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RIG-I/MDA5/MAVS Are Required To Signal a Protective IFN Response in Rotavirus-Infected Intestinal Epithelium

Alexis H. Broquet,* Yoshihiro Hirata,* Christopher S. McAllister,* and Martin F. Kagnoff*†

Rotavirus is a dsRNA virus that infects epithelial cells that line the surface of the small intestine. It causes severe diarrheal illness in children and ~500,000 deaths per year worldwide. We studied the mechanisms by which intestinal epithelial cells (IECs) sense rotavirus infection and signal IFN-β production, and investigated the importance of IFN-β production by IECs for controlling rotavirus production by intestinal epithelium and virus excretion in the feces. In contrast with most RNA viruses, which interact with either retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated gene 5 (MDA5) inside cells, rotavirus was sensed by both RIG-I and MDA5, alone and in combination. Rotavirus did not signal IFN-β through either of the dsRNA sensors TLR3 or dsRNA-activated protein kinase (PKR). Silencing RIG-I or MDA5, or their common adaptor protein mitochondrial antiviral signaling protein (MAVS), significantly decreased IFN-β production and increased rotavirus titers in infected IECs. Overexpression of laboratory of genetics and physiology 2, a RIG-I–like receptor that interacts with viral RNA but lacks the caspase activation and recruitment domains required for signaling through MAVS, significantly decreased IFN-β production and increased rotavirus titers in infected IECs. Rotavirus-infected mice lacking MAVS, but not those lacking TLR3, TRIF, or PKR, produced significantly less IFN-β and increased amounts of virus in the intestinal epithelium, and shed increased quantities of virus in the feces. We conclude that RIG-I or MDA5 signaling through MAVS is required for the activation of IFN-β production by rotavirus-infected IECs and has a functionally important role in determining the magnitude of rotavirus replication in the intestinal epithelium. The Journal of Immunology, 2011, 186: 1618–1626.

Epithelial cells that line the intestinal mucosal surface are a first line of defense against enteric pathogens (1–5). Microbial pathogens that infect the intestinal tract can cause structural and functional modifications of the epithelial barrier that are associated with diarrhea and systemic infection. Rotavirus, a dsRNA icosahedral RNA virus, is a major cause of severe diarrhea and results in significant morbidity, especially in infants and young children, with ~500,000 deaths worldwide annually (2, 6, 7). Severe rotavirus diarrhea also is more common in the elderly and immunocompromised.

Rotavirus infection of the small intestine most markedly damages epithelial cells located in the upper portion of intestinal villi (8, 9). After cell entry, RNA viruses generate dsRNA, which is a signature of virus infection, via RNA-dependent RNA synthesis. dsRNA activates cell type-specific pattern recognition receptors, which signal host cellular responses (10–13). For example, on interacting with dsRNA, dsRNA-activated protein kinase (PKR), a Ser/Thr kinase with dsRNA binding motifs in its N terminus, is activated (14). PKR phosphorylates eIF-2α and inhibits translation initiation (15). Although PKR is an IFN-regulated gene and can regulate apoptotic pathways important for eliminating virus-infected cells (16), it is not known to have a direct role in increasing the production of type I IFN that is important for host defense to virus infection (17).

Several host cellular proteins that interact with dsRNA signal host innate immune responses (10–13, 18, 19). For example, TLR3 is a pathogen recognition receptor (PRR) that transduces a signal to the adaptor molecule TRIF, which, in turn, signals the activation of IFN regulatory factor 3 (IRF3), type I IFN, and NF-κB (20). Mice genetically deficient in TRIF signaling have increased susceptibility to certain viral infections (e.g., mouse cytomegalovirus), indicating a significant role for the TLR3–TRIF signaling pathway in host-viral pathogenesis (20). However, signaling through the TLR–TRIF pathway is not essential for developing type I IFN responses because mice defective in TLR3–TRIF signaling can produce type I IFN responses to virus infection (21).

Currently, there is no evidence whether TLR3 has a role in signaling type I IFN production in rotavirus-infected intestinal epithelial cells (IECs). Further, how dsRNA produced in the cytoplasm during virus replication would access TLR3 is an open question, because interacting with dsRNA, dsRNA-activated protein kinase (PKR), a Ser/Thr kinase with dsRNA binding motifs in its N terminus, is activated (14). PKR phosphorylates eIF-2α and inhibits translation initiation (15). Although PKR is an IFN-regulated gene and can regulate apoptotic pathways important for eliminating virus-infected cells (16), it is not known to have a direct role in increasing the production of type I IFN that is important for host defense to virus infection (17).

