Respiratory and intestinal epithelial cells exhibit differential susceptibility and innate immune responses to contemporary EV-D68 isolates

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Abstract:

Enterovirus D68 (EV-D68) has been implicated in outbreaks of severe respiratory illness and acute flaccid myelitis (AFM) and is detected in patient respiratory samples and from stool and wastewater, suggesting both respiratory and enteric routes of transmission. Here, we used a panel of EV-D68 isolates, including a historical isolate and multiple contemporary isolates from AFM outbreak years, to define the dynamics of viral replication and the host response to infection in primary human airway cells and stem cell-derived enteroids. We show that some recent EV-D68 isolates have decreased sensitivity to acid and temperature compared with an earlier isolate and that the respiratory, but not intestinal, epithelium induces a robust type III interferon (IFN) response that restricts infection. Our findings define the differential responses of the respiratory and intestinal epithelium to contemporary EV-D68 isolates and suggest that some isolates have the potential to target both the human airway and gastrointestinal tracts.
Introduction

Enteroviruses (EVs) are a family of positive-stranded RNA viruses, including coxsackieviruses, echoviruses, enterovirus A71 (EV-A71), and enterovirus D68 (EV-D68) that are responsible for a broad spectrum of illness in humans. EVs, specifically EV-D68 and EV-A71, have been associated with acute flaccid myelitis (AFM), a polio-like illness causing paralysis in previously healthy individuals, primarily children, which has peaked in even numbered years from at least 2014 until 2018 (Messacar et al., 2015; Midgley et al., 2015; Mishra et al., 2019; Schubert et al., 2019). While 2020 was anticipated to be a peak year for AFM, to date there has not been a surge of cases reported, perhaps indicating that coronavirus infection-control measures such as social distancing and mask usage have also diminished exposure to other circulating pathogens (CDC, 2020). While EVs are traditionally spread via the fecal-oral route, previous work with EV-D68 isolates before the AFM outbreak in 2014 suggested reduced replication in acidic environments and improved replication at lower temperature than traditional EVs, suggesting suitability for respiratory tract replication (Oberste, 2004).

EV-D68 has undergone rapid evolution since the 1990s, leading to the emergence of four clades, termed A-D (Du et al., 2015; Tokarz et al., 2012). This degree of evolution has led to loss of neutralization from pre-existing antibodies, highlighting the potential significance of these changes (Imamura et al., 2014). Contemporary EV-D68 isolates exhibit different biologic properties than historical reference isolates, including replication in neuronal cells (Brown et al., 2018). EV-D68 is often detected in patient respiratory samples, however, EV-D68 has also been isolated from stool specimens and wastewater, suggesting that it may also be transmitted by the fecal-oral route (Bisseux et al., 2018; Pham et al., 2017; Weil et al., 2017). The viral and host determinants that influence EV-D68 tropism remain largely unknown, particularly in the respiratory and gastrointestinal epithelium. Moreover, whether there are differences in the replication dynamics and/or host responses to isolates circulating prior to AFM outbreaks versus contemporary isolates is also unclear.
The EV-D68 reference isolate Fermon is often used as a historic isolate, due to its isolation in the mid-1960s. However, this isolate has undergone decades of passage through cell lines and has thus likely undergone changes that make it well-adapted for replication in cell culture and less representative of its original sequence when it was isolated from a child with pneumonia (Schieble et al., 1967). These changes highlight the need to perform comparative studies using pre-outbreak and contemporary EV-D68 isolates in order to define the viral and host determinants of infection. In this study, we performed comparative studies of replication kinetics, temperature sensitivity, polarity of infection, and cellular responses to infection using a panel of EV-D68 isolates, including a historic isolate and multiple isolates from AFM outbreak years. To define host cell-type specific differences in EV-D68 replication and/or host responses, we performed comparative studies in primary human bronchial epithelial (HBE) cells grown at an air-liquid interface and in primary human stem-cell derived intestinal enteroids. We found that respiratory and intestinal cell lines were permissive to both historic and contemporary EV-D68 isolates, but that there were isolate-specific differences in temperature sensitivity at 33°C or 37°C. In contrast, primary HBE cells were largely resistant to EV-D68 replication, with only one isolate, KY/14/18953, able to replicate. KY/14/18953 and MA/18/23089 were able to replicate in human enteroids. Primary HBE, but not enteroids, mount a robust innate immune response to EV-D68 infection, characterized by the induction of type III interferons (IFNs) and to a lesser extent type I IFNs. Lastly, we show that inhibition of IFN signaling enhances EV-D68 replication in primary HBE, supporting a role for this signaling in the control of viral replication in the airway. Collectively, these data define the differential responses of the respiratory and intestinal epithelium to historic and contemporary EV-D68 isolates.

