Type III interferon signaling restricts enterovirus 71 infection of goblet cells

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Recent worldwide outbreaks of enterovirus 71 (EV71) have caused major epidemics of hand, foot, and mouth disease with severe neurological complications, including acute flaccid paralysis. EV71 is transmitted by the enteral route, but little is known about the mechanisms it uses to cross the human gastrointestinal tract. Using primary human intestinal epithelial monolayers, we show that EV71 infects the epithelium from the apical surface, where it preferentially infects goblet cells. We found that EV71 infection did not alter epithelial barrier function but did reduce the expression of goblet cell–derived mucins, suggesting that it alters goblet cell function. We also show that the intestinal epithelium responds to EV71 infection through the selective induction of type III interferons (IFNs), which restrict EV71 replication. Collectively, these findings define the early events associated with EV71 infections of the human intestinal epithelium and show that host IFN signaling controls replication in an IFN-specific manner.

INTRODUCTION

Enteroviruses are small (~30 nm) single-stranded RNA viruses that cause a broad spectrum of illnesses in humans. Disease manifestations of enterovirus infections can range from acute, self-limited febrile illness to meningitis, endocarditis, acute paralysis, and even death. Enterovirus 71 (EV71) has been associated with major epidemics of hand, foot, and mouth disease (HFMD) worldwide and severe neurological complications, including meningitis, encephalitis, and acute flaccid paralysis (1). First identified in 1969 (2), EV71 outbreaks have occurred throughout the globe, with epidemics most commonly occurring in the Asia-Pacific region. Between 2008 and 2012, outbreaks of EV71 in China have been associated with over 7,000,000 cases of HFMD and almost 2500 deaths (3). The pediatric population is at greatest risk of developing EV71–associated complications, with the vast majority of fatalities occurring in children below the age of two (3–6). There are currently no approved therapeutics to treat or prevent EV71 infections.

EV71 is transmitted by the fecal–oral route, where it targets the human gastrointestinal (GI) epithelium for host invasion. The mechanisms used by EV71 to cross the GI epithelial barrier remain largely unknown, owing, in part, to the lack of in vivo models to study EV71 infections by the enteral route. For example, modeling EV71 infections in mouse models is complex given the need to rely on the use of mouse-adapted viral strains, animals lacking functional interferon (IFN) signaling, and/or mice overexpressing the human homolog of the primary EV71 receptor SCARB2 (7–11). Previous work in non–human primate models parallels the central nervous system complication associated with EV71 infections in humans, including when infected by the enteral route (8, 12, 13). However, despite the development of these models, which provide platforms to determine the efficacy of EV71 vaccines and therapeutics in animals, the specific mechanisms by which EV71 crosses the human GI epithelial barrier have yet to be defined.

The human GI epithelium is a complex cellular barrier composed of multiple cell types. These diverse cell types are derived from leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5+) stem cells located within the base of intestinal crypts, which differentiate into absorptive and secretory lineages (14). Major advances in the development of ex vivo “mini-gut” enteroïd models, in which primary human intestinal crypts are isolated and cultured into epithelial structures that differentiate to contain the multiple cell types present in the human intestine, have expanded our understanding of enteric virus–GI interactions [reviewed in (15)]. In previous work, we used enteroïds isolated from human fetal small intestines to profile the susceptibility of the human intestine to enterovirus infections, using echovirus 11 (E11), coxsackievirus B (CVB), and EV71 as models (16). We showed that E11 exhibits a cell type specificity of infection and infects both enterocytes and enteroendocrine cells but is unable to infect goblet cells (16). However, during these studies, we noted that, in contrast to both E11 and CVB, EV71 replicated to low levels in human enteroïds, although the mechanistic basis for this remained unclear (16).

