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Activation of Innate Immune Defense Mechanisms by Signaling through RIG-I/IPS-1 in Intestinal Epithelial Cells 1

Yoshihiro Hirata,* Alexis H. Broquet,* Luis Menchén,* and Martin F. Kagnoff2*†

Intestinal epithelial cells (IECs) are a first line of defense against microbial pathogens that enter the host through the intestinal tract. Moreover, viral pathogens that infect the host via the intestinal epithelium are an important cause of morbidity and mortality. However, the mechanisms by which viral pathogens activate antiviral defense mechanisms in IECs are largely unknown. The synthetic dsRNA analog polyinosinic-polycytidylic acid and infection with live virus were used to probe the molecules that are activated and the mechanisms of signaling in virus-infected human IECs. Polyinosinic-polycytidylic acid activated IFN regulatory factor 3 dimerization and phosphorylation, increased activity of the IFN-stimulated response element, induced a significant increase in IFN-β mRNA transcripts and IFN-β secretion, and up-regulated the expression of IFN-regulated genes in IECs. Those responses were dependent upon activation of the dsRNA binding protein retinoic acid inducible gene I (RIG-I) and the RIG-I interacting protein IFN promoter stimulator-1, but not on dsRNA-activated protein kinase or TLR3, which also were expressed by IECs. Virus replication and virus-induced cell death increased in IECs in which RIG-I was silenced, consistent with the importance of the RIG-I signaling pathway in IEC antiviral innate immune defense mechanisms. The Journal of Immunology, 2007, 179: 5425–5432.

The single layer of intestinal epithelial cells (IEC)3 that lines the intestinal mucosa provides a first line of defense against enteric microbial pathogens (1–3). Microbial infection of the intestinal mucosa often is accompanied by structural and functional changes in the epithelium that activate mucosal inflammation and result in severe diarrhea (1, 3) and systemic infection. Pattern-recognition receptors, such as TLRs and nucleotide-binding oligomerization domain proteins, are important extracellular or endosomal membrane-associated and cytosolic epithelial cell sensors of microbial products, and can signal the onset of host innate immune responses (4–6). Such responses include the secretion of chemokines that chemoattract and activate target cells important for mediating innate mucosal defense (4, 5).

Enteric viruses can cause significant diarrheal illness. For example, intestinal rotavirus infection causes severe diarrhea in infants and young children and as many as 500,000 deaths worldwide each year (1, 7, 8). Other enteric viruses (e.g., norovirus) cause an acute gastroenteritis and have gained notoriety because of large outbreaks of gastroenteritis among passengers on cruise ships. Calicivirus and astrovirus also are known to infect the intestinal epithelium (1). Although virus-induced intestinal inflammation is often self-limited, severe dehydration frequently occurs in children, the elderly, and immunocompromised patients. The ability to prevent and alter the course of acute gastroenteritis caused by enteric viruses represents an important challenge in public health.

Enteric viruses are pathogenic following their uptake into host intestinal epithelial cells. After internalization, RNA viruses, such as rotavirus, generate dsRNA via RNA-dependent RNA synthesis. Single-stranded RNA viruses and some DNA viruses also produce dsRNA as part of their life cycle. The presence of dsRNA in the cell is a signature of virus infection and can activate cellular sensors that signal host cellular responses (9–11), which have a key role in controlling virus infection (12). The production of IFNs and IFN-regulated gene products are particularly important in this regard.

Several host cellular proteins that interact with dsRNA are linked to the recognition of internalized viruses and the signaling of innate immune responses (5, 9–13). dsRNA-activated protein kinase (PKR) is a Ser/Thr kinase (14) that contains dsRNA binding motifs in its N terminus and undergoes a conformational change that activates its kinase activity upon binding dsRNA. PKR phosphorylates eIF-2α and inhibits translation initiation (15). In addition, PKR can activate the transcription factor NF-κB, which is central to regulating cytokine expression and the ensuing inflammatory response (16), and PKR can regulate apoptotic pathways important for eliminating virus-infected cells (17). Although PKR is an important IFN-regulated gene, it does not appear to have a direct role in increasing IFN production in virus-infected cells (18).

