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

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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-2a 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 TLR3 is localized in the endosomal membrane in many cell types, including IECs (22).

Two members of the RIG-I–like receptor (RLR) family were recognized more recently as important for cell signaling activated by dsRNA (23, 24). These RNA helicases, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5

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Abbreviations used in this article: CARD, caspase activation and recruitment domain; ctrl, 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; PKR, dsRNA-activated protein kinase; poly(I:C), polyinosinic-polycytidylic acid; PRR, 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.

(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 (LGP2) is a third RLR family member. LGP2 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 IFNRs 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% CO_2 (HCA-7 and HT-29) or 95% air/5% CO_2 (MA-104) at 37°C.

Mice

Wild type (WT) C57BL/6J (B6) mice were from The Jackson Laboratory. $PKR^{-/-}$ (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 modulated 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 HCA-7 or nondifferentiated HT-29 cells were incubated overnight 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 (5×10^7 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 LGP2 were provided by Dr. T. Fujita (Kyoto University, Kyoto, Japan) (47, 48). DN-TLR3 (TLR3-ΔTIR) 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, LGP2, and MAVS (siGENOME mixture mix), and nontargeting siRNA (siRNA scrambled) were from Dharmacon (Lafayette, CO), as described previously (31). siRNA for TLR3, PKR, RIG-I, MDA5, and LGP2 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 LGP2, 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 (pISRE-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 24-well plates) were transfected with pISRE-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 RNeasy 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 mg/ml leupeptin, 1 mM PMSF, 5 mM $NaVO_4$). 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 Ab 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 Hanks' 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. 1*A*). 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. 1*A*).

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. 1*B*). Both rotavirus strains increased ISRE activity, upregulated IFN-β mRNA levels, and increased IFN-β secretion by those cells (Fig. 1*B–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. 2*A*, 2*B*). 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. 2*A*, 2*B*). 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. 2*A*, 2*B*; Supplemental Fig. 2*A*, 2*B*). Similar results were found using HT-29 cells (Supplemental Fig. 3*A*, 3*B*).

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. 2*C*, 2*D*). 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. 3*C*). 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. 3*A*, 3*B*). Consistent with this, downstream IRF3 phosphorylation also was decreased (Fig. 3*C*). 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. 3*A–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. 4*A*). Silencing both RIG-I and MDA5 led to even greater virus titers in SA11-5S–infected cells (Fig. 4*A*). Consistent with signaling through MAVS, but not the TLR3/TRIF pathway, HCA-7 and HT-29 cells trans-

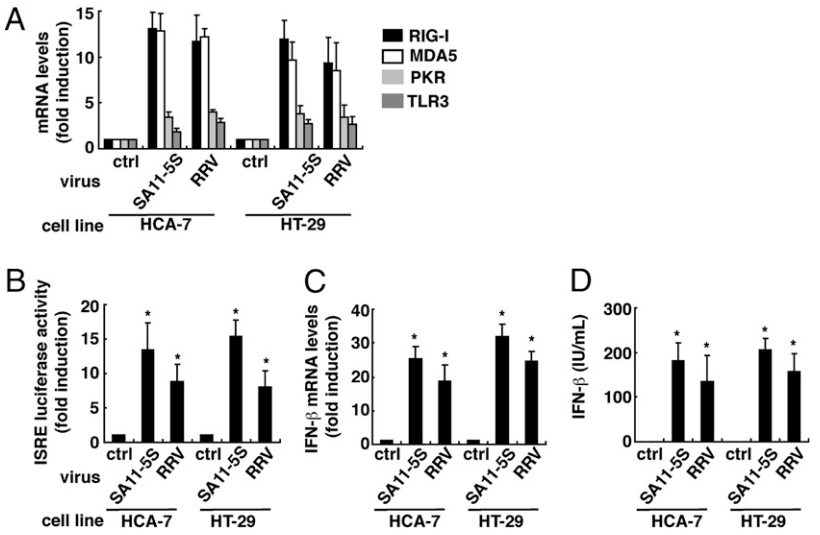

FIGURE 1. Rotavirus infection upregulates RIG-I and MDA5 mRNA expression in IECs and activates the IFN-β response. *A*, Expression of viral RNA sensors in IECs. mRNA expression of RIG-I, MDA5, PKR, and TLR3 was assessed in IECs left uninfected (control [ctrl]) or infected with rotavirus SA11-5S or RRV 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 (*B*) ISRE activation (fold induction relative to ctrl), (*C*) IFN-β mRNA levels (fold induction relative to ctrl), and (*D*) IFN-β secretion. Data are mean ± SEM of three independent experiments. $*p < 0.05$ versus ctrl.

