TLR3, TRIF, and Caspase 8 Determine Double-Stranded RNA-Induced Epithelial Cell Death and Survival In Vivo

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TLR3, TRIF, and Caspase 8 Determine Double-Stranded RNA-Induced Epithelial Cell Death and Survival In Vivo

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TLR3 signaling is activated by dsRNA, a virus-associated molecular pattern. Injection of dsRNA into mice induced a rapid, dramatic, and reversible remodeling of the small intestinal mucosa with significant villus shortening. Villus shortening was preceded by increased caspase 3 and 8 activation and apoptosis of intestinal epithelial cells (IECs) located in the mid to upper villus with ensuing luminal fluid accumulation and diarrhea because of an increased secretory state. Mice lacking TLR3 or the adaptor molecule TRIF mice were completely protected from dsRNA-induced IEC apoptosis, villus shortening, and diarrhea. dsRNA-induced apoptosis was independent of TNF signaling. Notably, NF-κB signaling through IkB kinase β protected crypt IECs but did not protect villus IECs from dsRNA-induced or TNF-induced apoptosis. dsRNA did not induce early caspase 3 activation with subsequent villus shortening in mice lacking caspase 8 in IECs but instead caused villus destruction with a loss of small intestinal surface epithelium and death. Consistent with direct activation of the TLR3–TRIF–caspase 8 signaling pathway by dsRNA in IECs, dsRNA-induced signaling of apoptosis was independent of non-TLR3 dsRNA signaling pathways, IL-15, TNF, IL-1, IL-6, IFN regulatory factor 3, type I IFN receptor, adaptive immunity, as well as dendritic cells, NK cells, and other hematopoietic cells. We conclude that dsRNA activation of the TLR3–TRIF–caspase 8 signaling pathway in IECs has a significant impact on the structure and function of the small intestinal mucosa and suggest signaling through this pathway has a host protective role during infection with viral pathogens. The Journal of Immunology, 2013, 190: 000–000.

The mucosa of the small intestine contains finger-like projections termed villi that are lined by a single-cell layer of intestinal epithelial cells (IECs) that separate the intestinal lumen from the underlying immune cell-rich lamina propria. IECs are armed with numerous pattern recognition receptors (PRRs) that detect and activate host responses to protect against viral and bacterial pathogens and their products (1). dsRNA is a virus-associated molecular pattern that is recognized by the endosomal PRR TLR3, and intracellular cytoplasmic retinoic acid-inducible gene (RIG)–like sensors (2, 3). Interestingly, dsRNA signaling through TLR3 has been reported to induce small intestinal mucosal damage in mice (4).

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Abbreviations used in this article: BAX, Bcl-2–associated X; DC, dendritic cell; DTR, diphtheria toxin receptor controlled by CD11c promoter; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IKK, IκB kinase; IRF, IFN regulatory factor; Isc, short-circuit current; MAVS, mitochondrial antiviral-signaling protein; pIC, polyminosinic:polyribocytidylic acid; PRR, double-stranded RNA-activated protein kinase; PPR, pattern recognition receptor; RIG, retinoic acid-inducible gene; WT, wild-type.
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TLR3 signals through the adaptor molecule TRIF; whereas the RIG-like RNA sensors, RIG-I and Mda5, signal through the adaptor molecule mitochondrial antiviral signaling (MAVS, also known as IPS-1) (2). These pathways activate IFN regulatory factor 3 (IRF3) and NF-κB transcription factors and result in the production of type I IFNs and proinflammatory cytokines. Furthermore, activation of host signaling pathways can induce apoptosis, likely as a means to kill infected host cells to shut down virus production (5). Although NF-κB can have both pro- and antiapoptotic effects within cells, IRF3 has been shown to interact with Bcl-2–associated X (BAX) protein to directly induce apoptosis after viral infection (6). In addition, in vitro studies have shown that TRIF, unlike the TLR3 adaptor protein MyD88, interacts with caspase 8 through the receptor interacting protein 1 signaling molecule to directly induce apoptosis (7–10).

PRR signaling pathways can also induce TNF that can act upon other cells to either induce survival through NF-κB signaling or death through apoptosis or necroptosis (11). Interestingly, TNF injection induces IEC apoptosis in vivo in mice (12). This effect is dependent on TNFR-1 and more pronounced in the proximal than distal small intestine, possibly because of the expression pattern of the TNFR-1 (13). Signaling through TNFR-1 leads to the recruitment of a signaling complex that activates caspase 8, which then mediates apoptosis by activating effector caspases including caspase 3 (11).