TLR3 is another dsRNA interacting protein that activates innate immune responses (9–11, 19). In response to dsRNA, TLR3 transduces a signal to the adaptor molecule Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), and downstream of TRIF signals the activation of IFN regulatory factor 3 (IRF3) and NF-κB (20). Mice genetically deficient in TRIF signaling have increased susceptibility to mouse cytomegalovirus infection, indicating a significant role for the TLR3-TRIF signaling pathway in viral pathogenesis (21). TLR3 is generally localized in the endosomal membrane.
dsRNA and activate antiviral defense. Cardif/MAVS/VISA as a major mechanism by which IECs detect interacting protein IFN promoter stimulator-1 (IPS-1, also termed the present study, we define signaling through RIG-I and the RIG-I cells as assessed by cell viability and virus replication (24, 25). In these RNA helicases mediated antiviral effects in virus-infected infection (24, 25). When transfected into murine fibroblasts, both of abrogate ISRE activity and IFN secretion in response to virus in- tective (DN) constructs of these RNA helicases that lack CARDs can in independent experiments.

A third group of proteins, the RNA helicases, are important for dsRNA-mediated cell signaling (9, 10). Retinoic acid inducible gene I (RIG-I) and myeloma differentiation associated gene 5 (MDA5) have DExD/H box helicase domains that unwind dsRNA through their ATPase activity. Both proteins also contain caspase activation and recruitment domains (CARDs) that enable protein-protein interactions for signal transduction. Overexpression of these proteins activates the IFN-stimulated response element (ISRE) and NF-kB driven transcription (24, 25). Dominant negative (DN) constructs of these RNA helicases that lack CARDs can abrogate ISRE activity and IFN secretion in response to virus infection (24, 25). When transfected into murine fibroblasts, both of these RNA helicases mediated antiviral effects in virus-infected cells as assessed by cell viability and virus replication (24, 25). In the present study, we define signaling through RIG-I and the RIG-I interacting protein IFN promoter stimulator-1 (IPS-1, also termed Cardif/MAVS/VISA) as a major mechanism by which IECs detect dsRNA and activate antiviral defense.

Materials and Methods

Human intestinal epithelial cell lines

The human colon cell lines HCA-7, HT-29, and SW480 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine (26). Cells were maintained in 95% air-5% CO2 at 37°C.

Reagents

Polyinosinic-polycytidylic acid (poly(I:C)) was from Calbiochem. Rabbit anti-IRF3 Ab was from IBL. Rabbit anti-IFNγ, anti-phospho-IRF3, anti- phospho-PKR, and anti-phospho-STAT1 Abs were from Cell Signaling Technology. Mouse monoclonal anti-β-actin and 2-aminopurine (2-AP) were from Sigma-Aldrich. Rabbit anti-PKR was from Santa Cruz Biotechnology. Mouse IgG2a monoclonal anti-α-IFN receptor chain 2 Ab (α-IFNR) (5 μg/ml) or anti-α-IFN receptor chain 2 Ab (α-IFNR) (5 μg/ml) for 12 h. IFN-β, IP-10, and ISG56 mRNA levels were determined by real-time RT-PCR and shown as fold-induction relative to control cultures. Values are mean ± SD of triplicates cultures in a representative experiment. Similar results were obtained in three independent experiments. * p < 0.05; ** p < 0.01.

Cell stimulation

Poly(I:C) dissolved in PBS (pIC-P) or PBS containing LipofectAMINE 2000 (LF; Invitrogen) (pIC-L) was added to IEC cultures, as indicated, for 24 h. Controls contained PBS or PBS with LF alone. 2-AP (10 mM) was added 1 h before the addition of poly(I:C), as indicated in some experiments.