fected with siRNA for MAVS, but not those transfected with TRIF siRNA, had significantly increased virus titers (Fig. 4*B*). Conversely, transfecting IECs with plasmids that overexpress RIG-I, MDA5,

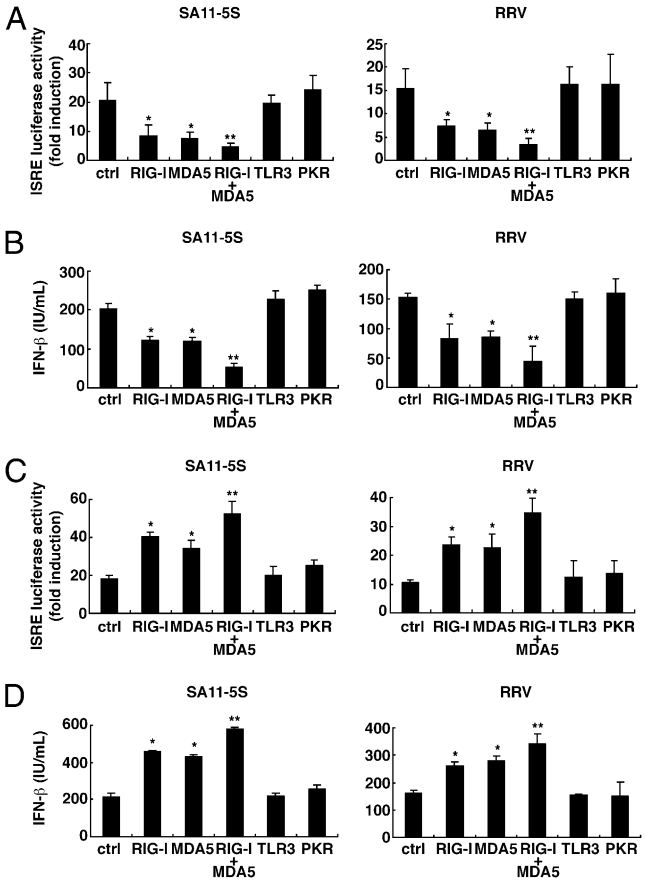

FIGURE 2. Role of viral RNA sensors RIG-I, MDA5, TLR3, and PKR in ISRE activation and IFN-β secretion in rotavirus-infected IECs. After transfection with the indicated siRNAs (*A*, *B*) or expression plasmids (*C*, *D*), HCA-7 cells were infected with rotavirus SA11-5S or RRV for 24 h. *A*, ISRE activation (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.

or both resulted in a significant decrease in virus titers in infected IECs (Fig. 4*C*).

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. 5*A*). IECs transfected with siRNA for LGP2 and infected with SA11-5S or RRV had significantly increased ISRE activation and IFN-β production (Fig. 5*B*, 5*C*). Conversely, overexpression of LGP2 in IECs significantly decreased ISRE activation and IFN-β production in rotavirus-infected IECs (Fig. 5*D*, 5*E*). Consistent with this, titers of rotavirus were significantly decreased in rotavirus-infected IECs transfected with LGP2 siRNA (Fig. 5*F*) and significantly increased in rotavirus-infected IECs transfected with a LGP2 expression plasmid (Fig. 5*G*). 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. 6*G*). 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. 6*A*) but not *MAVS*$^{-/-}$ mice (Fig. 6*B*, 6*C*) 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. 6*B*, 6*C*). IFN-β was not detectable in the sera of rotavirus-infected *MAVS*$^{-/-}$ mice (Fig. 6*D*) but increased to similar levels in infected