Results

EV-D68 replication in lung and intestinal cell lines varies with isolate and temperature
We sought to evaluate the replication competency of a panel of EV-D68 isolates, including a historical 2009 isolate and five contemporary isolates from outbreaks in the AFM peak years of 2014 and 2018 in cell lines representing the respiratory and intestinal tracts (details of viral isolates can be found in Supplemental Table 1). To do this, we used the MD/09/23229 isolate, collected in 2009 and in clade A, as a reference isolate prior to the 2014 outbreak and multiple isolates associated with AFM outbreak seasons, including 2014 and 2018. These isolates are inclusive of multiple clades, B1, B2, B3, and D1, (Du et al., 2015; Hadfield et al., 2018; Sagulenko et al., 2018; Sun et al., 2019) that have been associated with peak-year AFM outbreaks. In addition, KY/14/18953 and US/IL/18952 isolates are paralytogenic in mouse models (Brown et al., 2018; Hixon et al., 2017). We evaluated replication at 33°C and at 37°C in Calu-3 cells, a lung adenocarcinoma cell line and in Caco-2 cells, a colon adenocarcinoma cell line. We found that while all isolates replicated to some degree at 33°C in Calu-3 cells, two isolates, MD/09/23229 and MA/18/23089, were unable to efficiently replicate at 37°C (Figure 1A). In Caco-2 cells, all isolates were able to replicate at 33°C (Figure 1B). When infections were performed at 37°C, MA/18/23089 and KY/14/18953 continued to replicate well over background in Caco-2 cells (Figure 1B). In contrast, infections in Calu-3 cells performed at 37°C severely restricted the replication of several isolates, including MD/09/23229, IL/14/18952, and MO/14/18949, whereas the replication of KY/14/18953 was less severely restricted (Figure 1A). Collectively, these studies suggest that select EV-D68 isolates exhibit cell type-specific sensitivity to temperature in cell lines (summarized in Figure 1C).

Some contemporary EV-D68 isolates have increased acid tolerance

We found that all EV-D68 isolates were capable of replicating in gastrointestinal-derived cell lines. However, in addition to cellular tropism, enteric viruses must be stable in acidic environments to infect the GI tract. Previous work suggested that select EV-D68 isolates were destabilized...
following exposure to low pH (pH 4-6) as a mechanism of genome release during vial entry (Liu et al., 2018). However, whether this instability might influence the enteric route of transmission is unclear. In order to define the stability of EV-D68 virions in various conditions that mimic the environment in the GI tract, we exposed historical and contemporary isolates of EV-D68 to simulated intestinal fluids of the stomach and fed and fasted states of the small intestine over short (30 min) and long (60-120 min) exposure times. These fluids reflect not only the differential pH of the GI tract, but also contain bile acid and phospholipids that better recapitulate some aspects of the GI luminal content. To compare the stability of EV-D68 to other members of the enterovirus family that are transmitted primarily via the fecal-oral route, we performed similar studies with echovirus 11 (E11) and EVA71. E11 and EVA71 were stable in both fed state small intestine (FeSSIF pH 5) and fasted state small intestine (FaSSIF pH 6.5) for all exposure times tested (Figure 2A, 2B). However, whereas E11 exhibited significant reductions in titer when exposed to fasted state simulated gastric fluid (FaSSGF pH 2.0), EV71 was less impacted by this exposure (Figure 2A, 2B). None of the EV-D68 isolates tested were able to withstand the most acidic fluid (FaSSGF, pH 2.0) (Figure 2C-F). However, whereas EV-D68 isolates were generally stable in FaSSIF pH 6.5 conditions (Figure 2C-F), there were isolate-specific differences in stability in FeSSIF pH 5 conditions, with KY/14/18953 and to a lesser extent MA/18/23089 exhibiting some stability in this condition (Figure 2D-F). These data suggest that some contemporary isolates of EV-D68 exhibit enhanced stability in low pH conditions.

Comparison of EV-D68 growth characteristics in primary human airway epithelial cells and stem cell-derived enteroids

We found that many EV-D68 isolates efficiently infected airway- and intestinal-derived cell lines, which occurred in a temperature-dependent manner (Figure 1). However, given that cell lines do not fully recapitulate the complexities of the airway and intestinal epithelium, we performed similar studies in primary cell models. To model the human airway, we used primary human bronchial
epithelial cells (HBE) grown at an air liquid interface (ALI). HBE have increased similarity to the human respiratory tract with respect to polarization, functional cilia, and mucus production than cell line-derived respiratory models and thus provide a more physiological system to study EV-D68 infections in the human airway. We infected HBE cells from at least two independent donors from either the apical (Figure 3A, C, E, G) or basolateral (Figure 3B, D, F, H) domains and measured viral titer in the apical (Figure 3A-D) and basolateral (Figure 3E-H) supernatants to determine whether EV-D68 exhibited a polarity of entry and/or release. We found that one isolate, KY/14/18953, infected similarly from the apical and basolateral domains at 33°C or 37°C (Figure 3A-D), but exhibited preferential release from the apical surface (Figure 3A-H). In contrast, MD/09/23229 replicated more efficiently from the basolateral surface and exhibited a temperature preference for 33°C (Figure 3A-D). However, similar to KY/14/18953, it was also released preferentially from the apical surface (Figure 3A-H). Another contemporary isolate, IL/14/18952, infected best from the basolateral surface at 33°C while the contemporary isolate MA/18/23089 infected primary HBE inefficiently from either domain or temperature (Figure 3A-H, summarized in 3I). These data suggest that some isolates exhibit a preferential polarity of infection and are released primarily via the apical surface.