The online version of this article contains supplemental material.

Abbreviations used in this article: CARD, caspase activation and recruitment domain; cflr, control; DN, dominant negative; IEC, intestinal epithelial cell; IRF3, IFN regulatory factor 3; ISRE, IFN-stimulated response element; LGP2, laboratory of genetics and physiology 2; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; PRR, dsRNA-activated protein kinase; poly(I:C), polyinosinic-polycytidylic acid; PKR, pathogen recognition receptor; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I–like receptors; RRV, rhesus rotavirus; siRNA, small interfering RNA; WT, wild type.

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(MDA5, also known as helicard or IFIH1), each contain a C-terminal DExD/H box RNA helicase domain that is a characteristic amino acid signature motif of many RNA binding proteins, as well as two N-terminal caspase activation and recruitment domains (CARDs). Interaction of the DExD/H box RNA helicase domain with viral dsRNA induces the unwinding of RNAs by means of energy derived from ATP hydrolysis and, at the same time, induces conformational changes in RIG-I and MDA5 that promote the CARD-mediated downstream signaling cascade. This leads to the activation of the adaptor molecule mitochondrial antiviral signaling protein (MAVS; also termed IPS-1/Cardif/VISA) (25–29). Definitive evidence for the role of RIG-I and MDA5 in signaling activated by dsRNA viruses was obtained using mice or cells from mice deficient in either RIG-I or MDA5 (21, 30). Using an IEC line (HT-29), we observed that RIG-I was important for the activation of type I IFN production after cells were transfected with the dsRNA analog polyinosinic-polycytidylic acid [poly(I:C)], and this also appeared to be the case when those cells were infected with vesicular stomatitis virus or a strain of rotavirus (31).

Laboratory of genetics and physiology 2 (LG2P) is a third RLR family member. LG2P lacks the MAVS-interacting CARD domains found in RIG-I and MDA5, and has been proposed to both function as a negative regulator of RIG-I/MDA5 signaling by competing with those molecules for engagement with viral RNAs and as a positive regulator of RLR signaling depending on the virus and the cell type that is infected (32–34).

Activation of the RIG-I/MDA5–MAVS pathway, or the TLR3–TRIF pathway, results in the downstream dimerization and phosphorylation of IRF3, activation of the IFN-stimulated response element (ISRE), and expression and production of type I IFN. Type I IFN secreted by infected cells acts in an autocrine and paracrine manner on type I IFN receptors expressed on cell membranes, leading to the downstream transcription and expression of IFN-stimulated genes whose products can act to inhibit viral infection and alter other cellular functions (35, 36).

Type I IFN modulates infection with a number of different viruses (37). However, the importance, if any, of host IEC-produced type I IFN for modulating rotavirus infection of intestinal epithelium, which is the initial and major site of contact and entry for rotavirus, is not known. In one study, IEC lines (i.e., Caco-2 and HT-29) that were pretreated with exogenous IFN-α manifested increased resistance to rotavirus infection in vitro (38). However, pretreatment of suckling mice with a mixture of type I IFNs did not protect against diarrhea associated with rotavirus infection (39). Furthermore, the importance of type I IFN in controlling rotavirus infection can vary with the infecting strain of rotavirus (40) and its ability to subvert the function of critical molecules in host signaling pathways.

We report here that rotavirus sensing through RIG-I and MDA5, and signaling through the common downstream adaptor molecule MAVS, but not signaling through the TLR3/TRIF pathway or PKR, upregulates the type I IFN-β response in IECs. Importantly, this determines the magnitude of rotavirus production by infected IECs and rotavirus excretion in feces.

Materials and Methods

Cells lines

Two human colon epithelial cell lines, HCA-7 and HT-29 (41), were grown in DMEM. Embryonic African green monkey kidney cells (MA-104) used to titer rotavirus were grown in Eagle’s MEM. Media were supplemented with 10% heat-inactivated FBS and 2 mM l-glutamine. Cell lines were maintained in 90% air/10% CO₂ (HCA-7 and HT-29) or 95% air/5% CO₂ (MA-104) at 37°C.