We investigated the mucosal signaling pathways activated by dsRNA that cause small intestinal damage in vivo. We report that dsRNA rapidly induces intestinal villus epithelial cell apoptosis and cell loss in the small intestine that results in marked villus shortening and significant diarrhea, with subsequent recovery. dsRNA-induced IEC apoptosis in vivo was strictly dependent on TLR3 and TRIF signaling through caspase 8 in IECs, and independent of other dsRNA signaling pathways, as well as TNF, IL-15,
dendritic cells (DCs), NK cells, and other hematopoietic cells. Interestingly, this pathway proved to be host protective because, in the absence of downstream activation of caspase 8, dsRNA caused marked destruction of the small intestinal mucosa and death.

**Materials and Methods**

**Reagents**

Low m.w. polyinosinic-polycytidylic acid (pIC) was from InvivoGen (San Diego, CA) and used in all studies unless indicated otherwise. pIC sodium salt was from Sigma-Aldrich (St. Louis, MO). R848, ODN 1826, and LPS from Escherichia coli O111:B4 were from InvivoGen. pIC was heated to 50°C for 10 min and allowed to cool to room temperature to anneal. Cleaved caspase 3 (Asp175) and cleaved caspase 8 (Asp387) (DSB2) Ab was from Cell Signaling Technology (Danvers, MA). Recombinant mouse TNF was purchased from PeproTech (Rocky Hill, NJ).

**Mouse strains and treatment**

IEC-specific caspase-8 knockout mice (casp8<sup>−/−</sup>) were generated in our laboratory by breeding Casp8<sup>−/−</sup> mice (14) with villin-Cre mice (15) as described by Günther et al. (16). Wild-type (WT) C57BL/6J, TNF<sup>−/−</sup>, IFNAR<sup>−/−</sup> mice (21) mice from The Jackson Laboratory, IL-15<sup>−/−</sup> mice (30), MA VS<sup>−/−</sup> mice (25), CD11c-diphtheria toxin receptor (DTR) (26), IκBα<sup>−/−</sup> (27), IκBε<sup>−/−</sup> (28), TLR4<sup>−/−</sup> (27), and casp8<sup>−/−</sup> mice were maintained in specific pathogen-free conditions at University of California, San Diego. Mice were given a single i.p. injection of 30 mg/kg pIC, 10 mg/kg R848, 5 mg/kg ODN 1826, or 20 mg/kg LPS in sterile PBS or saline solution, or using sterile PBS or saline injection alone as a control. In one series of experiments, pIC was injected in the retro-orbital sinus of anesthetized mice. Recombinant TNF was injected i.p. Mice were sacrificed at 3–6 h after injection unless otherwise indicated. All animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

**Histology and immunohistochemistry**

Proximal small intestine starting 2 cm distal to the pylorus and continuing for the first third of the small intestine was processed as Swiss rolls (32). Distal small intestine Swiss rolls were taken from the distal third of the small intestine. Tissues were fixed in 10% formalin and embedded in paraffin. Sections were examined in our laboratory under an Olympus BX41 microscope. Digital images were captured using the PictureFrame Application 2.3 (Optronics).

**Ussing chamber studies**

Segments of proximal small intestine were cut along the mesenteric border and mounted in Ussing chambers (Physiological Instruments, San Diego, CA), exposing 0.09 cm² of tissue area to 4 ml circulating oxygenated Ringer’s buffer maintained at 37°C. The buffer consisted (in millimolars) of 140 Na<sup>+</sup>, 5.2 K<sup>+</sup>, 1.2 Ca<sup>2+</sup>, 0.8 Mg<sup>2+</sup>, 120 Cl<sup>−</sup>, 25 HCO<sub>3</sub><sup>−</sup>, 2.4 HPO<sub>4</sub><sup>2−</sup>, and 0.4 HP<sup>−</sup>. In addition, glucose (10 mM) was added to the serosal buffer as a source of energy, osmotically balanced by mannitol (10 mM) in the mucosal buffer. Agar–salt bridges were used to monitor the potential difference across the tissue and to inject the required short-circuit current (Isc) to maintain the potential difference at 0. This was registered by an automated voltage clamp and continuously recorded by computer. Baseline Isc values were obtained at equilibrium, 15 min after the tissues were mounted and expressed as μA/cm². Conductance (G) was also determined at baseline as an indicator of ion flux and expressed as mS/cm². FITC-labeled dextran (4 KDa; Sigma-Aldrich) was used as a probe to assess macromolecular permeability and was added (2.2 mg/ml final concentration) to the luminal buffer once equilibrium was reached. Serum samples (240 μl) were taken at 30-min intervals for 2 h and replaced with fresh buffer to maintain constant volume. Fluorescence was measured by end point assay (Victor4; PerkinElmer), and the flux of FITC–dextran from the mucosa to the serosa was calculated as the average value of two consecutive stable flux periods (60–90 and 90–120 min) and expressed as pmol/cm²h (33).