Although the crypt–based model used in our previous work has many advantages over standard cell line–based models, the culturing of crypts in Matrigel induces the formation of three-dimensional structures wherein the luminal (apical) domain faces inward and the basolateral domain faces the culture medium. This affects the polarity by which viruses infect enteroids, restricts the ability to determine whether there is a polarity of viral entry and/or release, and precludes an assessment of alterations that may be induced to the epithelium by infection, such as loss of barrier function. Here, we developed a monolayer model using isolated human fetal crypts cultured on permeable porous membrane inserts, which leads to the formation of a single-cell monolayer containing all the distinct cell types present in the GI epithelium. Using this model, we found that EV71 exhibits a distinct apical polarity for its infection of the epithelium. We found that, unlike E11, which preferentially targets enterocytes and abolishes epithelial structure and barrier function, EV71 preferentially infects goblet cells, and infection reduces the expression of goblet cell–derived mucins. Last, we show that EV71 infection specifically induces the type III IFNs IFN-λ2/3 and that type I and III IFNs restrict enterovirus replication in a virus–specific manner, with type I IFN exerting the greatest restriction of E11 and type III IFNs preferentially restricting EV71. Moreover, we show that
type III IFN signaling restricts EV71 infection, as treatment of monolayers with the Janus kinase 1/2 (JAK1/2) inhibitor ruxolitinib inhibits EV71-induced IFN-stimulated gene (ISG) induction and increases EV71 infection. Our findings thus define the events associated with EV71 infections in the GI tract, which could lead to the identification of novel therapeutic targets and/or strategies to prevent or treat the pathogenesis and morbidity associated with infections by this virus.

RESULTS

Crypt-based monolayer model

Previously, we grew enteroids generated from intestinal crypts isolated from human fetal small intestines cultured in Matrigel and infected these with E11, CVB, and EV71 (16). Here, we found that EV71 replicated poorly in comparison to other enteroviruses. However, the basis for this low level of infection was unclear. Given that enteroids cultured in Matrigel develop an apical surface facing into the lumen (Fig. 1A and fig. S1A), which is not accessible from the culture medium, we theorized that the low levels of EV71 replication in this model might result from the need to infect from the basolateral surface. We therefore determined whether direct culturing of isolated crypts on porous membrane Transwell inserts would provide a model to assess EV71 infection of the apical and basolateral surfaces in an intact monolayer setting. To do this, we isolated intestinal crypts from human fetal small intestines and plated them directly onto T-clear Transwell inserts in the presence of factors required to promote stem cell differentiation [R-spondin, Noggin, epidermal growth factor (EGF), Wnt3A, and the Rho kinase inhibitor Y-27632] (fig. S1B). Similar models have been used from crypts isolated from the adult GI tract, which often requires growth as enteroids in Matrigel before disruption and subsequent Transwell plating [reviewed in (17)]. We found that fetal small intestine–derived crypts plated directly on Transwell inserts developed into complete monolayers within 2 to 3 days after plating and exhibited distinct apical and basolateral domains that contained distinct intestinal cell types such as mucin-2 (MUC2)–positive goblet cells and chromogranin A (CHGA)–positive enteroendocrine cells at the same ratio as crypts cultured in Matrigel (Fig. 1A and fig. S1C). Using RNA sequencing (RNA-seq) and reverse transcription quantitative polymerase chain reaction (RT-qPCR), we found that crypts plated directly in Transwell inserts exhibited similar transcriptional profiles, with no significant differences in these profiles as assessed by DESeq2 analysis in R (18) (Fig. 1B), and expressed markers of enterocytes [sucrase-isomaltase, alkaline phosphatase (ALPL), goblet cells (MUC2, MUC5AC, MUC13, and MUC17), enteroendocrine cells (CHGA), and Paneth cells (regenerating islet-derived protein 3)], near equivalent to crypts plated directly in Transwell inserts and exhibited intact barrier function as assessed by high (more than approximately 1000 ohm) transepithelial resistance (TER) values (Fig. 1E).

EV71 preferentially infects HIE from the apical surface

It is unknown whether enteroviruses exhibit a preferential polarity of binding or infection in primary HIE. To address this, we performed binding and infection assays from either the apical or basolateral surfaces in primary HIE. These studies revealed significant differences in the capacity of E11 and EV71 to bind and infect in a polarized manner. Whereas E11 exhibited an enhanced capacity to infect from the basolateral surface as assessed by the production of viral RNA (vRNA) by RT-qPCR at 24 hours postinfection (p.i.), EV71 exhibited a much stronger preference for apical infection (Fig. 2A). Consistent with this, we found that EV71 preferentially binds to the apical surface of HIE as assessed by a qPCR-based binding assay (Fig. 2B). To determine whether E11 and EV71 also exhibit a polarity of release, we infected HIE with EV71 or E11 from the apical or basolateral surfaces, respectively, and titrated released progeny viral particles from medium isolated from the apical or basolateral compartments. These studies revealed that E11 was released from both the apical and basolateral compartments, although its release was skewed toward the basolateral compartment (Fig. 2C). In contrast, EV71 was solely released from the apical compartment, and no viral particles were detectable in the basolateral compartment (Fig. 2C).