Mice

Wild type (WT) C57BL/6J (B6) mice were from The Jackson Laboratory. PKR K296R (B6 background) and TLR3−/− (B6 background) mice were provided by Dr. E. Raz (University of California, San Diego [UCSD], La Jolla, CA). TRIF−/− (B6 background) mice were provided by Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA). MAVS−/− mice (C57BL/6J/129 mixed background) (42) and the corresponding WT mice were provided by Dr. Chen (University of Texas Southwestern Medical Center, Dallas, TX). All mouse strains were maintained at the UCSD. All animal studies were approved by the UCSD Institutional Animal Care and Use Committee.

Reagents

Trypsin (type IX-S, from porcine pancreas, 13–20 U/mg benzoyl L-arginine ethyl ester) and mouse anti-β-actin mAb were from Sigma-Aldrich (Milwaukee, WI). Rabbit anti-IRF3 Ab was from IBL (Minneapolis, MN). Rabbit anti-MAVS Ab was from Bethyl Laboratories (Montgomery, TX). Rabbit anti-TRIF Ab and anti–phospho-IRF3 Ab were from Cell Signaling Technology (Boston, MA).

Rotavirus infection

Rotavirus strain SA11-5S was provided by Dr. J.T. Patton (National Institutes of Health, Bethesda, MD). Rotavirus strains SA11-4F and rhesus rotavirus (RRV) were provided by Dr. M.K. Estes (Baylor College of Medicine, Houston, TX). SA11-5S, generated from parental strain SA11-4F, expresses a C-terminal truncated NSP1 protein in infected cells. SA11-4F, in contrast with SA11-5S, rapidly degrades the transcriptional factor IRF3 in IECs (43). Whereas RRV NSP1 variably modified IRF3 in fibroblasts and dendritic cells in a cell type-specific manner (44, 45), in preliminary studies, we found RRV signaling through IRF3 was largely intact in RRV-infected HT-29 and HCA-7 cells. Virus was grown in MA-104 cells infected at low multiplicity, and incubated for 72 h in presence of trypsin (0.44 μg/ml), after which cells were lysed by freezing and thawing to achieve virus release. Extracted virus was titrated by plaque assay as described previously (46). For infection of cultured cells, differentiated HT-29 or nondifferentiated MA-104 cells were infected in serum-free medium and then infected for 1 h at 37°C with trypsin-activated rotavirus (0.44 μg/ml trypsin for 30 min at 37°C) at the indicated multiplicity of infection. After adsorption to the cell surface, the virus inoculum was removed, cells were washed, and the infection was allowed to proceed for the indicated times in serum-free medium containing trypsin (0.44 μg/ml). Adult mice 8–10 wk of age were fasted the day before infection, orally administrated 50 μl of 2.5% sodium bicarbonate 15 min before infection, and then administered rotavirus (5 × 10⁷ PFU/g body weight) by oral gavage for the indicated time periods before sacrifice.

Quantification of IFN-β by ELISA

Human and mouse IFN-β were assayed using the HuIFN-β ELISA kit (Fujirebio, Tokyo, Japan) and the Verikine mouse IFN-β ELISA kit (PBL, Piscataway, NJ), respectively, according to the manufacturer’s instructions.

Plasmids, small interfering RNA, and transfection

Expression plasmids for TLR3, PKR, RIG-I, dominant negative (DN) RIG-I (lacks CARD domains), MDA5, DN-MDA5 (lacks CARD domains), and LG2P were provided by Dr. T. Fujita (Kyoto University, Kyoto, Japan) (47, 48). DN-TL3 (TLR3–4TRIF) and a PKR inactive mutant (PKR K296R) were generated in our laboratory (31). All expression plasmids were derivatives of the same vector (pEF-BOS). Each expression plasmid was verified as functional by transfecting HCA-7 cells followed by Western blotting with anti-FLAG Ab (Sigma-Aldrich). Each DN plasmid inhibited the activity of its respective target in cells transfected with the relevant WT plasmid alone, and together with the DN plasmid, and subsequently stimulated with known agonists of the WT plasmid. Small interfering RNA (siRNA) oligonucleotides for silencing TLR3, PKR, RIG-I, MDA5, LG2P, and MAVS (siGENOME mixture mix), and nontargeting siRNA (siRNA scrambled) were purchased from Dharmacon (Lafayette, CO), as described previously (31). siRNA for TLR3, PKR, RIG-I, MDA5, and LG2P were confirmed to inhibit the activity of their respective target by transfecting HCA-7 cells with each siRNA or scrambled siRNA as a control and the corresponding WT pEF-tag plasmid followed by Western blotting with anti-FLAG Ab. The efficiency of siRNA knockdown in HCA-7 cells was 98–100% for TLR3, PKR, MDA5, and LG2P, and ≥90% for RIG-I. The efficiency of siRNA knockdown of MAVS and TRIF in HCA-7 cells was 90 and 70%, respectively. siRNA (100 nM) or plasmids (2 μg/ml) were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) 48 or 24 h before rotavirus infection, respectively.
Reporter assay