Next, we determined whether EV-D68 could infect GI-derived primary cells, particularly given that all isolates replicated to high titers in a GI-derived adenocarcinoma cell line (Caco-2). To do this, we used human primary stem cell-derived enteroids, which we used previously to define the cellular tropism of other enteroviruses in the GI epithelium (Drummond et al., 2017; Good et al., 2019). We found that only one isolate, KY/14/18953, replicated in human enteroids, which occurred in a temperature-independent manner but that there were very low levels of infection by other isolates tested, although MD/09/232229 exhibited some capacity to replicate to low levels (Figure 4A-B). A limitation of the above-described model is that enteroids grown in Matrigel exhibit an “inside out” polarity, with the luminal surface facing inward. As we have previously shown that some enteroviruses such as EV-A71 exhibit preferential infection of the
apical domain, we next determined whether EV-D68 exhibited a similar polarity, which might explain the low levels of infection in enteroids grown in Matrigel (Good et al., 2019). To address this, we cultured intestinal crypts on Transwell inserts, which allows for the development of a monolayer containing diverse intestinal cell types (Good et al., 2019). Similar to our studies in HBE, we infected intestinal monolayers from the apical (Figure 4C, 4E) or basolateral (Figure 4D, 4F) domains and sampled the apical (Figure 4C-D) or basolateral (Figure 4E-F) supernatant for infectious virus. We found that KY/14/18953 replicated to high titers when inoculated from either the apical or basolateral surfaces but exhibited a preferential release into the apical compartment (Figure 4C-F), similar to what was observed in primary HBE. In contrast to our findings in Matrigel-derived enteroids, we found that MA/18/23089 replicated to high titers when infection was initiated from the apical surface, with slightly lower titers from the basolateral domain (Figure 4C-F). However, similar to KY/14/18953, this isolate also exhibited preferential release into the apical compartment (Figure 4C-F). Collectively, these data show that some contemporary isolates of EV-D68, particularly KY/14/18953, can replicate to higher titers in both primary HBE and enteroids (summarized in Figure 3l and 4G). In contrast, the historical isolate MD/09/23229 replicated to low titers in primary HBE, which only occurred at 33°C and was unable to replicate in enteroids (Figure 3l and 4G).

**EV-D68 infection induces cell type-specific antiviral signaling**

To define the cellular response to EV-D68 infection in HBE and in enteroids, we first performed RNAseq-based whole transcriptional profiling using select EV-D68 isolates, the historic strain MD/09/23229 and, due to successful replication under all tested conditions, KY/14/18953. Consistent with our infectious titer data, HBE cells infected from the basolateral surface had higher viral RNA (vRNA) fragments per kilobase per million reads mapped (FPKM) reads than those infected apically (Supplemental Figure 1A). However, despite near-equivalent viral input, HBE cells infected with MD/09/23229 had higher vRNA FPKM values than those infected with
KY/14/18953 (Supplemental Figure 1A), despite higher infectious titers in cells infected with KY/14/18953. We found that vRNA FPKM values in enteroids infected with KY/14/18953 were significantly higher than those observed in HBE and that these values were independent of temperature, as we obtained similar values in enteroids infected at 33°C or 37°C (Supplemental Figure 1A). Next, we performed differential expression analysis to identify transcripts induced by EV-D68 infection. Despite significant differences in the levels of infection, HBE infected with either MD/09/23229 or KY/14/18953 from the basolateral surface induced similar numbers of transcripts, with MD/09/23229 inducing 178 (Supplemental Figure 1B, Supplemental Table 3) and KY/14/18953 inducing 189 (Supplemental Figure 1C, Supplemental Table 3). Consistent with the low levels of vRNA present in HBE infected from the apical surface, relatively very few transcripts were induced under these conditions, with MD/09/23229 inducing 37 (Supplemental Figure 1B, Supplemental Table 3) and KY/14/18953 inducing 30 (Supplemental Figure 1C, Supplemental Table 3). Of the transcripts induced by basolateral infection, approximately half were shared between HBE infected with MD/09/23229 or KY/14/18953 (92 total, Supplemental Table 4). These transcripts were enriched in interferon stimulated genes (ISGs) (Supplemental Figure 1F, Supplemental Table 4). In contrast, there were very few transcripts induced by both HBE and enteroids infected with KY/14/18953, with only 9 transcripts shared between these conditions, despite enteroids inducing a greater total number of transcripts (332 total) (Supplemental Figure 1E, Supplemental Table 5). Of these transcripts, five included ISGs (MX2, IFIT3, IFIT1, IFI27, and IFITM1), which were induced in all conditions tested (Supplemental Figure 1G). Consistent with the induction of ISGs, HBE infected with MD/09/23229 or KY/14/18953 from the basolateral surface, and to a lesser extent the apical surface, potently induced the expression of the type III IFNs IFN-λ3, but not type I or II IFNs (Supplemental Figure 1H). In contrast, KY/14/18953 infection of enteroids elicited no significant induction of these transcripts, despite the higher levels of vRNA present in these samples.
(Supplemental Figure 1A, 1H). Taken together, these data suggest that there are cell type-specific differences in the response of HBE and enteroids to EV-D68 infection.