**Cytokine detection**

Serum samples were collected 1h after treatment and stored at −80°C. Cytokine measurements used the mouse ultrasensitive TNF kit or proinflammatory 7-plex kit from Meso Scale Discovery (Gaithersburg, MD), and cytokine levels were determined using a Meso Scale Discover Sector Imager 6000.

**RNA extraction and quantitative real-time PCR**

Total cellular RNA was extracted using TRizol (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. One microgram of total RNA was used for iScript (Bio-Rad Laboratories, Hercules, CA) reverse transcription, and the cDNA was used for quantitative real-time PCR using SYBR green Master mix (Applied Biosystems, Foster City, CA) and GAPDH forward primer 5'-ATCAACACGCTCCCTATTGACC-3' and GAPDH reverse primer 5'-CCATGACTCCAGGATACANTCAG-3' or IFN-β forward primer 5'-CTGAGACCCTAATGGGAAGG-3' and IFN-β reverse primer 5'-CTTGAAGTCCGCGTATGCGTT-3'. Denaturation was 5 min at 95°C, followed by 40 cycles of amplification at 95°C for 30 s and 60°C for 1 min using an ABI StepOnePlus (Applied Biosystems). IFN-β induction was calculated using the ΔΔCt method, and values were normalized to PBS-treated mice.

**NK and DC depletion**

To deplete NK cells, mice were injected with 200 μg purified anti-NK1.1 (PK136) mAb (eBioscience, San Diego, CA) or control IgG2 24 h before injection of pIC. NK1.1<sup>−/−</sup> cell depletion was confirmed by immunohistochemistry as described above. For DC depletion, DTR (26) mice were treated with 1 mg purified anti-NK1.1 mAb (Biomar) or Armenian hamster negative control (AbD Serotec) and then washed and stained with Cy3 goat anti-Armenian hamster (Jackson Immunoresearch Laboratories).

**Microscopy analysis**

Villus lengths were measured in each Swiss roll, and the 10 longest villi were averaged to give a final mean villus length. Cleaved caspase 3–stained cells that were completely black were counted as positive cells, and 10 representative villi were counted and averaged to provide a value for each mouse. Samples were examined in our laboratory under an Olympus BX41 microscope. Digital images were captured using the PictureFrame Application 2.3 (Optronics).

**Results**

dsRNA induces IEC apoptosis in the small intestine

We used the synthetic dsRNA viral nucleic acid mimic pIC as a probe of the host’s antiviral strategies in the small intestine. Administration of pIC i.p. resulted in villus shortening in the proximal small intestine, which was maximal between 3 and 6 h after injection and recovered thereafter (Fig. 1A–C). The dramatic reduction in villus length correlated with decreased numbers of IECs per villus (Supplemental Fig. 1A–C). The relatively rapid loss of IECs and ensuing recovery suggested a dsRNA-activated pathway that causes IEC loss, perhaps as a host response to rid the epi-
thelium of virus-infected cells while concurrently maintaining a relatively intact IEC layer.

To determine whether apoptosis explained the decreased numbers of IECs, we evaluated cleaved caspase 3 staining of small intestinal tissue sections (Fig. 1D–F) (34). IEC apoptosis preceded villus shortening with a peak at 2 h after pIC i.p. (Fig. 1G). Apoptotic IECs predominately localized in the mid to upper villus region, with little apoptosis in the lower villus region and no increase in apoptosis in either the crypt region (i.e., the site of epithelial stem cells) or the subepithelial lamina propria (Fig. 1E). Cleavage of caspase 8, an initiator caspase, was also observed at 2 h posttreatment (Supplemental Fig. 1D, 1E). Apoptosis of villus IECs and villus shortening were less pronounced but still significant in the distal small intestine (Supplemental Fig. 1F–I), likely reflecting shorter villi with decreased numbers of IECs in the distal small intestine.

