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CHARACTERIZING EPITHELIAL RESPONSES TO ROTAVIRUS INFECTION IN THE GUT

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CHARACTERIZATION OF EPITHELIAL RESPONSES TO ROTAVIRUS INFECTION IN THE GUT

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An abstract of a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy Program in Immunology and Molecular Pathogenesis Graduate Division of Biological and Biomedical Sciences.

September 8, 2010

ABSTRACT

CHARACTERIZATION OF EPITHELIAL RESPONSES TO ROTAVIRUS INFECTION IN THE GUT

By Amena H. Frias

Rotavirus (RV) is the leading cause of diarrhea in young children worldwide. RV targets intestinal epithelial cells (IEC), which clear infection within 7 days via pathways not strictly dependent on adaptive immunity. We hypothesized that IEC sense RV via innate pattern-recognition receptors (PRRs) and mount anti-viral immune responses involving Type 1 IFN-α/β. We sought to determine if RV structural components induce epithelial anti-viral gene expression and define the role of type 1 IFN in the RV-induced innate immune response. Model epithelia (HT29 IEC) were treated with RV (MOI 1-10), UV-irradiated RV (UV-RV) which was non-infectious but structurally intact, RV components including purified dsRNA and nucleic-acid free capsid shells (also called virus-like particles, or VLPs), and RV in presence of neutralizing antibodies to IFN-α/β. Gene transcription was assessed using microarray or qRT-PCR. Viral protein and anti-viral marker expression (STAT1, IRF 3/7, PKR), as well as PARP cleavage and DNA fragmentation (apoptotic events), were detected via western blotting or immunostaining. Secretion of IFN-β and IL-8 was measured using ELISA, and cell morphology was observed under a light microscope. Epithelia stimulated apically with trypsinized (protease-treated) RV exhibited upregulation of anti-viral markers including IFN-β but not IFN-α, and these trends were largely mimicked by UV-RV. RV dsRNA and VLPs poorly induced anti-viral signaling in comparison to UV-RV. Blockade of IFN-β signaling dramatically abrogated RV-induced anti-viral gene expression and prevented apoptosis. Surprisingly, impaired IFN-β activity also correlated with modest suppression of viral protein synthesis, particularly in a PKR-dependent manner. These data suggest IEC detect RV components via a trypsin-dependent, apical pathway of infection, and subsequently activate Type 1 IFN responses that promote anti-viral signaling, apoptosis, and viral replication in infected cells.

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TABLE OF CONTENTS

LIST OF TABLES

(listed in order of appearance as they appear in the chapter)

LIST OF FIGURES

(listed in order of appearance as they appear in the chapter)

CHAPTER 2

CHAPTER 3

CHAPTER 1: INTRODUCTION

Acute gastroenteritis afflicts billions of humans worldwide and causes up to 6 million deaths annually . A large number of these cases are due to infection from viruses such as rotavirus (RV), which particularly afflicts very young children . RV infection targets the small intestine, particularly the intestinal epithelial cells (IEC), and causes severe diarrheal illness which can ultimately lead to death (1). Since IEC are the main cell type infected by RV and have been shown to mount host immune responses to other types of pathogens, it seems likely they may play a role in mediating immunity to viruses such as RV.

The intestinal epithelium has long been recognized for its contribution to mucosal immunity by serving as a protective barrier to invading pathogens. More recently, it's also been shown the intestinal epithelium mounts anti-microbial immune responses (2). Evidence indicates such processes are mediated by epithelial pattern-recognition receptors (PRRs) which detect microbial components and subsequently activate innate immune signaling pathways leading to pathogen clearance and long-term protection against repeat infection(s). While a number of investigations have demonstrated intestinal epithelia use such PRR-medicated mechanisms to respond to gut bacteria (2-6), whether such a system exists with regards to enteric viruses such as RV has not been characterized.

Thus, we hypothesized that analogous to the case for bacteria, IEC mount host immune responses to viruses such as RV via PRR-mediated pathways. Using an *in vitro* cell culture model, we aimed to: 1) determine whether structural components of RV (dsRNA and/ or proteins) induced IEC innate immunity involving type 1 IFN, a well-

known anti-viral cytokine, and 2) characterize the function of epithelial type 1 IFN in mediating anti-rotaviral immunity. We showed UV-inactivated RV (UV-RV), which was rendered structurally intact but non-replicative, induced anti-viral immune responses in IEC that were comparable to or greater than that induced by live RV. Such anti-viral responses involved type 1 IFNs which induced epithelial anti-viral gene expression and apoptosis. Interestingly, however, RV was also found to exploit these type 1 IFN responses during early stages of infection to increase pathogenicity. In conclusion, this body of data suggests that IEC detect RV via PRR-mediated pathways and subsequently mount type 1 IFN-mediated immune responses promoting anti-viral signaling, apoptosis and viral replication in infected cells.

1.1 Intestinal epithelial response to bacteria

Within the past decade, it has become clear that the intestinal epithelium plays additional roles in mucosal immune defense besides serving as a mere protective barrier to foreign pathogens. Studies have shown that the intestinal epithelium also mediates immune responses to enteric microbes such as bacteria (2). For instance, in response to bacterial agents of gastroenteritis such as *S. typhimurium*, intestinal epithelial cells (IEC) secrete defensins with direct anti-bacterial activity and cytokines that recruit immune cells to the site of infection. These immune cells include neutrophils, which facilitate bacterial clearance, as well as dendritic cells and lymphocytes that likely help to elicit an adaptive immune response to the invading pathogen. Such investigations further indicate that cellular PRRs (pattern-recognition receptors) mediate such bacterial-epithelial

interactions by detecting bacterial components and subsequently activating immune signaling pathways leading to pathogen clearance and long-term prevention against re-infection. For example, TLR5, a PRR located basolaterally on IEC, engages flagellin subunits released by *S. typhimurium* and induces innate signaling that leads to induction of pro-inflammatory immune responses (3-5). Another example of an epithelial PRR-mediated anti-bacterial response is the Nod 1/2-*S. flexneri* interaction. Nod 1/2, intracellular peptidoglycan receptors, are activated upon epithelial infection from the aflagellate pathogen *S. flexneri*; such an engagement leads to downstream activation of (NF)-κB, a major inducer of pro-inflammatory gene expression (6). Similar to the case for bacteria, it is likely that epithelial PRRs may also play a role in mounting anti-viral immune responses to intestinal viruses such as RV.

1.2 Epidemiology of rotaviruses

Viral gastroenteritis, caused by enteric etiologic agents such as calciviruses, adenoviruses, astroviruses and rotaviruses, is a leading cause of childhood death worldwide and thus places a significant medical and economic burden on society (7, 8). RV, one of the most well-understood enteric viruses, is the primary cause of diarrheal disease in children less than 5 years old worldwide (7, 9). Globally, RV causes 600,000-875,000 deaths annually, with a disproportionate amount of RV-related mortality occurring in developing countries as compared to developed countries. However, significant morbidity due to RV infection still occurs in developed countries (10). During

2006, for example, RV-related illness in the US caused 55,000-70,000 hospitalizations, 200,000-272,000 emergency department visits and 410,000 physician office visits (9).

RV infection spreads from person-to-person via the fecal-oral route and has a complex distribution. Rotaviruses are serotypically diverse, classified into 7 groups (A-G) which are further divided into subgroups and serotypes based on antigenic protein differences (10, 11). Groups A-C infect humans, while all groups infect animals (12). Group A is the primary pathogen worldwide, while Groups B and C cause annual epidemics in Chinese adults and episodes of sporadic disease, respectively (10, 13). RV strains occur with varying frequency according to geographic location and time. For instance, RV G1 strains are generally more common in western Europe and the US than in other parts of the world. In addition, G1-G4 strains were more predominant during the early 1990s, while more recently, G9 rotaviruses have become more prevalent (14). For reasons which are still not clear, RV infection also exhibits seasonal or non-seasonal patterns associated with type of climate, peaking during the winter in temperate climates but occurring year-round in the tropics (14, 15).

1.3 Rotavirus structure and replication

1.3.1 Rotavirus structure

Under electron-microsopy, RV exhibits a large (1000 Å), complex, wheel-like appearance (10, 12, 14). RV particles are 70 nm in diameter, non-enveloped, and consist of several concentric capsid protein layers protecting an inner viral genome. Part of the *Reoviridae* family of viruses, RV contains an 11 dsRNA segmented genome which

encodes for different rotaviral proteins (10, 11). RV proteins are generally divided into two groups, structural VP proteins (VP 1-4, 6, 7) and non-structural NSP proteins (1-6) (14). VP proteins provide structural support to RV and also mediate cell entry (9, 14). VP 1, 2 and 3 proteins function as RNA-dependent RNA polymerases, scaffolding proteins, and guanylyltransferases and methylases, respectively, and together comprise the innermost layer protecting the genome. VP6 serves as the major structural protein of RV, making up over 51% of the virus (11), and constitutes the intermediate layer (15). VP7, a glycoprotein found on the outermost layer of RV, contains spoke-like VP4 hemagluttinin proteins, which play a role in cellular attachment during infection (10, 15). VP4 and VP7 proteins induce neutralizing antibody responses and are also the basis for the binary classification system for naming rotaviruses (9). The functions of NSP proteins, which are produced during infection, are only partially known (15). In general, however, NSP proteins are recognized for playing various roles in viral replication and pathogenesis (14). For instance, NSP1 associates with the cellular cytoskeleton and suppresses the host immune response during infection (15). Additionally, NSP4, a viral enterotoxin and glycoprotein, is recognized for its role in inducing diarrhea (15, 16).

1.3.2 Rotavirus replication

The rotaviral replication cycle is incompletely understood and remains a critical area of investigation. Upon protease cleavage of VP4 into VP5 and VP8 subunits, infectious RV attaches to the cell surface via a multi-step process involving binding to sialylated and non-sialylated receptors (17). The identity of the primary cellular receptor

for RV remains controversial, however several candidates have been proposed, including sialic acid residues, glycoconjugates (glycoproteins, glycolipids, and glycosphingolipids), or a receptor specifically engaging the lipophilic RV VP5 subunit. Following engagement with a primary receptor, RV interacts with integrins such as very late antigen-2 (VLA-2), very late antigen-4 (VLA-4), and complement receptor 4 (CR4) (16). Next, RV enters the cell via either direct membrane penetration or calcium (Ca2+)-dependent endocytosis (12, 16). Inside endosmes, where conditions are favorable for uncoating (i.e., high protease and low Ca2+ levels), the virus partially sheds its outermost layer (17). Double-layered rotaviral particles subsequently enter the cell cytoplasm and function as molecular machines, transcribing 5'mRNA with the help of a viral RNA-dependent RNA polymerase that converts viral dsRNA to mRNA. These newly synthesized mRNAs are then extruded into the cytoplasm, where they undergo replication to produce new viral dsRNA as well as translation (14, 17). After this step, RV replication and packaging occurs, presumably in electron-dense structures (viroplasms) located near the ER and nucleus . Newly formed double-layered viral particles leave viroplasms and then bud into the ER, where they acquire a transient envelope and an outermost capsid protein layer (14). To exit the cell, infectious rotaviral virions use either cellular lysis or a non-vesicular transport mechanism which bypasses the Golgi complex and facilitates release from the apical surface (14, 16).

1.4 Rotavirus pathogenesis

The triple-layered capsid structure of RV confers stability, facilitating fecal-oral transmission and efficient delivery into the small intestine (14). RV targets the epithelial cells of the small intestine, specifically the mature enterocytes located on the tips of the villi (12, 18). RV infection disrupts the digestive and absorptive function of the small intestinal epithelium, causing diarrheal illness which typically clears within 7 days (18, 19). Pathological changes associated with RV infection, exclusively limited to the small intestine, generally include villous atrophy and blunting, mononuclear infiltration of the lamina propria, vacuolization of epithelial cells and mild inflammation (17). Clinical symptoms associated with RV infection include fever and vomiting for 2-3 days followed by non-bloody diarrhea, and typically manifest in children rather than adults (12, 14). In the most extreme cases, rotaviral disease can cause severe dehydration that is life-threatening. Lastly, repeat infections with RV are usually seen to be less severe than the primary disease (12).

1.4.1 Rotaviral induction of diarrhea

While it is not well understood how RV causes diarrhea, several theories have been proposed. One the most commonly accepted mechanisms of RV-induced diarrhea is malabsorption of fluid and nutrients resulting from epithelial damage (12, 18). Specifically, malabsorption would occur due to enterocyte destruction and decreased absorption of sodium, water and mucosal disaccharidases (18). Secondly, RV NSP4, a viral enterotoxin, is also cited as a causative factor. NSP4, a secreted fragment of NSP4,

or NSP4 peptides have been observed to elicit diarrhea in a murine model (18). Specifically, NSP4 seems to cause diarrhea via Ca2+-dependent signaling pathways which lead chloride (Cl-) secretion and subsequent loss of water (9, 12, 14). Third, stimulation of the enteric nervous system (ENS) has been correlated with rotaviral diarrhea, as several drugs known for blocking ENS activity attenuate RV-induced secretion in the intestine (18). Other possible causes of rotaviral diarrhea include increased gut levels of prostaglandin PGE2, increased paracellular permeability due to weakening of tight junctions between cells, alterations in intestinal motility and villous ischemia (12, 14, 18). To date, it seems likely diarrhea resulting from RV infection is not due to a single process, but rather multiple causes contributing simultaneously (12).

1.4.2 Extraintestinal spread of rotavirus infection

RV infection was generally thought to be limited to the small intestine, however recent studies indicate viremia (infectious virus in the blood) and extraintestinal infection occurs frequently in humans and animals (20). In children, rotaviral RNA and proteins have been found in the blood and nonintestinal tissues including liver, heart, lung and nervous system. Due to technical limitations in clinical laboratories and difficulties in obtaining tissue for analysis, however, human data on RV-infected blood and extraintestinal tissue has been deficient. Hence, much of this information has been collected from experimental animal models. RV proteins were detected in the blood (antigenemia) of infected mice, rats, rabbits, calves and gnotobiotic piglets. Further, viremia was observed in RV-infected mice, rats and piglets, and infectious RV, along

with RV RNA or antigen, was found in rodent extraintestinal tissues (i.e., stomach, liver, lungs, kidneys, heart, bladder, pancreas, spleen and mesenteric lymph node). Infection of cell types other than IEC also indicate RV spreads beyond the intestine, as RV gene expression was evident in murine immune cells such as B cells, macrophages and dendritic cells, as well as rat lung and monocytic cells located within a blood vessel (21). Interestingly, immunocompromised individuals exhibited RV in the liver and kidneys (22), suggesting an important role for mucosal immunity in restricting virus infection to the gut. Whether a causal association exists between rotaviral viremia and/ or extraintestinal infection and development of systemic disease such as hepatitis, myocarditism, pancreatitis, pneumonia or encephalopathy are questions that need to be addressed by future investigation (20).

1.4.3 Genetic reassortment of rotavirus

The segmented nature of the RV genome suggests that, during a mixed infection between different rotaviral strains, genetic reassortment would theoretically occur (23). In fact, *in vitro* and *in vivo* studies have demonstrated when 2 different rotaviruses coinfect the same cell, genetic reassortment occurs at a high frequency and produces progeny viruses containing mixed segmented genomes from the parental strains (15). Some human rotaviruses, for example, were found to have mixed genotypes which seemed to originate from two different human rotavirus strains, Wa (VP7 serotype 1) and DS-1 (VP7 serotype 2). There have also been cases of suspected inter-species transmissions between humans and animals, with some human RV strains exhibiting

genomes which shared a high degree of homology with animal RV strains (17). The ability of RV to genetically reassort can thus lead to emergence of new rotaviral strains, consequently presenting challenges to present or future vaccines designed to target the most common human genotypes of RV (9, 15).

1.5 Rotavirus vaccine development

1.5.1 Live, attenuated rotavirus vaccines

RV vaccines have been in the development process since the early 1980s. Several strategies have been adopted to create a safe, effective, and low-cost vaccine that prevents disease in developed countries as well as more challenging settings, such as developing parts of the world (24). The earliest RV vaccines were created using the "Jennerian" approach, in which animal rotaviruses were used to protect humans against future infections with human RV. For example, a bovine RV vaccine candidate, RIT4237, was highly effective (> 80%) in preventing severe diarrhea in Finnish children; however, this vaccine was not pursued due to failure in clinical trials of developing countries such as Africa (14). Following these initial studies, a tetravalent RV vaccine called Rotashield was developed which consisted of a rhesus rotavirus (RRV) strain that had been modified to include 4 distinct human VP7 serotypes (15, 24). Rotashield was deemed to be highly effective (80-100%) in preventing severe diarrhea during clinical trials in the US, Finland and Venezuela, but was subsequently withdrawn due to an association with an increased risk (25-fold) for infant intussception (internal collapse of the intestine). In 2006, after almost a decade since the withdrawal of Rotashield, two

new live, attenuated rotaviral vaccines (RotaTeq and Rotarix) were licensed in the US, Europe, and many countries in Central and South America (14). The Rotarix vaccine was developed using a tissue-culture adapted human rotavirus, while RotaTeq consists of 5 different bovine-human reassortant rotaviral strains exhibiting seven different (VP4/ VP7) serotypes. To date, these vaccines show more promise than their predecessors and are proving to be highly effective (> 70% protection against any RV diarrhea and > 90% protection against severe RV diarrhea), safe, and most importantly, not associated with intussception (15). For example, in a Children's Hospital of Philadelphia study conducted in 2009, a dramatic decline in RV-related cases was observed since the introduction of RotaTeq in 2006. In the 13 years preceeding the licensure of RotaTeq in the US, the number of RV-related hospitalizations were near or above 100. During 2005 and 2006, 271 and 167 cases were reported, respectively. Most strikingly, only 36 cases were identified in 2007, reflecting an 87% reduction from 2005. The authors reasoned that herd immunity (resisting spread of disease via vaccination of a majority of people) may have contributed to effectiveness of this vaccine, as rates of protection seemed to exceed rates of vaccination in the population (25).

1.5.2 Inactivated rotavirus and VLP vaccines

Although safety and efficacy studies of recently introduced live, attenuated, RV vaccines are encouraging, efforts to develop alternative, non-replicating rotaviral vaccines are underway (9, 26). In both developed and developing countries, alternative vaccine candidates, such as inactivated RV (IRV) and subunit virus-like particle (VLP),

show an ability to yield immune responses in children comparable to live RV vaccines. IRVs induce strong serum antibody responses and protect against oral challenge in experimental animal models, are quicker and cheaper to develop relative to live vaccines, and are not associated with intussception or other adverse events (26). RV VLP vaccines, consisting of various types of VLPs (empty capsid shells of RV) assembled in a baculovirus-based insect cell expression system, are also considered safe and effective (26, 27). These vaccines produce robust antibody responses and elicit protection in animal models, but are not feasible because they are difficult and expensive to produce in the laboratory (26). Thus, in comparison to IRVs, RV VLP vaccines are a less attractive choice for use in routine childhood immunization

1.6 Adaptive and innate immune responses to rotavirus

1.6.1 Experimental animal models

Understanding of RV immunity has largely been generated from studies in experimental animal models. The two most widely used animal models are gnotobiotic pig and mouse models. The gnotobiotic pig model is useful for studying determinants of protection against rotaviral disease, while the mouse model allows various effector mechanisms of anti-rotaviral immunity (i.e., B, T or innate cell function) to be elucidated. Pigs remain susceptible to re-infection with RV for a longer period of time than mice, can be infected by human as well as porcine rotaviruses, and closely resemble humans in terms of gastrointestinal physiology and development of the mucosal immune system (14, 17). Studies of pigs infected with human RV reveal, for example, that high numbers of

intestinal IgA and RV-specific antibody-secreting cells (ASCs) correlate with protection against future challenge. The pig model is also helpful for evaluating vaccine candidates; key investigations have shown live RV infection is the most efficient inducer of protective immunity and implicate an array of mucosal immunity markers including anti-rotavirus intestinal IgA, enteric rotavirus-reactive antibody secreting cells (ASCs), and IgA memory B cells (14, 28). Unlike the mouse model, however, pigs are more difficult and expensive to maintain, cannot be genetically manipulated, lack availability of B and T cell depleting reagents (i.e. monoclonal Abs), and are difficult to analyze in large numbers simultaneously (1, 14).