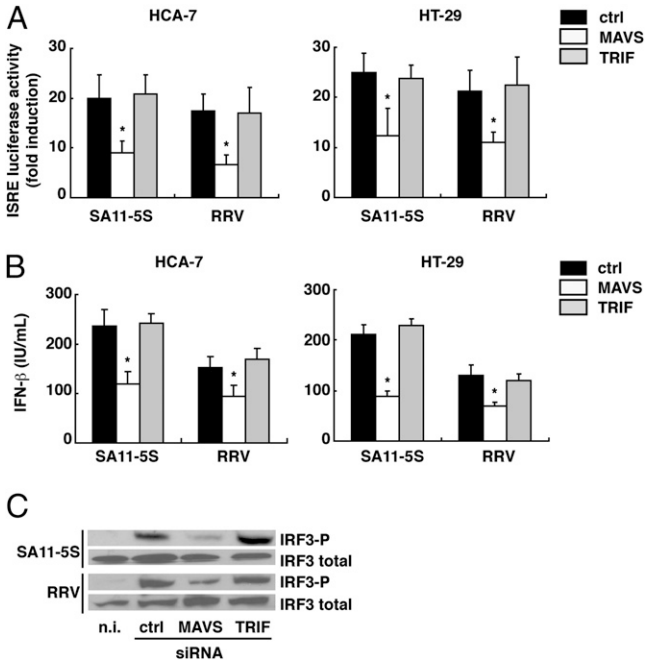

FIGURE 3. Role of the adaptor molecules MAVS and TRIF in ISRE activation, and the IFN-β response in rotavirus-infected IECs. IECs were transfected with the indicated siRNAs (control [ctrl], MAVS, or TRIF) and 24 h later infected with rotavirus SA11-5S or RRV. Cells were analyzed 24 h postinfection for (*A*) ISRE activation (fold induction relative to ctrl) and (*B*) IFN-β secretion. Data are mean ± SEM of four independent experiments. $*p < 0.05$ versus ctrl. *C*, After transfection with the indicated siRNAs (ctrl, MAVS, or TRIF) for 24 h, HCA-7 cells were infected with rotavirus SA11-5S or RRV for 24 h or not infected (n.i.). Total cellular protein was analyzed by immunoblot for IRF3 phosphorylation. Results are from a representative experiment. Similar results were seen in three independent experiments.

WT, $TLR3^{-/-}$, $TRIF^{-/-}$, and $PKR^{-/-}$ mice (Fig. 6*D*). Four days postinfection, $MAVS^{-/-}$, but not $TRIF^{-/-}$, mice had significantly increased titers of rotavirus in isolated IECs compared with WT-infected controls (Fig. 6*E*) and significantly greater virus shedding in the feces (Fig. 6*F*). Rotavirus-infected $MAVS^{-/-}$ mice did not develop diarrhea or significant histologic abnormalities of small IECs, but did manifest an increased cellular infiltrate in the lamina propria of many villi (Fig. 6*G*). Nonetheless, virus clearance occurred over a similar time frame postinfection in infected $MAVS^{-/-}$ and WT mice (Fig. 6*F*). 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 small intestine (data not shown) and had increased titers of virus in the intestinal epithelium and feces (Supplemental Fig. 4*A*, 4*B*). 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

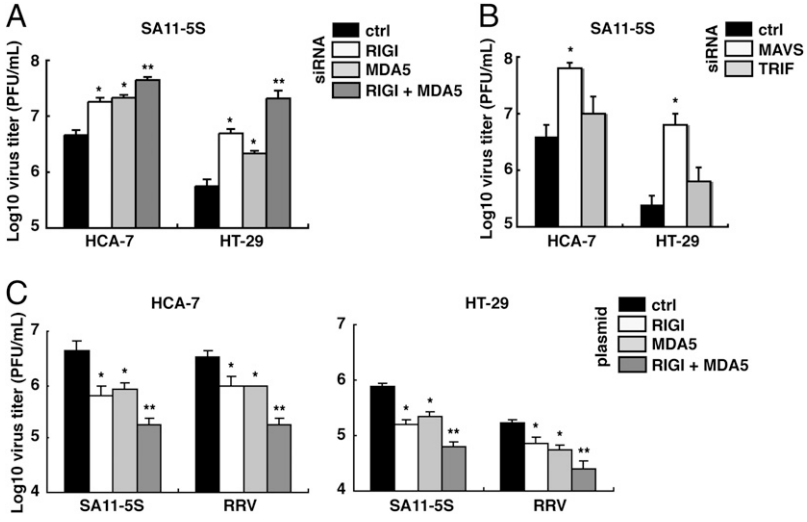

FIGURE 4. Expression levels of RIG-I and MDA5 in IECs significantly affect rotavirus production. HCA-7 and HT-29 cells were transfected with the indicated siRNAs (*A*, *B*) or expression plasmids (*C*) and then infected for 24 h with the indicated rotavirus strains. Virus titers were determined by plaque assay. Data are mean ± SEM of triplicate samples from a representative experiment. Similar results were obtained in five independent experiments. $*p < 0.05$ versus control [ctrl]; $**p < 0.05$ versus transfection with RIG-I or MDA5 siRNA or expression plasmids alone.