pIC-treated mice had significant fluid accumulation in the small intestine and diarrhea that peaked at 4–6 h and returned to normal thereafter (Fig. 1H–J). To address the cause of this increase in intestinal fluid accumulation, Ussing chamber studies were done to measure the small intestinal secretory state and mucosal permeability. Baseline Isc, indicative of active ion secretion, was elevated in mice treated with pIC compared with PBS controls (Fig. 1K). In contrast, conductance indicative of ion permeability (Fig. 1L) and FITC–flux indicative of macromolecular permeability (Fig. 1M) were not significantly altered by pIC treatment. Taken together, these data suggest that increased luminal fluid accumulation in the proximal small bowel resulted from an increased secretory state in these mice in the context of intact tight junctions and mechanisms of endocytosis.

We next investigated whether the mode of pIC delivery determined IEC apoptosis, because injection by the i.p. route suggested the possible involvement of i.p. macrophages or other cell subsets specific to the peritoneal cavity in inducing villus shortening and IEC apoptosis. However, there was no dependence on i.p. injection, as either i.p. or retro-orbital sinus injection of pIC induced significant villus shortening in the proximal small intestine and IEC apoptosis (Supplemental Fig. 1J–M, 1N, 1O). Moreover, Cy3-labeled pIC was detected in the lamina propria of proximal small intestinal villi after retro-orbital injection (Supplemental Fig. 1P, 1R). Administration of pIC by oral gavage did not induce villus shortening or IEC apoptosis (Supplemental Fig. 1M–O).

TLR3-TRIF signaling is required for IEC apoptosis

We found that TLR3 was required for dsRNA-induced intestinal damage as reported before by others (4). Indeed, TLR3−/− mice were completely resistant to dsRNA-induced IEC apoptosis and villus shortening (Fig. 2A, 2B, 2D, 2E). Furthermore, dsRNA did not induce IEC apoptosis or villus shortening in mice lacking TRIF, the downstream adaptor molecule for TLR3 (Fig. 2C, 2F).
Villus length and the number of IECs with cleaved caspase 3 staining per villus were quantified and confirmed the essential involvement of TLR3–TRIF signaling in pIC-activated villus shortening and apoptosis in the proximal small intestine (Fig 2G, 2H). Neither TLR3<sup>−/−</sup> nor TRIF<sup>−/−</sup> mice developed small intestinal fluid accumulation or diarrhea. There was no difference in IEC proliferation between WT and TLR3<sup>−/−</sup> mice after pIC, indicating that pIC did not damage the proliferative capacity of crypt cells (Fig. 2I–L).

We assessed the requirement for other RNA sensors to determine whether they had a contributory role in pIC-activated IEC apoptosis in the small intestine. MAVS (IPS-1), the signaling adaptor for RIG-like receptors, was not required for villus shortening or caspase 3 cleavage (Fig. 2M, 2N). PKR is an IFN-inducible protein kinase that can act as a general translation inhibitor and induce apoptosis in response to activation by dsRNA (35). Mice deficient in PKR had similar levels of villus shortening and numbers of caspase 3–cleaved cells as WT mice (Fig. 2M, 2N), indicating that PKR-dependent translation inhibition or signaling was not required for pIC-activated IEC apoptosis and villus shortening.

Apoptosis of small intestinal IECs induced by TLR ligands

High levels of TNF result in IEC apoptosis (12) and, consistent with this, i.p. injection of 5 µg, but not 1 µg, of TNF induced IEC apoptosis in WT mice (Fig. 3E–G). Because several TLR ligands that signal through MyD88 induce the release of TNF (2), we evaluated IEC apoptosis induced by other TLR ligands and found that pIC induced significantly higher levels of IEC apoptosis than the TLR7 ligand R848, TLR9 ligand ODN, and TLR4 ligand LPS (Fig. 3 A–D, 3G). Moreover, pIC induced TNF levels orders of magnitude lower than those TLR ligands, with LPS inducing the highest TNF levels (Fig. 3H). Although others observed higher serum TNF levels after pIC (4) we established that this was likely due to endotoxin contamination of pIC in their studies. However, we found such levels of endotoxin contamination did not significantly affect IEC apoptosis or villus shortening (Supplemental Fig. 2).