Although mice do not develop RV-induced diarrhea after 14 days of age, they remain susceptible to RV infection throughout their life (14). Adult mice, in particular, can be readily infected with RV and shed detectable levels of viral antigen in their stool that correlates with infection (1). Thus, the adult mouse model is useful for studying various aspects of anti-rotaviral host defense, including innate and adaptive immune responses to RV. Studies in passive transfer mouse models show protection to RV can be mediated via administration of neutralizing antibodies to viral proteins VP4 and VP7. Further, B lymphocytes are considered the primary determinants of protection against reinfection from natural infection, whereas CD8+ T cells mediate viral clearance during primary infection. Interestingly, CD4+ T cells provide support to B and T cells during infection, but have also been shown to have direct anti-rotaviral activity in recombinant VP6-immunized mice. Lymphocytes homing to the intestine, particularly of B-cells, has also been demonstrated to be important for anti-RV immunity (14). In general, RV infection of mice which lack B and T cells results in chronic infection, highlighting the

importance of adaptive immunity in generating protection to RV (29, 30). However, studies of RV infection in severe combined immunodeficient mice (SCID), in which 40% of infected mice clear RV infection (30), and other investigations showing RV clearance in athymic Balb/c mice does not require antibodies (31), suggest the innate immune response also plays a significant role in mediating anti-rotaviral immunity.

1.6.2 Role of type 1 IFNs in innate anti-rotaviral immunity

In vivo as well as *in vitro* studies have shown innate cytokines such as type 1 IFNs (IFN α/ β), which are well-known for possessing anti-viral properties, appear to modulate anti-rotaviral immunity (14, 32). Levels of type 1 IFNs increase in RV-infected children and animals, and administration of exogenous type 1 IFN, particularly IFN-α, reduces RV-induced diarrhea in cattle and pigs (14). *In vitro*, pretreatment of cultured cells with type 1 IFNs limits infection (33). Interestingly, loss of type 1 IFN receptors in mice does not impair rotaviral clearance (20), thus implying type 1 IFNs may not be absolutely required for controlling RV. However, deficiency of STAT1, an important transducer of IFN signaling, correlates with increased viral shedding in mice during RV infection (34). Further, impaired IFN signaling in mice has been linked to extraintestinal spread of RV infection and development of systemic diseases such as lethal biliary atresia (BA) and pancreatitis (14, 20), suggesting a role for IFN in confining RV to the intestine. Also, RV-encoded NSP1 was recently observed to suppress IFN signaling via degradation of interferon regulator factor 3 and 7 (35, 36), further supporting the notion that type 1 IFNs pose a significant hindrance to RV.

1.6.3 Intestinal epithelial response to rotavirus

Studies in polarized human intestinal epithelial cell lines (i.e., Caco-2, HT-29) have shown RV infection induces a number of cellular responses including disruption of cellular protein trafficking, structural and functional damage of tight junctions, cytoskeletal rearrangement (i.e., F-actin disassembly) dependent on intracellular Ca2+ levels, alterations in cell-cell interactions (16), and apoptosis (37, 38). In addition, RV infection elicits activation of cellular immune signaling pathways, including proinflammatory and Type 1 IFN responses. For example, intestinal epithelia activate transcription factors (NF)-κB, STAT1, IRF3/ 7 and ISGF3, or secrete cytokines IL-8, RANTES, GRO-α, GM-CSF and IFN-α in response to RV (16). Also, RV-infected epithelia, specifically Caco-2 cells, upregulate mRNA expression of hundreds of genes relative to mock-infected controls, including a group of genes associated with Type 1 IFN responses (39). More recently, it was demonstrated RV induces activation of protein kinase R (PKR), an IFN-induced gene which is important for mediating anti-viral host defense mechanisms (40). The generation of such immune responses, and how they relate to RV pathogenesis or immunity, remains an ongoing area of investigation.

1.7 Cellular anti-viral defense mechanisms

Although studies suggest IEC activate immune signaling pathways in response to RV, the question of how such processes are initiated and whether they contribute to anti-rotaviral immunity are not completely understood. Indeed, a body of literature supports a paradigm that suggests cells are capable of mounting anti-viral immune responses. These

investigations indicate cells use pattern-recognition receptor (PRR)-mediated mechanisms to detect viral components and subsequently activate signaling pathways that promote viral clearance and adaptive immunity (32, 41). One of the most well-characterized cellular anti-viral responses is to viral dsRNA, a common replication intermediate of the viral life cycle. Viral dsRNA is initially recognized by PRRs in the membranes and/ or cytosol, such as toll-like receptor 3 (TLR-3), melanoma differentiation associated gene 5 (Mda-5) or retinoic acid inducible gene 1 (RIG-1). Upon engagement of viral dsRNA, PRRs activate downstream signaling cascades leading to IRF (3/ 7) transcription factor activation and type 1 IFN (α/ β) production (41). IRF3 and IRF7 are functionally similar, however IRF3 promotes IFN-β transcription, whereas IRF7 induces IFN-α and IFN-β (42). Following secretion from the infected cell, type 1 IFNs engage their receptors (IFNAR) in an autocrine or paracrine fasion, subsequently eliciting Jak/ STAT signaling pathways which produce over hundreds of anti-viral genes, known as interferon-stimulated genes (ISGs). Combinatorially, these ISGs serve to create an "anti-viral state" in cells which limits viral replication and spread (32, 41, 43). The three most well-known ISGs are Mx (myxovirus resistance gene), PKR (protein kinase R) and OAS (2'-5' oligoadenylate synthetase). Mx is an IFN-inducible GTPase which interferes with viral assembly, specifically via sequestration of viral ribonucleoproteins to subcellular compartments. PKR and OAS function as additional dsRNA sensors, activating apoptosis upon detection of viral dsRNA. PKR, a serine-threonine kinase, induces apoptosis by inhibiting cellular translation, whereas OAS promotes apoptosis via mediating degradation of cellular and viral mRNA (41). It is generally thought that IFN-induced apoptosis promotes viral clearance in the host by

preventing viral use of cellular machinery for replication and spread (32). However, type 1 IFNs also possess other functions which could potentially contribute to anti-viral immunity. For example, type 1 IFNs enhance NK cell cytoxicity and activity, induce MHC 1 expression, upregulate costimulatory molecules on DCs, and promote expansion of specific memory CD8+T cell subsets (41, 44). Thus, based on the evidence, it seems plausible to assume IEC may use PRRs to sense and mediate anti-viral immune responses to RV.

CHAPTER 2:

Intestinal epithelia activate anti-viral signaling via intracellular sensing of rotavirus structural components

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Key Words: Type I interferon, dsRNA, pattern-recognition receptors, STAT-1, IRF 3/7

Abbreviations: RV: rotavirus, UV-RV: irradiated rotavirus, VLPs: virus like particles, IEC: intestinal epithelial cell, IRF: Interferon regulatory factor, PRR: pattern-recognition receptor, STAT: signal transducer and activator of transcription, PKR: protein kinase R

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ABSTRACT

Rotavirus (RV), a leading cause of severe diarrhea, primarily infects intestinal epithelial cells (IEC) causing self-limiting illness. In order to better understand innate immunity to RV, we sought to define the extent to which IEC activation of anti-viral responses required viral replication or could be recapitulated by inactivated RV or its components. Using model human intestinal epithelia, we observed that RV-induced activation of signaling events and gene expression typically associated with viral infection was largely mimicked by administration of UV-inactivated RV. Use of anti-IFN neutralizing antibodies revealed that such replication-independent anti-viral gene expression required type I interferon signaling. In contrast, RV-induction of NF-κB-mediated IL-8 expression was dependent upon viral replication. The anti-viral gene expression induced by UV-RV was not significantly recapitulated by RV RNA or RV VLP even though the latter could enter IEC. Together, these results suggest that RV proteins mediate viral entry into epithelial cells leading to intracellular detection of RV RNA that generates an anti-viral response.

INTRODUCTION

Rotavirus (RV) is the most common cause of severe dehydrating diarrheal disease in young children worldwide, causing up to 100 million cases and > 600,000 deaths each year (19, 45). RV infections are also common in adults, especially those who interact with children, but generally cause only mild symptoms (12). RV, a member of the *Reoviridae* family of viruses, is a non-enveloped double- stranded (ds) RNA virus containing three concentric protein layers (14, 46). Its genome consists of 11 dsRNA segments that each code for different rotaviral proteins. Rotaviral proteins are divided into two groups, namely, structural proteins (VP 1-4, 6-7) that compose the viral structure and non-structural proteins (NSP1-6), which are synthesized during infection and function to facilitate viral replication or pathogenesis (14). VP4, a spike-shaped hemagglutinin protein that emanates through the outermost (VP7) protein layer, plays a role in cellular attachment and must be cleaved by proteases normally present in the host intestine for rotavirus to be infectious (14, 46). NSP proteins play a role in driving RV-induced diarrhea and suppressing host immune responses (14).

RV infection is generally localized to the gastrointestinal tract and typically resolves within 7 days (19, 29). RV infection of mice lacking functional B and T lymphocytes often results in chronic infection, highlighting the importance of adaptive immunity in host defense to this pathogen (29, 30). However, Franco and Greenberg (30) observed that 40% of SCID mice (on a C57BL/6 background) cleared RV infection. Additionally, Eiden et al. (31) observed clearance of RV in athymic Balb/c mice that was not accompanied by anti-RV antibodies. These findings suggest an important role for innate immunity in controlling RV infection. Such innate immune control of RV may be

mediated by intestinal epithelial cells (IEC) since these cells are the predominant target of RV infection. The role of IEC in clearing RV infection is likely independent of RV-induced diarrhea in that the kinetics of clearance during primary infection are similar in newborn and adult mice even though only the former exhibit diarrhea (14). Numerous studies have noted that RV robustly induces NF-κB mediated epithelial expression of the chemokine IL-8, yet given that one of the hallmarks of RV infection is the absence of neutrophil infiltration (47-49), it is hard to envision IEC production of this chemokine plays a major role in clearance of RV. Rather, based on the large body of data on host responses to viral infections in general, it seems more plausible that type 1 interferon (IFN) responses contribute to innate immune-mediated clearance of RV. In accordance, gene-profiling studies of RV-infected IEC observed elevated expression of a panel of genes related to type I IFN responses (39). Pre-treatment of cultured cells with type 1 IFNs limited RV infection (33). Levels of type I IFNs increase in RV-infected children and animals and administration of exogenous type I IFN reduces disease sequellae in cattle and pigs (14, 50-52). Moreover, RV uses the non-structural protein NSP1 to suppress IFN signaling (35, 36). Mutations in NSP1 that ablate RV's ability to interfere with IFN-related signaling attenuate RV's spread to uninfected cells (35), further supporting the notion that IFN signaling is a potential hindrance to this pathogen. While mice deficient in type I and II IFN receptors are able to clear RV (20), loss of the transcription factor STAT1 , which mediates much of the gene expression induced by type I IFN, severely impairs control of RV (34). Thus, although there is considerable redundancy in host defense mechanisms against RV, it seems likely that IEC activation of STAT1 and induction of genes regulated by type I IFN play an important role in host

defense against this virus. Therefore, we sought to investigate the mechanism by which IEC activate such anti-viral signaling events in response to RV infection.

Viral infection has long been known to alter host gene expression by inducing endoplasmic reticulum (ER) stress, sometimes called “ER overflow,” in which the rapid switch in translation from host-encoded to viral-encoded proteins activates host stress-related transcription factors (53). This model predicts that a UV-inactivated virus, which cannot replicate or express its genes, would not alter host gene expression. In accordance, inactivation of the RV genome via UV-irradiation markedly reduced RV’s ability to induce NF-κB activation and IL-8 secretion (48, 49). More recently, it has become appreciated that a large portion of microbe-induced gene expression does not require a viable microbe *per se* but, rather, results from host pattern-recognition receptors (PRR) such as the toll-like receptors (TLR) detecting various microbial components. For example, IEC detection of motile bacteria is largely mediated by TLR5 detection of bacterial flagellin (5). However, IEC appear to be hyporesponsive to a number of other TLR agonists, highlighting the stark differences in the mechanism by which different microbes might be detected by IEC (54). IEC have been observed to respond to synthetic dsRNA, poly (I:C), resulting in expression of both IL-8 and a panel of genes associated with type I IFN responses, suggesting IEC have the potential to respond to RV via detection of viral RNA (40). However, whether such observations could apply to an actual virus-IEC interaction remained unclear. Thus, the goal of this study was to define the extent to which IEC response to RV results from viral replication or, rather is largely a consequence of IEC detection of RV components. We observed that RV-induced IEC

anti-viral signaling was almost entirely driven by detection of viral components likely resulting from VP4-mediated viral entry followed by detection of RV RNA.

RESULTS

Apical infection by rotavirus induces epithelial anti-viral signaling

Epithelial cell-mediated innate immune responses play a potentially important role in protecting the host against rotavirus (RV). Yet, although some studies of RV-induced innate immune activation have focused on activation of NF-κB mediated genes such as IL-8, rotavirus-induced anti-viral signaling in intestinal epithelial cells has not been extensively studied. Thus, we infected human HT-29 intestinal epithelial cells (IEC) with RV (MOI 0.5-1) and temporally assayed signaling events typically associated with a variety of viral infections. Mock-infected control samples were exposed to low levels of trypsin, which may activate protease activated receptors (PARs), however such treatment was included because it is required for cleavage of RV capsid protein VP4 and thus allows for robust infectivity (55). RV infection induced transient phosphorylation of transcription factors IRF3 and STAT1 with a maximal response being observed between 8-16 hours post-inoculation (hpi) (Figure 1A). RV also induced epithelial secretion of IFN-β (Figure 1B) and, in accordance with other studies, IL-8 (Figure 1C). Interestingly, RV-induced IL-8 production was relatively delayed suggesting it may not reflect immediate IEC sensing of the virus. These signaling events, which were not observed in mock-infected cells, roughly correlated with levels of the viral protein VP6 and are consistent with the finding that viral replication is required for RV-induced NF-κB activation (49). We next performed these experiments in polarized IEC, which result when IEC are cultured on collagen-coated permeable supports, in the hope that doing so might provide insight into mechanisms underlying such RV-induced anti-viral signaling. For example, use of polarized IEC previously enabled us to uncouple *Salmonella*

invasion from activation of innate immunity, ultimately allowing definition of mechanisms underlying the latter process (56). Consistent with previous findings (57), RV infection was markedly more efficient when the virus was administered to the apical surface of epithelia (Figure 1D). Such reduced infectivity of basolaterally-administered RV correlated with marked attenuation of IRF7, STAT1, and PKR activation and induced secretion of IFN-β and IL-8 (Figure 1 D, E, F). Such preferential infection and induction of anti-viral signaling by apical RV argues against RV activating innate immune signaling via a basolateral pattern-recognition receptor, as occurs in response to *Salmonella* (3), but seems consistent with the possibilities that anti-viral signaling might be triggered by an apical receptor, an intracellular receptor, or result from viral replication causing ER stress.

Inactivated rotavirus induces anti-viral gene expression similar to live rotavirus

To determine the extent to which RV-induced anti-viral signaling required viral replication or could be mimicked by structural components of RV, we examined the epithelial response to UV-irradiated RV (UV-RV), which is structurally intact but rendered non-replicative (58, 59). The inability of UV-RV to replicate in IEC was verified by monitoring levels of VP6 over time (Figure 2A). In accordance with previous studies, such UV-inactivation of the RV genome substantially reduced induction of IL-8 (Figure 2C). However, in contrast, signaling events typically associated with viral infection including activation of STAT1 and IRF3/ 7 and induction of IFN-β secretion, were elicited at least as robustly by UV-RV (Figure 2 A, B). Similar activation of anti-

viral signaling by RV and UV-RV was also observed in polarized epithelia in response to apical stimulation (data not shown). To confirm these events were indeed induced by UV-RV as opposed to UV cross-linked cell debris that might have been present in our virus preparation, we performed a control experiment showing that UV-irradiation of a mock viral preparation, which contained MA104 cell debris but not RV, did not elicit anti-viral signaling induced by UV-RV (Figure 2 D). Next, we determined if trypsinization, which is known to be required for viral entry, is also required for anti-viral signaling in response to UV-RV. Indeed, robust activation of anti-viral signaling by both RV and UV-RV required the stimulating agonist to be treated with trypsin prior to IEC stimulation (Figure 3). Lastly, we observed that, analogous to the case for RV, activation of innate immune signaling in response to UV-RV was more robust when UV-RV was applied to the apical rather than basolateral surface of polarized epithelia (Figure 4). Together, these results suggest that UV-RV induces anti-viral signaling via a mechanism similar to live virus, and further supports the notion that type I IFN is activated by IEC detection of RV structural components rather than viral replication.

We next sought to define the extent to which RV-induced gene expression in general can be mimicked by UV-RV and determine the role of type I IFN in RV-induced changes in IEC gene expression. IEC were mock-infected or exposed to RV, UV-RV, or RV in the presence of neutralizing antibodies to type 1 IFN (anti-IFN α/β) for 24 h, at which time gene expression was assayed by cDNA microarray. The gene chip employed for this purpose permits simultaneous examination of 12 different samples allowing us to assay 4 different experimental conditions in biological triplicates, thus permitting statistical analysis to be performed directly on the microarray data. Such microarray

analyses indicated that RV upregulated 1190 genes by at least 1.3 fold relative to mock-treated uninfected cells (cut-off was arbitrarily chosen based on our previous experience with microarray-based studies of IEC (40)). The entire microarray data set is available on-line (posted on GEO) and some of the common means of examining microarray data such as unsupervised clustering analysis are shown in supplemental data (Supplemental Figure 1). In Figure 5A, we sought to display our microarray data in a manner that would most facilitate addressing our central questions. Specifically, we generated a "heat map" that displays gene expression in each of the 12 samples (4 conditions, 3 replicates) relative to the average expression of mock-treated cells. The relative uniformity of the 3 replicates in each condition indicates the high degree of similarity among our biological replicates. Genes were ordered (top to bottom) based on their relative dependence upon type I IFN (ratio of expression upon exposure to RV alone vs. RV in the presence of anti-IFN α/β). The majority of genes that were induced by RV are thus type I IFN-dependent in that their expression was reduced by the neutralizing antibody. The heat map shows that the vast majority of such IFN-dependent gene expression did not appear to require viral replication in that almost of all of these genes were similarly induced by both RV and UV-RV. Such type-I IFN dependent, replication-independent, RV-induced gene expression included a panel of classic anti-viral genes such as IRF7, IFN-β, STAT1, Mx1, OAS-2 and MHC I (Table I). Use of qRT-PCR verified the upregulated mRNA levels of some of these genes (Figure 5 B, C, D, E). In contrast to the induction of such classic anti-viral genes, expression of IL-8 was partially dependent upon viral replication (Figure 2C) and independent of type I IFN (Table I). Thus, a large portion of RV-

induced gene expression in IEC, particularly upregulation of genes typically associated with viral infection, is independent of viral replication and dependent upon type I IFN.