**EV-D68 infection of primary human airway cells preferentially induces type III IFNs**

Our RNASeq-based studies pointed to cell type-specific differences in the response of primary HBE and enteroids to EV-D68 infection. To further define the cellular response to EV-D68 infection, we performed multianalyte Luminex-based assays for 37 pro-inflammatory cytokines in cells infected with historical and contemporary EV-D68 isolates at 33°C at both 24h and 48h post-infection. In HBE, we also directly compared the impact of the polarity of infection on cytokine induction. At 24h p.i., all EV-D68 isolates induced the type III IFNs IFN-λ1 and IFN-λ2, with little to no significant induction of the type I IFNs IFN-β and IFN-α2 (Figure 5A-G). Of note, despite the low levels of viral replication in HBE infected from the apical surface (Figure 3), we observed near-equivalent levels of IFN induction under these conditions (Figure 5A-G). At 48h p.i., levels of type III IFNs further increased to very high levels (>10ng/mL) (Figure 5A, 5D-G). In addition, at the later time point, we observed a significant induction of IFN-β, but not IFN-α2 (Figure 5A-C). In contrast to EV-D68-infected HBE, EV-D68 infection in enteroids did not induce detectable changes in any of the cytokines tested, including IFNs (Figure 5A, 5D-G). These data suggest that the airway and intestinal epithelium induce cell type-specific responses to EV-D68 infection.

Prior reports have suggested that *in vitro* respiratory virus replication differences at 33°C and 37°C may be related to increased IFN responses at higher temperatures (Foxman et al., 2015). To determine whether differential temperature-dependent IFN responses explained differences in EV-D68 replication in HBE at 33°C and 37°C, we again utilized Luminex-based multiplex assays against 37 pro-inflammatory cytokines, including type I and III IFNs. To do this, we compared infection of primary HBE cells with EV-D68 isolates MD/09/23229 and KY/14/18953 at 33°C and 37°C for either 24 or 48 hpi. Despite differences in the efficiency of replication in HBE...
at 33°C and 37°C, we did not detect any significant differences in the induction of type I (IFN-β) or III (IFN-λ1, IFN-λ2) IFNs under these conditions (Supplemental Figure 2A-D). In addition, we found that Calu-3 lung epithelial and Caco-2 intestinal epithelial cell lines did not mount an IFN-mediated immune response to EV-D68 infection at either temperature (Supplemental Figure 2E-F).

**IFN signaling restricts EV-D68 replication in primary human airway cells**

We observed robust IFN-mediated antiviral signaling in HBE cells infected with EV-D68 despite very low to undetectable levels of replication, suggesting that this antiviral response restricts EV-D68 infection. To test this, we infected HBE cells with EV-D68 in the presence of a selective small-molecule inhibitor of JAK1/2 signaling (ruxolitinib). Treatment of HBE with ruxolitinib significantly decreased the secretion of IFN-b and IFN-I1 in response to EV-D68 infection (Figure 6A-B) and also significantly reduced ISG induction (Figure 6C). Consistent with this, there was a significant increase in MD/09/23229 infectious titers at 48 hpi as compared to DMSO-treated controls, with less robust enhancement of KY/14/18953 (Figure 6D).

**Discussion**

In this study, we define differences in the dynamics of EV-D68 replication and pH stability using a panel of isolates from AFM peak years and a pre-outbreak isolate. In addition, utilizing two primary human cell models representing common tissue sites targeted by enteroviruses in humans, we define differences in epithelial responses to EV-D68 between the respiratory and GI tracts. Collectively, this work details the varied responses of the respiratory and intestinal epithelium to historic and contemporary EV-D68 isolates and defines the role of type III IFN signaling in the control of EV-D68 infection in the respiratory, but not intestinal, epithelium.
We found that most isolates of EV-D68 efficiently replicated in both respiratory and intestinal epithelial cell lines, although there were some isolate-specific differences in temperature sensitivity. By comparison, primary HBE cells were less permissive to EV-D68 infection. One isolate, KY/14/18953, replicated very efficiently in primary HBE from either the apical or basolateral domains at both 33°C and 37°C, but exhibited a preferential release into the apical compartment. Another isolate, IL/14/18952, infected preferentially from the basolateral surface, but similarly was released into the apical compartment. The historic isolate MD/09/23229 and contemporary isolate MA/18/23089 replicated to comparably lower levels in all conditions but shared an apical release preference. Despite high levels of infection in the intestinal-derived Caco-2 cell line, only one isolate, KY/14/18953, replicated to high titers in stem cell-derived enteroids, although the contemporary isolate MA/18/23089 also replicated to low, but detectable, levels which improved when access to the apical surface was available. These studies point to key differences in the susceptibility of different primary epithelial-derived cell models to EV-D68 infection and suggest that host factors likely influence this tropism. For example, although attachment factors have been identified for EV-D68, including sialic acid and decay accelerating factor (DAF), some contemporary strains of EV-D68 including KY/14/18953 do not bind to sialylated receptors and their role in mediating infection is unknown (Baggen et al., 2016; Blomqvist et al., 2002). In addition, the neuron-specific intercellular adhesion molecule 5 (ICAM-5/telencephalin) has been identified as a potential receptor for several historic and contemporary isolates of EV-D68, but restricted expression in other cell types makes it unclear what role it might play in the epithelium (Hixon et al., 2019; Wei et al., 2016).