Quantification of IFN-β by ELISA

ELISA for IFN-β used a HulIFN-β ELISA kit (Fujirebio) according to the manufacturer’s instructions. The detection limit of the assay was 2.5 U/ml.

Plasmids, small interfering RNA (siRNA), and transfection

DN RIG-I (RIG-C), wild type TLR3, and PKR expression vectors were provided by Dr. T. Fujita (Kyoto University, Kyoto, Japan) (25, 27). DN TLR3 (TLR3-ΔTIR) was constructed by deleting the TIR domain from a full-length human TLR3 expression vector. Kinase mutant PKR (PKR K296R) was generated by replacing lysine residues with arginine using site-directed mutagenesis. All vectors were derivatives of the same back- bone vector (pEF vector). Each of these DN vectors was shown to inhibit the activity of its respective target in cells transfected with the relevant wild type vector alone or together with the DN vector, and subsequently stimulated with known agonists of the wild type construct. Reporter plasmids

**FIGURE 1.** Induction of type I IFN responses in IECs. HT-29, HCA-7, and SW480 cells were incubated with poly(I:C) (1 or 10 μg/ml) in the presence (pIC-L) or absence (pIC-P) of LF or with LF alone (control) for 24 h. A, IFN-β mRNA levels were determined by real-time PCR and are expressed as fold-induction relative to control cultures. Values are mean ± SD of triplicates cultures in a representative experiment. Similar results were obtained in three independent experiments. B, HT-29, HCA-7, and SW480 cells were stimulated with poly(I:C) (10 μg/ml) for 24 h as described above, after which IFN-β in culture supernatants was measured by ELISA. Values are mean ± SD of triplicates cultures in a representative experiment. Similar results were obtained in three independent experiments. C, HT-29 cells were incubated with poly(I:C) (10 μg/ml) in LF for the indicated times, after which mRNA levels of IP-10 and ISG56 were determined by real-time PCR. Data are fold-induction relative to control cultures incubated with LF alone. Values are mean ± SD of triplicates cultures in a representative experiment. Similar results were obtained in three independent experiments. D, HT-29 cells were stimulated with pIC-L (10 μg/ml) for 12 h, after which medium was replaced with fresh medium including mouse IgG (5 μg/ml) or anti-α-IFN receptor chain 2 Ab (α-IFNR) (5 μg/ml) for 12 h. IFN-β, IP-10, and ISG56 mRNA levels were determined by real-time RT-PCR and shown as fold-induction relative to control cultures. Values are mean ± SD of triplicates cultures in a representative experiment. Similar results were obtained in three independent experiments. * p < 0.05; ** p < 0.01.
for the ISRE (pISRE-Luc) and for the internal control (pRL-TK) were from Stratagene and Promega, respectively. siRNA oligonucleotides for silencing RIG-I (DDX58), TLR3, TRIF, PKR (PRKR), and IPS-1 (KIAA1271) (siGENOME mixture mix) and nontargeting siRNA (si control) were from Dharmacon. siRNA (100 nM) was transfected using LF 48 h before poly(I:C) stimulation or viral infection. For vector-mediated silencing, the pSUPER RNAi system (Oligoengine) was used. Two sequences for RIG-I, Sequence 1: 5'-H11032-AATTCATCAGAGATAGTCA-3' and Sequence 2: 5'-H11032-GGAAGAGGTGCAGTATATT-3' were inserted into pSUPERpuro vectors that contain a puromycin resistance gene to generate pS-RIG-1 and pS-RIG-2 respectively. The vector containing sequences for luciferase (pS-luc) was used as a control. For selection of pSUPER-transfected cells, HT-29 cells in 10 cm dishes were transected with 10 µg of pSUPER vector for 24 h with Effectene (Qiagen), after which cells were treated with puromycin (3 µg/ml) for 24 h, and with fresh medium without puromycin for an additional 24 h. Cells were removed with 0.25% trypsin and plated for experiments.