RV components fail to recapitulate RV-induced IEC anti-viral signaling

Next, we sought to better define the structural determinant of RV that played an important role in activating IEC anti-viral signaling. Specifically, we compared epithelial responses to UV-RV, RV virus-like particles (VLPs), and purified RV RNA. The VLPs used here are protein shells comprised of 4 major RV structural proteins (VP 2/4/6/7) that lack nucleic acid (60). RV VLPs were used at protein concentrations of 0.5-5 μg/ml, which is equivalent to the concentration of UV-RV that corresponds to MOIs of 1-10. Purified RV RNA was used at concentrations of 0.5-5 μg/ml, which is approximately 100 times the amount of RNA in UV-RV that corresponds to MOIs of 1-10. In contrast to UV-RV, neither RV VLPs or RNA induced detectable elevations in levels of phospho-STAT1 or IRF7 (Figure 6 A). RV VLPs and RNA also failed to recapitulate the induction of IFN-β or IL-8 elicited by UV-RV (Figure 6 B, C). To more broadly understand the extent to which these components of UV-RV might recapitulate its ability to activate gene expression in epithelial cells, we measured changes in epithelial gene expression via microarray analysis. A modest concentration of UV-RV (MOI 0.5) induced 401 genes by > 1.3 fold relative to mock-treated control cells including key anti-viral genes such as Mda-5, IFN-β, MHC I (Tables II and III). Only a small portion of the genes upregulated by UV-RV were similarly induced by treatment with RV VLPs or RNA (Table II). In accordance, induction of anti-viral gene expression

by RV viral components was also not observed to be comparable to UV-RV (Table III), and these trends were confirmed by measuring mRNA synthesis of select anti-viral genes via qRT-PCR (Figure 6 D, E). Thus, neither RV RNA nor VLPs could substantially recapitulate the changes in gene expression induced by inactive but structurally intact RV.

RV and UV-RV display similar cell entry kinetics

To better understand the interaction of UV-RV and RV VLPs with polarized epithelia and activation of epithelial anti-viral signaling in response to RV, we examined the interaction of RV, UV-RV, and RV VLPs with model epithelia via confocal microscopy for up to 4 hpi (Figure 7 A, B). RV and UV-RV (MOI 10), and RV VLPs roughly equivalent to the estimated protein concentration of the RV preparation, were applied to the apical surface of epithelia for 1 and 4 hpi, followed by washing off of non-adsorbed or non-adhered materials. As expected, planar images of IEC taken 3 μm below the apical surface revealed the presence of RV, UV-RV and RV VLPs in the sub-apical region within 1 hpi (Figure 10A). RV increased in abundance by 4 hpi, reflecting viral replication, while UV-RV levels decreased likely due to degradation of viral proteins and an inability to replicate (Figure 7B). Thus, consistent with models by which RV is internalized by IEC, RV's primary structural proteins are sufficient to mediate its entry into IEC. Taken together, we interpret our results to suggest that RV activation of anti-viral signaling requires viral structural proteins to mediate entry of viral RNA into epithelial cells where it can be detected by host pattern-recognition receptor(s).

DISCUSSION

The ability of adult humans and mice to efficiently clear rotavirus without major sequellae serves as an example of effective mucosal immunity. Thus, understanding host immunity to RV may provide insights into understanding the pathogenesis of viruses that are not dispatched in such an expedient manner. Such adept handling of RV likely reflects considerable redundancy in the mechanisms that protect the host against RV. In accordance, and in contrast to the case for many viruses, mice lacking either adaptive immunity or type I IFN still exhibit substantial control of RV infection (20, 30). Although not required for clearance, type I IFNs modulate the course of RV infection in a variety of experimental systems (14, 33, 50, 51) and deletion of STAT1, a transducer of IFN-related signals, severely impairs control of RV (34). Thus, activation of STAT1/ type I IFN signaling is likely one important means of host defense against RV. Therefore, the goal of this study was to investigate how the predominant cell type infected by RV, the intestinal epithelial cell (IEC), activates such anti-viral signals in response to RV infection.

We observed that, in contrast to RV activation of NF-κB, which is driven by synthesis of viral proteins that may cause ER stress (49), RV-induced anti-viral signaling was almost entirely driven by IEC detection of viral components. Indeed, while adding RV and inactivated RV (UV-RV) at the same MOI initially delivered similar levels of viral components, UV-RV antigens were degraded within a few hours while RV replication resulted in increasing levels of viral proteins over the ensuing 24 h. Yet, a greater level of anti-viral signaling was observed in response to UV-RV, largely reflecting higher induction of type I IFN. That such higher levels of viral proteins in RV-

infected cells did not result in higher IFN levels than was seen in cells exposed to UV-RV likely reflects the ability of RV to suppress IFN signaling/ expression via NSP1-mediated degradation of IRF 3, 5, and 7 (35, 36). Such ability of RV to suppress what might otherwise be a very robust anti-viral signal in response to the presence of even modest amounts of viral components speaks to the potential of these innate immune signals to limit RV infection.

While a large portion of RV-induced IEC gene expression was dependent upon type I IFN, including numerous genes typically associated with viral infection, RV nonetheless induced numerous genes whose induction did not require type I IFN. Such RV-induced gene expression was not substantially recapitulated by UV-RV suggesting it results from ER stress and/ or requires higher levels of viral components than was achieved by treatment with UV-RV. RV replication-dependent induced genes includes several NF-κB regulated pro-inflammatory genes including the neutrophil chemoattractant IL-8, which was one of the genes most highly induced by RV. That RV infection is not typically associated with cellular inflammation makes the role of such chemokine induction somewhat enigmatic. Yet, given that purified bacterial flagellin, which primarily activates NF-κB mediated pro-inflammatory gene expression provided mice with temporary protection against RV infection (61), we speculate that ER stress-mediated NF-κB activation may also be a redundant mechanism by which IEC limit RV infection.

The potent induction of anti-viral signaling observed in response to UV-RV was not mimicked by RV VLPs or RV RNA. The failure of RV VLPs to activate anti-viral signaling could conceivably reflect that RV non-structural proteins, which are not present

in VLPs, play a role in activation of innate immunity. However, it seems more likely that IEC detection of RV RNA is required to generate robust anti-viral signaling. The failure of RV-RNA to robustly activate anti-viral signaling likely resulted from lack of significant uptake by IEC. In accordance, IEC uptake and responses to synthetic dsRNA, poly(I:C), required that poly(I:C) be administered at high concentrations and correlated with its uptake (40). *In vivo*, and perhaps *in vitro*, free RNA that is not rapidly taken up by IEC would likely be quickly degraded by RNAses. In contrast, surface expression of VP4 on RV, UV-RV, and RV VLPs results in efficient internalization upon encountering the sialic acid residues abundant on the apical surface of gut epithelia (57), enabling intracellular detection of RV dsRNA. The identity of such sialylated proteins, and the question of whether they engage other proteins during RV internalization, remains elusive. Highlighting the difference in the way that free dsRNA would interact with epithelia, we note that in contrast to RV and UV-RV, which preferentially enter IEC and activate anti-viral signaling when applied to the apical surface, uptake of poly(I:C) and subsequent signaling was much greater when administered to the basolateral surface (40). Following RV entry, its RNA could be recognized by endosomal pattern-recognition receptors (PRR) such as TLR3 or by cytosolic detectors of dsRNA such as RIG-I and Mda-5. Protein kinase R (PKR) is known to play a role in RV-induced gene expression but whether PKR is a true dsRNA receptor or is purely a participant in the dsRNA-activated signaling cascade remains unclear.

In conclusion, inactivation of the RV genome does not ablate but rather augments its induction of anti-viral signaling in IEC. These findings parallel observations made by us and others that treatment of epithelial cells with heat-killed bacteria and/ or purified

bacterial components induce greater activation of innate immune signaling than live bacteria. Such a finding supports the general paradigm that innate immune responses are largely driven by host PRR and do not require viable microbes *per se*, but rather, anti-microbial responses occur despite the complex mechanisms employed by microbes to suppress them. Consequently, inactivated microbes and/ or their components might not only be useful for eliciting adaptive immune responses to protect against future infection, but may also help an infected host overcome the microbial innate immune suppression that can hinder pathogen clearance.

MATERIALS AND METHODS

Reagents

Rabbit anti-RV sera to purified rhesus rotavirus was prepared as described previously (62). Mab to VP6, clone 6E7, was previously described (63). Antibodies to total and phosphorylated STAT1, IRF3 and PKR were obtained from Cell Signaling Technology (Beverly, MA). Total IRF7 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to human interferon alpha and beta (anti-IFN α/β) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Reference Reagent Laboratory through ATCC (Manassas, VA). Anti-β-actin and psoralen AMT (4'-aminomethyl-4,5', 8-trimethylpsoralen) were purchased from Sigma-Aldrich (St. Louis, MO). The ELISA kit used to assay IFN-β was obtained from PBL Biomedical Laboratories (Piscataway, NJ). IL-8 was assayed via R&D systems Duoset IL-8 reagents (Minneapolis, MN).

Cell culture

Human intestinal epithelial cells (HT29), herein referred to as "IEC," were cultured as previously described on both standard tissue-culture plastics or collagen-coated permeable supports to result in polarized IEC (64).

Preparation of rotavirus and components

Rhesus RV (RRV) was propagated in MA104 cells as previously described (65). Virus was prepared in bulk, aliquoted, and stored at –80°C until use. To prepare UV-inactivated RV (UV-RV), aliquots of RRV were pre-treated with 40 µg/ml psoralen AMT and then irradiated by long-wave UV-light (365 nm) for two hours as previously described (58). RV virus-like particles (VLPs) were isolated from Sf9 cells that had been infected with baculovirus recombinants expressing cDNAs encoding RV Rf VP2, and SA11 VP4, VP6, and VP7 as previously described (27). RV RNA was extracted from purified virions as previously described (66, 67).

Cell treatment with RV and its components

Prior to infection, RV, UV-RV, RV VLPs and RV RNA were diluted in serum-free medium (SFM) and incubated with 10 µg/ml trypsin (Mediatech, Inc., Manassas, VA), except where indicated otherwise, for 30 min in a 37^{o}C water bath. Control samples were treated with an equivalent amount of trypsin diluted in SFM (Mock) or SFM alone. Where indicated, neutralizing antibodies to human interferon alpha and beta (anti-IFN α/β) (1:100) were added to some preparations of trypsinized RV prior to infection. IEC, HT29 cells, were grown to 90-100% confluence in 6 well plates or collagen-coated permeable supports, washed several times with SFM, and inoculated with virus, viral components, or mock controls for 1 h at 37^{o}C/ 5% $C0_2$ to allow for adsorption. Following adsorption, cells were washed again several times with SFM and then incubated with 2 µg/ml trypsin in SFM for 0-48 hours post-inoculation (hpi). Cells

stimulated with RV in the presence of anti-IFN α/β were treated with 2 μg/ml trypsin in SFM plus the same concentration of Type 1 IFN antibodies used during viral adsorption. For experiments comparing RV VLPs and RNA, longer cell stimulation was required to allow enough time for RNA to enter cells; thus, following adsorption these cells were not washed with SFM and instead components were retained in the presence of 2 μg/ml trypsin for up to 24 h. At the indicated time points, supernatants were collected and stored at -20^{o}C for IL-8/ IFN-β ELISA. Cells were washed several times with PBS and resuspended in radioimmunoprecipitation assay II buffer (RIPA II) (20mM Tris-HCl, 2.5 mM EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1 % SDS, 50 mM NaF, 10 mM $Na_2P_2O_7$, and 2 mM $NaV0_4$ plus protease inhibitor cocktail) for western blotting or TRIzol for RNA isolation and subsequent qRT-PCR/ microarray analysis.

Immunoblotting

At various time points (0-48 hpi), cells were rinsed several times in PBS, lysed in RIPA II buffer and cleared by centrifugation (10 min at 15, 000 x g, 4^{o}C). Total protein concentrations were estimated by BioRad Protein Assay. Cell lysates were assayed for anti-viral markers (IRF3, IRF7, STAT1 and PKR) by 12% SDS-PAGE immunoblotting and membranes were stripped and probed for β-actin as a loading control. Immunoblots were visualized with the ECL system (GE Healthcare, Piscataway, NJ).

ELISA

Supernatants were collected at different time points (0-48 hpi) and stored at -20^{o}C until use. Human IL-8 secretion in the supernatants was measured by the Duoset kit from R&D systems (Minneapolis, MN). The human IFN-β enzyme immunoassay kit from PBL Biomedical Laboratories (Piscataway, NJ) was used for the quantification of IFN-β in the supernatants, according to the manufacturer's instructions.

Microarray analyses

Cells were exposed to RV or the indicated component for 24 h, washed several times with PBS, resuspended in TRIzol, subjected to DNase I digestion and purified for RNA by using a commercially available RNeasy Minikit from Qiagen (Valencia, CA). RNA concentration was measured using spectrophotometry and quality was assessed by Agilent BioAnalyzer analysis. Microarray analyses were performed at the Emory Biomarker Microarray Core, where qualified RNA samples were reverse-transcribed, amplified, labeled, and used to probe human HT-12 chips purchased from Affymetrix, Inc. (Santa Clara, CA). Briefly, samples were assayed using a Molecular Devices Gene Pix (4100A) and raw fluorescence readings were processed by an algorithm designed to reduce spurious readouts of gene activation. Microarray data was quantile normalized using freely available scripts written in R (http://R-project.org). Significantly altered genes were identified using SAM (Significance of Analysis of Microarray) analyses and assessed by hierarchal clustering and principle component analysis using Spotfire

Decision Site for Functional Genomics software to determine relatedness of gene expression patterns resulting from cell stimulation by RV or the indicated component.

qRT-PCR

At the indicated time points, total RNA was extracted from cells using TRIzol reagent and reverse-transcribed using a commercial kit (TaqMan Reverse Transcription kit; PerkinElmer, Boston, MA) according to the manufacturer's directions. The RT cDNA reaction products were subjected to quantitative real-time quantitative PCR (qRT-PCR) (SYBR Green PCR Core kit; PerkinElmer, Boston, MA) with primers for IFN-β (Sense 5'-CTCTCCTGTTGTGCTTCTCC-3', Antisense 5'-GTCAAAGTTCATCCTGTCCTTG-3'), Mda-5 (Sense 5'-TCAGCCAAATCTGGAGAAGG-3', Antisense 5'-CTTCATCTGAATCACTTCCC-3'), STAT1 (Sense 5'- GTTAGACAAACAGAAAGAGC-3', Antisense 5'-TCTGTTGTGCAAGGTTTTGC-3'), OAS-2 (Sense 5'-CAACAAATGCTTCCTAGAGC-3', Antisense 5'- ACGAGATCGGCATCAGAGCC-3') (Invitrogen; Carlsbad, CA), and 18S ribosomal RNA (Sense 5'-CGGCTACCACATCCAAGGAA-3', Anti-sense 5'-GCTGGAATTACCGCGGCT-3') (PerkinElmer; Boston, MA) as previously described (68). Expression level of anti-viral genes was normalized to 18S rRNA levels of the same sample. Fold difference was the ratio of the normalized value of each sample to that of uninfected control cells. All PCR samples were performed in triplicate.

Confocal microscopy

IEC were grown to confluence on collagen-coated permeable supports, apically treated with the indicated stimuli, and fixed in 10% formalin for 15 min. Cells were washed 3X in PBS, permeabilized in 0.5% Triton X-100/ PBS for 10 min at RT, and blocked overnight in 3% BSA in PBS (4^{o}C). Cells were incubated for 1 h with polyclonal rabbit anti-RRV (1:10,000) or monoclonal mouse anti-VP6 (1:100) in blocking buffer, washed with PBS 3X, and probed with anti-rabbit and anti-mouse FITC secondary antibody in PBS (1:50) (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively, for 1 h at RT. Alexa-conjugated phalloidin stain (Alexa Fluor 633; Invitrogen, Carlsbad, CA) was used as a counterstain for F-actin and was included in the secondary antibody preparation at a dilution of 1:500. After staining with secondary antibody and phalloidin, cells were washed 3X in PBS and mounted on slides with fluorescent anti-fade medium (VectaShield; Burlingame, CA). Stained cell monolayers were examined using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M axiovert and ×63 or ×100 Pan-Apochromat oil lenses. Fluorescent dyes were imaged sequentially in frame-interlace mode to eliminate cross talk between channels. Images shown are representative of at least 3 experiments, with multiple images taken per slide.

Table I. Fold change induction of anti-viral markers relative to mock

	RV	UV-RV	RV + anti-IFN α/β
TLR3	1.5 ± 0.07	2.2 ± 0.37	1.13 ± 0.09
Mda-5	8.7 ± 1.2	11.1 ± 0.84	4.24 ± 0.71
RIG-I	3.7 ± 0.33	6.6 ± 0.39	2.41 ± 0.29
IRF3	1.3 ± 0.06	1.3 ± 0.06	1.16 ± 0.07
IRF7	5.9 ± 0.24	7.7 ± 0.90	3.92 ± 0.20
IFN-α	1.1 ± 0.06	1.5 ± 0.04	0.95 ± 0.08
IFN-β	11 ± 0.88	31.6 ± 4.4	6.24 ± 0.84
STAT1	10.5 ± 1.1	12.8 ± 0.85	4.42 ± 0.28
Mx1	35.6 ± 0.44	45.2 ± 3.9	12.3 ± 0.37
PKR	3.3 ± 0.18	3.3 ± 0.22	1.72 ± 0.18
OAS-2	43.6 ± 2.3	76.5 ± 4.2	11.6 ± 1.2
MHC I	11.6 ± 0.92	24.1 ± 1.7	4.1 ± 0.64
IL-8	60 ± 5.9	24 ± 2.2	58 ± 5.3
IFITM1	44.4 ± 1.9	112.6 ± 4.9	8 ± 0.92
IFITM3	28.9 ± 2.3	37.3 ± 2.9	6.4 ± 0.83
CXCL10	35.5 ± 3.7	108 ± 11.3	17.6 ± 2.3
ISG15	10.4 ± 3.0	15.4 ± 3.3	5.03 ± 0.90

Table I. Anti-viral gene expression in cells treated with RV, UV-RV, and RV + anti-IFN α/β. Intestinal epithelial **(**HT29) cell monolayers were stimulated with RV and UV-RV (MOI 1), RV (MOI 1) plus Type 1 IFN antibodies (anti-IFN α, anti-IFN β), and mock treatments as described in Figure 5. At 24 hpi, lysates were collected in TRIzol to assess global transcription of genes (Figure 5A), including anti-viral markers of interest as indicated above. mRNA expression of select anti-viral genes was confirmed via qRT-PCR analysis (Figure 5 B, C, D, E). Fold induction values greater than standard deviation calculations reflect statistically significant differences from mock ($P < 0.05$).

Table II. Genes induced by UV-RV, RV VLPs, and RV RNA relative to mock

	# genes	Avg. induction
UV-RV	401	1.5
RV VLPs	64	1.4
RV RNA lo	72	1.4
RV RNA hi	97	1.5

Table II. Cellular Gene Transcription Induced by UV-RV, RV VLP, and RV RNA. Intestinal epithelial **(**HT29) cell monolayers were stimulated for 24 h with UV-RV (MOI 0.5), RV VLPs (2.5 ug/ ml), and RV RNA at low (0.1 μg/ml) and high (1 μg/ml) concentrations (denoted as RV RNA "lo" and "hi," respectively). Mock-treated samples received equivalent amounts of trypsin in SFM. Experiments were performed in biological duplicates. Lysates were collected in TRIzol to assess mRNA expression via microarray as described in text. The number of genes induced by UV-RV, RV VLPs and RV RNA with > 1.3 fold change is shown.