Reporter plasmids for the ISRE (pSRE-luciferase) and the internal control plasmid (pRL-TK) were from Stratagene (La Jolla, CA) and Promega (Madison, WI), respectively. IECs (5 × 10^5 cells in 2-well plates) were transfected with pSRE-luciferase (300 ng) and pRL-TK (10 ng) using lipofectamine 2000. DN vectors and/or their controls (900 ng total) or siRNA oligonucleotides (100 nM) were included, as indicated. Post-infection, cell lysates were prepared and luciferase activity was measured using the Dual Assay Kit (Promega) and a luminometer. Luciferase activity was normalized using Renilla luciferase as an internal control, and the fold induction of luciferase activity above control was calculated.

RNA extraction and real time PCR

Total cellular RNA was extracted using RNase Mini Kits from Qiagen (Valencia, CA), followed by DNase I (Qiagen) treatment according to the manufacturer’s instructions. Reverse transcription used 1 μg RNA and the Improm II Reverse Transcription System (Promega). cDNA was PCR amplified using the primers indicated (Supplemental Table I). For real-time PCR, cDNA was mixed with 2× SYBR green Master mix (Applied Biosystems, Foster City, CA). Denaturation was 5 min at 95°C followed by 40 cycles of amplification at 95°C for 30 s and 60°C for 30 s using an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Immunoblot analysis

IECs were washed in ice-cold PBS and lysed in lysis buffer (50 mM Tris HCl, pH 8.0, 1% Nonidet P40, 150 mM NaCl, 100 μg/ml leupeptin, 1 mM PMSF, 5 mM NaVO₄). Cell lysates were centrifuged at 15,000 × g for 10 min at 4°C. Aliquots (20 μg) were mixed with 4× SDS sample buffer, boiled, and separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and probed with the indicated primary antibody followed by HRP-conjugated secondary Ab, and developed using the ECL plus kit (GE Healthcare, Buckinghamshire, U.K.).

Isolation of mouse IECs

Proximal small intestine starting 4 cm distal to the pylorus (i.e., jejunum) was cut open longitudinally and intestinal content was removed by washing with PBS. Intestine was cut into 2- to 3-mm pieces and rocked in Hank’s balanced salt solution (HBSS) containing 30 mM EDTA for 10 min at 37°C. Supernatant was removed and centrifuged. After washing in ice-cold PBS, the resulting pellet was snap-frozen in liquid nitrogen.

Rotavirus titers

Virus titers in cultured IEC lysates and supernatants, isolated mouse IECs, and fecal pellets were assessed by plaque assay on MA-104 cells as previously described (46).

Histology

Proximal small intestine starting 4 cm distal to the pylorus (i.e., jejunum) was processed as Swiss rolls (49). Tissues were fixed in 10% formalin and embedded in paraffin, and 5-μm sections were stained with H&E.

Statistical analysis

Statistical analysis used Student t test. A p value <0.05 was considered statistically significant.

Results

Expression of viral RNA sensors and type I IFN production in rotavirus-infected IECs

RIG-I, MDA5, TLR3, and PKR can act as cellular PRRs that enable the host’s detection of RNA viruses. We found that HCA-7 and HT-29 cells constitutively expressed basal mRNA levels for each of these (Supplemental Fig. 1A). During rotavirus infection, levels of RIG-I and MDA5 mRNA, but not those of TLR3 and PKR, were significantly increased in HCA-7 and HT-29 cells (Fig. 1A).

To determine whether rotavirus increased ISRE activity and the expression and production of IFN-β by IECs, HCA-7 and HT-29 cells were infected with rotavirus SA11-5S or RRV for 24 h at a multiplicity of infection of 2 PFU/cell (Supplemental Fig. 1B). Both rotavirus strains increased ISRE activity, upregulated IFN-β mRNA levels, and increased IFN-β secretion by those cells (Fig. 1B–D). In contrast, rotavirus SA11-4F, whose NSP1 protein degrades IRF3 (43), did not increase ISRE activity, IFN-β mRNA, or IFN-β secretion in HCA-7 or HT-29 cells (data not shown).