Previous work with EV-D68 before the emergence of AFM suggested that due to preferences for replication at 33°C and sensitivity to acid in vitro, it was more suited to be a respiratory pathogen behaving similarly to rhinoviruses as opposed to other enteroviruses (Liu et al., 2018; Oberste, 2004). While previous studies have evaluated acid stability of EV-D68, we utilized biologically relevant solutions with complexities other than acidity, such as bile acid and...
phospholipids, that more closely mimic the gastrointestinal environment. Our studies using multiple contemporary isolates after the emergence of AFM suggest that these isolates are relatively stable at 37°C and also have improved acid stability. However, none of the EV-D68 isolates tested were stable during even short incubations with the most acidic fluid, the simulated fasted state stomach fluid, at a pH of 2. Our data also indicate that many isolates of EV-D68, even those associated with AFM outbreaks, are unable to replicate efficiently in human enteroids. However, one contemporary isolate, KY/14/18953, replicated to high levels in human enteroids and we observed replication of the contemporary isolate MA/18/23089 when primary intestinal enteroids were cultured on Transwell inserts. The basis for the very high capacity of KY/14/18953 to replicate in enteroids is unknown, but this isolate is genetically unique, and it is one of the very few members of the newly defined clade D, which thereby exhibits significant sequence variation in the VP1 region often used for receptor binding. These data suggest that the intestinal epithelium might serve as a site of EV-D68 transmission, particularly for some isolates.

The pro-viral factors that mediate EV-D68 infection in the epithelium remain largely unknown, but our studies suggest that the induction of IFN signaling plays a major role in restricting replication in the airway epithelium. We have shown previously that type III IFNs are preferentially induced by enterovirus infections in human enteroids and that this signaling restricts replication (Drummond et al., 2017; Good et al., 2019). In addition, type III IFNs are also the dominant IFNs induced in response to influenza, RSV, measles, and mumps infections of respiratory epithelial cells (Crotta et al., 2013; Fox et al., 2015; Galani et al., 2017; Jewell et al., 2010; Okabayashi et al., 2011). Although the type I IFN IFN-β was induced in response to EV-D68 infection, its induction was delayed compared to type III IFNs. Of note, we observed significant induction of IFNs even when levels of infection were not detectable, highlighting the potency by which the airway epithelium responds to these infections. The induction of IFNs is likely one mechanism by which the airway restricts EV-D68 replication, which is supported by our findings that treatment of HBE with ruxolitinib increased infection. However, it should be noted...
that ruxolitinib only partially recovers infection, suggesting other cellular pathways in addition to IFN also restrict infection. Surprisingly, despite robust IFN induction in response to EV-D68 infection of primary HBE, primary human enteroids did not mount any detectable IFN response to EV-D68 infection, suggesting that there are important differences in the capacity of the respiratory and airway epithelium to sense and respond to EV-D68 infection. The lack of antiviral signaling in infected enteroids would appear to be specific for EV-D68, as we have shown previously that enteroids infected with other enteroviruses including CVB, echoviruses, and EV71 respond via the induction of type III IFNs (Drummond et al., 2017; Good et al., 2019). While the mechanistic basis for this is unknown, differences in viral antagonism strategies and/or host detection mechanisms may explain these differences.

Currently there are no available no virus-specific treatments or vaccines to prevent AFM, which is a critically important emerging illness with significant morbidity to young children. Further understanding how EV-D68 targets the airway and/or gastrointestinal epithelium are critical to improve our understanding of how is transmitted, particularly given increases in its circulation. Our work presented here provides important insights into the dynamics of EV-D68 replication in the human airway and intestinal epithelium and provide ideal models to develop and test anti-EV-D68 therapeutics.

Materials and Methods

Cell culture

HeLa cells were provided by Dr. Jefferey Bergelson, Children’s Hospital of Philadelphia, Philadelphia, PA, and grown in MEM, with 5% FBS, non-essential amino acids, and penicillin/streptomycin. Calu-3 cells (HTB-55) were obtained from the ATCC grown in MEM w/ 10% FBS and 1% pen/strep and Caco-2 cells (BBE clone, CRL-2101) were obtained from the ATCC and grown in DMEM with 10% FBS and 1% pen/strep.
Human intestinal enteroids were derived as previously described by isolation of intestinal crypts from small intestine (Drummond et al., 2017) 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 and frozen. Enteroid lines were thawed, passaged, and maintained as previously described (Stewart et al., 2020) in Matrigel. Experiments with enteroids were performed on a Matrigel coating or on Transwell inserts, as detailed in the text. Crypt culture medium was 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, 1% N-acetylcysteine (100 mM; Sigma-Aldrich), 1% N-2 supplement (100×; Invitrogen), 2% B27 supplement (50×; Invitrogen), Gibco Heps (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, R-spondin, and Noggin as produced by preconditioned media from WRN cells obtained from the ATCC (CRL-3276) and described previously (Miyoshi and Stappenbeck, 2013) and hEGF (50 ng/ml; Thermo Fisher Scientific) (Egan et al., 2016; Shaffiey et al., 2016) and was changed every 48-72 hours throughout culturing.