Table III. Fold change induction of anti-viral markers relative to mock

	UV-RV	RV VLPs	RV RNA lo	RV RNA hi
Mda-5	1.8 ± 0.18	1.4 ± 0.21	1.2 ± 0.03	1.4 ± 0.00
IFN-β	6.4 ± 0.12	1.2 ± 0.06	1.1 ± 0.02	1.3 ± 0.21
MHC I	1.7 ± 0.14	1.2 ± 0.02	1.1 ± 0.03	1.3 ± 0.12

Table III. Antiviral Gene Expression in Cells Treated with UV-RV, RV VLPs and RV RNA. Cells were stimulated with UV-RV, RV VLPs, RV RNA and mock treatments as described in Table 2. After 24 h, lysates were collected in TRIzol to assess global transcription of genes (depicted in Table 2), including anti-viral markers of interest as indicated above. mRNA expression of select anti-viral genes was confirmed via qRT-PCR analysis (Figure 6 D, E). Fold induction values greater than standard deviation calculations reflect statistically significant differences relative to mock ($P < 0.05$).

Figure 1.

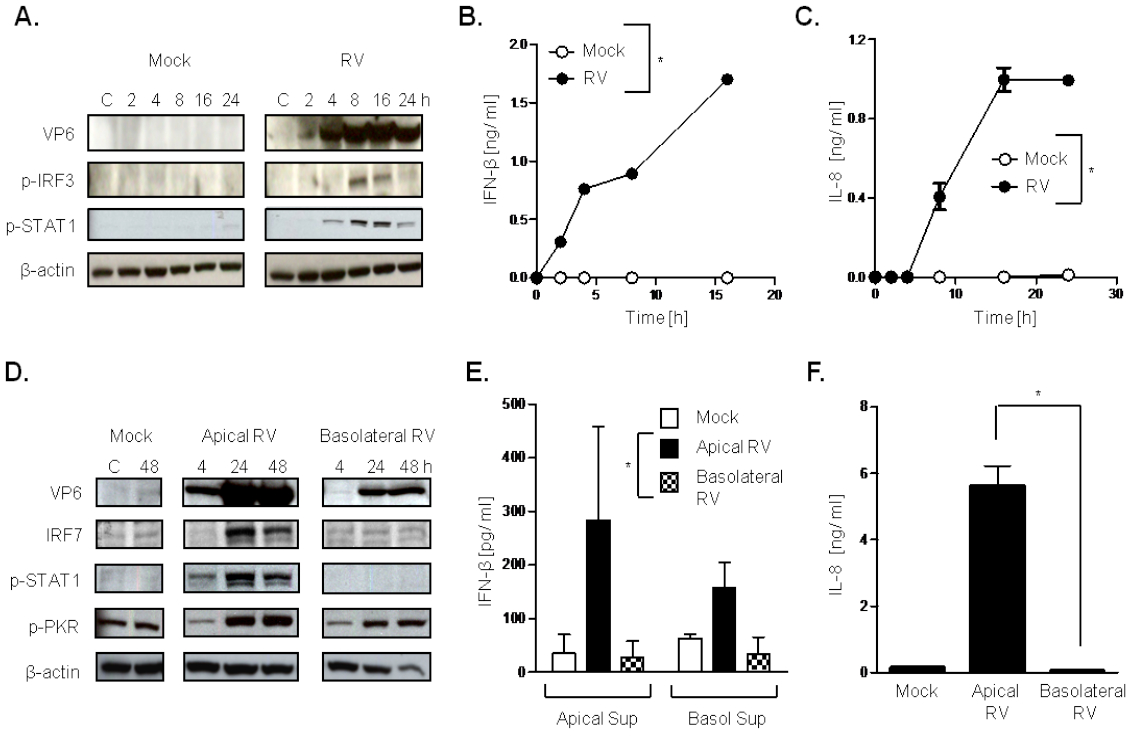

A.
Mock
RV
C 2 4 8 16 24
C 2 4 8 16 24 h
VP6
p-IRF3
p-STAT1
β-actin
B.
Mock
RV
IFN-β [ng/ml]
Time [h]
C.
Mock
RV
IL-8 [ng/ml]
Time [h]
D.
Mock
Apical RV
Basolateral RV
C 48
4 24 48
4 24 48 h
VP6
IRF7
p-STAT1
p-PKR
β-actin
E.
Mock
Apical RV
Basolateral RV
IFN-β [pg/ml]
Apical Sup
Basol Sup
F.
IL-8 [ng/ml]
Mock
Apical RV
Basolateral RV

Figure 2.

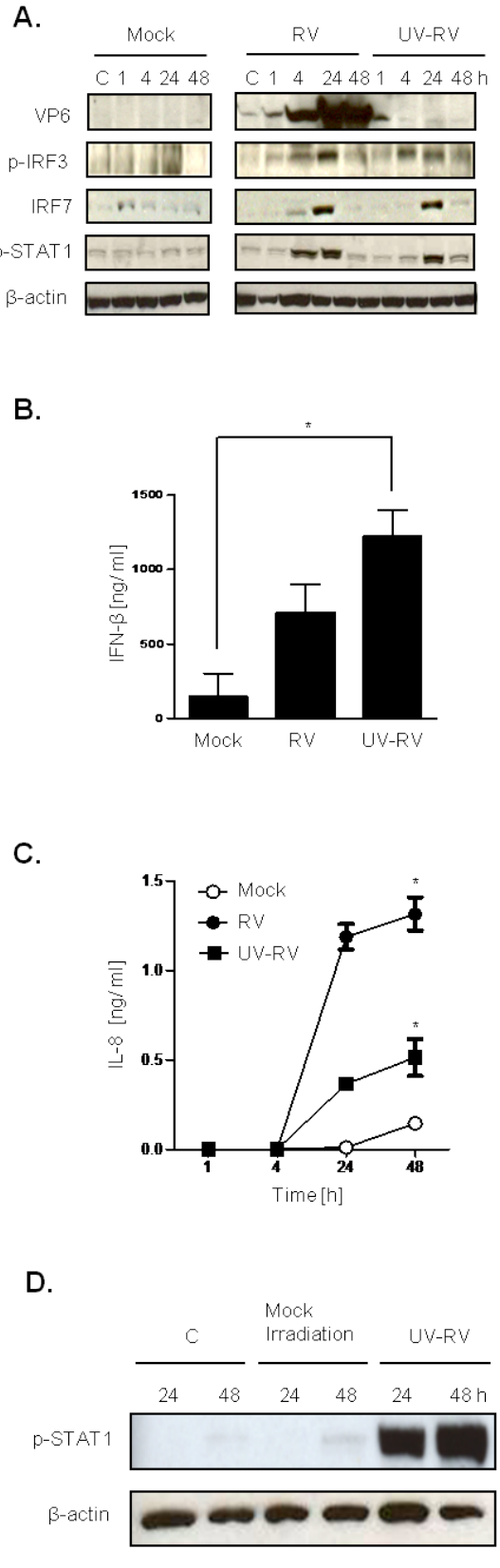

A.
Mock
RV
UV-RV
C 1 4 24 48
C 1 4 24 48 1 4 24 48 h
VP6
p-IRF3
IRF7
p-STAT1
β-actin
B.
*
1500
1000
500
0
IFN-β [ng/ml]
Mock
RV
UV-RV
C.
Mock
RV
UV-RV
1.5
1.0
0.5
0.0
IL-8 [ng/ml]
*
*
1
4
24
48
Time [h]
D.
C
Mock Irradiation
UV-RV
24
48
24
48
24
48 h
p-STAT1
β-actin

Figure 3.

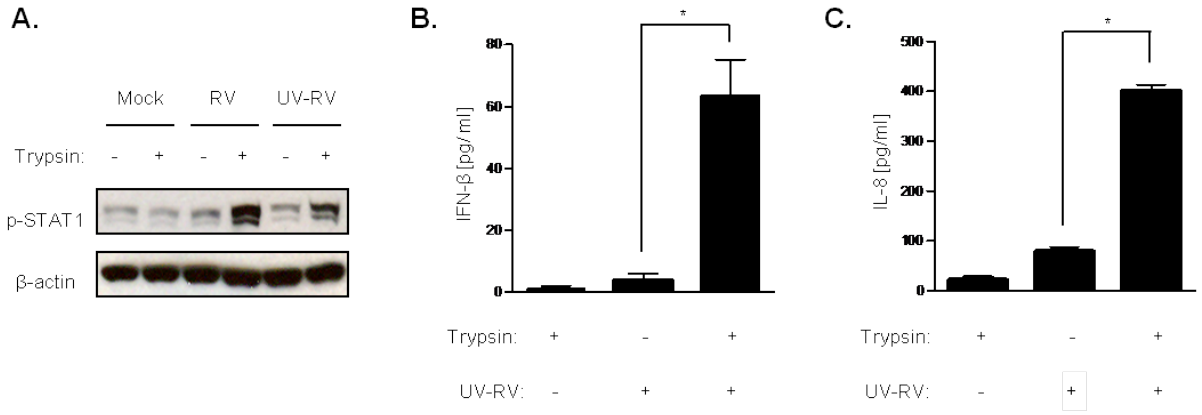

A.
Mock
RV
UV-RV
Trypsin: - + - + - +
p-STAT1
β-actin
B.
IFN-β [pg/ml]
80
60
40
20
0
*
Trypsin: + - +
UV-RV: - + +
C.
IL-8 [pg/ml]
500
400
300
200
100
0
*
Trypsin: + - +
UV-RV: - + +

Figure 4.

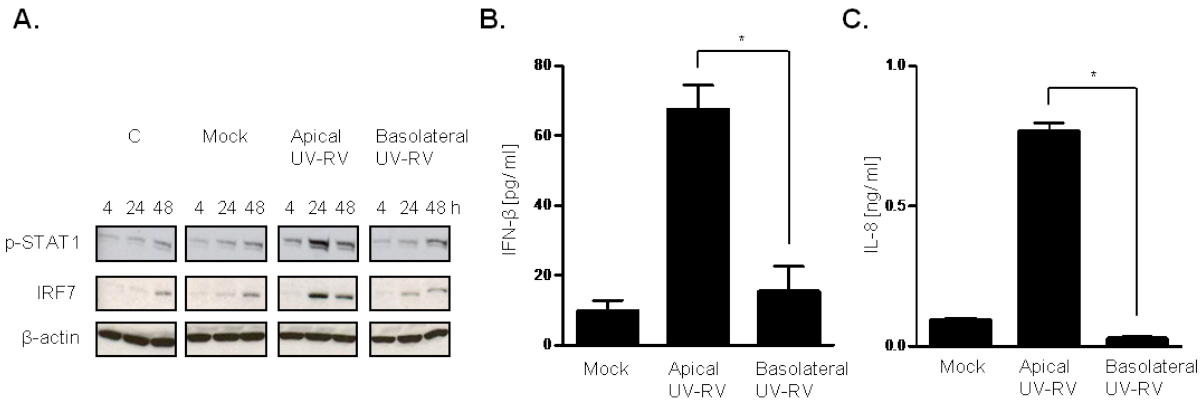

A.
C
Mock
Apical UV-RV
Basolateral UV-RV
4 24 48 4 24 48 4 24 48 4 24 48 h
p-STAT1
IRF7
β-actin
B.
IFN-β [pg/ml]
80
60
40
20
0
*
Mock
Apical UV-RV
Basolateral UV-RV
C.
IL-8 [ng/ml]
1.0
0.5
0.0
*
Mock
Apical UV-RV
Basolateral UV-RV

Figure 5.

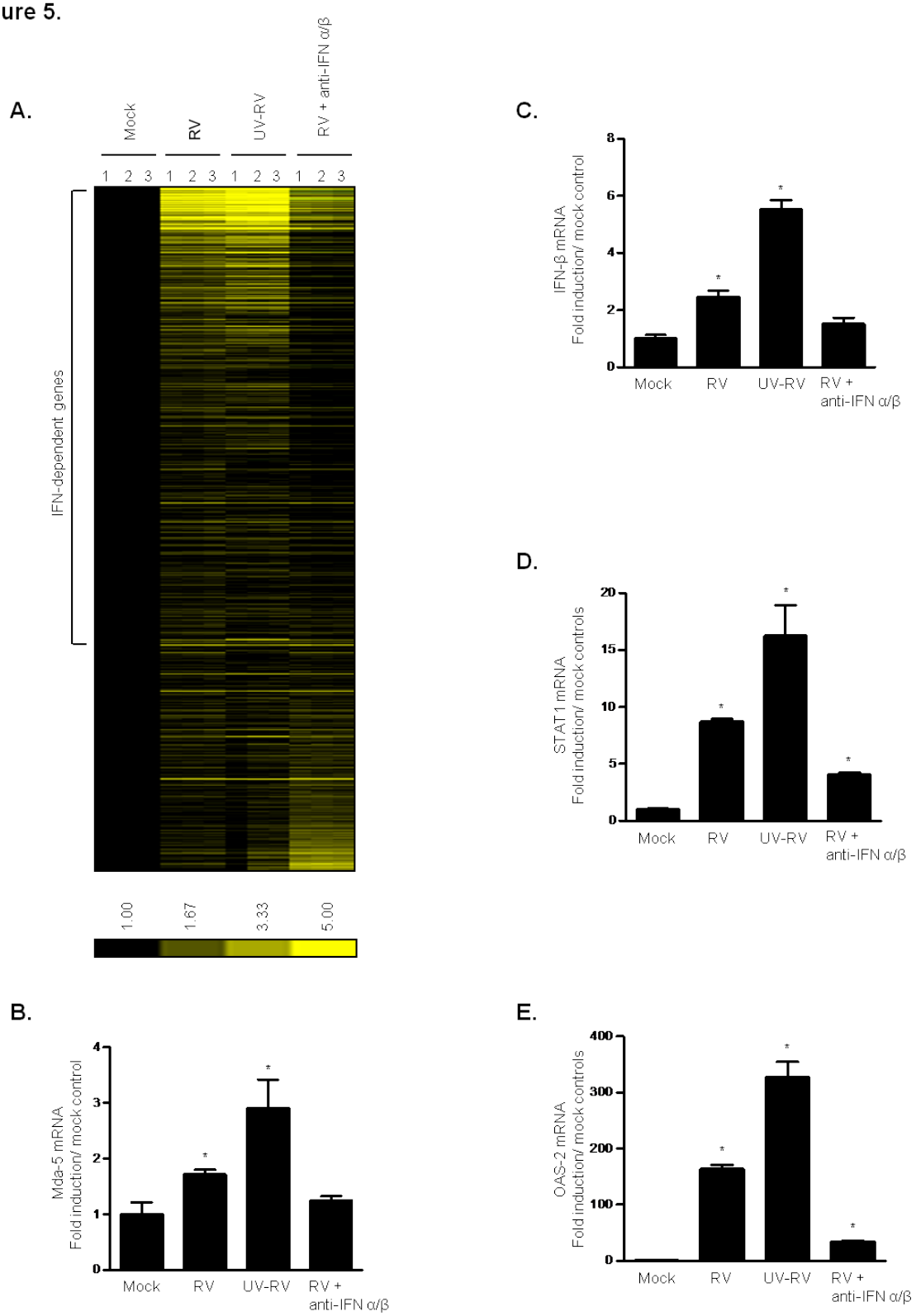

A.
Mock
RV
UV-RV
RV + anti-IFN α/β
1 2 3 1 2 3 1 2 3 1 2 3
IFN-dependent genes
1.00
1.67
3.33
5.00
B.
Mda-5 mRNA
Fold induction/ mock control
Mock
RV
UV-RV
RV + anti-IFN α/β
C.
IFN-β mRNA
Fold induction/ mock control
Mock
RV
UV-RV
RV + anti-IFN α/β
D.
STAT1 mRNA
Fold induction/ mock controls
Mock
RV
UV-RV
RV + anti-IFN α/β
E.
OAS-2 mRNA
Fold induction/ mock controls
Mock
RV
UV-RV
RV + anti-IFN α/β

Figure 6.