**Reporter assay**

IECs (5 × 10⁶ cells in 24-well plates) were transfected with 300 ng pISRE-luc and 10 ng of pRL-TK using LF for 24 h (48 h in experiments using siRNA). DN vectors and/or their controls (900 ng total) or siRNA oligonucleotides (100 nM) were included, as indicated. After stimulation with poly(I:C), cell lysates were prepared. Luciferase activity was measured using the Dual Luciferase 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 RT-PCR

Human intestinal epithelial cells (1 x 10^6) in 12-well plates were stimulated with poly(I:C) for the indicated time periods, after which total cellular RNA was extracted using RNeasy (Qiagen), followed by DNase I treatment according to the manufacturer's instructions. Reverse transcription used 1 μg RNA and the Impron II Reverse Transcription System (Promega). cDNA was PCR amplified using the following primers:

- IFN-β: sense 5'-TGCTCTCCTGTGGCTTCCTC-3', antisense 5'-TGTCTGATGGTCATCGCCG-3'; IsoG56: sense 5'-TAGCCACACATGCTTACAGCAGAC-3', antisense 5'-CTTTTACCATCCCCCTTCA-3'; TLR3: sense 5'-TGCTCTCCTGTGGCTTCCTC-3', antisense 5'-GACCTCCATCATCACCTCTT-3'; RIG-I: sense 5'-CAGTATATTCTAGGCGGAG-3', antisense 5'-GCCCATTTCCCTTCTGTGC-3'; PKR: sense 5'-GGATTGGGCAAGTCTTCTTC-3', antisense 5'-ATCCTACACCTCCAAACACG-3'.

The amplification profile for qualitative RT-PCR was 15 min denaturation at 95°C, followed by 40 cycles of amplification at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. For real-time quantitative PCR, cDNA was mixed with 2 μl SYBR Green Master mix (Applied Biosystems). 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 the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Immunoblot analysis

IECs were cultured and stimulated with poly(I:C) in 12-well plates. Cells were washed in ice-cold PBS and lysed in lysis buffer (50 mM Tris-Cl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 100 μg/ml leupeptin, 1 mM PMSF, 5 mM Na_3VO_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. For IRF3 detection by native-PAGE, aliquots (10 μg) were mixed with 2× native sample buffer (125 mM Tris-HCl, pH 6.8, 30% glycerol) and separated on Ready Gels (BioRad) using native-PAGE buffer (25 mM Tris-Cl (pH 8.4), 192 mM glycine). Proteins were transferred to PVDF membranes and probed with the indicated primary Ab followed by HRP-conjugated secondary Ab, and developed using the ECL plus kit (GE Healthcare).
EMSA

IECs in 6 cm dishes were stimulated with pIC-L or pIC-P (10 μg/ml) for 24 h or IFN-α2 (1000 U/ml) for 2 h, after which nuclear extracts were prepared using a nonionic detergent method (28). Nuclear extracts (10 μg) were assayed for ISRE binding using a [32P]-labeled double-stranded oligonucleotide corresponding to the ISRE of the ISG15 gene (‘5‘-GATCG GAAAGGGAAACCGAAACTGAAGCC-3‘). Binding reactions were done for 20 min at RT in binding buffer (10 mM Tris–Cl (pH 7.5), 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 mM MgCl2, 4% glycerol, and 50 μg/ml poly(dI-dC)). Specificity was assessed by using an excess (1000x) of nonlabeled probe. Extracts were electrophoresed on 4% polyacrylamide gel at 100 V for 1 h. Oct-1 binding activity was used as a loading control. Gels were dried and exposed to Kodak BioMax MS film at −80°C.