pIC-induced IEC apoptosis is independent of TNF, IL-6, and IL-1 signaling

We definitively excluded the involvement of TNF in pIC-induced apoptosis using mice deficient in TNF (TNF<sup>−/−</sup>) that did not produce TNF following dsRNA treatment (Fig. 3L). TNF<sup>−/−</sup> mice were as sensitive as WT mice to dsRNA-induced IEC apoptosis (Fig. 3I–K). These results confirmed that TNF has no role in pIC-induced small intestinal IEC apoptosis. We also confirmed that IL-6 and IL-1β, two cytokines that were increased after pIC, are not required for dsRNA-induced IEC apoptosis using IL-6<sup>−/−</sup> and IL-1R<sup>−/−</sup> mice (Supplemental Fig. 3).
IEC apoptosis is independent of IL-15 and NK cells

IL-15 signaling and NK cell-mediated killing were reported to be required for dsRNA-induced small intestinal villus shortening and epithelial cell injury (4). To evaluate the role of IL-15 in our model, we used mice deficient in IL-15 production. IL-15−/− mice demonstrated the same level of sensitivity to pIC as WT mice (Fig. 4A-D). To assess the role of NK1.1+ cells in dsRNA-induced IEC apoptosis, those cells were depleted using Ab to NK1.1. We observed the same level of IEC apoptosis in the villi of IKKα−/− and WT mice in villus shortening or apoptosis (Fig. 4J-M).

Antiviral signaling through MAVS can activate IRF3 in a manner that results in IRF3 interacting with BAX and localizing to mitochondria to induce apoptosis (6). Because TLR3 signaling also activates IRF3 (37), we tested whether this pathway was involved in pIC-activated IEC apoptosis. We found no evidence to suggest the involvement of IRF3 or BAX, because neither IRF3−/− nor BAX−/− mice manifested differences in villus length or IEC apoptosis compared with WT mice after pIC treatment (Fig. 4N-R).

NF-κB signaling in IECs does not affect villus IEC apoptosis but is required to protect IECs in the small intestinal crypts from apoptosis

TLR3–TRIF signaling can activate the transcription factor NF-κB (37). To assess the involvement of NF-κB signaling in pIC-induced apoptosis of IECs, we used mice conditionally deficient in IκB kinase (IKKα and IKKβ) in small intestinal IECs (27, 38). The magnitude of IEC apoptosis in the villi of IKKαβEC−/− mice had increased levels of IEC apoptosis in the small intestinal crypts (Fig. 5B, 5F). To assess the contribution of IKKα and IKKβ to this phenotype, we used IKKαEC−/− and IKKβEC−/− mice deficient selectively in IKKα or IKKβ, respectively, in the IECs and determined that increased crypt apoptosis after pIC was dependent on the absence of IKKβ signaling (Fig. 5D, 5F). Notably, this same pattern of IKKβ dependence for crypt, but not IEC, apoptosis was observed in response to TNF (Fig. 5G-J).

TLR3 signaling in cells of nonhematopoietic origin is required for IEC apoptosis

NK1.1 cell depletion demonstrated that NK1.1+ cells were not involved in pIC-activated IEC apoptosis (Fig. 4E-H). To exclude the contribution of other immune cell subsets, we assessed the sensitivity of immunodeficient RAG1−/− mice that lack functional B and T cells and certain intraepithelial lymphocyte (IEL) subsets (39) to dsRNA-induced IEC apoptosis. RAG1−/− mice were not protected from pIC-induced villus shortening and IEC apoptosis (Supplemental Fig. 4D-F). We also assessed the sensitivity of DC-depleted mice (26) to pIC-activated IEC apoptosis. CD11c-DTR mice treated with diphtheria toxin to deplete DCs showed similar...
Interestingly, DC-depleted mice had significantly decreased serum TNF levels after pIC treatment (Supplemental Fig. 4P). DC depletion was confirmed by immunohistochemistry (Supplemental Fig. 4Q, 4R).