We next performed growth curves from HIEs infected with EV71 from either the apical or basolateral surfaces. For these studies, we used neutral red (NR) containing EV71 particles to distinguish between EV71 particles that remained attached to the cell surface from those that were actively replicating. This technique involves labeling of vRNA with NR, a compound that cross-links the vRNA if exposed to light (19, 20), thus generating viral particles that are rendered noninfectious when exposed to light. To perform growth curves, we pre-adsorbed NR-EV71 to cells from the apical or basolateral surfaces under semi-dark conditions, exposed to light immediately after binding (0 hours) or following viral entry and genome release (6 hours p.i.), and then infected for an additional 24 to 96 hours. NR-EV71 particles that remained at the cell surface would thus be rendered noninfectious at the 6-hour light exposure. Using HIEs prepared from three independent human tissues and infected as described, we found that EV71 vRNA production peaked by ~24 hours p.i. and then was rapidly reduced by 48 to 72 hours p.i., with levels diminishing significantly by 96 hours p.i. (Fig. 2D). This trend was specific for apical infection, as only a single preparation exhibited any detectable vRNA when infection was initiated from the basolateral surface (Fig. 2D). In parallel, we collected cell supernatants from the apical or basolateral compartments and measured infectious particle release over a 24- to 96-hour period. Consistent with our vRNA data, we found that the levels of infectious EV71 release were highest at 24 hours p.i., with levels diminishing between 48 and 96 hours p.i. (Fig. 2E). Of note, even when low levels of infectious EV71 particles were released following infection of the basolateral surface, this release was only detectable in the apical compartment (Fig. 2E). Together, these data show that EV71 exhibits a strong preference to infect HIEs from the apical surface and that infectious particles also exhibit an apical polarity of release.

EV71 infection of HIE does not alter epithelial barrier function

We showed previously that E11 infection of human enteroids grown in Matrigel induced significant damage to the epithelium, including the reorganization of tight junctions (16). Consistent with this, we found that infection of HIE with E11 from the basolateral surface induced a significant loss of epithelial barrier function, as indicated by the loss of TER values from ~2000 to ~200 ohm (Fig. 3A). In contrast,
Fig. 1. Establishment of human fetal small intestine–derived monolayer model. (A) Confocal micrographs of isolated crypts grown in Matrigel (left) or on Transwell T-clear inserts (right) for 6 days. Immunofluorescence images from monolayers immunostained for cytokeratin-19 (Cyt-19) (an epithelial marker) (green, top) and actin (red, top) or chromogranin A (CHGA; an enteroendocrine marker) (green, bottom) and mucin-2 (MUC2; a goblet marker) (red, bottom) are shown. In all, 4′,6-diamidino-2-phenylindole (DAPI)–stained nuclei are shown in blue. At the top and right of the upper panel are XYZ or XZY images obtained by serial sectioning. (B) Volcano plot of RNA-seq–based differential expression analysis of three independent preparations of isolated crypts plated directly on Matrigel or onto Transwell inserts 6 days after plating. No transcripts were detected as being significantly differentially expressed between culture conditions (gray circles, P > 0.05) as assessed by DESeq2 analysis. (C) RT-qPCR for the indicated markers [alkaline phosphatase (ALPL), sucrase-isomaltase (SI), CHGA, MUC2, regenerating islet-derived protein 3 (REG3A), and leucine-rich repeat-containing G protein–coupled receptor 5 (LGR5)] in three matched independent human enteroid cultures (shown as independent symbols) plated in Matrigel or T-clear Transwell inserts. Data are shown as means ± SD as a fold change from Matrigel-plated enteroids. Significance was determined using a standard t test, ***P < 0.01; ns, not significant. (D) Confocal micrographs of isolated crypts grown on Transwell T-clear inserts for 6 days. Immunofluorescence images from HIE immunostained for E-cadherin (E-cad) (an adherens junction marker in enterocytes; green), ZO-1 (a tight junction marker in enterocytes; red), and actin (magenta) are shown. DAPI-stained nuclei are shown in blue. At the top and right of the upper panel are XYZ or XZY images obtained by serial sectioning. (E) Transepithelial resistance (TER; in ohm) values from five independent HIE cultures (ENT-1 to ENT-5 in gray; two to three Transwells were averaged per preparation). Average TER values from all preparations are shown in red.
EV71 infection (from either the apical or basolateral surfaces) had no effect on TER values (Fig. 3A), even when infection was allowed to proceed for up to 4 days (Fig. 3B). Likewise, we found that E11 and EV71 also exhibited differences in their impact on epithelial morphology, with E11 infection inducing loss of actin cytoskeletal integrity, which was not present in EV71-infected HIE (Fig. 3C). These data highlight differences between enteroviruses on their impact on intestinal epithelial structure and function and show that EV71 infection does not alter epithelial barrier function.