RIG-I and MDA5, and not TLR3 or PKR, are important for signaling type I IFN responses in rotavirus-infected IECs

To determine the major pathway by which rotavirus infection initiates activation of IFN-β production in IECs, we transfected cells with siRNA constructs shown to silence the expression of RIG-I, MDA5, TLR3, or PKR before rotavirus infection. siRNA for RIG-I and MDA5 significantly attenuated ISRE activation and IFN-β production in HCA-7 cells infected with either SA11-5S or RRV, whereas siRNA for TLR3 and PKR did not (Fig. 2A, 2B). In a complementary approach, DN plasmids for RIG-I and MDA5, but not for TLR3 and PKR, also significantly decreased rotavirus-stimulated ISRE activation and IFN-β production in HCA-7 cells (Supplemental Fig. 2A, 2B). Silencing both RIG-I and MDA5 in combination, with either siRNA or DN plasmids, was significantly more effective for inhibiting ISRE activation and IFN-β production than silencing either helicase alone (Fig. 2A, 2B, Supplemental Fig. 2A, 2B). Similar results were found using HT-29 cells (Supplemental Fig. 3A, 3B).

To determine whether overexpressing RIG-I, MDA5, TLR3, or PKR in IECs increases ISRE activation and IFN-β production in rotavirus-infected cells, HCA-7 cells were transfected with plasmids expressing WT RIG-I, MDA5, TLR3, or PKR. Overexpression of RIG-I and MDA5, but not TLR3 or PKR, resulted in significantly increased ISRE activation and IFN-β production in response to rotavirus infection (Fig. 2C, 2D). Moreover, overexpression of both RIG-I and MDA5 in combination further increased ISRE activation and IFN-β production compared with either alone. Similar results were found using HT-29 cells (Supplemental Fig. 3C). These data highlight the importance of RIG-I and MDA5, compared with TLR3 and PKR, for signaling the IFN-β response after rotavirus infection of IECs.

Rotavirus activates the MAVS pathway

After sensing dsRNA, RIG-I and MDA5 interact with MAVS through their respective CARD domains, leading to downstream activation of IRF3 and IFN-β production (11–13, 25–28). To determine the importance of MAVS in rotavirus-infected IECs, we used siRNA to silence MAVS in HCA-7 and HT-29 cells before infection with rotavirus SA11-5S or RRV. ISRE activity and IFN-β production were significantly decreased in rotavirus-infected cells in which MAVS was silenced (Fig. 3A, 3B). Consistent with this, downstream IRF3 phosphorylation also was decreased (Fig. 3C). In contrast, silencing TRIF, the downstream adaptor molecule for TLR3, did not abrogate activation of the ISRE, IRF3 phosphorylation, or IFN-β production in rotavirus-infected IECs (Fig. 3A–C).

Signaling through RIGI and MDA5 controls rotavirus production in IECs

To determine the functional importance of rotavirus signaling through RIG-I or MDA5 on the production of virus by IECs, we assessed rotavirus titers 24 h postinfection of IECs that overexpress or underexpress RIG-I, MDA5, or both helicases, and in cells that underexpress MAVS or TRIF. Cells transfected with siRNA for RIG-I or MDA5 had significantly increased virus titers compared with cells transfected with control siRNA (Fig. 4A). Silencing both RIG-I and MDA5 led to even greater virus titers in SA11-SS–infected cells (Fig. 4A). Consistent with signaling through MAVS, but not the TLR3/TRIF pathway, HCA-7 and HT-29 cells trans-
FIGURE 1. Rotavirus infection upregulates RIG-I and MDA5 mRNA expression in IECs and activates the IFN-β response. A, Expression of viral RNA sensors in IECs. mRNA expression of RIG-I, MDA5, PKR, and TLR3 was assessed in IECs left uninfected (control [ctrl]) or infected with rotavirus SA11-5S or RRV for 24 h. B–D, HCA-7 and HT-29 cells were infected with rotavirus SA11-5S or RRV for 24 h or left uninfected (ctrl) and analyzed for ISRE activation (fold induction relative to ctrl), IFN-β mRNA levels (fold induction relative to ctrl), and IFN-β secretion. Data are mean ± SEM of five independent experiments. *p < 0.05 versus ctrl.