To determine whether other cells of hematopoietic origin contribute to dsRNA-induced IEC apoptosis after pIC, we generated TLR3\(^{-/-}\) bone marrow chimera mice. As shown in Fig. 6, WT mice reconstituted with TLR3-deficient bone marrow (TLR3\(^{-/-}\)BM→WT) were as sensitive to pIC-induced IEC apoptosis as WT mice (Fig 6A, 6C). In addition, TLR3\(^{-/-}\) mice reconstituted with WT bone marrow (WT BM→TLR3\(^{-/-}\)) did not show increased IEC apoptosis in response to pIC (Fig. 6B, 6D). Numbers of cleaved caspase 3-positive IECs per villus in these mice demonstrated that IEC apoptosis was not dependent on hematopoietic cells of WT origin (Fig. 6E). Successful bone marrow transfer was apparent from assaying serum TNF levels in mice after treatment with pIC (Fig. 6F). WT mice showed TNF levels consistent with previous experiments and TLR3\(^{-/-}\) mice showed no induction of TNF after pIC, whereas TLR3\(^{-/-}\)BM→WT mice had levels of TNF comparable to TLR3\(^{-/-}\) mice and WT BM→TLR3\(^{-/-}\) mice had TNF levels comparable to WT mice (Fig. 6F).

Caspase 8 coordinates IEC apoptosis and preserves the integrity of the intestinal epithelium following dsRNA treatment

We observed activated caspase 8 at early time points following pIC treatment (Supplemental Fig. 1D, 1E). TRIF has been shown to activate apoptosis through caspase 8, the same initiator caspase that mediates TNF-induced apoptosis (8, 10). To assess the importance of caspase 8 in pIC-activated IEC apoptosis and subsequent small intestinal damage, we generated mice that lack caspase 8 in IECs. Control mice with floxed caspase 8 (casp8\(^{fl}\)) behaved like WT mice following pIC treatment and had numerous mid to upper levels of IEC apoptosis as WT mice in response to pIC (Supplemental Fig. 4K–O). Interestingly, DC-depleted mice had significantly decreased serum TNF levels after pIC treatment (Supplemental Fig. 4P). DC depletion was confirmed by immunohistochemistry (Supplemental Fig. 4Q, 4R).

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villus IECs that were positive for activated caspase 3 (Fig. 7B, 7C). However, mice lacking caspase 8 in IECs (casp8<sup>Aiec</sup>) did not have significant levels of activated caspase 3 in villus IECs indicative of epithelial apoptosis (Fig. 7E). Interestingly, by 2.5 h posttreatment, these mice manifested destruction of the small intestinal villi, with a loss of the villus epithelium (Fig. 7F) and died within 6 h of treatment (data not shown). These results demonstrate that caspase 8 is required to regulate IEC apoptosis following pIC-activated TLR3–TRIF signaling in villus IECs. In the absence of caspase 8, signaling through TLR3–TRIF causes villus destruction and death.

**Discussion**

We elucidated the signaling pathways and mechanisms by which the viral dsRNA mimic pIC selectively causes small intestinal mucosal damage in vivo. Small intestinal mucosal changes after encountering pIC were characterized by a rapid onset of epithelial cell apoptosis, the loss of IECs from villus structures, accompanying marked loss of villus height, and significant diarrhea with recovery over the following 24–48 h. IEC apoptosis and villus shortening after pIC treatment were strictly dependent on signaling through the TLR3–TRIF pathway and caspase 8 as demonstrated using TLR3<sup>−/−</sup>, TRIF<sup>−/−</sup>, and caspase 8<sup>Aiec</sup> mice. Although this apoptotic pathway has been studied in vitro (40), we demonstrate an in vivo system where it dramatically impacts the structure and function of the small intestine.

TNF is also well known for its ability to induce IEC apoptosis (12), but dsRNA-induced IEC apoptosis was completely independent of TNF as well as other cytokines like IL-6 and IL-1. Moreover, pIC-induced apoptosis of small intestinal villus IECs in vivo was independent of the TRIF signaling arm of TLR4 and dsRNA signaling pathways that use the MAVS adaptor molecule or PKR.

