.C.), a Burroughs Wellcome Investigators in the Pathogenesis of Infectious Disease Award (to C.B.C.), NIH Grant K08DK101608 (to M.G.), and the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center (UPMC) Health System (M.G.). The authors would also like to acknowledge the UPMC Tissue and Research Pathology Services/Health Sciences Tissue Bank, which receives funding from NIH Grant P30CA047904.

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