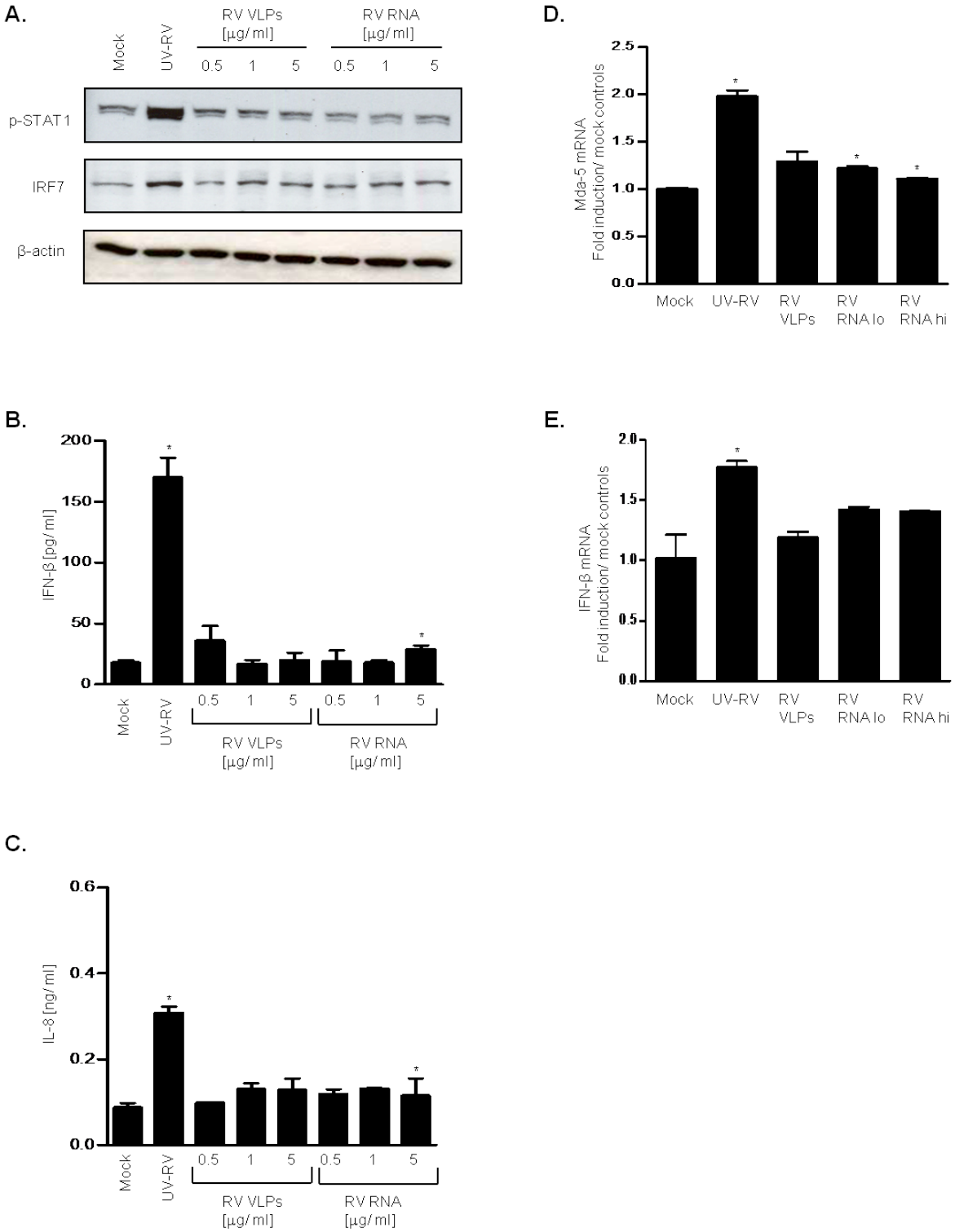
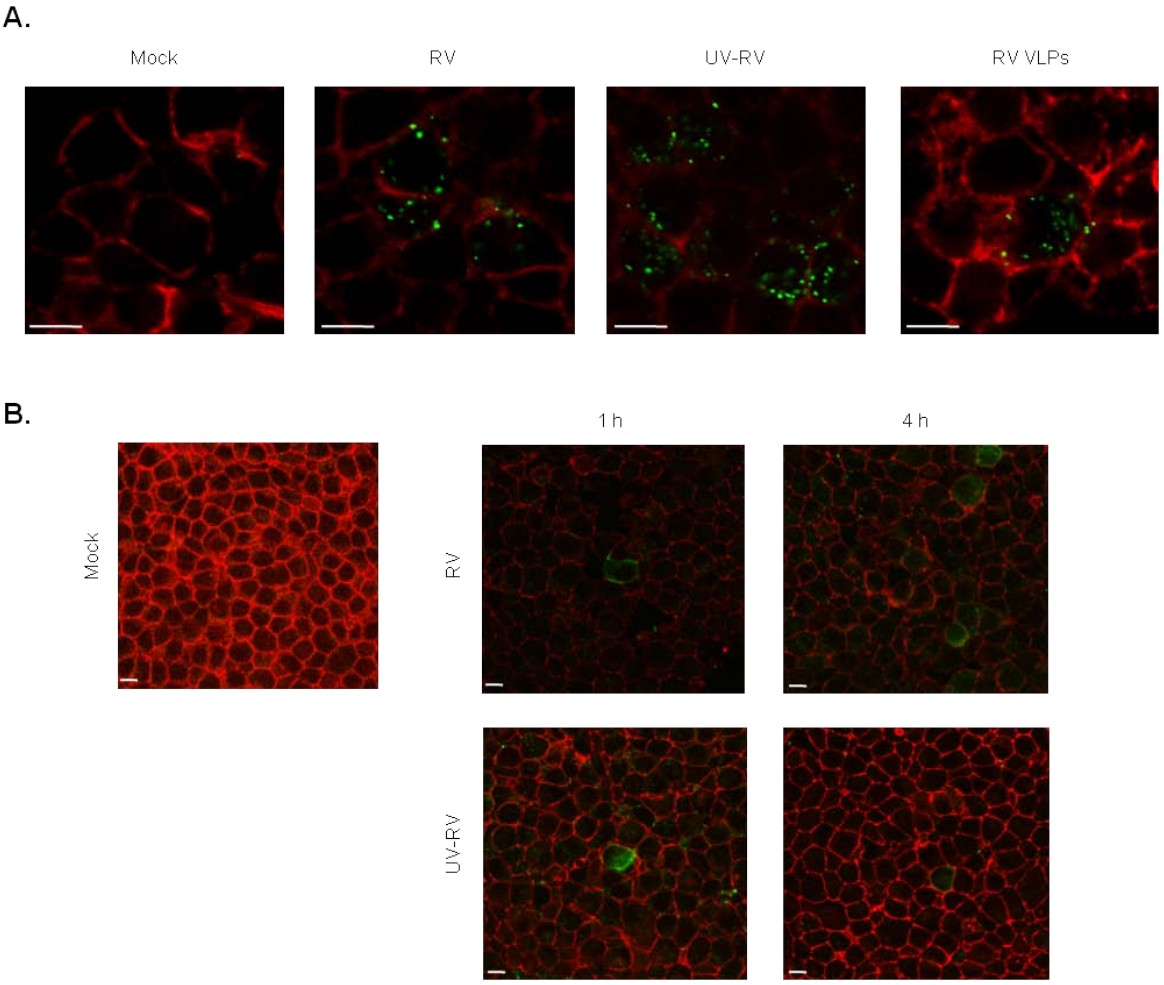
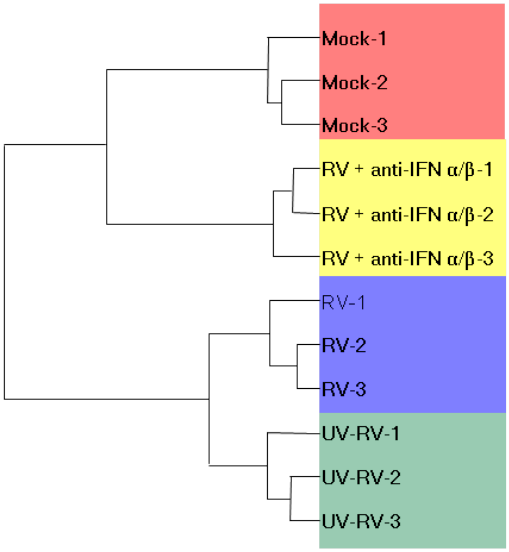

A.
Mock
UV-RV
RV VLPs [μg/ml]
RV RNA [μg/ml]
0.5 1 5 0.5 1 5
p-STAT1
IRF7
β-actin
D.
Mda-5 mRNA Fold induction/ mock controls
2.5
2.0
1.5
1.0
0.5
0.0
Mock
UV-RV
RV VLPs
RV RNA lo
RV RNA hi
B.
IFN-β [pg/ml]
200
150
100
50
0
Mock
UV-RV
0.5 1 5 0.5 1 5
RV VLPs [μg/ml]
RV RNA [μg/ml]
E.
IFN-β mRNA Fold induction/ mock controls
2.0
1.5
1.0
0.5
0.0
Mock
UV-RV
RV VLPs
RV RNA lo
RV RNA hi
C.
IL-8 [ng/ml]
0.6
0.4
0.2
0.0
Mock
UV-RV
0.5 1 5 0.5 1 5
RV VLPs [μg/ml]
RV RNA [μg/ml]

Figure 7.

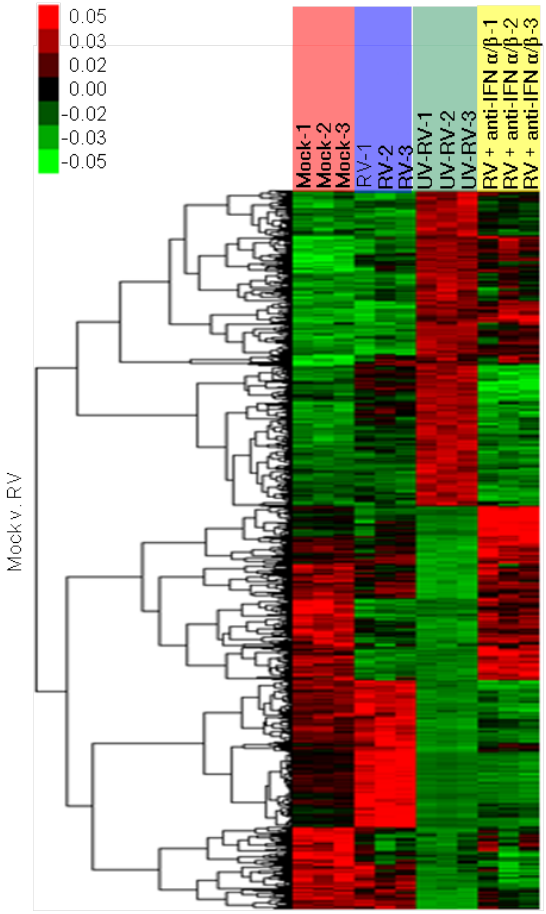


Supplemental Figure 1

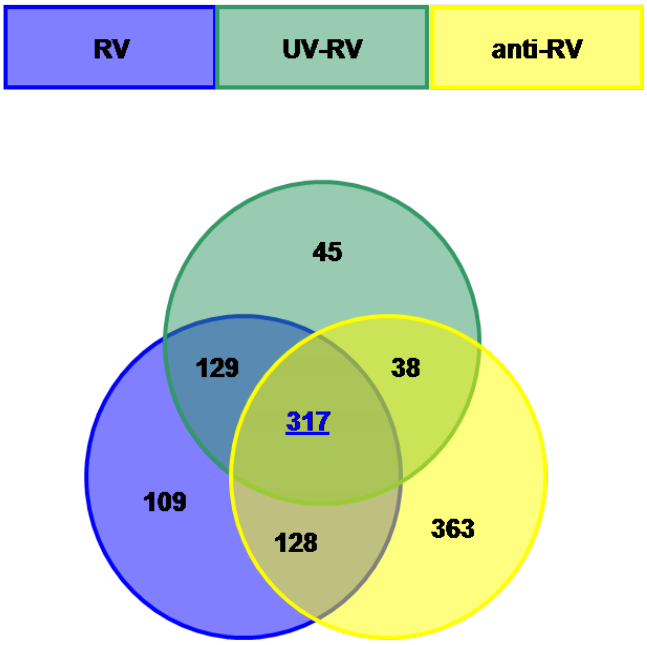
A.



B.



C.



SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGEMENTS

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DISCLOSURE

The authors declared no conflicts of interest.

FIGURE LEGENDS

Figure 1. Anti-viral protein expression in RV-infected intestinal epithelia. Human intestinal epithelia (HT29) were grown to confluence in 6 well plates (A, B, C) or collagen-coated permeable supports (D, E, F) and infected with RV (MOI 0.5-1). Control samples were treated with trypsin diluted in SFM (Mock) or SFM alone (C). Cell lysates and supernatants were collected 0–48 hours post-inoculation (hpi). Western blot analyses were performed to assess viral protein (VP6) synthesis and protein expression of the indicated anti-viral markers in cell lysates (A, D). ELISA assays were used to measure IFN-β (B, E) and IL-8 (C, F) secretion in supernatants over time. Data in A, B, D, and E are results of a single experiment and representative of 3 separate experiments that gave similar results. Data in C and F reflects the mean +/- standard error of the mean (SEM) of 3 parallel experiments. Statistically significant differences $P < 0.05$ are denoted as starred values (*).

Figure 2. Anti-viral protein expression exhibited in RV-infected and UV-RV stimulated epithelia. Confluent intestinal epithelia (HT29) were grown in 6 well plates and treated with RV and UV-RV (MOI 1). Control samples were exposed to trypsin diluted in SFM (Mock), irradiated cellular debris from a mock preparation of UV-RV (Mock Irradiation), or SFM alone (C). Cell lysates and supernatants were collected at various time points (0–48 hpi). Western blot analyses were performed to assess viral protein (VP6) synthesis and protein expression of the indicated anti-viral markers in cell lysates (A, D). ELISA assays were used to measure secretion of IFN-β (B) and IL-8 (C) in supernatants at 48 hpi. Data in A, B and D are results of a single experiment and

representative of 3 separate experiments that gave similar results. Data in C is the mean +/- SEM of 3 parallel experiments. Statistically significant differences $P< 0.05$ are denoted as starred values (*).

Figure 3. Anti-viral protein expression exhibited in epithelia treated with RV and UV-RV in the presence or absence of trypsin. Intestinal epithelial monolayers (HT29) grown in 6 well plates were treated with RV and UV-RV (MOI 1) in the presence or absence of trypsin (24 hpi). Control samples received equivalent amounts of trypsin diluted in SFM (Mock) or SFM alone. Trypsin and trypsin-free treatments are denoted as (+) and (-) symbols, respectively. Western blot analysis was used to detect anti-viral gene expression in cell lysates (A). ELISA assays were performed to measure secretion of IFN-β (B) and IL-8 (C) in supernatants at 24 hpi. Data in A shows results of a single experiment and is representative of 3 separate experiments that gave similar results. Data in B and C is the mean +/- SEM of 3 parallel experiments. Statistically significant differences $P< 0.05$ are denoted as starred values (*).

Figure 4. Anti-viral protein expression exhibited in epithelia treated apically and basolaterally with UV-RV. Intestinal epithelial monolayers (HT29) were grown on collagen-coated permeable supports and infected either apically or basolaterally with UV-RV (MOI 1). Control samples received equivalent amounts of trypsin diluted in SFM (Mock) or SFM alone (C). Cell lysates and supernatants were collected at various time points (0–48 hpi). Western blot analyses were performed to assess protein expression of the indicated anti-viral markers in cell lysates (A). ELISA assays were used to measure

IFN-β (B) and IL-8 (C) secretion in supernatants at 48 hpi. Data in A shows results of a single experiment and is representative of 3 separate experiments that gave similar results. Data in B and C reflects the mean +/- SEM of 3 parallel experiments. Statistically significant differences $P < 0.05$ are denoted as starred values (*).

Figure 5. Transcription profiles of epithelia stimulated with RV, UV-RV, and RV in the presence of Type 1 IFN (α/β) antibodies. Intestinal epithelial **(**HT29) cell monolayers were grown in 6 well plates and infected with RV and UV-RV (MOI 1), and RV (MOI 1) plus Type 1 IFN antibodies (anti-IFN α/β). Control samples received equivalent amounts of trypsin diluted in SFM (Mock). Experiments were performed in biological triplicates. At 24 hpi, cell lysates were extracted for RNA and microarray analyses were performed to assess global transcription of genes. Heat map illustration of genes induced by RV and UV-RV with > 1.3 fold change relative to mock (A). qRT-PCR results used to confirm mRNA synthesis of select anti-viral genes at 24 hpi (B, C, D, E). Data in A shows results of 3 parallel experiments. Data in B, C, D, and E reflects the mean +/- SEM of 3 parallel experiments. Statistically significant differences $P < 0.05$ are denoted as starred values (*).

Figure 6. Anti-viral protein expression exhibited in epithelia treated with UV-RV, RV VLPs and RV RNA. Confluent intestinal epithelia (HT29) were grown in 6 well plates and treated with UV-RV (MOI 0.5-1), RV RNA (0.5-5 μg/ml), and VLPs (0.5–5 μg/ml). Control samples received equivalent amounts of trypsin diluted in SFM (Mock). Cell lysates and supernatants were collected at various time points (0–24 hpi). Western

blot analyses were used to detect anti-viral gene expression in cell lysates (A). ELISA assays were performed to measure IFN-β (B) or IL-8 (C) secretion in supernatants at 24 hpi. qRT-PCR analyses were utilized to confirm mRNA synthesis of select anti-viral genes from microarray experiments (see Table III). Data in A are results of a single experiment and representative of 3 separate experiments that gave similar results. Data in B, C, D, and E shows the mean +/- SEM of 3 parallel experiments. Statistically significant differences $P < 0.05$ are denoted as starred values (*).

Figure 7. RV, UV-RV, and VLP cell entry during early stages of infection. Intestinal epithelial monolayers (HT29) were grown on collagen-coated permeable supports and treated apically with RV and UV-RV (MOI 10), and an amount of VLPs roughly equivalent to the estimated protein concentration in the RV preparation. Control samples were apically treated with an equivalent amount of trypsin in SFM (Mock) for 4 hpi. At 1 and 4 hpi, cells were fixed, stained and examined via confocal fluorescence microscopy for presence of rotaviral proteins (green) and F-actin (red) in the sub-apical region of the cells (3 μm below the apical surface). Sub-apical images of cells stimulated with RV, UV-RV, and RV VLPs at 1 hpi, magnification 60X (A). Sub-apical images of cells treated with RV and UV-RV at 1 and 4 hpi, magnification 40X (B). Data in A and B are results of a single experiment and representative of 3 separate experiments. Scale reflects distance of 10 μm.

Supplemental Figure 1. As described in Figure 5, confluent intestinal epithelia **(**HT29) were infected with RV and UV-RV (MOI 1), and RV (MOI 1) plus Type 1 IFN

antibodies (anti-IFN α/β) for 24 hpi. Control samples received equivalent amounts of trypsin diluted in SFM (Mock). Experiments were performed in biological triplicates. Cell lysates were extracted for RNA and microarray analyses were performed to assess global transcription of genes. Microarray data was subjected to an unsupervised clustering analysis. Dendogram and Venn diagram analyses were performed to reflect high reproducibility among replicates and degree of gene overlap between the experimental conditions, respectively (A, C). Heat map illustration depicts data in a standard format (B).

CHAPTER 3:

Rotavirus-induced IFN-β promotes anti-viral signaling, apoptosis and viral replication in intestinal epithelial cells

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Key Words: STAT-1, IRF7, PKR, PARP

Abbreviations: RV: rotavirus, IEC: intestinal epithelial cell, IRF: Interferon regulatory factor, STAT: signal transducer and activator of transcription, PKR: protein kinase R, PARP: Poly (ADP-ribose) polymerase, 2AP: 2 amino-purine

ABSTRACT

Rotavirus (RV), a leading cause of diarrhea, primarily infects intestinal epithelial cells (IEC). RV-infected IEC produce IFN-β and express hundreds of IFN-dependent genes. We thus hypothesized that type 1 IFN plays a key role in helping IEC limit RV replication and/or protect against cell death. To test this hypothesis, we examined IEC (HT29 cells) infected with RV (MOI 1) ± neutralizing antibodies to IFN α/β via microscopy and SDS-PAGE immunoblotting. We hypothesized neutralization of IFN would be clearly detrimental to RV-infected IEC. Rather, we observed that blockade of IFN function rescued IEC from the apoptotic cell death that otherwise would have occurred 24-48 h following exposure to RV. This resistance to cell death correlated with reduced levels of viral replication, particularly at early times (< 8h) following infection and eventuated in reduced production of virions. The reduction in RV replication that resulted from IFN neutralization correlated with, and could be recapitulated by blockade of IFN-induced PKR activation, suggesting involvement of this kinase. These results suggest non-mutually exclusive possibilities that IFN signaling is usurped by RV to promote replication and induction of cell death may be a means by which IFN signaling possibly clears RV from the intestine.

INTRODUCTION

Rotaviruses (RV) are the leading cause of severe dehydrating diarrhea in young children worldwide, causing up to 100 million cases and > 600,000 deaths annually (19, 45). Part of the *Reoviridae* family of dsRNA viruses, rotaviruses primarily infect epithelial cells of the small intestine, causing self-limiting illness that is typically cleared within 7 days (14, 19, 29). While RV infection is associated with B and T cell responses that help assure complete viral clearance and protect against re-infection (14), mice lacking adaptive immunity maintain considerable control, and sometimes complete clearance, of the virus, indicating that innate immunity is important for controlling this viral pathogen (30, 31). Given that IEC are the main cell type targeted by RV *in vivo* and the first line of defense against pathogens in the gut in general (2, 16), it seems likely that IEC play a role in mediating innate immunity to RV. Like most viral infections of cells, RV infected IEC produce type I IFN. Moreover, a substantial portion of the overall gene expression elicited in RV-infected IEC was shown to be dependent on type 1 IFN signaling in that induction was reduced by neutralizing antibodies to type 1 IFN (69). Additionally, Type 1 IFN levels have also been shown to increase in RV-infected humans and animals (52).