FIGURE 2. Role of viral RNA sensors RIG-I, MDA5, TLR3, and PKR in ISRE activation and IFN-β secretion in rotavirus-infected IECs. After transfection with the indicated siRNAs (A, B) or expression plasmids (C, D), HCA-7 cells were infected with rotavirus SA11-5S or RRV for 24 h. A, ISRE activation (fold induction relative to control [ctrl] scrambled siRNA); B) IFN-β production. Uninfected cells produced no IFN-β (not shown); (C) ISRE activation (fold induction relative to ctrl plasmid pEF-BOS lacking insert); (D) IFN-β secretion. Uninfected cells produced no IFN-β (not shown). Data are mean ± SEM of five independent experiments. *p < 0.05 versus infected cells transfected with ctrl siRNA or ctrl plasmid (ctrl), **p < 0.05 versus transfection with RIG-I or MDA5 alone.

LGP2 can counterregulate signaling through RIG-I and MDA5

LGP2 has a C-terminal DExD/H box RNA helicase domain that interacts with viral RNA but lacks the CARD domains found in RIG-I and MDA5 and, as a result, does not signal through MAVS. Thus, LGP2 has been postulated to function as a DN in terms of downstream signaling (32, 50). LGP2 mRNA levels were significantly increased in rotavirus-infected HCA-7 and HT-29 cells compared with control uninfected cells (Fig. 5A). IECs transfected with siRNA for LGP2 and infected with SA11-5S or RRV had significantly increased ISRE activation and IFN-β production (Fig. 5B, 5C). Conversely, overexpression of LGP2 in IECs significantly decreased ISRE activation and IFN-β production in rotavirus-infected IECs (Fig. 5D, 5E). Consistent with this, titers of rotavirus were significantly decreased in rotavirus-infected IECs transfected with LGP2 siRNA (Fig. 5F) and significantly increased in rotavirus-infected IECs transfected with a LGP2 expression plasmid (Fig. 5G). Taken together, these results indicate that LGP2 in rotavirus-infected IECs counterregulates the activity of RIG-I and MDA5.

Decreased IFN-β production and increased rotavirus replication and shedding in MAVS−/− mice

For in vivo infections, we used an adult rotavirus infection model (51–53). Mice were infected between 8 and 10 wk of age with RRV or SA11-5S. In contrast with infection of neonatal mice, adult mice infected with rotavirus do not develop diarrhea or significant mucosal damage, although they develop a significant host immune response (51, 52, 54) (Fig. 6G). Because MAVS was required for signaling the upregulated IFN-β response downstream of both RIG-I and MDA5 in IECs in vitro, mice lacking MAVS (MAVS−/−) were used to determine the importance of the RIG-I/MDA5–MAVS pathway for signaling the IFN-β response in the intestinal epithelium in vivo postinfection with rotavirus by the enteric route. IFN-β mRNA levels were upregulated in IECs isolated from the proximal small intestine (jejunum) of SA11-5S− or RRV-infected WT (Fig. 6A) but not MAVS−/− mice (Fig. 6B, 6C). In contrast, IFN-β mRNA levels in small IECs of SA11-5S− and RRV-infected TLR3−/−, TRIF−/−, and PKR−/− mice did not differ significantly from those in infected WT mice (Fig. 6B, 6C). IFN-β was not detectable in the sera of rotavirus-infected MAVS−/− mice (Fig. 6D) but increased to similar levels in infected...
and WT mice (Fig. 6F). In additional experiments, we found that WT mice infected with rotavirus SA11-4F, whose NSP1 protein degrades IRF3, did not upregulate IFN-β mRNA in the intestinal epithelium and feces (Supplemental Fig. 4A, 4B). Together, these results indicate the importance of IFN-β for controlling rotavirus infection in IECs and for influencing the amount of virus excreted in the feces.

Discussion

After entering the host by the enteric route, rotavirus infects epithelial cells that line the small intestinal mucosa. Understanding how the host epithelium responds to rotavirus infection is critical for understanding the pathogenesis of this infection and for identifying potential therapeutic targets. We found that the RLRs RIG-I and MDA5 alone and together are the major sensors of rotavirus infection in IECs. Moreover, those helicases mediate an essential functional role in signaling the epithelial cell type I IFN-β response and determine the magnitude of virus production in IECs and virus excretion in the feces.