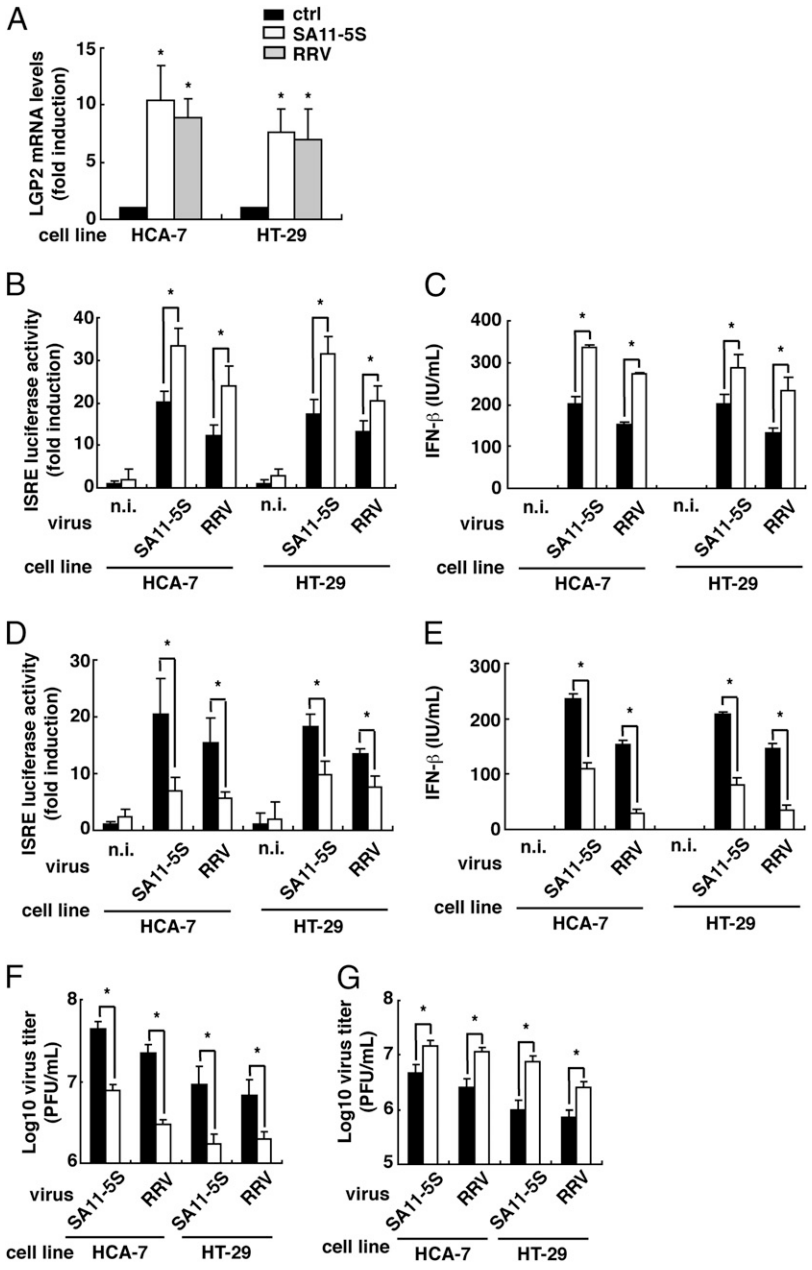

FIGURE 5. LGP2 counterregulates signaling through RIG-I and MDA5 in rotavirus-infected IECs. *A*, LGP2 mRNA levels (fold induction relative to control [ctrl]) in HCA-7 and HT-29 cells infected with rotavirus for 24 h or left uninfected (ctrl). $*p < 0.05$ versus ctrl. *B* and *C*, HCA-7 and HT-29 cells transfected with ctrl siRNA (black bars) or LGP2 siRNA (white bars) were infected with rotavirus SA11-5S or RRV for 24 h or left uninfected (n.i.), and ISRE activation (fold induction relative to n.i.) (*B*) and IFN-β production (*C*) were determined. *D* and *E*, HCA-7 and HT-29 cells transfected with plasmid ctrl (black bar) or LGP2 expression plasmid (white bar) were infected with rotavirus SA11-5S or RRV for 24 h or left uninfected (n.i.), and ISRE activation (fold induction relative to n.i.) (*D*) and IFN-β production (*E*) were determined. *F*, Rotavirus titers after HCA-7 and HT-29 cells were transfected with ctrl siRNA (black bar) or LGP2 siRNA (white bar) and then infected with rotavirus SA11-5S or RRV for 24 h. *G*, Rotavirus titers after HCA-7 and HT29 cells were transfected with plasmid ctrl (black bar) or LGP2 expression plasmid (white bar) and then infected with rotavirus SA11-5S or RRV for 24 h. Data are mean ± SEM of five independent experiments. *B–G*, $*p < 0.05$ versus infected IECs transfected with the corresponding ctrl siRNA or ctrl plasmid (ctrl).