That RV-induced epithelial gene expression exhibits substantial dependence upon type IFN is in accordance with the broad role played by IFN in anti-viral innate immunity (32). Type 1 IFN, produced in an autocrine or paracrine manner, activates Jak/STAT signaling pathways which induce expression of hundreds of anti-viral genes, also known as interferon-stimulated genes (ISGs). ISGs combinatorially function to limit viral replication and spread in infected and neighboring uninfected cells (32, 41-43). One of

these ISGs, IRF7 (interferon regulatory factor 7), is a transcription factor which serves to promote further type 1 IFN production (42). Another ISG, PKR (protein kinase R), serves as a sensor of viral dsRNA and may facilitate viral clearance via promotion of apoptosis (32, 70). Such broad anti-viral action of type I IFNs are in accordance with observations that interference with type I IFN signaling greatly impairs the ability of mice to clear several classes of viruses (34), particularly in mice lacking adaptive immunity (71). In contrast, mice lacking type I IFN exhibit relatively normal clearance of RV even upon a Rag-/- background (20) making the role of RV-induced type I signaling unclear (20, 72). On the other hand, pre-treatment of mice with type I IFN can reduce diarrhea *in vivo* (50, 51) and limit rotavirus infection *in vitro* (33). Although studies in RV-infected mice show that loss of type 1 IFN receptors does not alter diarrhea or rate of viral clearance (72), STAT1 deficiency correlates with increased viral shedding in feces during RV infection (34). The fact that RV has adopted strategies for thwarting type 1 IFN responses and thus increasing infectivity, such as by encoding NSP1 proteins which dampen type 1 IFN production (35, 36) or preventing STAT1 accumulation in the nucleus (73), also supports the notion that type 1 IFNs pose a significant threat to RV.

We recently reported that the structural components of RV induce type 1 IFN in IEC, suggesting that RV-induced type 1 IFN responses are activated via PRR-mediated pathways with a likely role for recognition of viral nucleic acids (69). Here, we sought to determine the role of RV-induced type 1 IFN in affecting the outcome of the RV-IEC interaction. We observed that neutralizing the type 1 IFN response resulted in a dramatic impairment of anti-viral signaling. Surprisingly, such ablation of antiviral signaling reduced viral replication and prevented RV-induced IEC cell death. Taken together, our

data provides insight into how epithelial type 1 IFN-mediated responses contribute to anti-rotaviral immunity and also reveals a new mechanism by which RV exploits these processes to enhance pathogenicity.

RESULTS

RV-induced type 1 IFNs elicit IEC anti-viral signaling and apoptosis

Infection of intestinal epithelial cells (IEC) with rotavirus (RV) results in a substantial remodeling of IEC gene expression with significant induction of over 1000 genes (39, 69). The majority of such RV-induced gene expression in IEC is dependent upon type I IFN in that it was blocked by neutralizing antibodies to IFN-α and IFN-β (69). Here, we observed that such neutralization of type I IFN also reduced RV-induced phosphorylation of STAT-1 and PKR and synthesis of IRF7 that followed RV infection in IEC (Figure 1A), in further accordance with the notion that type I IFN plays a key role in the IEC response to RV (33, 39, 69). To determine the relative roles of IFN-β or IFN-α in the RV-induced IEC response, we examined these signaling events in the presence of upon selective antibody to IFN-α or IFN-β. We observed that the effect of adding both antibodies together was largely mimicked by antibody to IFN-β while antibody to IFN-α was without significant effect (Figure 1B). The failure of anti-IFN-α to block RV-induced responses did not reflect inability of IEC to respond to IFN-α nor the ability of the antibody to neutralize its target as IEC responded to recombinant IFN-α and the response was completely neutralized by anti-IFN-α (Figure 1C). Rather, the inability of anti-IFN-α to affect RV-induced signaling seemed to simply reflect the lack of a role for IFN-α in this infection as qRT-PCR did not detect any induction of expression in the IFN-α gene in response to RV infection (data not shown). Conversely, the blockade of RV-induced signaling by antibody to IFN-β likely shows a role for RV-induced IFN-β

expression rather than a non-specific action of the antibody, as this antibody effectively neutralized the action of IFN-β and did not have a marked effect on activity of recombinant IFN-α (Figure 1D). Thus, IFN-β mediates a significant portion of RV-induced signaling in IEC.

In light of the prominent role of type I IFN in mediating RV-induced signaling and gene expression in IEC, we expected that suppression of type I IFN activity might result in increased viral replication and/or impair the ability of IEC to withstand the cytotoxic effects of the virus, which, under the conditions used here, normally become apparent around 24-48 h following initiation of infection (Figure 2). Neutralization of type I IFN did not cause a significant increase in RV levels as assessed by levels of the viral protein VP6 (Figure 1 A, B). Rather, the most striking effect of IFN neutralization was that it prevented the loss of cells from the culture plate that otherwise occurred by 48 hpi in response to RV infection (Figure 2A). Closer examination of these cells under an inverted light microscope revealed that RV-infected epithelia exhibited altered shape, membrane fusion, and cell lysis that was also absent when type I IFN was neutralized. It has been observed that one means by which type 1 IFN impedes viral infection is via induction of apoptosis in infected and neighboring uninfected cells (32). Thus, we sought to determine the extent to which the effects of IFN neutralization in preventing IEC loss correlated with effects on IEC apoptosis. Again, IEC were infected with RV alone or RV + anti-IFN α/β and then examined at 0-48 hpi for evidence of apoptosis. First, we measured levels of cleaved Poly (ADP-ribose) polymerase (PARP), a downstream substrate of the caspase-3 signaling pathway, which was selected as a marker for apoptosis after verification in separate control experiments that it could be readily

detected in IEC treated with staurosporine, a potent inducer of apoptosis (74) (data not shown). RV infection induced PARP cleavage that was prevented by type I IFN neutralization (Figure 2B). Next, we assessed apoptosis via TUNEL assay. Similarly, apoptosis of RV-infected IEC also appeared to occur in an IFN-dependent manner (Figure 2C). Together, these results support the notion that RV-induced changes in cell morphology are part of an apoptotic process and suggest that type IFN promotes IEC apoptosis in response to RV infection.

RV exploits epithelial type 1 IFN responses to promote viral replication and spread

In some viral infections, type I IFN does not alter replication in infected cells *per se* but rather reduces total viral loads by preventing the spread of viral infection from infected to uninfected cells (35, 75). One means by which type I IFN might prevent viral spread is by promoting apoptosis thus preventing viruses from using cellular machinery for assembly and release (32). Thus, to better understand the role of type I IFN in such a context, we next examined the consequences of blocking IFN activity in RV-infected IEC using methods that allowed us to assess the relative amount of cells that contained virus. Specifically, IEC were exposed to RV in the absence or presence of anti-IFN α/β for 0-24 hpi and the presence of RV was assayed via immuno-fluorescence microscopy. This technique afforded detection of a small but easily observable population of RV infected cells 4 hours following exposure to the virus (Figure 3A). The number of infected cells increased markedly by 8 hpi, and increased slightly further by 24 hpi. Contrary to our original hypothesis that neutralization of IFN would increase viral spread, we observed

that blocking type I IFN signaling markedly reduced the number of infected cells at 4 and 8 hpi; however, greater numbers of infected cells could be seen by 24h. These findings were further supported by quantitation of fluorescence via NIH image J analysis (Table 1). The reduced level of viral proteins observed via immuno-fluorescence at 4h was also seen in western blot of cell lysates (Figure 3B) generated in parallel and was in accordance with results of Figure 1. The lack of a consistent increase in viral protein synthesis observed via western blotting at 24-48h could possibly be due to a difference in number of infected cells vs. total level of viral antigens, or, reflect that the lysates were subjected to greater dilution prior to analysis in order to normalize the protein levels of the samples (since cell loss was greatly reduced by neutralization of type 1 IFN, as described in Figure 2). We also assessed levels of viral antigens in cell-free supernatants that were released by RV-infected epithelia by western blot and ELISA, which was done without normalizing level of total protein (Figure 3B and Table 2, respectively). Neutralization of type I IFN reduced the level of RV antigens released into the supernatant. Together, these results indicate that blockade of type I IFN signaling reduces viral replication, particularly during early stages of infection. In addition, lack of type 1 IFN activity also correlates with reduced IEC apoptosis and consequently greater numbers of surviving infected cells.

We next sought to begin to investigate the mechanism by which blockade of type I IFN reduced RV replication in IEC. In considering candidate mechanisms, we noted that one of the kinases whose activation was IFN-dependent, namely PKR, has been shown to correlate with increased infectivity of reoviruses, which belong to the same *Reoviridae* family of dsRNA viruses as rotavirus (76, 77). Thus, we investigated whether

RV might, similar to reovirus, exploit type 1 IFN signaling to promote PKR-dependent replication. Epithelial cells were infected with RV or RV + 2AP (2 amino-purine), a PKR inhibitor, and subsequently examined for spread of viral infection via immuno-fluorescence microscopy. PKR inhibition reduced the number of RV-infected cells at all time points assayed (Figure 4 and Table 3). To verify that the PKR inhibitor had not simply blocked all signaling in IEC *per se,* we verified that an event previously shown to be independent of PKR, namely flagellin-induced IL-8 secretion (40), was not blocked by PKR inhibition (data not shown). These results support the notion that activation of PKR may be one means by which induction of type I IFN signaling is exploited by RV.

DISCUSSION

Like many cell types infected with viruses, intestinal epithelial cells (IEC) generate type I IFN upon infection with rotavirus (RV). Such IFN induction is responsible for induction of over 500 genes in RV-infected IEC (69). Herein, we observed that type I IFN, in particular IFN-β, also plays a predominant role in activating some of the phosphorylation events commonly associated with viral infection. Based on the presumption that all immune responses should be considered beneficial unless proven otherwise, we expected that blockade of IEC type I IFN signaling *in vitro* would enhance RV infection in a manner that would suggest an obvious role for such signaling in antiviral immunity *in vivo*. In contrast, neutralization of type I IFN primarily modulated RV infection in a way that seemed consistent with the possibility that RV exploits type I IFN to promote its replication and cause a pathologic response in the host. Specifically, we observed that blockade of type I IFN markedly attenuated the rate of RV replication particularly in the first 8 hours following inoculation suggesting that the type I IFN response promotes RV replication. Additionally, we observed that RV-induced type 1 IFN promoted cell death. Since, for other viruses that cause acute infections, replication rates *in vitro* often correlate with virulence *in vivo*, and that RV-induced cell death is thought to play a role in causing clinical manifestations of RV infection (37, 38), these results suggest that the type I IFN response may be considerably detrimental to RV-infected hosts.

The notion that some viruses have evolved mechanisms to take advantage of IFN signaling has been suggested previously with Smith and colleagues (76, 77) in particular finding that the IFN-associated PKR activity promoted replication of reovirus, which

shares considerable similarity with RV. Our observation that pharmacological inhibition of PKR suppressed RV replication suggests that RV may exploit IFN signaling in a manner similar to that used by reovirus. While taking advantage of signaling induced by type I IFN may thus be a strategy used by a variety of viruses, there are many more reports of viruses interfering with type I IFN signaling in a variety of ways (78), likely reflecting the broad ability of type I IFN to suppress viral infection. Indeed, elegant *in vitro* studies by Patton and colleagues demonstrate that RV employs this strategy in that one of its non-structural proteins, namely NSP1, suppresses type 1 IFN production via degradation of IFN-inducing IRF transcription factors (35, 36). Absence of NSP1-mediated IRF degradation was associated with reduced viral spread (35) suggesting that inability to suppress IFN signaling might impair RV fitness *in vivo*. Our observation that the relatively small amount of UV-irradiated rotavirus induced greater IFN signaling than live virus present in IEC 24 h following infection speaks to the ability of RV to suppress IFN signaling. But, nonetheless, it should be noted that RV suppression of IFN signaling is not absolute, as RV infection still results in detectable activation of IFN and numerous IFN-activated genes (69). Thus, one possibility of reconciling our findings with those of Patton and colleagues is that a small amount of type I IFN signaling, perhaps just enough to activate a threshold level of PKR activation, provides the optimal environment for RV and thus any alterations in IFN signaling (i.e., increase or decrease) may reduce RV infectivity.

Another potential way of interpreting our findings is to view RV-induced cell death as a means of innate immunity that is effective *in vivo,* albeit harder to appreciate *in vitro*. *In vivo*, under normal conditions, apoptotic epithelial cells shed into the gut lumen

in a manner that preserves gut barrier function (79, 80). Thus, one can envisage that using this process might be a safe, efficient means of eliminating RV infected cells and thus IFN promotion of apoptosis may, in fact, be of benefit to the host. In this context, one could view the loss of RV-infected IEC from the cell culture plate observed herein as a means of viral clearance as, *in vivo*, these cells would be flushed out of the intestine in the fecal stream. The high regenerative capacity of the intestine would likely allow for a considerable level of viral clearance by this mechanism before loss of barrier function and subsequent inflammation would ensue, which is in accordance with observations that RV infection is not associated with histopathologic inflammation (14). However, such a mechanism of RV clearance, if indeed operative *in vivo*, might not only provide a benefit to the host but would also seem likely to aid RV in its dissemination to new hosts, presumably via fecal-to-oral route (12). Thus, RV-induced IFN-mediated apoptosis may, in fact be mutually beneficial to both RV and the host, in accordance with the notion that ancient pathogens have co-evolved with their hosts.

In considering the relative importance of various *in vitro* observations discussed herein, we note that, in contrast to the case for most other viral infections, loss of the type I IFN receptor and subsequent ablation of all type I IFN responses, does not have a dramatic alteration on the course of infection (20, 72). Our favored interpretation of this observation is that, overall, the type I IFN response is utilized by both RV and the host to promote, respectively, viral replication and clearance. Thus, the net result of eliminating type IFN signaling is rather modest although it seems to modulate local dynamics of the infectious process. In this scenario, it might be possible to modulate the course of infection by RV and other viruses by better understanding and subsequently more precise

manipulation of type I IFN signaling and innate immunity in general.

MATERIALS AND METHODS

Abs and Reagents

Rabbit and guinea-pig anti-RV sera were provided as a kind gift from Jon Gentsch at the CDC. Antibodies to total and phosphorylated STAT1 and PKR were obtained from Cell Signaling Technology (Beverly, MA). Total IRF7 antibodies were purchased from Santa Cruz Technology (Santa Cruz, CA). Human interferon alpha and beta (IFN α/β) and antibodies to human interferon alpha and beta (anti-IFN α/β) and were obtained from the National Institute of Allergy and Infectious Disease (NIAID) Reference Reagent Laboratory through ATTC (Manassas, VA). β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO), respectively. Cleaved PARP antibody was obtained from Cell Signaling (Beverly, MA). 2-aminopurine was purchased from SIGMA (St. Louis, MO).

Cell culture and RV propagation

Model intestinal epithelia (HT29) were cultured as previously described on standard tissue-culture plastics (64) or Lab Tek Chamber slides (Nalge Nunc International, Rochester, NY). Rhesus rotavirus (RRV) was propagated in MA104 cells and titered as previously described (65, 81).

Cell infection with RV and type I IFN antibodies

Prior to infection RV was diluted in serum-free medium (SFM) to an MOI 1 and incubated with 10 ug/ ml trypsin (Mediatech, Inc., Manassas, VA, #25-054-CI) for 30 min in a 37^{o}C water bath. Control samples were treated with an equivalent amount of trypsin diluted in SFM (Mock) or SFM alone. Where indicated, neutralizing antibodies to human interferon alpha and beta (anti-IFN α/β) (1:100) were added to some preparations of trypsinized rotavirus prior to infection. Cell monolayers were washed several times with SFM and inoculated with virus alone or virus plus anti-IFN α/β for 1h at 37^{o}C/ 5% CO_2 to allow for adsorption. Following adsorption, cells were washed again several times with SFM and then incubated with 2 ug/ ml trypsin in SFM or SFM only for 0-48 h post-inoculation (hpi). Cells stimulated with RV in the presence of anti-IFN α/β were treated with 2 ug/ ml trypsin in SFM plus the same concentration of type I IFN antibodies used during viral adsorption. At various time points from 0-48 hpi, supernatants were collected and stored at -20^{o}C for ELISA. Cells were washed several times with PBS and resuspended in radioimmunoprecipitation assay II buffer (20mM Tris-HCl, 2.5 mM EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1 % SDS, 50 mM NaF, 10 mM $Na_2P_2O_7$, and 2 mM $NaV0_4$ plus protease inhibitor mixture) (RIPA II) for western blotting.

Cell stimulation with type I IFNs alone or in the presence of type I IFN antibodies

Prior to stimulation, type I IFNs (α/β) were diluted in SFM to a concentration of 200 IU/ ml. Where indicated, type I IFN antibodies (anti-IFN-α, anti-IFN-β) were added to type I

IFN preparations (1:100). Confluent cells were washed 3X in SFM and treated with type I IFNs (200 IU/ ml) or type I IFNs plus anti-IFN α/β for 0-48 h. At the indicated time points, cells were washed 3X with PBS and resuspended in RIPA II buffer for western blotting or TRIzol for qRT-PCR analysis as described above.

Cell infection with RV and 2-aminopurine

RV was diluted in SFM alone (MOI 1) or SFM containing 2mM 2-aminopurine (2AP). Control samples were treated with an equivalent amount of trypsin diluted in SFM (Mock) or SFM alone. Confluent cells were washed 3X with serum-free DMEM and infected with RV alone or RV plus 2-aminopurine (2 mM) for 1h/ 37C/ 5% C02. Cells were washed 3X with serum-free DMEM and incubated with 2 ug/ml trypsin or 2ug/ml trypsin containing 2mM aminopurine for 0-24 hpi. At the indicated time points cells were fixed, stained and mounted onto microscope slides for visualization via immunofluorescent microscopy as described elsewhere.

Western blotting

Cells were grown to confluence and stimulated with indicated stimuli as described above. At various time points from 0-48 hpi, cells were washed 3X in PBS, lysed in RIPA II buffer as described above and cleared by centrifugation (10 min at 15, 000 x g, 4^{o}C). Total protein concentrations were estimated for lysates by BioRad Protein Assay. Equal amounts of protein were assayed for antiviral and apoptotic markers (IRF7, STAT1 and

PKR, cleaved PARP) and viral proteins by 12% SDS-PAGE immunoblotting and membranes were stripped and probed for β-actin (control). Immunoblots were visualized with the ECL system (Amersham Biosciences, Piscataway, NJ).

ELISA

Confluent cells were treated with indicated stimuli as described above. At various time points from 0-48 hpi, cell-free supernatants were collected and analyzed for viral protein levels via double antibody sandwich ELISA. The assays were performed using anti-rhesus rotavirus polyclonal antibodies from rabbits and guinea-pigs. Briefly, microtiter plates were coated overnight at RT with rabbit anti-RV, washed several times with 0.05% Tween/ PBS, and blocked for 1h/ RT with 1% BSA/ PBS. Next, plates were washed again as before and incubated for 1h/RT with standards and samples that were diluted in PBS. Standards were prepared from rhesus rotavirus that was propagated and titrated in MA104 cells as described previously (65, 81). Samples were prepared from supernatants that were diluted in PBS. Following incubation, guinea-pig anti-RRV was added to the plates for 1h/ RT. Next, plates were washed and treated with horseradish peroxidase conjugated donkey anti-guinea pig antibody (Jackson Immunoresearch, Westgrove, PA) for 1h at RT. After several washes, TMB substrate and STOP solution (KPL, Gaithersburg, MD) were added to the plates. Absorbance readings were taken at 450 nm on a microplate reader (Molecular devices, Sunnyvale, CA).