**EV71 infects goblet cells**

Because we observed differences in the impact of E11 and EV71 infections on epithelial barrier function, we next determined whether these viruses exhibited differences in the specific cell types infected in HIE. We showed previously that E11 preferentially infects enterocytes and can also infect enteroendocrine cells but is unable to infect goblet cells (16). To determine whether EV71 also exhibits a cell type specificity, we first performed immunofluorescence microscopy for double-stranded vRNA (a replication intermediate) and the virally encoded capsid protein VP1 in HIE infected with EV71 from the apical surface for 24 hours (a time when we observed peak levels of replication). These studies revealed colocalization of EV71 vRNA and VP1 to punctate structures in select cells throughout the monolayer (Fig. 4A). The cells that were positive for EV71 vRNA and VP1 exhibited characteristics of goblet cells, such as a highly polarized nuclear localization and large cytoplasmic space (Fig. 4A, enlarged panel at right). Follow-up studies confirmed that EV71 vRNA was exclusively localized to MUC2-positive goblet cells (Fig. 4, B and C). As an additional confirmation for the goblet cell specificity of EV71 infection, we also performed immunofluorescence microscopy for VP1 using an immunostaining technique that distinguishes between VP1 localized on the extracellular surface and VP1 localized intracellularly (21). These studies confirmed the presence of intracellular VP1 only in cells exhibiting goblet cell morphology (Fig. 4D). Of note, the primary receptor for EV71, SCARB2 (11), was expressed in goblet cells, where it localized to intracellular vesicles (fig. S2A). In addition, we found that EV71 infection of HIE was inhibited by treatment of monolayers with dynasore, an inhibitor of dynamin, consistent with the role of clathrin- and SCARB2-dependent entry of EV71 into cultured cells (fig. S2B) (22). In further support of EV71 infection of goblet cells, we also found that EV71 infection of HIE led to significant decreases in the expression of the goblet cell–expressed mucins MUC1 and MUC2 but had no impact on the expression of the enterocyte-enriched transcription factor CDX2, as assessed by RT-qPCR, suggesting that infection might alter aspects of goblet cell function (Fig. 4E). Collectively, these studies show that EV71 specifically infects via the apical surface of HIE and exhibits preferential infection of goblet cells.
Type III IFNs are induced in response to EV71 infection of HIE

Our EV71 growth curve studies revealed that the peak of EV71 replication was at 24 hours p.i., with levels of infection declining after this time point (Fig. 2, D and E). These data suggest that the host innate immune response to EV71 might suppress viral replication at an early stage to control its replication. To determine whether this is the case, we performed RT-qPCR analyses for two ISGs that we previously showed were induced in HIE in response to E11 infection (16) in HIE infected with EV71. These studies showed that these ISGs, CXCL10 and IFI44L, were induced by EV71 infection of HIE at 24 hours p.i., with induction diminishing by 48 to 72 hours p.i. (Fig. 5A). Similarly, we found that EV71 and E11 infection highly induced the expression of the ISG IFIT1 as assessed by confocal microscopy, with elevated levels of IFIT1 observed in infected cells (Fig. 5B).

We next determined whether type I and/or type III IFNs were responsible for this induction of ISGs by performing Luminex singleplex assays for IFN-β, IFN-λ1, or IFN-λ2/3 (the high degree of sequence homology between these IFNs make them indistinguishable in this assay). We found that EV71 infection of HIE led to the specific induction of type III IFNs, specifically IFN-λ2/3, at both 24 and 48 hours p.i., with no detectable IFN-λ1 induced and very low levels of IFN-β induced at 24 hours p.i. (Fig. 5C). Of note, IFNs were present in medium collected from the apical chamber following infection, and we were unable to detect any IFNs from medium collected from the basolateral chamber. Likewise, E11 infection also induced the preferential secretion of type III IFNs, but unlike EV71, low levels of IFN-λ1 were also produced in response to infection (fig. S3A). These data suggest that type III IFNs, specifically IFN-λ2/3, are induced in response to EV71 infection of HIE.