(59). The specific characteristics of rotavirus that allow it to signal through both RIG-1 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.

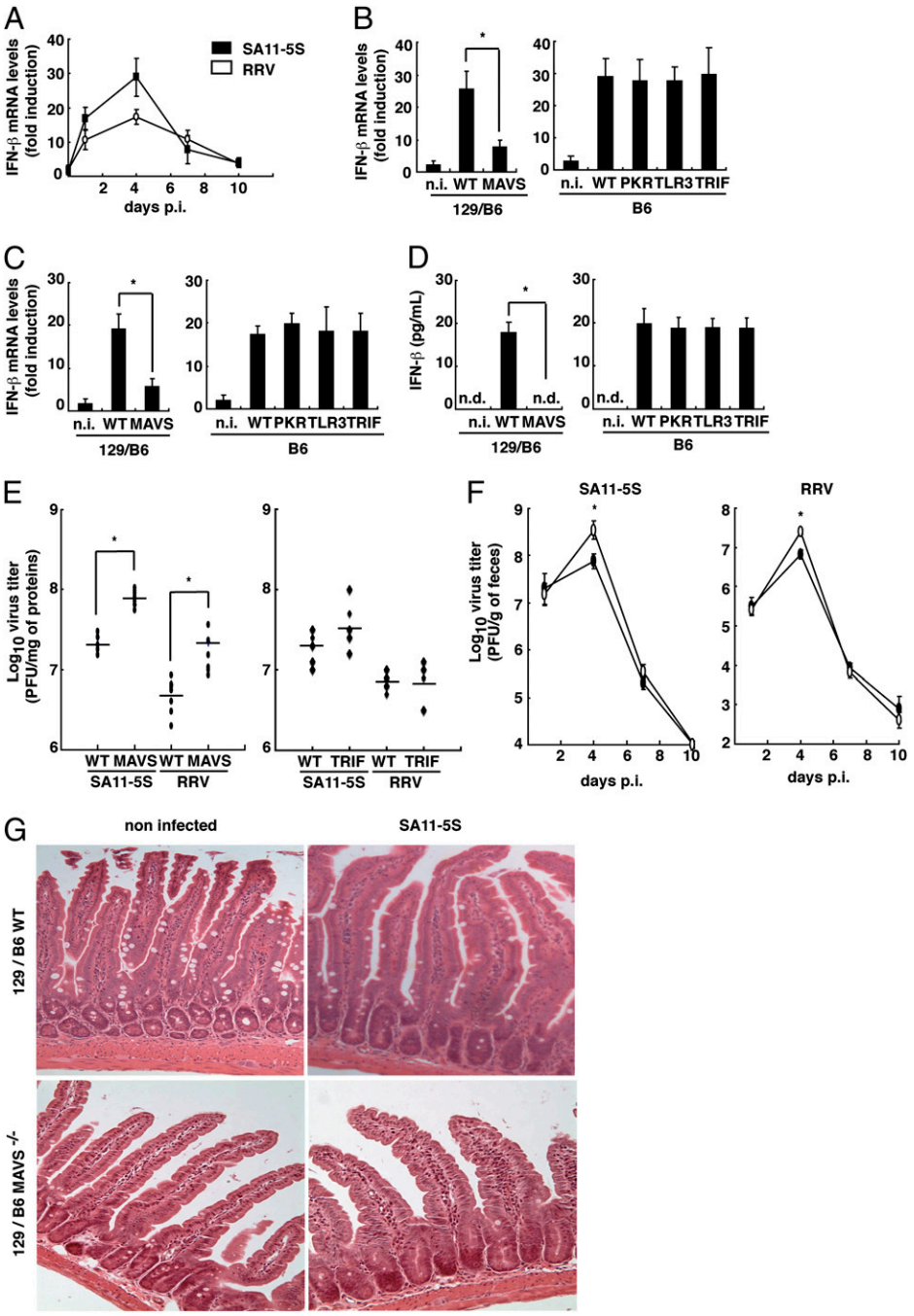

FIGURE 6. Decreased epithelial cell IFN-β response and increased IEC virus replication and shedding in adult rotavirus-infected mice lacking MAVS. *A*, Time course postinfection (p.i.) of increased IFN-β mRNA levels in WT mice infected with rotavirus SA115S or RRV (fold induction relative to control [ctrl]). *B* and *C*, IFN-β mRNA levels in IECs from infected WT mice and genetically mutant mice lacking PKR, TLR3, TRIF, and MAVS were assayed 4 d postinfection with rotavirus SA11-5S (*B*) or RRV (*C*); values are fold induction over the relevant ctrl IECs from B6 or 129/B6 mice that were not infected (n.i.). *D*, Serum IFN-β levels 4 d postinfection of WT mice and mice lacking PKR, TLR3, TRIF, or MAVS with rotavirus SA11-5S were determined by ELISA. n.d., none detected. *A*, $n = 6$–9 mice per data point. *B* and *C*, $n = 7$–10 mice per group. *D*, $n = 6$–9 mice per group. $*p < 0.05$. *E*, Rotavirus titers were determined in IECs from WT, MAVS$^{-/-}$, and TRIF$^{-/-}$ mice infected for 4 d with rotavirus SA11-5S or RRV. Horizontal line is geometric mean. $*p < 0.05$. $n = 5$–8 mice. *F*, Fecal titers of rotavirus in WT (closed circles) and MAVS$^{-/-}$ (open circles) mice infected for the time indicated with rotavirus SA11-5S or RRV. Data are mean ± SEM. $*p < 0.05$. $n = 4$ mice per time point. *G*, 129/B6 WT and MAVS$^{-/-}$ mice were left uninfected or infected with rotavirus SA11-5S for 4 d. Sections of proximal small intestine were stained with H&E. Original magnification ×200.

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 *ifnβ* gene and a large family of *ifnα* 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 *ifnα* 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