Induction of type I IFN in IECs is an important antiviral mechanism (41, 42). However, pIC-induced IEC apoptosis in the villi and villus shortening was independent of this pathway. Furthermore, although IRF3 can induce apoptosis in response to viral infection through a BAX-dependent pathway in fibroblast cell lines (6), we found no role for BAX and IRF3 in pIC-induced apoptosis of small intestinal IECs.

dsRNA-induced small IEC apoptosis, mucosal remodeling, and recovery after pIC in vivo required signaling downstream of TLR3–TRIF through caspase 8. In the absence of an intact signaling pathway through caspase 8, there was severe damage to the epithelium and small intestinal mucosa. This suggests the caspase 8 apoptotic pathway has an important function as a protective mechanism that maintains the integrity of the intestinal epithelium after dsRNA-activated TLR3–TRIF signaling. Moreover, the lack of significant caspase 3 activation in pIC-treated villus IECs from caspase 8<sup>Aiec</sup> mice suggests IEC death and loss by an alternative death pathway such as necroptosis, as seen after TNF treatment (16, 40, 43).
is the case in vivo for hepatocytes where both TNF and D-galactose require two stimuli to induce apoptosis (e.g., the activation of IECs in the small intestine occurred in the absence of additional sensitivity to dsRNA-induced apoptosis of mid to upper villus IECs). Activation of NF-κB pathway protected crypt IECs but not villus IECs from pIC-induced apoptosis. Moreover, the marked sensitivity to dsRNA-induced apoptosis of mid to upper villus IECs, villus shortening, and loss of small intestinal villus epithelial cells, villus remodeling, and diarrhea, therapeutic approaches designed to inhibit TLR3 signaling could result in decreased virus clearance, as was reported in TLR3−/− mice infected with murine rotavirus (61).

The mechanisms, which we show are responsible for pIC-induced small IEC death and villus shortening, differ from those proposed by Zhou et al. (4, 62). Their model was based on a mix of in vitro and in vivo experiments in which pIC signaling through TLR3 was proposed to increase IL-15 production by IECs and RAE1, a ligand for NKG2D on IECs. Furthermore, IEC-produced IL-15 was proposed to increase NKG2D on IELs, with co-culture of isolated IECs and IELs, resulting in killing of IECs in vitro at 24 h. Support for their model of pIC-induced small intestinal injury was based on an in vivo finding that Ab to IL-15Rα partially decreased villus shortening in pIC-injected mice (62). Whereas dsRNA has been reported to induce IL-15 in vivo (63), using IL-15−/− mice we showed that pIC-induced apoptosis and villus shortening after pIC was totally independent of IL-15. Zhou et al. (62) further reported decreased small intestinal villus damage in response to dsRNA after NK1.1 cell depletion, whereas NK1.1 cell depletion did not affect dsRNA-induced IEC apoptosis or villus shortening in our studies. In addition, using bone marrow chimera mice we showed that cells of hematopoietic origin were not required for IEC apoptosis following pIC treatment.

Finally, the relevance of our findings can be envisioned to extend beyond viral RNA triggers of TLR3−/−TRIF signaling. In this regard, mRNA released from necrotic cells was shown to activate TLR3 signaling (64). Furthermore, RNA from necrotic cells in rheumatoid arthritis synovial tissues activated TLR3-dependent signaling (65). In other studies, endogenous RNA from injured tissues and cells activated TLR3 signaling and resulted in cell death. For example, endogenous RNA, derived from lung tissue or necrotic neutrophils, signaled through TLR3 in the absence of exogenous virus and increased lung damage and death in a model of hyperoxia lung injury (66). TLR3 also functioned as an endogenous sensor of tissue necrosis, independent of viral activation, in models of septic peritonitis and ischemic gut injury (67). Importantly, injury and mortality were attenuated in anti-TLR3 Ab treated and TLR3−/− mice. Taken together with our results, these studies indicate that dsRNA associated with either virus infection or tissue injury and inflammation can have a dramatic impact on mucosal tissues.

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Cells generated in the stem cell compartment in the crypts of the small intestine proliferate and migrate upward to populate the villi where they differentiate and ultimately are shed from the upper villus tips. Apoptosis of IECs after pIC was largely confined to the mid to upper villus region. Activation of NF-κB in IECs is important both for the induction of IEC innate proinflammatory chemokines and cytokines and for IEC resistance to apoptosis (27, 38, 44, 45). Using mice conditionally deficient in IKKα, IKKβ, or both in IECs, we showed that activation of the canonical NF-κB pathway protected crypt IECs but not villus IECs from pIC-induced apoptosis as well as TNF-induced apoptosis. Moreover, the marked sensitivity to dsRNA-induced apoptosis of mid to upper villus IECs in the small intestine occurred in the absence of additional stimuli. This differs from many other cell types and tissues that require two stimuli to induce apoptosis (e.g., the activation of death receptors and translation inhibition) (11). For example, this is the case in vivo for hepatocytes where both TNF and D-galactosamine, a translation inhibitor, are required to induce apoptosis (46). Even in certain cancer cells, dsRNA-induced apoptosis through TLR3 requires a translation inhibitor (47). In those cells, prosurvival signals from NF-κB signaling appear to protect from the activation of apoptosis by caspase 8 (11, 48). It is possible that the different susceptibility of crypt and villus IECs to apoptosis may reflect differences in the negative regulation of NF-κB signaling in these cells, the state of proliferation or differentiation of those cells, or different NF-κB isoforms throughout the crypt villus axis, where an increased fraction of p50 homodimers may act to negatively regulate NF-κB targets in the villi (38, 49, 50). In this regard, NF-κB signaling likely is tightly regulated in villus IECs because these cells ultimately must undergo detachment-dependent apoptosis, termed anoikis, at the villus tips as a part of their lifecycle and NF-κB signaling has been shown to decrease anoikis (51).