EV71 is sensitized to the antiviral effects of type III IFNs

Because E11 and EV71 both induced type III IFNs in infected HIE, we next determined whether these viruses were sensitive to the antiviral effects of type I and III IFNs in a virus- or IFN-specific manner. HIE were pretreated with recombinant IFN-β or IFN-λ (IFN-λ3, which was induced by both E11 and EV71) for 24 hours and then infected with E11 or EV71 from the basolateral or apical surfaces, respectively. We found that, whereas E11 was more potently restricted by IFN-β treatment, as detected by RT-qPCR for vRNA and viral titration, EV71 was more potently restricted by IFN-λ treatment (Fig. 6, A and B). Next, we determined whether HIE exhibited differences in their ability to respond to exogenous type I and III IFNs and whether these IFNs induced ISGs with differing kinetics, as has been shown in adult enteroids at early time points of exposure (23), which could explain the differential control of E11 and EV71. To do this, we first performed RNA-seq transcriptional profiling from HIEs treated with recombinant IFN-β or IFN-λ for 24 hours. Differential expression analysis revealed that fetal-derived HIE potently respond to IFN-β and IFN-λ and induced the expression of canonical ISGs to similar levels (Fig. 6, C and D). Moreover, differential expression analysis between IFN-β- and IFN-λ–treated HIE showed that very few transcripts were differentially regulated by IFN-β, with none of these transcripts being known ISGs (fig. S3B). A kinetic profiling of the responsiveness of HIE to recombinant IFN-β and IFN-λ confirmed these findings and showed that there
were no significant differences in the kinetics by which fetal HIE respond to type I or III IFN treatment (fig. S4). Collectively, these data show that enteroviruses display differential susceptibility to the antiviral effects of IFNs, with EV71 exhibiting an enhanced sensitivity to type III IFNs.

**Type III IFNs control EV71 in HIE**

Because we found that EV71 infection of HIE preferentially induced type III IFNs and that treatment of HIE with recombinant IFN-λ restricted EV71 infection, we next determined whether IFNs controlled EV71 replication during the course of HIE infection. To do

Fig. 4. EV71 infects goblet cells. (A) Confocal micrographs from mock or EV71 apically infected HIE immunostained for vRNA (green) and VP1 (red) at 24 hours p.i. Zoomed images from areas in white boxes are shown at the right. (B) Confocal micrographs from mock or EV71 apically infected HIE immunostained for vRNA (green) and MUC2 (red) at 24 hours p.i. Zoomed images from areas in white boxes are shown at the bottom. (C) Quantification of the extent of colocalization between vRNA and MUC2-positive or MUC2-negative cells as assessed by image analysis. Data were generated from three independent HIE preparations from at least five independent fields. Significance was determined using an unpaired t test, ***P < 0.001. (D) Confocal micrographs from mock or EV71 apically infected HIE immunostained for intracellular (VP1<sup>in</sup>; green) or extracellular (VP1<sup>out</sup>; red) at 24 hours p.i. Zoomed images from areas in white dashed boxes are shown at the right. (E) MUC1, MUC2, and CDX2 expression as assessed by RT-qPCR at the indicated times after infection (from the apical surface). Monolayers were infected with NR-labeled EV71 exposed to light immediately after adsorption (0 hours) or at 6 hours p.i., and then, infection was allowed to proceed for the indicated time (in hours). Data are shown as a fold change from HIE exposed to light at 0 hours and are from three or four independent HIE preparations. Significance was determined using a one-way ANOVA with Dunnett's test for multiple comparisons (compared to 0-hour controls) in **P < 0.01. In (C) and (E), data are shown as means ± SD. In (E), independent preparations are shown as an individual symbol averaged from technical replicates.
this, we used a selective small-molecule inhibitor of JAK1/2 signaling (INCB018424/ruxolitinib). First, we confirmed that ruxolitinib was active in HIE and that it inhibited ISG induction downstream of IFN signaling. We found that treatment of HIE with 5 µM of ruxolitinib inhibited ISG induction in four independent preparations of HIE exposed to 500 ng of recombinant IFN-α (fig. S3C), confirming that this inhibitor potently suppressed IFN-induced ISG expression in HIE. Next, we determined whether ruxolitinib would inhibit EV71-induced ISG induction and whether this treatment would sensititize HIE to EV71 infection. We found that treatment of HIE with ruxolitinib significantly reduced ISG induction in EV71-infected HIE (Fig. 6E). We also found that ruxolitinib treatment enhanced EV71 replication in HIE, with a significant increase in EV71 titers in ruxolitinib-treated HIE (Fig. 6F). These data support a role for type III IFNs in the control of EV71 infection of HIE.
DISCUSSION