Microscopy

Confluent cells grown on 8-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) were treated with the indicated stimuli as described above. Cell morphology was observed under an inverted microscope (magnification 100X). For immunofluorescent microscopy, unless indicated otherwise, stimulated cells were fixed in ice-cold ethanol (95% EtOh) for 10 min/ RT, washed 3X in PBS-0.01% Tween and permeabilized in 0.1% Triton-X for 8 min/ RT. Cells were washed 3X in PBS-0.01%, followed by incubation in blocking buffer (3% BSA/ PBS) for 1h/ RT and primary antibody (rabbit anti-RV, 1:10,000) in blocking buffer O/N at 4C. Cells were washed 3X with PBS-0.01% Tween, incubated with secondary antibody (anti-rabbit conjugated to FITC, 1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking buffer for 1h/ 37^{o}C in a humidified chamber, and washed 3X with PBS-0.01% Tween. Cells were counterstained with DAPI (1:1000/ PBS) for 10 m/ RT in the dark and washed 3X as described previously. Stained cells were mounted on slides with fluorescent anti-fade medium (VectaShield; Burlingame, CA) and viewed under a fluorescent microscope. To quantitate fluorescence levels per image threshold analysis was performed using ImageJ v1.36b software (http://rsb.info.nih.gov/ij/).

TUNEL assay

Confluent cells grown on 8-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) were treated with the indicated stimuli. Cells were fixed in 4% paraformaldehyde for 1h/ RT, permeabilized in 0.1% Triton-X/ 0.1% sodium citrate for 2

min on ice, and labeled by an InSitu Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN), using TUNEL according to manufacturer's guidelines. Following labeling, samples were counterstained for nuclei with SYTO83 (Invitrogen, Carlsbad, California) diluted in PBS (1:5000) for 15 m/ RT in the dark, mounted onto slides with fluorescent anti-fade medium as described above, and viewed under a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY).

Table 1. Percent threshold of fluorescence in RV-infected cells vs. RV-infected cells with anti-IFN α/β

	1'	4'	8'	24'
Mock	0	0	0	0
RV	0.01	0.5	4.69	6.1
RV + anti-IFN α/β	0.01	0.01	1.64	8.78

*Intestinal epithelia (HT29) were infected with RV (MOI 1) alone, RV + anti-IFN α/β, and control samples which received equivalent amounts of trypsin diluted in SFM (Mock). At various time points from 0-24 hpi, cells were permeabilized, fixed and stained for expression of RV proteins via immunofluorescence microscopy (see Figure 3A). Threshold levels of fluorescence per image, quantitated via NIH Image J analysis with a gray-scale cut-off value of 111/ 255, are shown. Percentage of pixels containing green fluorescence are shown.

Table 2. Viral levels in supernatants of RV-infected cells vs. RV-infected cells with anti-interferon antibodies

	4'	24'	48'
Mock	0	0	0
RV	0	6.2x10^7	4.0x10^7
RV + anti-IFN α/β	0	3.4x10^7	3.7x10^7

*Intestinal epithelia (HT29) were infected with RV (MOI 1) alone, RV + anti-IFN α/β, and control samples which received equivalent amounts of trypsin diluted in SFM (Mock). At various time points from 0-48 hpi, supernatants were collected and assayed for levels of rotaviral proteins (pfu/ ml) via ELISA. Mock samples had no detectable levels of and are not shown.

Table 3. Percent threshold of fluorescence in RV-infected cells vs. RV-infected cells treated with 2AP

	1'	4'	8'	24'
Mock	0	0	0	0
RV	0.07	0.08	0.23	16.73
RV + 2AP	0	0	0.13	1.92

*Intestinal epithelia (HT29) were infected with RV (MOI 1) alone, RV + 2AP, and control samples which received equivalent amounts of trypsin diluted in SFM (Mock). At various time points from 0-24 hpi, cells were permeabilized, fixed and stained for expression of RV proteins via immunofluorescence microscopy (see Figure 4). Threshold levels of fluorescence per image, quantitated via NIH Image J analysis with a gray-scale cut-off value of 50/ 255, are shown. Percentage of pixels containing green fluorescence are shown.

Figure 1.

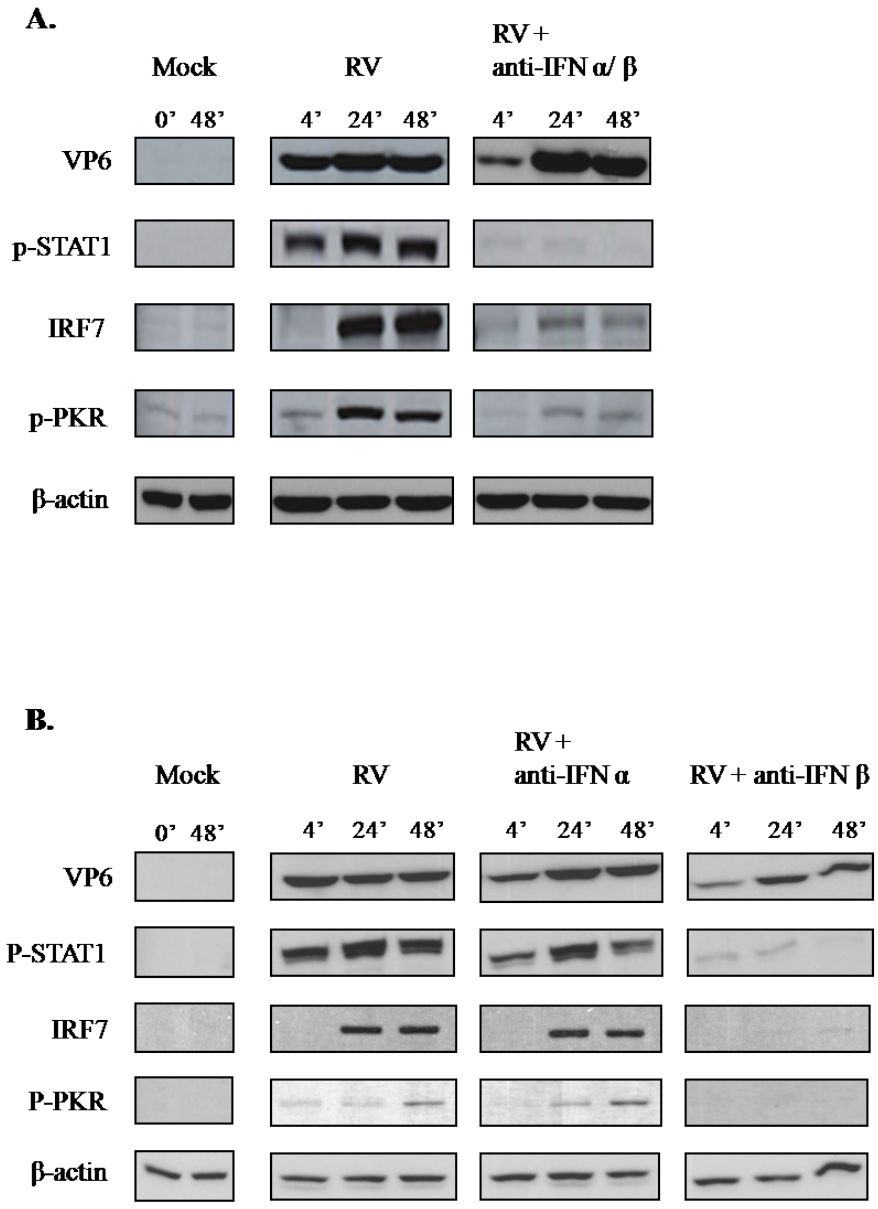
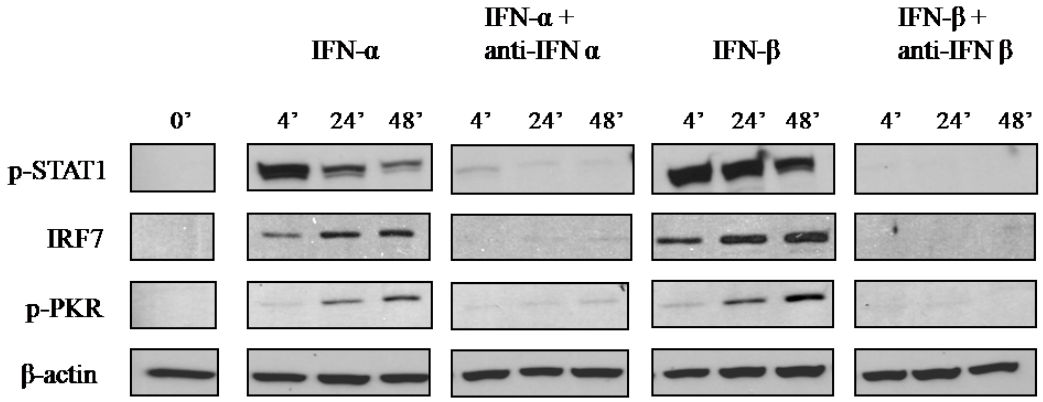

A.
Mock
RV
RV +
anti-IFN α/ β
0' 48'
4' 24' 48'
4' 24' 48'
VP6
p-STAT1
IRF7
p-PKR
β-actin
B.
Mock
RV
RV +
anti-IFN α
RV + anti-IFN β
0' 48'
4' 24' 48'
4' 24' 48'
4' 24' 48'
VP6
P-STAT1
IRF7
P-PKR
β-actin

Figure 1., cont.

C.



D.

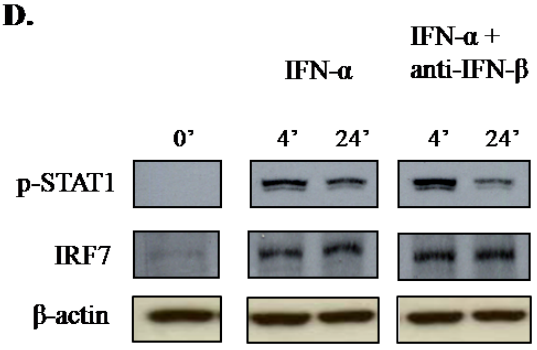
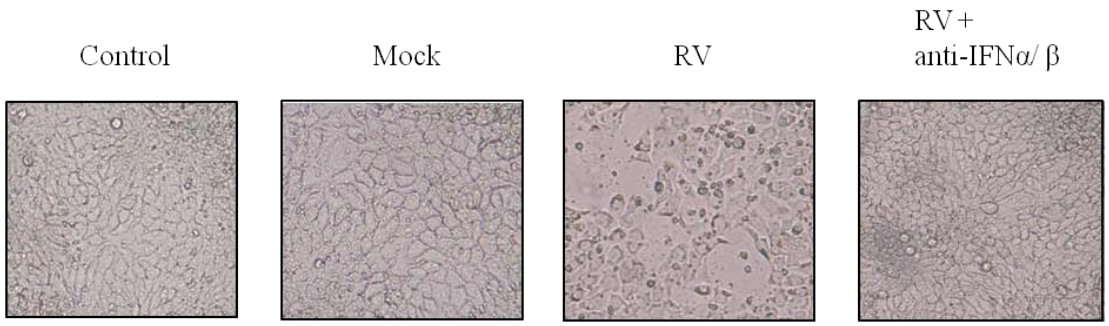
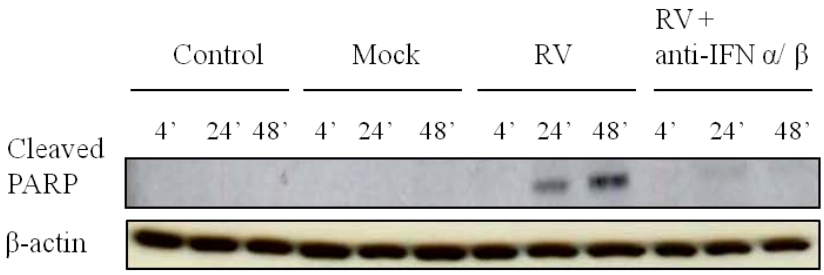


Figure 2.

A.


Control
Mock
RV
RV +
anti-IFNα/ β

B.


Control
Mock
RV
RV +
anti-IFN α/ β
4' 24' 48' 4' 24' 48' 4' 24' 48' 4' 24' 48'
Cleaved
PARP
β-actin

C.

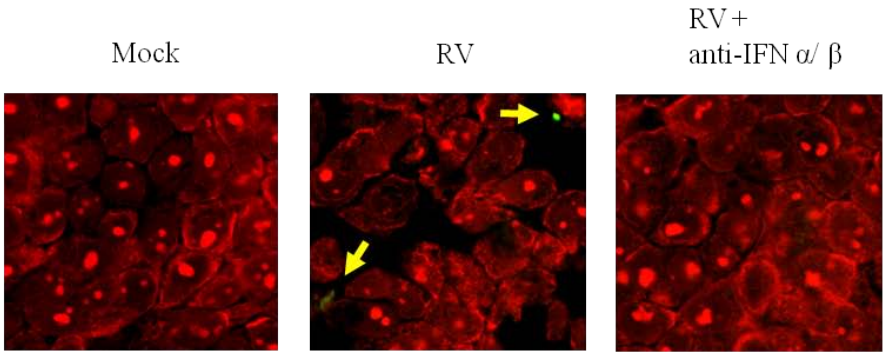

Mock
RV
RV +
anti-IFN α/ β

Figure 3.

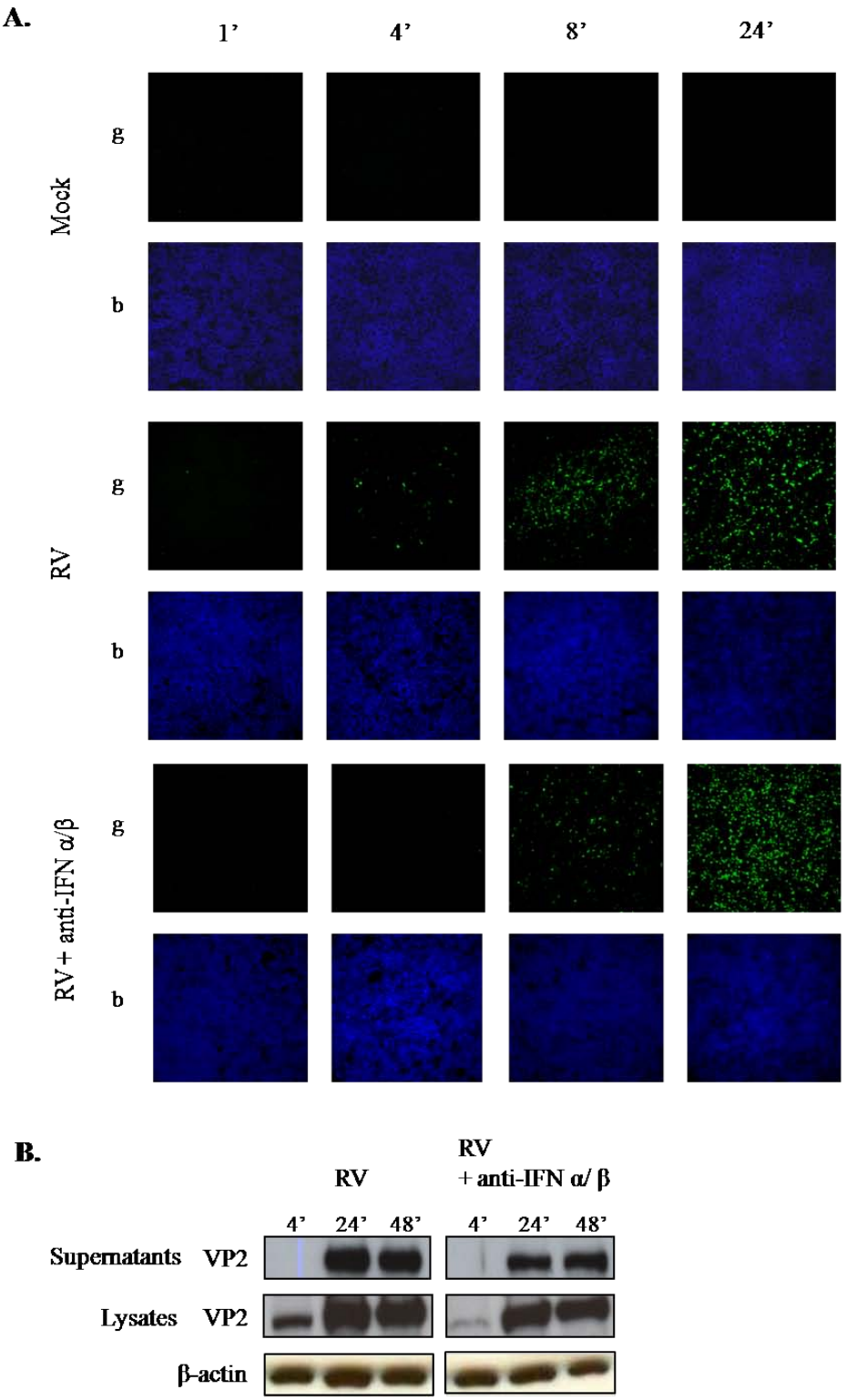

A.
1'
4'
8'
24'
Mock
RV
RV + anti-IFN α/β
g
b
B.
RV
RV
+ anti-IFN α/ β
4'
24'
48'
4'
24'
48'
Supernatants VP2
Lysates VP2
β-actin

Figure 4.

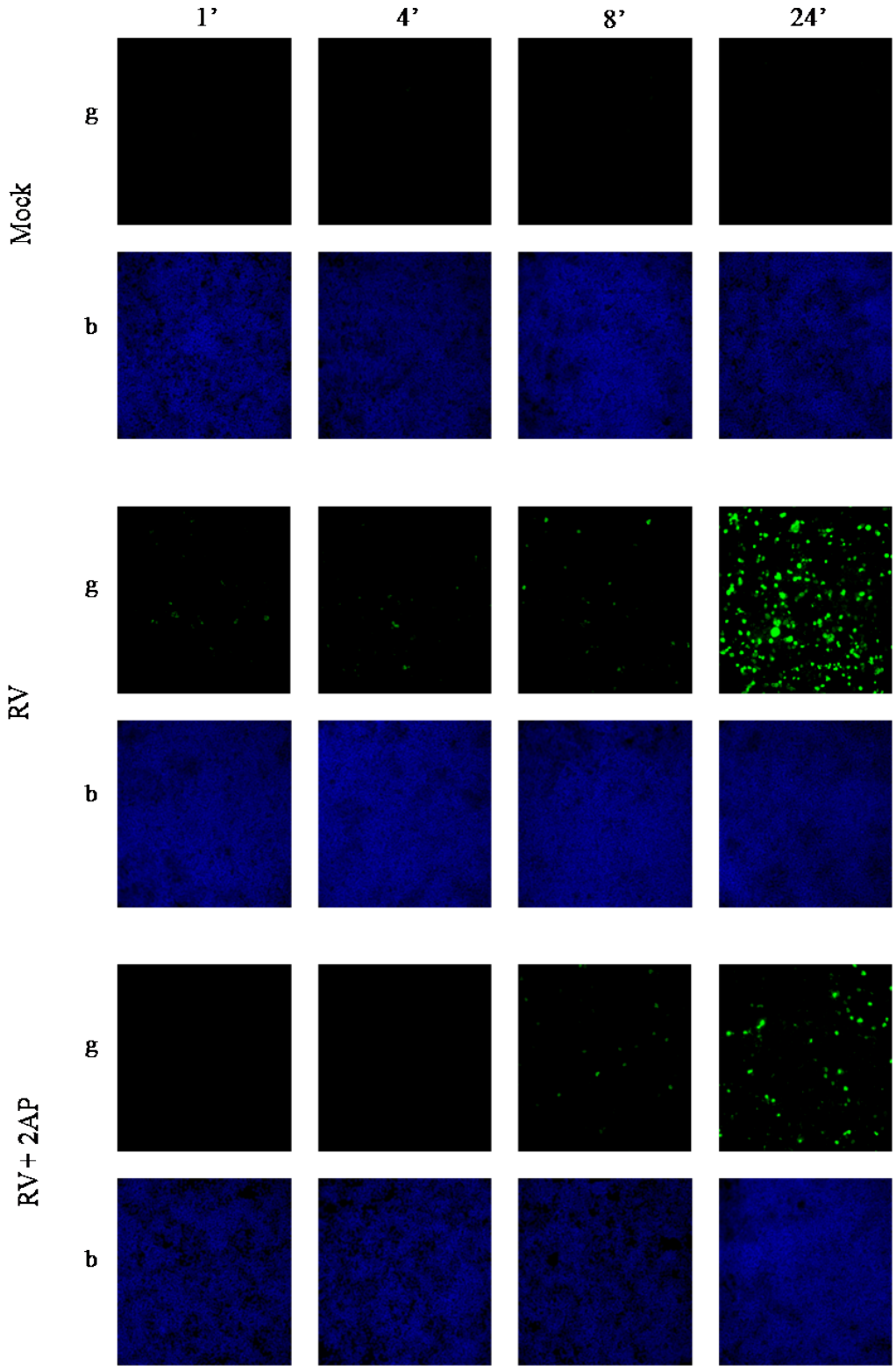


FIGURE LEGENDS

Figure 1. RV-induced type I IFN, particularly IFN-β, elicits epithelial anti-viral gene expression. Intestinal epithelial **(**HT29) cell monolayers were infected with RV (MOI 1), RV plus both type I IFN antibodies (anti-IFN α/β), RV plus either type 1 IFN antibody (anti-IFN-α or anti-IFN-β), and control samples which received equivalent amounts of trypsin diluted in SFM (Mock) or SFM alone (0) (A, B). In parallel experiments, confluent HT29 cells were treated with type 1 IFNs (IFN-α or IFN-β) alone or in the presence of either anti-IFN-α or anti-IFN-β (C, D). At various time points from 0-48 hpi, cell lysates were collected and analyzed for expression of viral proteins (VP6) and antiviral markers via western blot analysis. Data shown are results from a single experiment and representative of 3 separate experiments that gave similar results.