Human Bronchial Epithelial Cells (HBE)

Primary HBE cells were differentiated from human lung tissue by following an IRB–approved protocol and were maintained at an air-liquid interface with differentiation media changed twice per week, as described previously (Myerburg et al., 2010). Differentiation media (BEGM/Ultroser G; Pall Corporation, Crescent Chemical Company, Islandia, NY) was comprised of 5 μg/ml insulin, 10 μg/ml transferrin, 0.07 μg/ml hydrocortisone, 0.6 μg/ml epinephrine, 0.8% vol/vol bovine hypothalamus extract, 0.5 mg/mL BSA, 0.5 μM ethanolamine, 15 ng/ml retinoic acid, 0.5 ng/ml...
human epidermal growth factor, 10 nM triiodothyronine, 0.5 μM phosphoethanolamine, and 0.5% vol/vol Ultroser G (USG) in Dulbecco's MEM (DMEM)/F12. Cells were cultured for 3-6 weeks in order to differentiate and achieve a mucociliary phenotype on phase contrast microscopy prior to all experiments. Mucus was removed by extensive washes in 1x PBS prior to infection.

Viruses and Infections

Experiments were performed with a panel of EV-D68 viruses described in Supplemental Table 1. Viruses were grown in HeLa cells at 33°C in 5% CO₂ until CPE was observed, purified by ultracentrifugation over a 30% sucrose cushion as previously described (Morosky et al., 2016). Purity of all viral stocks was confirmed by Sanger sequencing of VP1 using enterovirus-specific primers, as described previously (Oberste et al., 2003). Plaque assays were performed in HeLa cells overlayed with 1% agarose, incubated for 72 h, and plaques counted after staining with crystal violet. Viruses were obtained from the ATCC or were provided by the Center for Disease Control and Prevention (CDC) as noted in Supplemental Table 1.

For infections, cells were infected with 10⁶ plaque-forming units (PFU) of indicated viral isolates. Virus was adsorbed to the cell surface (apical or basolateral as indicated) for 1 hour at room temperature, cells were then washed with PBS, and then media replaced prior to placement back in the incubator at the indicated temperature for the indicated times. For viral replication analysis, aliquots of media were collected at indicated times post-infection and virus was detected via TCID50 assays in HeLa cells. For HBE growth experiments, media was applied to the apical surface at the indicated timepoint and incubated for 30 minutes at the experimental temperature prior to collection.

Simulated intestinal fluids
Simulated gastric fluid powders fasted state gastric fluid (FaSSGF), fasted state small intestinal fluid (FaSSIF), and fed state small intestine (FeSSIF) (Biorelevant) were prepared as described by the manufacturer. $10^6$ PFU/mL of the indicated virus was incubated in FaSSGF, FaSSIF, FeSSIF, or DMEM for the indicated time at 37°C. A one mL aliquot was collected, neutralized to pH 7.0 with 2.5M sodium hydroxide, and then replication competence was assessed via TCID50 assay.

**qPCR**

Total RNA was isolated from cells using the Sigma GenElute Total Mammalian RNA Miniprep Kit, according to the manufacturer protocol with the addition of a Sigma DNase digest reagent. RNA (1mg total) was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) and diluted to 100 μl in ddH2O for subsequent qPCR. 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 ΔC_Q method, normalized to human actin. Primer sequences can be found in Supplemental Table 2.

**RNASeq**

Total RNA was extracted as described above. RNA quality and concentration were determined by NanoDrop, then 1 μg of RNA was used for library preparation with TruSeq Stranded mRNA Library Preparation Kit (Illumina) per the manufacturer’s instructions. Illumina NextSeq 500 was used for sequencing. RNA-seq FASTQ data were processed and mapped to the human reference genome (hg38) with the CLC Genomics Workbench 20 (Qiagen). Differential gene expression was analyzed with the DESeq2 package in R (Drummond et al., 2015). Raw sequencing files have been deposited in Sequence Read Archives and are publicly available (PRJNA688898).

**Luminex assays**
Luminex profiles utilized the Human Inflammation Panel 1 37-plex assay kit (Bio-Rad) per the manufacturer’s protocol using the laboratory multianalyte profiling system (MAGPIX) developed by Luminex Corporation (Austin, TX).

**Inhibitor treatments**

HBE cells or enteroids on MG coats were incubated with 5 μM ruxolitinib or dimethyl sulfoxide (DMSO) control for 1 hour at 37°C and then infected with the indicated EV-D68 isolate in the presence of ruxolitinib or DMSO.