The events associated with EV71 infection of the human GI tract are largely unknown. Here, we show that EV71 preferentially infects HIE from the apical surface, where it preferentially replicates in MUC2-positive goblet cells. We also show that, unlike E11, an enterovirus that targets enterocytes, EV71 infection of HIE has no impact on epithelial barrier function or cytoskeletal morphology, but infection reduces the expression of goblet cell–associated mucins, suggesting

**Fig. 6.** EV71 is suppressed by recombinant type III IFN treatment of HIE. (A and B) E11 and EV71 infection from HIE pretreated with 500 ng of IFN-β or IFN-λ1 for 24 hours and then infected with E11 or EV71 for 24 hours. In (A), replication was assessed by vRNA production by RT-qPCR, and in (B), viral titration by plaque assay was performed. In (A) and (B), data are shown as means ± SD. (C and D) Volcano plots of HIE treated with 500 ng of IFN-β (C) or IFN-λ1 (D) denoting ISGs (red circles) or non-ISGs (yellow circle) differentially expressed by treatment \((P < 0.05)\). Gray circles are genes whose differential expression was not significant. Data are from three independent HIE preparations. A one-way ANOVA with a Dunnett’s test for multiple comparisons was used to determine significance. In (A) and (B) and (E) and (F), each independent preparation is shown as an individual symbol averaged from technical replicates.
that its replication may alter some aspect of goblet cell function. We further show that EV71 infection of HIE specifically induces the type III IFN IFN-λ2/3, which serve to limit EV71 replication. Collectively, these findings provide important insights into the mechanisms by which EV71 and other enteroviruses bypass the GI barrier and point to an important role for type III IFNs in the host response to enterovirus infections within the GI tract.

Our data indicate that enteroviruses exhibit a distinct cell type specificity by which they infect the human GI tract in a virus-specific manner. Whereas E11 specifically targets enterocytes and also infects enteroendocrine cells (16), EV71 preferentially infects goblet cells. Although it is possible that EV71 also replicates in other cell types present in HIE at levels that are below the limit of detection of our assays, our data point to an enrichment of EV71 replication in goblet cells. The mechanistic basis for the differential cell type specificity between E11 and EV71 remains unclear, although the cell type–specific expression and localization of viral receptors are likely to play a key role. Although the receptor for E11 is unknown, all EV71 isolates tested to date use SCARB2 as a primary receptor (11, 24). SCARB2, also known as lysosomal integral membrane protein II, is an integral membrane protein that specifically localizes to lysosomes and secretory granules (25). We found that SCARB2 was expressed in goblet cells, where it localized to intracellular vesicles. Goblet cells are characterized by the presence of large secretory vesicles that function to transport mucus to the apical surface of the epithelium. The targeting of goblet cells by EV71 for intestinal infection is therefore likely driven at least, in part, by the enrichment of SCARB2 to secretory vesicles within these cells, which might expose the receptor through apical mucus release.

It is also possible that EV71 uses other apically localized attachment factors for its initial binding to the epithelial surface, much like CVB relies on decay-accelerating factor to attach to the apical surface (26), before it reaches SCARB2. EV71 has been shown to interact with sialic acid–linked glycans, which might facilitate its initial attachment to the apical surface of the epithelium (27). However, this binding is unlikely to be a primary determinant for goblet cell infection. The cell type–specific nature of enterovirus infections also suggests that the host response to infection may differ depending on the specific cell types targeted by a given virus. In support of this, our data also point to important differences in the impact of E11 and EV71 infection of epithelial structure and barrier function, which could markedly affect viral pathogenesis in a virus-specific manner.