1. Nagler-Anderson, C. 2001. Man the barrier! Strategic defences in the intestinal mucosa. *Nat. Rev. Immunol.* 1: 59–67.
2. Wilhelmi, I., E. Roman, and A. Sánchez-Fauquier. 2003. Viruses causing gastroenteritis. *Clin. Microbiol. Infect.* 9: 247–262.
3. Kelly, D., S. Conway, and R. Aminov. 2005. Commensal gut bacteria: mechanisms of immune modulation. *Trends Immunol.* 26: 326–333.
4. Chae, S., L. Eckmann, Y. Miyamoto, C. Pothoulakis, M. Karin, and M. F. Kagnoff. 2006. Epithelial cell I κ B-kinase β has an important protective role in *Clostridium difficile* toxin A-induced mucosal injury. *J. Immunol.* 177: 1214–1220.
5. Kagnoff, M. F. 2006. Microbial-epithelial cell crosstalk during inflammation: the host response. *Ann. NY Acad. Sci.* 1072: 313–320.
6. Ramig, R. F. 2004. Pathogenesis of intestinal and systemic rotavirus infection. *J. Virol.* 78: 10213–10220.
7. Widdowson, M. A., J. S. Bresee, J. R. Gentsch, and R. I. Glass. 2005. Rotavirus disease and its prevention. *Curr. Opin. Gastroenterol.* 21: 26–31.
8. Rollo, E. E., K. P. Kumar, N. C. Reich, J. Cohen, J. Angel, H. B. Greenberg, R. Sheth, J. Anderson, B. Oh, S. J. Hempson, et al. 1999. The epithelial cell response to rotavirus infection. *J. Immunol.* 163: 4442–4452.
9. Ciarlet, M., and M. K. Estes. 2001. Interactions between rotavirus and gastrointestinal cells. *Curr. Opin. Microbiol.* 4: 435–441.
10. Sen, G. C., and S. N. Sarkar. 2005. Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev.* 16: 1–14.
11. Haller, O., G. Kochs, and F. Weber. 2006. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* 344: 119–130.
12. Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. *Nat. Immunol.* 7: 131–137.
13. Meylan, E., and J. Tschopp. 2006. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol. Cell* 22: 561–569.
14. Williams, B. R. 2001. Signal integration via PKR. *Sci. STKE* 2001: re2.
15. Lyles, D. S. 2000. Cytopathogenesis and inhibition of host gene expression by RNA viruses. *Microbiol. Mol. Biol. Rev.* 64: 709–724.
16. Hsu, L. C., J. M. Park, K. Zhang, J. L. Luo, S. Maeda, R. J. Kaufman, L. Eckmann, D. G. Guiney, and M. Karin. 2004. The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature* 428: 341–345.
17. Yang, Y. L., L. F. Reis, J. Pavlovic, A. Aguzzi, R. Schäfer, A. Kumar, B. R. Williams, M. Aguet, and C. Weissmann. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* 14: 6095–6106.
18. Tian, B., P. C. Bevilacqua, A. Diegelman-Parente, and M. B. Mathews. 2004. The double-stranded-RNA-binding motif: interference and much more. *Nat. Rev. Mol. Cell Biol.* 5: 1013–1023.
19. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.
20. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol.* 169: 6668–6672.
21. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, et al. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101–105.
22. Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171: 3154–3162.
23. Takeuchi, O., and S. Akira. 2008. MDA5/RIG-I and virus recognition. *Curr. Opin. Immunol.* 20: 17–22.
24. Wilkins, C., and M. Gale, Jr. 2010. Recognition of viruses by cytoplasmic sensors. *Curr. Opin. Immunol.* 22: 41–47.
25. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6: 981–988.
26. Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167–1172.
27. Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122: 669–682.
28. Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu. 2005. VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol. Cell* 19: 727–740.
29. Johnson, C. L., and M. Gale, Jr. 2006. CARD games between virus and host get a new player. *Trends Immunol.* 27: 1–4.
30. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19–28.