**Statistics**

Statistical analysis was performed with GraphPad Prism software version 8.4.3. Experiments were performed at least three times and primary cells from at least two genetically distinct donors were utilized for each experiment. Data are presented as mean ± standard deviation. Student’s t test or one-way analysis of variance (ANOVA) was used to determine significance, as indicated in figure legends, for normally distributed data. Growth curve analysis was completed using two-way ANOVAs. P values of <0.05 was considered significant and are indicated in the figure legends.
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Non-redundant Front-Line Antiviral Protection against Influenza Virus Infection without


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Supplemental Table 1: List of viral isolates used in the study

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<th>Accession #</th>
<th>Source</th>
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Supplemental Table 2: RT-qPCR Primers

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<tr>
<td>IFIT1</td>
<td>CAACCAAGCAAATGTGAGGA</td>
<td>AGGGGAAGCAAAGAAAATGG</td>
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<tr>
<td>actin</td>
<td>ACTGGGACGACATGGAGAAAA</td>
<td>GCCACACGCAGCTC</td>
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</table>
Figure 1. EV-D68 replication in lung and intestinal cell lines varies with strain and temperature. Calu-3 cells (A) or Caco-2 cells (B) were infected with the EV-D68 strains MD/09/23229 (orange), MO/14/18949 (cyan), IL/14/18952 (burgundy), CO/18/23087 (green), MA/18/23089 (pink), or KY/14/18953 (blue) at an MOI of 5 and incubated at 33°C or 37°C. The supernatant was sampled at the indicated hours post-infection (hpi) and titrated by TCID50. Data are shown as mean ± standard deviation from three replicates. Dotted line denotes limit of assay detection. (C), Summary table denotes titers at 72 hpi, + corresponds to $10^3$, ++ $10^4$, +++ $10^5$, and ++++ $10^6$. Significance was determined by a Two-way ANOVA with multiple comparisons. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 compared to the KY/14/18953, which exhibited the highest replication levels.
**Figure 2.** Select contemporary EV-D68 strains exhibit increased acid tolerance. (A), Enterovirus 71 (EV71), (B), echovirus 11 (B), or (C-F) the indicated EV-D68 isolates (10⁶ PFU/mL) were incubated with control medium (DMEM), pH 2 FaSSGF (Gastric), pH 5 FeSSIF (Fed SI), or pH 6.5 FaSSIF (Fast SI) solution and incubated at 37°C for the indicated times. An aliquot of the virus/fluid mixture was collected, neutralized with sodium hydroxide, and then evaluated for infectivity via TCID50 assays. Titers are shown as mean ± standard deviation from three independent replicates. Significance was assessed at 120 min post-incubation using a One-Way ANOVA compared to DMEM-incubated controls, *p<0.05, ns, not significant.
Figure 3. Comparison of EV-D68 strain-specific growth characteristics in primary human airway epithelial cells. Primary human bronchial epithelial (HBE) cells grown at an air-liquid interface were infected with 10^6 PFU of the indicated EV-D68 strains: MD/09/23229 (orange), IL/14/18952 (burgundy), MA/18/23089 (pink), or KY/14/18953 (blue) and incubated at 33°C (A, B, E, F) or 37°C (C, D, G, H) for the indicated hours post-infection (hpi). HBE were infected from either the apical (A, C, E, G) or basolateral (B, D, F, H) surfaces. Supernatants were sampled at the indicated hpi from both apical (A-D) and basolateral (E-H) compartments and titers determined by TCID50 assays. Titers are shown as mean ± standard deviation from three independent replicates. Dotted line denotes limit of assay detection. (I), Summary table denotes titer at 48 hpi as collected from the apical compartment (A-D), - indicates no detectable replication, + corresponds to 10^3, ++ 10^4, +++ 10^5, and ++++ 10^6. Significance determined by two-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 compared to the KY/14/18953, which exhibited the highest replication levels.
Figure 4. Comparison of EV-D68 strain-specific growth characteristics in primary human enteroids. Primary enteroids grown on Matrigel (A-B) or transwells (C-F) were infected with 10^6 PFU of the indicated EV-D68 strains: MD/09/23229 (orange), IL/14/18952 (burgundy), MA/18/23089 (pink), or KY/14/18953 (blue) and incubated at 33°C (A) or 37°C (B-F) for the indicated hours post-infection (hpi). For intestinal cells grown on transwells, cells were infected from either the apical (C, E) or basolateral (D, F) surfaces. Supernatants were sampled at the indicated hpi from both apical (C-D) or basolateral (E-F) compartments and titers determined by TCID50 assays. Titers are shown as mean ± standard deviation from three independent replicates. Dotted line denotes limit of assay detection. (G), Summary table denotes titer at 72 hpi, + corresponds to 10^3, ++ 10^4, +++ 10^5, and ++++ 10^6, NT not tested. Significance determined by One-way ANOVA. *p<0.05, **p<0.005, ns, not significant.