Our findings implicate type III IFNs as key contributors in the control of enterovirus infections in the GI tract. These findings are consistent with the work of others who have shown that human rotaviruses (28–31), reoviruses (32), and noroviruses (32–34) are also controlled by intestinal-derived type III IFNs. However, unlike other enteric viruses such as rotavirus, which controls the production of type III IFNs during infection through viral antagonism (28), our findings show that E11 and EV71 infection induce the secretion of type III IFNs at the protein level, suggesting that enteroviruses may lack this mechanism or be less proficient at suppressing this pathway. In cell lines, even those of intestinal lineages, EV71 and other enteroviruses potently antagonize the host innate immune response (35). This suggests that mechanisms of evasion may differ in primary cells, particularly those isolated from the GI tract. Our data also show that EV71, but not E11, is more potently restricted by type III IFNs than type I IFNs. Similar to E11, rotaviruses are also more sensitive to exogenous treatment with type I IFNs (28). The mechanistic basis for these differences in sensitivity is unclear, but our data suggest that, at least in the fetal GI tract, these differences are unlikely to be the result of differences in the magnitude or kinetics of ISG induction between type I and III IFNs. Instead, these differences may result from differences in the cell type–specific nature of enteric virus infections, with rotaviruses (36) and E11 (16) preferentially infecting enterocytes, whereas EV71 targets goblet cells. However, our immunofluorescence staining of IFIT1, which revealed increased expression of this ISG in EV71-infected goblet cells and in neighboring uninfected enterocytes, would suggest that both cell types respond to type III IFNs induced during the course of infection. Future studies dissecting the role of IFNs in the unique cell types of the HIE will provide important clues into the differential role that type I and III IFNs might play in the GI tract.

Our studies suggest that enteroviruses have evolved diverse mechanisms to infect distinct cell types in the GI epithelium, which might affect many aspects of their pathogenesis, including the role that type III IFNs play in restricting infection and spread. Further defining the events associated with EV71 infection in the GI tract could lead to the identification of novel therapeutic targets and/or strategies to prevent or treat the pathogenesis and morbidity associated with infections by this virus.

**MATERIALS AND METHODS**

**Cell culture and human crypt isolation**

Human fetal intestinal crypts were isolated from the entire small intestine and cultured as described previously (16). Human fetal tissue (<24 weeks of gestation) that resulted from elective terminations were obtained from the University of Pittsburgh Biospecimen Core through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. All tissue was genetically normal. Approximately 100 isolated crypts were plated into each well of a 24-well T-clear (0.4-μm pore size) Transwell insert and were grown in crypt culture medium composed of Advanced DMEM/F12 (Invitrogen) with 20% HyClone ES (embryonic stem) Cell Screened Fetal Bovine Serum (Thermo Fisher Scientific), 1% penicillin/streptomycin (Invitrogen), 1% l-glutamine, gentamycin, 0.2% amphotericin B, 1% N-acetylcysteine (100 mM; Sigma-Aldrich), 1% N-2 supplement (100×; Invitrogen), 2% B27 supplement (50×; Invitrogen), Gibco Hepes (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 0.05 mM; Invitrogen), ROCK Inhibitor Y-27632 (1 mM, 100×; Sigma) and supplemented with the following growth factors: WNT3a (100 ng/ml; Thermo Fisher Scientific), R-spondin (500 ng/ml; R&D Systems), Noggin (100 ng/ml; PeproTech), and EGF (50 ng/ml; Thermo Fisher Scientific) (37, 38) for the remainder of the respective experiments, with medium changes occurring every 48 hours. Unless otherwise stated, monolayers of HIE were used in studies at 6 days after plating and when TER values were >600 ohm.

**Viral infections**

Experiments were performed with EV71 (1095) or E11 (Gregory) that were expanded as described previously (39). In some cases, experiments were performed with light-sensitive NR viral particles, which were generated as described previously (21). Briefly, EV71 was propagated in the presence of NR (10 μg/ml) in the semi-dark and was subsequently purified in semi-dark conditions by ultracentrifugation over a sucrose cushion, as described (39).
For infections, wells were infected with $10^6$ plaque-forming units (PFU) of the indicated virus. Virus was preadsorbed to the apical or basolateral surfaces for 1 hour at room temperature (basolateral infections were initiated by inverting the Transwell inserts). Infections were then initiated by shifting to 37°C and allowed to proceed for the times indicated. For NR virus experiments, particles were exposed to light (on a light box) for 20 min at 6 hours p.i. and then infected for the indicated number of hours after light exposure. In some cases, cells were exposed immediately following adsorption (0 hours), which served as a control. E11 and EV71 plaque assays were performed in HeLa cells overlayed with 1.0 or 0.8% agarose, respectively; plaques were enumerated following crystal violet staining.

Binding assays were performed by preadsorbing $10^6$ PFU of the indicated virus to the apical or basolateral surfaces for 60 min at room temperature, followed by extensive washing with 1× phosphate-buffered saline (PBS). Following washing, RNA was isolated immediately, and RT-qPCR was performed, as described below.

**qPCR and complementary DNA synthesis**

Total RNA was prepared from HIE using the Sigma GenElute Total Mammalian RNA Miniprep Kit, according to the protocol of the manufacturer and using the supplementary Sigma DNase digest reagent. RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad), following the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed in a 20-μl reaction and subsequently diluted to 100 μl for use. RT-qPCR was performed using the iQ SYBR Green Supermix or iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was determined on the basis of a ΔΔCq method, normalized to human actin. Primer sequences can be found in table S1.