Viral infection, plaque assay, and cell killing assay

Puromycin-resistant HT-29 cells transfected with the indicated pSUPER siRNA vector were seeded in 24-well plates. Vesicular stomatitis virus (VSV, Indiana strain) at various multiplicities of infection (MOI) was added to culture medium for 1 h and replaced with fresh medium for 24 h. Simian rotavirus (SA11–5S), donated by Dr. J. T. Patton (National Institute of Health), was activated by 0.44 μg/ml trypsin for 30 min at 37°C, and then added to HT-29 cells at a MOI of 2. This strain has a truncated nonstructural protein 1 and can activate IRF3 and stimulate the IFN-β promoter (29). Culture supernatant was recovered for plaque assays after which the cells were fixed with 5% formalin and stained with crystal violet. To determine the magnitude of cell killing in individual wells by colorimetric assay, dye was resolubilized in methanol, and absorbance determined at 595 nm. Viral yield was assayed by serial dilution of recovered supernatant and plaque assay using L929 cells for VSV or MA104 cells for rotavirus.

Statistical analysis

Statistical analysis used Student’s t test. p < 0.05 was considered statistically significant.

Results

Poly(I:C) up-regulates type I IFN mRNA levels and IFN-β secretion by IECs

The dsRNA analog poly(I:C) was used to probe mechanisms of the IEC response to dsRNA. We focused on the regulated expression of IFN-β, because type I IFN is a signature of host cellular responses to viral infection. IFN-β mRNA transcript levels were up-regulated in a dose dependent manner in three different IEC lines (HT-29, HCA-7, SW480) stimulated by poly(I:C). Because IECs are not phagocytic cells, poly(I:C) was coadministered with LF (pIC-L) to facilitate its intracellular entry. IFN-β mRNA levels were not increased in IECs stimulated with poly(I:C) in PBS alone (pIC-P), or with LF alone (Fig. 1A). This was also the case for IL-8 mRNA (data not shown), which is a prototypic NF-κB target gene in IECs (30). Paralleling the mRNA results, pIC-L, but not pIC-P stimulation increased IFN-β secretion by IECs (Fig. 1B).

We examined the time-dependent expression of prototypic ISRE-stimulated genes in pIC-L stimulated cells. IP-10 and ISG56 mRNA levels increased by 24 h (Fig. 1C) and this was the case also for ISG15 (not shown). The contribution of type I IFN produced by pIC-L stimulated IEC to an autocrine/paracrine up-regulation of IFN-β, IP-10, and ISG56 gene expression was determined. Neutralizing Ab for type I IFN inhibited pIC-L stimulated increases in IFN-β and IP-10 mRNA by 80% and 40%, respectively, but did not inhibit ISG56 expression (Fig. 1D), indicating that pIC-L can up-regulate type I IFN regulated genes in IECs both directly and indirectly through the production of type I IFN that mediates autocrine and/or paracrine activities on IEC.

Poly(I:C) activates the ISRE and IRF3

Type I IFNs regulate gene expression through the activation of ISREs in the promoter regions of target genes. The IFN-β promoter also contains ISRE-like elements in its positive regulation domain I and III (31, 32). To determine whether poly(I:C) acti-
and RIG-I in IECs were relatively low compared with PKR (Fig. 3A). Furthermore, RIG-I, but not TLR3 and PKR mRNA levels were increased by pIC-L stimulation (Fig. 3A).

To determine the role of RIG-I, TLR3, and PKR in signaling poly(I:C) stimulated type I IFN responses, we assessed pIC-L stimulated ISRE activation in HT-29 and SW480 cells transfected with DN expression vectors for those proteins. DN-RIG-I inhibited pIC-L mediated ISRE activation in HT-29 cells, whereas DN-TLR3 and DN-PKR did not (Fig. 3B). Similar results were obtained with SW480 cells (Fig. 3C). In a complementary approach, we assessed pIC-L stimulated ISRE activity in HT-29 cells transfected with siRNA for RIG-I, TLR3, or PKR. Consistent with the data obtained with DN expression vectors, RIG-I siRNA inhibited pIC-L-mediated ISRE activation in HT-29 cells, whereas siRNA for TLR3 and PKR did not (Fig. 3D). To determine whether these results were paralleled by changes in poly(I:C) stimulated IFN-β secretion, IFN-β secretion was assessed in HT-29 and HCA-7 cells transfected with each of the siRNAs. IFN-β secretion in response to pIC-L was significantly decreased in RIG-I but not TLR3 siRNA transfected cells (Fig. 3E). In contrast, IFN-β secretion was increased in cells transfected with PKR siRNA and stimulated with pIC-L.