Figure 5. EV-D68 infection of primary human airway cells induces a preferential type III IFN response. (A), Luminex-based multianalyte profiling of 37 cytokines and chemokines in primary human bronchial (HBE) cells or enteroids (ENT) infected with $10^5$ PFU of the indicated EV-D68 strains MD/09/23229, IL/14/18952, MA/18/23089, or KY/14/18953 from the apical or basolateral surfaces and incubated at 33°C (HBE) or 37°C (ENT). Supernatants were collected from the apical compartment at 24 and 48 hours post-infection (hpi). Shown is a heatmap based on cytokines induced relative to mock infected controls (key at right), with blue denoting significantly increased cytokines in comparison to uninfected. Grey denotes little to no change (scale at top right). Data are based on three independent experiments. Levels of IFN-b (A, C), IFN-l1 (D, E), or IFN-l2 (F, G) infected from the apical (B, D, F) or basolateral (C, E, G) are shown. Symbols represent individual biological replicates from unique donor cells. Statistical significance was determined using a Kruskall-Wallace test, *p<0.05, **p<0.01.
Figure 6. IFN signaling restricts EV-D68 replication in primary human airway cells. (A, B) Levels of IFN-b (A) or IFN-l1 (B) as determined by Luminex-based assays in medium harvested from primary human bronchial epithelial (HBE) cells pretreated with the JAK1/2 inhibitor ruxolitinib (5 mM, Ruxo) or DMSO control for 1 hour and then infected with EV-D68 strains MD/09/23229 or KY/14/18953 as indicated in the presence of inhibitor for 48 hours at 33°C. Symbols represent individual biological replicates from at least two unique donors. (C), Induction of the interferon stimulated genes (ISGs) CXCL10 or IFIT1 in control (DMSO)- or Ruxo-treated HBE infected with the EV-D68 strains MD/09/23229 or KY/14/18953 as assessed by RT-qPCR. Symbols represent individual biological replicates from at least two unique donors. (D), Viral titers in control (DMSO) or Ruxo-treated HBE infected with MD/09/23229 or KY/14/18953 for 48 hours at 33°C. Statistical significance was determined using a Student’s t-test, *p<0.05, **p<0.01, ns not significant.
Supplemental Figure 1. EV-D68 infection of primary human airway cells induces robust antiviral signaling. Whole genome RNAseq-based transcriptional profiling from total RNA isolated from primary human bronchial epithelial (HBE) cells grown at an air-liquid interface or primary human enteroids infected with EV-D68 isolates MD/09/23229 and KY/14/18953 was performed in HBE infected from the apical or basolateral domains of in human enteroids infected at 33°C or 37°C. (A) Heatmap of vRNA FPKM (fragments per kilobase per million reads mapped) values for apical and basolateral infection of HBE with the indicated isolate and of enteroid infection at 33°C and 37°C with KY/14/18953. Key is at right. Purple indicates high viral reads and white indicates low viral reads. (B-E), Venn diagrams denoting the overlap in differentially regulated transcripts in HBE infected from the apical or basolateral domains with MD/09/23229 (B) or KY/14/18953 (C), shared between both isolates following basolateral infections (D), and between HBE and enteroids infected with KY/14/18953 (E). (F) Heatmap of select interferon stimulated genes (ISGs) in primary HBE infected with the indicated isolates of EV-D68 from the apical or basolateral domains or in mock-infected controls. Scale at right. Red indicates higher expression and blue indicates lower expression. (G), Heatmap of transcripts upregulated in infected HBE and enteroids by both strains based on log2 RPKM values. Key is at right. Red indicates higher RPKM values, blue represents low RPKM values, and grey represents no reads. (H) Heatmap of transcripts (based on log2 RPKM) associated with type I, II, or III interferons (IFNs) in HBE cells infected apically and basolaterally at 33°C with the indicated strains or in enteroids infected with KY/14/18953 at 33°C or 37°C. Scale at right, Red indicates higher RPKM values, blue represents low RPKM values, and grey represents no reads.
Supplemental Figure 2. IFN induction in response to EV-D68 infection is independent of temperature. (A), Luminex-based multianalyte profiling of 37 cytokines and chemokines in primary human bronchial (HBE) cells infected with 10^6 PFU of EV-D68 strains MD/09/23229 or KY/14/18953 at 33°C or 37°C. Supernatant was collected from the apical compartment at 24 and 48 hours post-infection (hpi). Shown is a heatmap based on cytokines induced relative to mock infected controls (key at right), with blue denoting significantly increased cytokines in comparison to uninfected. Grey denotes little to no change (scale at top right). Data are based on three independent experiments. Levels of IFN-b (B), IFN-l1 (C), or IFN-l2 (D) from HBE infected at 33°C or 37°C are shown. Symbols represent individual biological replicates from unique donor cells. (E, F), Levels of IFN-l1 as determined by Luminex-based assays in Calu-3 (E) or Caco-2 (F) cells infected with MD/09/23229 or KY/14/18953 at 33°C or 37°C. Symbols represent individual biological replicates. Statistical significance was determined using a Student’s t-test, not significant (ns).