31. Hirata, Y., A. H. Broquet, L. Menchén, and M. F. Kagnoff. 2007. Activation of innate immune defense mechanisms by signaling through RIG-I/IPS-1 in intestinal epithelial cells. *J. Immunol.* 179: 5425–5432.
32. Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J. Immunol.* 175: 5260–5268.
33. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, et al. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* 175: 2851–2858.
34. Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J. Immunol.* 178: 6444–6455.
35. Samuel, C. E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14: 778–809 (table of contents).
36. Taniguchi, T., and A. Takaoka. 2002. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr. Opin. Immunol.* 14: 111–116.
37. McCartney, S. A., and M. Colonna. 2009. Viral sensors: diversity in pathogen recognition. *Immunol. Rev.* 227: 87–94.
38. Bass, D. M. 1997. Interferon gamma and interleukin 1, but not interferon alfa, inhibit rotavirus entry into human intestinal cell lines. *Gastroenterology* 113: 81–89.
39. Angel, J., M. A. Franco, H. B. Greenberg, and D. Bass. 1999. Lack of a role for type I and type II interferons in the resolution of rotavirus-induced diarrhea and infection in mice. *J. Interferon Cytokine Res.* 19: 655–659.
40. Feng, N., B. Kim, M. Fenaux, H. Nguyen, P. Vo, M. B. Omary, and H. B. Greenberg. 2008. Role of interferon in homologous and heterologous rotavirus infection in the intestines and extraintestinal organs of suckling mice. *J. Virol.* 82: 7578–7590.
41. Berin, M. C., M. B. Dwinell, L. Eckmann, and M. F. Kagnoff. 2001. Production of MDC/CCL22 by human intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280: G1217–G1226.
42. Sun, Q., L. Sun, H. H. Liu, X. Chen, R. B. Seth, J. Forman, and Z. J. Chen. 2006. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* 24: 633–642.
43. Barro, M., and J. T. Patton. 2005. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. *Proc. Natl. Acad. Sci. USA* 102: 4114–4119.
44. Douagi, I., G. M. McInerney, A. S. Hidmark, V. Miriallis, K. Johansen, L. Svensson, and G. B. Karlsson Hedestam. 2007. Role of interferon regulatory factor 3 in type I interferon responses in rotavirus-infected dendritic cells and fibroblasts. *J. Virol.* 81: 2758–2768.
45. Sherry, B. 2009. Rotavirus and reovirus modulation of the interferon response. *J. Interferon Cytokine Res.* 29: 559–567.
46. Broquet, A. H., C. Lenoir, A. Gardet, C. Sapin, S. Chwetzoff, A. M. Jouniaux, S. Lopez, G. Trugnan, M. Bachelet, and G. Thomas. 2007. Hsp70 negatively controls rotavirus protein bioavailability in caco-2 cells infected by the rotavirus RF strain. *J. Virol.* 81: 1297–1304.
47. Iwamura, T., M. Yoneyama, N. Koizumi, Y. Okabe, H. Namiki, C. E. Samuel, and T. Fujita. 2001. PACT, a double-stranded RNA binding protein acts as a positive regulator for type I interferon gene induced by Newcastle disease virus. *Biochem. Biophys. Res. Commun.* 282: 515–523.
48. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5: 730–737.
49. Maaser, C., M. P. Housley, M. Iimura, J. R. Smith, B. A. Vallance, B. B. Finlay, J. R. Schreiber, N. M. Varki, M. F. Kagnoff, and L. Eckmann. 2004. Clearance of *Citrobacter* rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. *Infect. Immun.* 72: 3315–3324.
50. Saito, T., R. Hirai, Y. M. Loo, D. Owen, C. L. Johnson, S. C. Sinha, S. Akira, T. Fujita, and M. Gale, Jr. 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc. Natl. Acad. Sci. USA* 104: 582–587.
51. Jaimes, M. C., N. Feng, and H. B. Greenberg. 2005. Characterization of homologous and heterologous rotavirus-specific T-cell responses in infant and adult mice. *J. Virol.* 79: 4568–4579.