Poly(I:C) activates the RIG-I-IPS-1 pathway in IECs

IPS-1 is a RIG-I interacting protein that was recently reported to transduce the signal for IFN-β production in some cell types (9, 10, 12, 37–40). We investigated whether IPS-1 was important in the RIG-I signaling pathway in IECs. ISRE reporter activity activated by pIC-L was significantly decreased in IPS-1 siRNA-transfected HT-29 cells (Fig. 4A), whereas this was not the case when TRIF, an adaptor protein for TLR3 (20, 21) was silenced (Fig. 4A). In contrast, siRNA silencing of TRIF in HEK293 cells overexpressing TLR3 completely abrogated ISRE activity in response to poly(I:C) (data not shown). We further showed the importance of IPS-1 in IRF3 activation. As was the case with RIG-I siRNA, silencing IPS-1 signaling with siRNA abrogated IRF3 phosphorylation and dimerization in response to pIC-L (Fig. 4B). Consistent with this, IPS-1 siRNA significantly decreased IFN-β secretion in pIC-L stimulated cells (Fig. 4C) and decreased expression of the IFN target gene ISG15 (Fig. 4B). Thus, both IPS-1 and RIG-I are essential components of the pathways relevant to poly(I:C)-activated signaling of type I IFN in IECs.

RIG-I is important for antiviral responses of IECs

The prior results with poly(I:C) suggested RIG-I signaling may also be important for epithelial defense in IEC infected with viruses that produce dsRNA as part of their life cycle. To test this, HT-29 cells were transfected with pSUPER vectors (pS-luc, pS-RIG-1, or pS-RIG-2) that contain a puromycin resistance gene and generate gene specific siRNAs to knockdown expression of RIG-I in the transfected cells. Nontransfected cells were depleted by treatment with puromycin. Surviving cells were left uninfected or
incubated with increasing MOIs of VSV, which generates dsRNA in infected cells (41). HT-29 cells transfected with the pS-RIG-1 vector manifested significantly greater cell death, compared with control pS-luc transfected cells, at each MOI tested (Fig. 5A). Similar results were found using a different target sequence for RIG-I (pS-RIG-2) (Fig. 5A). In addition cells transfected with RIG-I siRNAs produced significantly more virus than control pS-luc transfected cells (Fig. 5B).

Additional experiments assessed the importance of RIG-I in virus infection using rotavirus as a dsRNA virus. Rotavirus infection-induced ISRE activation and IFN-β secretion were decreased by silencing of RIG-I, but not by silencing TLR3 or PKR (Fig. 5, C and D). Furthermore, RIG-I silenced HT-29 cells exhibited greater susceptibility to rotavirus induced cell death (Fig. 5E) and rotavirus replication was increased in RIG-I silenced cells (Fig. 5F). Thus, RIG-I has a central role both in signaling activation of type I IFN production by IECs and in IEC innate antiviral defense.

**Discussion**

Passage across the intestinal epithelium is an essential step in virus entry into the host. These studies define the RIG-I/IPS-1 signaling pathway as important in innate viral defense in IECs. Using the dsRNA analog poly(I:C), three dsRNA interacting proteins RIG-I, TLR3, and PKR were probing for their role in activating intracellular signaling pathways leading to type I IFN production by IECs. Silencing either RIG-I, or the RIG-I interacting protein IPS-1, but not TLR3 or its adaptor protein TRIF, or PKR, significantly abrogated IRF3 phosphorylation and dimerization, activation of the ISRE, IFN-β mRNA expression and protein secretion, and the expression of IFN target genes in poly(I:C) stimulated IECs. Moreover, our findings with poly(I:C) also appear to have functional importance for IEC mediated defense to live viruses. Thus, IECs in which RIG-I was silenced manifested greater viral replication and cell death after infection with either VSV or rotavirus.