Figure 2. RV-induced type I IFN induces apoptosis in intestinal epithelia. Intestinal epithelial **(**HT29) cell monolayers were infected with RV (MOI 1) and RV plus type I IFN antibodies (anti-IFN α/β). Control samples received equivalent amounts of trypsin diluted in SFM (Mock) or SFM alone (0). At 48 hpi, cell morphology was observed under an inverted microscope (100X) for evidence of cell death (A). From 0-48 hpi, cell lysates were collected and analyzed for expression of cleaved PARP, an apoptotic marker, via western blot analysis (B). At 24 hpi, cells were fixed, permeabilized, and stained for DNA fragmentation, another marker for apoptosis, via TUNEL assay (C). Fluorescent microscope pictures of cells stained for apoptosis (green, indicated by yellow arrows) and nuclei (red) are shown as merged images (63X). Data shown are results

from a single experiment and representative of 3 separate experiments that gave similar results.

Figure 3. RV-induced type I IFNs promote, not impair, viral protein synthesis and cell-to-cell spread. Intestinal epithelial **(**HT29) cell monolayers were infected with RV (MOI 1), RV plus type I IFN antibodies (anti-IFN α/β), and control samples which received equivalent amounts of trypsin diluted in SFM (Mock). For up to 24 hpi, cells were permeabilized, fixed and stained for viral protein expression (green) and nuclei (blue), and viewed under a fluorescent microscope, magnification 20X (A). Fluorescence per image was quantitated using NIH Image J analysis (see Table 1). In separate experiments, cells were mock-treated and infected with RV or RV + anti-IFN-α/β for 0-48 hpi, and supernatants or lysates were collected and analyzed via western blot analysis for viral protein expression (VP2) (B). Viral protein concentration in the supernatants was quantitated by ELISA (see Table 2). Data shown are results from a single experiment and representative of 3 separate experiments that gave similar results.

Figure 4. Rotaviral spread is PKR-dependent. Intestinal epithelial **(**HT29) cell monolayers were infected with RV (MOI 1), RV plus PKR inhibitor 2 amino-pourine (2AP), and control samples which received equivalent amounts of trypsin diluted in SFM (Mock). For up to 24 hpi, cells were permeabilized, fixed and analyzed under a fluorescent microscope for viral protein expression (green) and nuclei (blue), magnification 20X. Fluorescence per image was quantitated using NIH Image J analysis

(see Table 3). Data shown are results of a single experiment and representative of 3 separate experiments that gave similar results.

CHAPTER 4: DISCUSSION

In this body of work, the evidence suggests intestinal epithelial cells (IEC) were capable of recognizing enteric viruses and subsequently mounting host immune responses. Such epithelial responses could possibly aid in clearance of viral infection and protect against future repeat infection(s). Specifically, IEC detected structural components of rotavirus (RV), a common etiologic agent of childhood diarrhea, and elicited innate immune signaling pathways involving type 1 IFN, a cytokine well-known for having potent anti-viral properties (32, 41). These anti-rotaviral immune responses appeared to be mediated by epithelial pattern-recognition receptors (PRRs), which is consistent with literature indicating IEC use a similar system for sensing and mounting appropriate immune responses to gut bacteria (2-6). Further, type 1 IFNs were found to induce anti-viral signaling and apoptosis in RV-infected IEC, mechanisms which could potentially contribute to anti-rotaviral immunity. Interestingly, however, the data presented here also illuminates a mechanism whereby RV possibly exploits type 1 IFN activity to enhance pathogenicity. During early infection, viral replication and spread was enhanced in association with type 1 IFN signaling, suggesting RV may benefit from type 1 IFN *in vivo*. Taken together, these investigations not only advance understanding of epithelial immune responses to RV, but also provide insight into how IEC may defend themselves against other gut viruses which remain difficult to study in a laboratory setting. Lastly, this data contributes to knowledge of RV pathogenesis and thus has potentially important implications for rotaviral vaccine or drug development.

UV-inactivated rotavirus (UV-RV), rendered non-replicative but structurally intact, elicited innate immune responses in IEC that were comparable to live RV, with

some important differences (Chapter 2). Both UV-RV and RV upregulated protein expression of anti-viral markers (p-IRF3, IRF7, STAT1 or PKR) and induced IFN-β secretion via a trypsin-dependent, apical pathway of infection (Figure 2-1 to 2-4). Even more striking, UV-RV and RV exhibited similar transcription profiles, including induction of over 1,000 genes (Figure 2-5). However, with regards to an array of select anti-viral markers dependent upon type 1 IFN signaling for expression, UV-RV was more often a stronger inducer of anti-viral gene signaling than RV. These findings indicate a potential for RV to evade epithelial anti-rotaviral immune responses. Indeed, suppression of host anti-viral immunity, such as type 1 IFN activity, has been shown for a number of viruses, including poxviruses, herpesviruses, adenoviruses, or influenza viruses, and others (78). In addition, RV has been shown to interfere with the type 1 IFN response by encoding NSP1 proteins which degrade IRF transcription factors (35, 36).

Another distinction between IEC immune responses elicited by UV-RV and RV was observed for secretion of IL-8, a classic pro-inflammatory cytokine (40). Consistent with other reports (48), UV-RV was a poorer inducer of IL-8 than RV (Figure 2-2), indicating optimal production of IL-8 requires viral replication. Interestingly, although IL-8 is considered to be a potent chemoattractor for leukocytes such as neutrophils (48, 82), RV infection is generally not associated with neutrophil influx (47-49), thus making the role of IL-8 in anti-rotaviral immunity unclear. It's possible RV-induced IL-8, while not supportive of neutrophil activity, may be mediating other aspects of anti-rotaviral immunity. For example, IL-8 is also known to promote recruitment or activation of other immune cells such as macrophages, T cells, and intraepithelial lymphocytes, and plays a role in expanding B cell populations (47, 83, 84). Thus, IL-8 may be mediating such

responses to help clear RV infection. Alternatively, RV may be utilizing a yet unknown mechanism to block IL-8 function, as viruses are known to encode inhibitory proteins which impair cytokine activity (85). It has also been proposed that in contrast to other gut pathogens, RV may not produce a cofactor needed to promote neutrophil influx during infection (47, 86).

The above characterized IEC response to UV-RV suggests a potentially important role for pattern-recognition receptors (PRRs) in mediating anti-rotaviral immunity. UV-RV, consisting of RV dsRNA and proteins, was a robust inducer of epithelial anti-viral signaling, suggesting rotaviral components may be detected by cellular PRRs. Activation of cellular PRRs results from engagement with specific molecular structures on pathogens (pathogen associated molecular patterns, or PAMPs) and leads to induction of innate immune pathways which help to clear infection and shape adaptive immunity. During viral infections, PRRs such as TLR-3, Mda-5 and RIG-1, have been implicated in recognizing dsRNA, a common viral replication intermediate, and subsequently eliciting anti-viral, type 1 IFN-mediated immunity (41, 87). Hence, it seemed plausible that IEC may be using such PRRs to detect RV. Indeed, microarray analyses indicated these PRRs were significantly upregulated by UV-RV relative to mock, with Mda-5 exhibiting the largest increase in fold change induction (Table 2-1).

In addition to implicating PRRs in rotaviral recognition, further experiments demonstrated that such PRRs may be located intracellularly and within the apical region of the cell. For example, UV-RV receiving trypsin protease treatment induced greater epithelial anti-viral gene expression than non-trypsinized UV-RV (Figure 2-3). Since trypsinization is required for mediating cell entry and enhancing infectivity of RV (46),

such data seems to support a role for intracellular PRRs in mediating immune responses to RV. Thus, inside the cell, TLR-3 on endosomal membranes or cytosolic Mda-5 and RIG-1 (41) could serve to detect rotaviral dsRNA during infection and subsequently initiate anti-viral immune responses.

As described above, apical, but not basolateral, stimulation with UV-RV elicited robust IEC anti-viral signaling (Figure 2-4), indicating that an apical PRR may activate immune responses to RV. Indeed, polarization of PRRs has been observed in epithelial cells and is thought to facilitate appropriate and rapid immune responses to invading pathogens. For instance, TLR-5, a sensor of bacterial flagellin, has been detected in the basolateral domain of IEC which is adjacent to bodily tissues (3, 5). Due to such a location, TLR-5 may be more equipped to respond to invasive bacterial pathogens rather than apical commensal bacteria (2). With regards to sensing gut viruses such as RV, an apically located PRR would make sense, as the apical lumen of the small intestine would be the initial site of infection following RV entry into the gut. Taken together, it's possible that one or more PRRs may play a role in detecting RV. To address such questions, however, future studies will need to be performed *in vivo* to examine whether deletion of PRR(s) exacerbates RV infection, delays rotaviral clearance, or induces poorer protection against future challenge with RV.

Individual components of RV, including purified viral dsRNA and VLPs, did not recapitulate ability of UV-RV to induce anti-viral responses in IEC. Although fluorescence analysis indicated UV-RV and RV VLPs entered cells as efficiently as live virus (Figure 2-7), UV-RV retained a much higher capacity than VLPs to induce epithelial anti-viral gene expression (Table 2-3, Figure 2-6). The fact that UV-RV

contains viral proteins as well as dsRNA, while VLPs contain only proteins, strongly implicates viral dsRNA as the primary inducer of anti-rotaviral immunity. However, when experiments were conducted to see whether exogenously administered viral dsRNA elicited greater IEC anti-viral signaling than VLPs, dsRNA was also found to be a poor activator of anti-viral immune responses (Table 2-3, Figure 2-6). Thus, such results support a model whereby both RV proteins and RNA are needed to elicit optimal anti-viral immunity, as trypsinized proteins would facilitate delivery into the cell and allow PRR activation upon engagement with dsRNA.

The failure of RV VLPs to mimic UV-RV in this cell culture system can be intrepreted in a couple of ways. First, VLPs, assembled *in vitro* via a baculovirus vector-expression system and closely resembling the outermost layers of infectious RV (27), may not be as efficiently recognized by PRRs as native RV proteins. This could be due to some unknown difference(s) between VLPs and native rotaviral capsid shells, such as in terms of conformation. Thus, it's possible RV proteins do actually induce PRR-mediated signaling pathways *in vivo*, but we were just not able to demonstrate that such a response occurs with the VLPs used in this system. Second, although PRR detection of viral proteins has been described in the literature with regards to immune cells (87), it's plausible that in a different cell type such as IEC, rotaviral proteins are not efficiently detected by PRRs.

The finding that RV dsRNA, albeit exogenously administered, poorly induces epithelial anti-viral signaling (Table 2-3, Figure 2-6) seems to contradict the prediction that viral dsRNA is the primary component for inducing immunity. However, a lack of an IEC response to dsRNA may have been detected for a number of reasons. First, it's

plausible dsRNA was not detected by cellular PRRs due to degradation by RNases existing in the extracellular environment. Second, dsRNA may have not efficiently entered the cell, preventing recognition by intracellular PRRs. For example, we've reported elsewhere that Poly(I:C), a synthetic mimic of viral dsRNA, only elicits epithelial anti-viral signaling when administered at very high concentrations (40). Thus, if forced into the cell as was done in the Poly(I:C) studies, either by using high concentrations or via more non-physiological methods such as lipotransfection, perhaps RV dsRNA would elicit a more robust IEC anti-viral response. Third, if dsRNA was not degraded by extracellular RNases and also did not enter the cell, the observed lack of an epithelial response could be explained by an absence of dsRNA sensors on the cellular surface. Indeed, cell surface PRRs which detect viruses have been reported previously (87), however they seem to detect viral proteins rather than viral dsRNA.

As described earlier, RV upregulates IEC production of Type 1 IFN, a cytokine well-known for possessing anti-viral activity (41). In addition, a number of *in vitro* and *in vivo* studies suggest type 1 IFNs play a role in modulating RV infection (20, 33, 34, 39, 50-52). Therefore, the question was asked whether Type 1 IFNs mediate epithelial anti-rotaviral immunity (Chapter 3). Using neutralizing antibodies to Type 1 IFN (α/β), it was demonstrated that Type 1 IFN, particularly IFN-β, is primarily responsible for inducing anti-viral gene expression and apoptosis in RV-infected IEC (Figures 3-1, 3-2). Such IFN-induced processes are thought to contribute to anti-viral immune responses by promoting viral clearance and/ or modulating adaptive immunity (41). RV-induced type 1 IFNs were observed to upregulate expression of select anti-viral markers implicated in mediation of Type 1 IFN responses (Figure 3-1), including p-STAT1, IRF7, and PKR

(41). Previously, it was noted that RV-induced type 1 IFN signaling promoted expression of MHC-I, a protein important for facilitating CD8+T cell killing of virus-infected cells (88) (Table 2-1). Apoptosis of virus-infected cells was also found to occur in an IFN-dependent fashion, particularly during late phases of infection (24-48 hpi) (Figure 3-2). The literature proposes IFN-induced apoptosis facilitates viral clearance, as death of infected cells would theoretically prevent the virus from using cellular machinery for replication and spread (32). Although a correlation between IFN-induced apoptosis and reduced viral infection was not found in this *in vitro* system (Figures 3-2, 3-3, and Tables 3-1, 3-2) at 24-48 hpi, which seems to contradict such a model, it is possible that such a phenomenon may occur *in vivo*. For example, IFN-induced apoptosis may effectively work in conjunction with other aspects of the immune system, such as phagocytes, CD8+T cells and CD4 +T cells that promote B cell activity, to efficiently clear RV.

While it's possible type 1 IFNs help control RV, the described studies reveal that RV may use strategies to overcome type 1 IFN responses and subsequently enhance pathogenicity. Indeed, viruses have long been known to evade type 1 IFN-mediated immunity in a variety of ways (78). RV, in particular, encodes NSP1 proteins which dampen type 1 IFN production via degradation of IRF transcription factors (35, 36). Herein, we report increased RV replication and spread in correlation with type 1 IFN signaling. This trend was particularly evident during early stages of infection (4-8 hpi), when type 1 IFN responses are perhaps not exhibiting a maximal anti-viral effect (Figure 3-3, Table 3-1). In theory, initial type 1 IFN production by the original infected cell leads to autocrine and paracrine induction of over hundreds of genes with combinatorial anti-

viral activity (32, 41), and such a process may take some time to develop. In a sub-optimal type 1 IFN environment that provides lower resistance to viral infection, RV may be more efficient at usurping cellular machinery for replication and establishing an infection in the host.

Due to the observation that RV appears to benefit from the type 1 IFN response, albeit during early infection, additional studies were performed to see whether RV infectivity was enhanced in correlation with interferon-stimulated genes, or ISGs. Given that type 1 IFN responses produce over hundreds of ISGs, some well-known for anti-viral activity while others remain yet to be characterized in terms of function (32), it seemed possible that RV evolved to exploit one or more of these genes to enhance pathogenicity. Such exploitation of ISG activity has in fact been reported for reoviruses, which belong to the same *Reoviridae* family of dsRNA viruses as RV (14, 76, 77). The authors of these studies showed that reoviral replication increased in correlation with expression of PKR, an IFN-induced gene (77). Reovirus appeared to particularly benefit from synthesis of ATF4 transcription factor, which occurs downstream of PKR activation. The authors reasoned that since ATF4 is a pro-survival protein responsible for promoting recovery from cellular stress, its activation may help to create a favorable environment for reoviral growth (76). Similar to reovirus, rotaviral infectivity was also dependent upon PKR (Figure 3-4, Table 3-3). Thus, it's possible RV may take advantage of the same aspects of PKR signaling as reovirus, or perhaps benefit from entirely different parts of this pathway during infection. Future studies will need to be conducted to determine exactly how RV exploits PKR activity.

Taken together, the presented evidence has potentially important implications for rotaviral vaccine and drug development. Specifically, the finding that inactivated rotavirus elicits robust anti-viral immunity comparable to live virus lends further support to the idea of using inactivated rotavirus vaccines (IRV) for preventing RV infection. While IRVs induce robust serum antibody responses which protect against reinfection with infectious RV (26), we've shown here that IRVs are also strong inducers of innate immunity, particularly in the gut. Indeed, local activation of anti-rotaviral immune responses in the intestine, rather than systemic immunity, appears to be highly effective in mediating protection to RV (15). Additionally, since RV seemed to possibly exploit type 1 IFN-mediated processes for enhancing pathogenicity, perhaps an IRV would be more effective at eliciting robust anti-viral immunity than a live RV vaccine. Finally, based on the observation that RV benefited from PKR signaling during infection, perhaps this protein could be targeted by anti-rotaviral drugs to help treat infection.

To conclude, the presented work demonstrates that the intestinal epithelium uses a PPR-mediated sensing system to mount immune responses to gut viruses such as RV. In particular, these anti-viral responses appeared to be mediated by Type 1 IFNs that induced IEC anti-viral gene expression and apoptosis in IEC. Such IFN-induced processes could potentially contribute to anti-rotaviral immunity by facilitating viral clearance or shaping adaptive immunity (41). However, while capable of eliciting epithelial anti-viral immune responses, type 1 IFN signaling also appeared to be exploited by RV in a PKR-dependent manner. Thus, this data provides useful information for developing RV vaccines or anti-rotaviral therapies, and may also help create strategies for controlling other enteric viral infections.

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