The finding that after rotavirus infection both RIG-I and MDA5 initiate signaling that leads to upregulated IFN-β production was unexpected and revealed an important redundancy in the sensing of rotavirus infection in IECs. Silencing RIG-I or MDA5, or both, led to decreased IRF3 phosphorylation, decreased ISRE activation, less IFN-β production, and higher titers of rotavirus in IECs and in the feces postinfection, thereby revealing those helicases have an important functional role in activating the host epithelial cell response and important host defense mechanisms to rotavirus infection. Although signaling through RIG-I and MDA5 has been reported for West Nile virus, dengue virus, and reovirus type 3 in cultured undifferentiated mouse embryonic fibroblasts (55, 56), this is, to the best of our knowledge, the first demonstration of both RIG-I and MDA5 being used by a virus infecting its physiologically and clinically relevant target.

The detection of virus by RIG-I and MDA5 is determined by characteristics of the RNA molecules encountered by those PRRs postinfection (21). RIG-I, but not MDA5, is activated by 5'-triphosphorylated RNA found in the genome, for example, of influenza virus and other negative-strand RNA viruses (57, 58), whereas viruses that do not have triphosphorylated RNA genomes (e.g., picornaviruses) are recognized by MDA5 (21). The length of the dsRNA is also important, with short RNA fragments being recognized preferentially by RIG-I and long fragments by MDA5...
The specific characteristics of rotavirus that allow it to signal through both RIG-I and MDA5 are not known. Activation of both RIG-I and MDA5 may be a consequence of the special genome organization of rotavirus, which has 11 segmented dsRNA fragments of different sizes. Predictably, this would increase the number and type of RNA substrates available for interacting with those helicases. It is also known that the minus strand of the 11 rotavirus genes lacks the 5' cap present in the plus strand (60) and displays a 5' triphosphorylated end that could be recognized by RIG-I. Nonetheless, how rotavirus RNA gains access to RIG-I and MDA5 in the cytoplasm remains unknown because it replicates in double-layered particles within the host cell (61–63). However, nascent transcripts of rotavirus mRNA are reported to be extruded from particles into the cytoplasm (61, 62), where they may gain access to RIG-I and MDA5.

TLR3 and PKR can function as intracellular sensors of dsRNA, and both are expressed by IEC lines (64), including those studied in this paper. However, neither was important for activating the IFN-β response and controlling rotavirus production in IECs in vitro or in vivo. Whereas activation of PKR activated NF-κB and CXCL1 in T84 IECs (64), PKR is not known to upregulate type I IFN production. Moreover, mice lacking PKR and WT mice produce similar magnitude type I IFN responses when stimulated with a synthetic dsRNA analog, poly(I:C), and during virus infection (17). Although several viruses signal through TLR3 and activate type I IFN responses in various other cell types, this has not been shown for IECs (65). It was reported that i.p. injection of either poly(I:C) or dsRNA prepared from rotavirus causes TLR3-dependent small intestinal injury in mice (66). Because TLR3 is generally located in the endosomal compartment, whereas RIG-I and MDA5 are regarded as cytoplasmic PRRs, it is likely that rotavirus directly enters the cytoplasmic compartment of IECs (i.e., a nonphagocytic cell type) where it interacts with RIG-I and MDA5. In contrast, after direct i.p. injection of nonphysiologic stimuli such as poly(I:C) or rotavirus dsRNA, they would likely be taken up by phagocytic mononuclear cells that have a high capacity for endocytosis (22) and encounter TLR3 in the endosomal compartment.
A third RNA helicase, LGP2, abrogated the activation of the ISRE and IFN-β by RIG-I and MDA5 in rotavirus-infected IECs. The apparent ability of LGP2 to act as a negative regulator of RIG-I and MDA5 signaling (67) may reflect a capacity to sequester RNA from RIG-I and MDA5 coupled with the inability to signal through MAVS. In this regard, LGP2 lacks the CARD domains needed for downstream signaling through MAVS, which are present in RIG-I and MDA5. Consistent with a counterregulatory role, silencing LGP2 in IECs resulted in significantly increased levels of IFN-β and less rotavirus production, and IECs that overexpressed LGP2 produced less IFN-β and greater quantities of rotavirus. Whereas others reported that LGP2-deficient mice treated with either vesicular stomatitis virus or poly(I:C) produced increased type I IFN, infection of those mice with encephalomyocarditis virus decreased type I IFN production. This suggests that the functional role of LGP2 in regulating signaling may be more complex and depend on characteristics of the infecting virus or target cells, or both (68).