52. Vancott, J. L., M. M. McNeal, A. H. Choi, and R. L. Ward. 2003. The role of interferons in rotavirus infections and protection. *J. Interferon Cytokine Res.* 23: 163–170.
53. Warfield, K. L., S. E. Blutt, S. E. Crawford, G. Kang, and M. E. Conner. 2006. Rotavirus infection enhances lipopolysaccharide-induced intussusception in a mouse model. *J. Virol.* 80: 12377–12386.
54. Kordasti, S., C. Istrate, M. Banasaz, M. Rottenberg, H. Sjövall, O. Lundgren, and L. Svensson. 2006. Rotavirus infection is not associated with small intestinal fluid secretion in the adult mouse. *J. Virol.* 80: 11355–11361.
55. Fredericksen, B. L., B. C. Keller, J. Fornek, M. G. Katze, and M. Gale, Jr. 2008. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J. Virol.* 82: 609–616.
56. Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. García-Sastre, M. G. Katze, and M. Gale, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J. Virol.* 82: 335–345.
57. Hornung, V., J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, et al. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994–997.
58. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Näslund, P. Liljeström, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314: 997–1001.
59. Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J. Exp. Med.* 205: 1601–1610.
60. Patton, J. T., R. Vasquez-Del Carpio, M. A. Tortorici, and Z. F. Taraporewala. 2007. Coupling of rotavirus genome replication and capsid assembly. *Adv. Virus Res.* 69: 167–201.
61. Patton, J. T., R. Vasquez-Del Carpio, and E. Spencer. 2004. Replication and transcription of the rotavirus genome. *Curr. Pharm. Des.* 10: 3769–3777.
62. Pesavento, J. B., S. E. Crawford, M. K. Estes, and B. V. Prasad. 2006. Rotavirus proteins: structure and assembly. *Curr. Top. Microbiol. Immunol.* 309: 189–219.
63. Guglielmi, K. M., S. M. McDonald, and J. T. Patton. 2010. Mechanism of intraparticle synthesis of the rotavirus double-stranded RNA genome. *J. Biol. Chem.* 285: 18123–18128.
64. Vijay-Kumar, M., J. R. Gentsch, W. J. Kaiser, N. Borregaard, M. K. Offermann, A. S. Neish, and A. T. Gewirtz. 2005. Protein kinase R mediates intestinal epithelial gene remodeling in response to double-stranded RNA and live rotavirus. *J. Immunol.* 174: 6322–6331.
65. Vercammen, E., J. Staal, and R. Beyaert. 2008. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. *Clin. Microbiol. Rev.* 21: 13–25.
66. Zhou, R., H. Wei, R. Sun, and Z. Tian. 2007. Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. *J. Immunol.* 178: 4548–4556.
67. Komuro, A., and C. M. Horvath. 2006. RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *J. Virol.* 80: 12332–12342.
68. Satoh, T., H. Kato, Y. Kumagai, M. Yoneyama, S. Sato, K. Matsushita, T. Tsujimura, T. Fujita, S. Akira, and O. Takeuchi. 2010. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc. Natl. Acad. Sci. USA* 107: 1512–1517.
69. Deonarain, R., A. Alcamí, M. Alexiou, M. J. Dallman, D. R. Gewert, and A. C. Porter. 2000. Impaired antiviral response and alpha/beta interferon induction in mice lacking beta interferon. *J. Virol.* 74: 3404–3409.