Infection with enteric viruses like rotavirus causes significant loss of small intestinal villus epithelial cells, villus shortening, and concomitant diarrhea (52, 53) similar to that noted in this paper. Other Reoviridae also contain a dsRNA genome, whereas other viruses that induce gastroenteritis, including norovirus, produce dsRNA during their replication cycle (54–56). The likely relevance of the model using parenterally administered synthetic dsRNA to enteric virus infection is strengthened further because circulating dsRNA is found in rotavirus-infected humans and animals (57–60). Our findings with dsRNA suggest a host protective role for the TLR3−/−TRIF−/− caspase 8 activated apoptosis pathway in IECs in viral infection in vivo. This would result in a rapid shedding of apoptotic virus–infected cells with shortening of villi and a decreased epithelial surface area available for virus infection. Furthermore, the generation of fluid in the intestinal lumen by crypt secretory epithelium concurrent with less epithelial surface available for fluid absorption would culminate in diarrhea that flushes out virus. Conversely, although TLR3−/− and TRIF−/− mice are protected from dsRNA-induced epithelial cell apoptosis, villus remodeling, and diarrhea, therapeutic approaches designed to inhibit TLR3 signaling could result in decreased virus clearance, as was reported in TLR3−/− mice infected with murine rotavirus (61).

The mechanisms, which we show are responsible for pIC-induced small IEC death and villus shortening, differ from those proposed by Zhou et al. (4, 62). Their model was based on a mix of in vitro and in vivo experiments in which pIC signaling through TLR3 was proposed to increase IL-15 production by IECs and RAE1, a ligand for NKG2D on IECs. Furthermore, IEC-produced IL-15 was proposed to increase NKG2D on IELs, with co-culture of isolated IECs and IELs, resulting in killing of IECs in vitro at 24 h. Support for their model of pIC-induced small intestinal injury was based on an in vivo finding that Ab to IL-15Rα partially decreased villus shortening in pIC-injected mice (62). Whereas dsRNA has been reported to induce IL-15 in vivo (63), using IL-15−/− mice we showed that pIC-induced apoptosis and villus shortening after pIC was totally independent of IL-15. Zhou et al. (62) further reported decreased small intestinal villus damage in response to dsRNA after NK1.1 cell depletion, whereas NK1.1 cell depletion did not affect dsRNA-induced IEC apoptosis or villus shortening in our studies. In addition, using bone marrow chimera mice we showed that cells of hematopoietic origin were not required for IEC apoptosis following pIC treatment.

Finally, the relevance of our findings can be envisioned to extend beyond viral RNA triggers of TLR3−/−TRIF signaling. In this regard, mRNA released from necrotic cells was shown to activate TLR3 signaling (64). Furthermore, RNA from necrotic cells in rheumatoid arthritis synovial tissues activated TLR3-dependent signaling (65). In other studies, endogenous RNA from injured tissues and cells activated TLR3 signaling and resulted in cell death. For example, endogenous RNA, derived from lung tissue or necrotic neutrophils, signaled through TLR3 in the absence of exogenous virus and increased lung damage and death in a model of hyperoxia lung injury (66). TLR3 also functioned as an endogenous sensor of tissue necrosis, independent of viral activation, in models of septic peritonitis and ischemic gut injury (67). Importantly, injury and mortality were attenuated in anti-TLR3 Ab treated and TLR3−/− mice. Taken together with our results, these studies indicate that dsRNA associated with either virus infection or tissue injury and inflammation can have a dramatic impact on mucosal tissues.

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Disclosures

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