Type I IFNs in mice are characterized by a single ifn-β gene and a large family of ifn-α genes whose products share a common cell surface receptor (69). IFN-β was used as an indicator of type I IFN production by IECs based on its consistent regulated production by the rotavirus-infected cell lines used in this study, and the fact that most ifn-α genes appear to be partially dependent on IFN-β for their induction (69). However, whether decreased rotavirus production by IECs and decreased rotavirus shedding reflects a direct effect of IFN-β on rotavirus replication, or also requires IFN-α and/or the products of other IFN-stimulated genes that are activated after the production of type I IFNs, is not known. Consistent with our finding that virus clearance occurred over a similar time frame postinfection in infected WT and MAVS-/- mice, virus clearance was not delayed in IFNAR-deficient mice.
(39). It is clear, however, from the studies in this paper that type I IFN, produced by IECs in response to RIG-I and MDA5 sensing of rotavirus infection, and signaling through MAVS, has a pivotal functional role in determining the amount of virus produced by the intestinal epithelium. This conclusion was further supported by our experiments in vivo using a rotavirus strain (SA11-4F) that interferes with type I IFN production by degrading IRF3 (43), which is common to the signaling pathways initiated both by RIG-I and MDA5, as well as TLR3.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary data

Supplementary Figure 1. Basal Expression of viral RNA Sensors in IECs and Upregulated IFN-β Production in Response to Infection with Increasing MOI of Rotavirus.

A, PKR, TLR3, RIG-I, MDA5 mRNA were assessed in uninfected HCA-7 and HT-29 cells by RT-PCR. Number of PCR amplification cycles (cyc) is shown on the right. (B) HCA-7 cells were infected with SA11-5S (black circle) or RRV (open circle) at various MOI for 24 h and then analyzed for IFN-β production in the supernatant. Data are mean ±SEM of three independent experiments.

Supplementary Figure 2. Role of the viral RNA Sensors RIG-I, MDA5, TLR3 and PKR in ISRE Activation and IFN-β Secretion in Rotavirus Infected HCA-7 cells.

After transfection with the indicated DN plasmid for RIG-I, MDA5, TLR3 or PKR or a plasmid control pEF-BOS lacking insert (ctrl), HCA-7 cells were infected with rotavirus SA11-5S or RRV for 24 h. (A) ISRE activation (fold induction relative to ctrl) and B, IFN-β secretion. Data are mean ±SEM of five independent experiments. *: p<0.05 compared to infected cells transfected with a control plasmid (ctrl). **: p<0.05 compared to infected cells transfected with a DN plasmid for RIG-I or MDA5 alone.

Supplementary Figure 3. Role of the viral RNA Sensors RIG-I and MDA5 in ISRE Activation and IFN-β Secretion in Rotavirus Infected HT-29 cells.

HT-29 cells were transfected with the indicated siRNAs (A), DN plasmids (B), or WT expression plasmids (C) for RIG-I and MDA5 alone or in combination, or the relevant controls (siRNA scrambled or plasmid pEF-BOS lacking insert). Cells were infected with rotavirus SA11-5S or RRV for 24 h and ISRE activation (fold induction relative to ctrl) and IFN-β secretion were determined. Data are mean ±SEM of five independent experiments. *: p<0.05 compared to infected cells transfected with a control siRNA or control plasmid (ctrl).
**: p<0.05 compared to transfection with a siRNA, DN plasmid or expression plasmids for RIG-I or MDA5 alone.

**Supplementary Figure 4.** Increased mucosal rotavirus titers and increased rotavirus shedding in the feces of adult WT mice infected with rotavirus SA11-4F compared to SA11-5S. (A) Rotavirus titers were determined in the intestinal mucosa of WT mice infected for 4 days with rotavirus SA11-4F, a strain that did not induce IFN-β in the intestinal epithelium, or SA11-5S that was shown to induce IFN-β in the intestinal epithelium. Data are mean ±SEM. *: p<0.05. n= 8 mice. (B) Fecal titers of rotavirus from mice infected with rotavirus SA11-4F (open circles) or SA11-5S (closed circles). Data are mean ±SEM. *: p<0.05. n= 4 mice per time point.

**Supplementary Table 1.** Primer sequences used in this study.
A

Log_{10} virus titer (PFU/mg of proteins)

SA11-5S  SA11-4F

B

Log_{10} virus titer (PFU/g of feces)

days p.i.
### Supplementary Table 1

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| **Mouse** | **GAPDH** | sense | atcaacacccacctctgcc |
|           |           | antisense | ccgctgactgacagtgaatc |
| **IFN-β** | sense | ctgacagctgcaatgggc |
|           | antisense | ctgtagctcggagcagagag |