**RNA sequencing**

Total RNA was extracted as described above. RNA quality was assessed by NanoDrop and an Agilent bioanalyzer, and 1 μg was used for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) as per the manufacturer’s instructions. Sequencing was performed on an Illumina NextSeq 500. RNA-seq FASTQ data were processed and mapped to the human reference genome (hg38) using CLC Genomics Workbench 11 (Qiagen). Differential gene expression was performed using the DESeq2 package in R (18). Files from HIE used in the current study were deposited in sequence read archives.

**Immunofluorescence microscopy**

Monolayers grown on Transwell inserts were washed with PBS and fixed with 4% paraformaldehyde at room temperature, followed by 0.25% Triton X-100 to permeabilize cell membranes for a minimum of 15 min at room temperature. Cultures were incubated with primary antibodies for 1 hour at room temperature, washed, and then incubated for 30 min at room temperature with Alexa Fluor–conjugated secondary antibodies (Invitrogen). Slides were washed and mounted with Vectashield (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI). The following antibodies or reagents were used: recombinant anti–double-stranded RNA (dsRNA) antibody [provided by A. Brass, University of Massachusetts and described previously (40)], MUC2 (H-300, Santa Cruz Biotechnology), lysozyme C (E-5, Santa Cruz Biotechnology), E-cadherin (ECCD-2, Invitrogen), ZO-1 (ZMD.436, Invitrogen), cytokeratin-19 (EP1580Y, Abcam), VP1 (NCL-ENTERO, Leica), IFIT1 (PA3-848, Thermo Fisher Scientific), intestinal ALPL (ab186422, Abcam), and SCARB2 (EPR12081, Abcam) and Alexa Fluor 594– or Alexa Fluor 633–conjugated phalloidin (Invitrogen). Images were captured using a Zeiss LSM 710 inverted laser scanning confocal microscope or with a Leica SP8X tandem scanning confocal microscope with white light laser and contrast-adjusted in Photoshop. Image analysis was performed using Fiji. MUC2- and VP1-positive cells were counted using the ImageJ Cell Counter plugin.

**Recombinant IFN treatments**

HIE monolayers were treated with 100 to 500 ng of recombinant IFN-β, IFN-λ1, or IFN-λ3 (1598-IL-025, 5259-IL-025, and 8499-IF-010; R&D Systems) added to both the apical and basolateral compartments for ~20 hours before initiating infections, as described above.

**Inhibitor treatments**

HIE monolayers were treated with 5 μM ruxolitinib or dimethyl sulfoxide (DMSO) control for 1 hour at 37°C and then infected with NR-labeled EV71 or treated with IFN-λ3 in the presence of ruxolitinib or DMSO. Similarly, HIE monolayers were treated with 80 μM dynasore or DMSO for 30 min at 37°C before infecting with NR-labeled EV71 in the presence of dynasore or DMSO.

**Luminex assays**

Luminex profiling was performed using the Human Bio-Plex Pro Inflammation Panel 1 IFN-β, interleukin-29 (IL-29), and IL-28A sets (Bio-Rad) according to the manufacturer’s protocol using the laboratory multipanalyte profiling system (LabMAP), a system developed by Luminex Corporation (Austin, TX).

**Statistics**

All statistical analysis was performed using GraphPad Prism. Experiments were performed at least three times from independent intestines with technical replicates performed from independent Transwells (a total of 36 small intestines were used in this study) as indicated in the figure legends or as detailed here in the text. Data are presented as means ± SD. A Student’s t test, one-way analysis of variance (ANOVA), or two-way ANOVA was used to determine statistical significance, as described in figure legends. Parametric tests were applied when data were distributed normally based on D’Agostino-Pearson analyses. If data were not normally distributed (based on D’Agostino-Pearson analyses), then nonparametric tests were applied. P values of <0.05 were considered statistically significant, with specific P values noted in the figure legends.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/3/eaau4255/DC1

Fig. S1. Validation and model of human intestinal epithelial monolayers.

Fig. S2. SCARB2 localization in HIE and dynamin-dependent EV71 infection of HIE.

Fig. S3. IFN induction and signaling in HIE.

Fig. S4. Kinetics of ISG induction in HIE treated with recombinant IFNs.

Table S1. Primers used in this study.

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