The importance of RIG-I compared with TLR3 for signaling type I IFN responses in IECs likely reflects differences in the intracellular localization of those proteins. RIG-I is a cytosolic sensor of dsRNA. In contrast, in phagocytic cells (e.g., macrophages, microglial cells, and other professional APCs) TLR3 is localized in the endosomal membrane (11, 22). IECs are a non phagocytic cell type and consistent with this the uptake of poly(I:C) in IECs required that it be administered together with a membrane permeant molecule (i.e., LF). In IECs poly(I:C) predictably would encounter dsRNA interacting proteins in the cytosol whereas in phagocytic cells, poly(I:C) likely would encounter TLR3 in endosomes. In this regard TLR3 is known to be important role in signaling the production of type I IFN in response to poly(I:C) in phagocytic cell types (19, 25, 42, 43). Similarly, TLR3 may signal antiviral defense in IECs in situations where viral dsRNA encounters TLR3 in the endosomal compartment (43, 44). The relative importance of RIG-I compared with TLR3 signaling in IEC, compared with phagocytic cell types is not likely to reflect cell type specific usage of the pattern recognition receptors for dsRNA, such as reported in conventional compared with plasmacytoid DCs (45).

The dsRNA interacting protein PKR was constitutively expressed in IECs. Moreover, silencing PKR with a PKR siRNA did not decrease poly(I:C) stimulated activation of the ISRE, a finding also noted using a DN PKR vector. However, we found that poly(I:C) stimulation of PKR siRNA transfected cells resulted in increased type I IFN secretion. This increase in type I IFN secretion may be explained by the multiple functions of PKR, which include inhibition of protein synthesis resulting from phosphorylation of eIF2α (15). This suggests that PKR activation by dsRNA, at the protein level, may normally down modulate, rather than increase, type I IFN production in IECs. Although PKR can signal some cellular innate immune responses that are dependent on NF-κB activation (14, 17), activation of PKR has not been shown to increase IFN production in other studies. Moreover, in PKR knockout mice type I IFN gene expression was normally up-regulated in response to poly(I:C) or virus infection (18). Taken together, these data do not support a role for PKR activation in signaling increased transcription of type I IFN.

Poly(I:C) was reported to up-regulate IL-8 mRNA in the human IEC line T84 in the absence of an added membrane permeant, by signaling through PKR as determined using pharmacological inhibitors of PKR (e.g., 2-AP) (46). Although PKR signaling through NF-κB may have a role in the poly(I:C) stimulated IL-8 response, those studies did not assess type I IFN expression. Moreover, the specificity of inhibition by 2-AP for PKR in those studies was not confirmed using specific genetic approaches (46). Whereas, 2-AP inhibited poly(I:C) stimulated type I IFN production, we also found that 2-AP inhibition was not specific for PKR. Thus, in addition to PKR, 2-AP also inhibited STAT1 and IRF3 phosphorylation that are key in the activation of the IFN pathway (data not shown), and others have previously reported that 2-AP inhibited leptin induced STAT3, ERK, and JNK activation, independently of PKR (47). These findings strongly support the use of specific genetic approaches when defining relevant signaling pathways.

Whereas the present study focused on the RNA helicase RIG-I as a dsRNA sensing and signaling protein in IEC, we have also found that RIG-I and the RIG-I interacting molecule IPS-1 are expressed in normal human intestinal epithelial cells in vivo (data not shown). We note that MDA5, another RNA helicase that is structurally related to RIG-I, also signals type I IFN production in response to poly(I:C) (23, 24). MDA5 appears to recognize distinct dsRNAs from those that activate RIG-I and different viruses than studied herein (23, 24, 48). Preliminary data from our laboratory indicates that MDA5 is expressed by IECs and that RIG-I and MDA5 have complementary roles in mediating antiviral innate responses in IECs and antiviral responses to different viruses.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


