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Recognition of Double-Stranded RNA by TLR3 Induces Severe Small Intestinal Injury in Mice

Rongbin Zhou,2 Haiming Wei,2 Rui Sun, and Zhigang Tian3

The role of TLRs on intestinal epithelial cells (IECs) is controversial, and the mechanisms by which TLRs influence mucosal homeostasis are obscure. In this study, we report that genomic dsRNA from rotavirus, and its synthetic analog polyinosinic-polycytidylic acid (poly(I:C)), induce severe mucosal injury in the small intestine. Upon engaging TLR3 on IECs, dsRNA triggers IECs to secrete IL-15, which functions to increase the percentage of CD3+ CD8α+ intestinal intraepithelial lymphocytes (IELs) and enhances the cytotoxicity of IELs. Moreover, the CD3+CD8α+ IELs are proved as CD8αβ+ IELs. These results provide direct evidence that abnormal TLR3 signaling contributes to breaking down mucosal homeostasis and the first evidence of pathogenic effects mediated by CD8α+ IELs. The data also suggest that genomic dsRNA may be involved in the pathogenesis of acute rotavirus gastroenteritis. The Journal of Immunology, 2007, 178: 4548–4556.

The rotavirus causes ~110 million cases of gastroenteritis and >400,000 deaths in children annually in the world (1). Rotavirus infection alters the function of the small intestinal epithelium, resulting in diarrhea, and the diarrhea is generally considered to be associated with enterocyte destruction (2). However, the exact mechanisms by which the rotavirus causes enterocyte destruction remain obscure.

The intestinal intraepithelial lymphocytes (IELs)4 are mostly T cells dispersed as single cells within the epithelial cell layer and located at the interface between the Ag-rich outside world and the body. The majorities of IELs are CD8+ IELs and are simply classified as CD8α+ IELs or CD8αβ+ IELs. The CD8αβ+ IELs bear the hallmarks of adaptive immune cells. In contrast, the CD8α+ IELs possess many “unconventional” features that distinguish them from conventional CD8+ T cells and are considered innate immune cells (3–8).

The rotavirus is genetically comprised of 11 segments of dsRNA (9). The purified genomic dsRNA from rotavirus or reovirus are recognizable by TLR3 (10, 11), suggesting that genomic dsRNA may be involved in viral pathogenesis. TLRs constitute a family of at least 10 members that detect conserved molecular products derived from pathogens and commensal microflora (12). The TLRs, with the exception of TLR10, have been detected in primary intestinal epithelial cells (IECs) (13), but the role of TLRs on IECs is controversial. Although Rakoff-Nahoum et al. (14) has shown that persistent and basal TLR activation induced by commensal microflora plays a crucial role in the maintenance of intestinal epithelial homeostasis, experimental data from both human and animals suggest that abnormal TLR signaling may contribute to the disruption of epithelial homeostasis in human inflammatory bowel disease and other enteric bacterial infections (15–19). However, none of these studies provides direct evidence that TLR signaling breaks down epithelial homeostasis or provides an understanding of the mechanisms responsible.

Polyinosinic-polycytidylic acid (poly(I:C)), a ligand of TLR3 is a synthetic analog of viral dsRNA and has been used extensively to mimic viral infection. In this study, we report that both poly(I:C) and purified viral dsRNA induce severe mucosal damage via TLR3-dependent manner. This study also demonstrates that IL-15 and CD8α+ IELs play critical roles in disrupting epithelial homeostasis caused by abnormal TLR3 signaling.

Materials and Methods

Mice

Male C57BL/6 mice (6–8 wk old) were obtained from the Shanghai Experimental Animal Center. TLR3−/− and control mice were obtained from The Jackson Laboratory and maintained under specific pathogen-free conditions. The handling of mice and experimental procedures were conducted in accordance with guidelines for experimental animal from the University of Science and Technology of China and abided by the China and Anhui Province laws on animal protection.

Reagents

The polyclonal rabbit anti-AsGM1 Ab was purchased from Wako Pure Chemicals Industries (20), Anti-TLR3 polyclonal-blocking Ab (21), anti-IL-2 (JES6-1A12) Ab, and anti-TNF-α (MP6-XT3)-neutralizing Ab were purchased from eBioscience. Rabbit polyclonal anti-murine IFN-γ and anti-murine IFN-β were purchased from PBL Biomedical Laboratories. Anti-NK1.1 (PK136) mAb, which recognizes the mouse NK1.1 Ag (22), anti-CD4 (GK1.5)-, anti-CD8α (2.43)-, anti-IFN-γ (R4-6A2)-, and anti-IL-12 (R2-10F6)-neutralizing Ab were purchased from PeproTech. Chloroquine, 2-aminopurine, and sulfasalazine were purchased from Sigma-Aldrich.

Injection protocol

Poly(I:C) sodium (Sigma-Aldrich) was dissolved in pathogen-free saline and injected i.p. (5 or 30 μg/g wt). The CpG-oligodeoxynucleotides (class
The cell cultures were harvested and then frozen and thawed three times.

Preparation of IELs and lamina-propria lymphocytes (LPLs)
IELs were isolated as described previously (23). Briefly, Peyer’s patches were excised, and the small intestine and colon were opened longitudinally and cut into 5-mm long pieces. Then the specimens were washed twice in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. The pieces were then stirred at 37°C in prewarmed RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS for 30 min, followed by vigorous shaking for 30 s. This process was repeated, and the supernatants were passed through a small cotton-ghost wool column to remove cell debris and were then separated on a Percoll density gradient (Amersham Biosciences). A discontinuous density gradient (40 and 70%) was used. The cells that layered between the 40 and 70% fractions were collected as IELs. These IELs contained >90% CD3+ cells as determined by FACS analysis.

LPLs were isolated by a modified version of published protocols (24, 25). Briefly, following IEL isolation, residual epithelial cells were removed by shaking in PBS containing 1.3 mM EDTA at 37°C for 30 min. This step was repeated, and the supernatants were discarded. The intestinal tissue was then cut into 2- to 3-mm long pieces and dispersed in 100 ml RPMI 1640 containing 5% FCS at 37°C for 20 min, and any released cells were discarded. LPLs were then isolated after digestion in RPMI 1640 supplemented with 100 U/ml collagenase (Sigma-Aldrich), 1 mM CaCl2, 1 mM MgCl2, and 5% FCS at 37°C for 30 min. Released cells were then washed in PBS containing 5% FCS and subjected to Percoll fractionation as described above for isolation of IELs. IEL and LPL viabilities were assessed by trypan blue exclusion.

IEC preparation and culture
IECs were isolated and cultured as described previously (26). Peyer’s patches were excised, and small intestines were opened longitudinally and were washed in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. The tissue was then cut into 5-mm long fragments and incubated for 10 min at 22°C on a shaker platform in PBS containing 60 U/ml collagenase Xa (Sigma-Aldrich), 0.02 mg/ml dispase I (Sigma-Aldrich), and 0.2% D-glucose. Cells and small sheets of intestinal epithelium were separated from the denser intestinal fragments by harvesting supernatants after 2-min depositing in medium containing DMEM (Invitrogen Life Technologies) containing 10% FCS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS (Invitrogen Life Technologies). Cells were centrifuged five times at 800 rpm for 3 min in DMEM plus 2% FCS. Supernatant fluids were discarded. The remaining pellet mainly contained cells in intestinal crypts and small sheets of intestinal epithelium (organoids). The purity of these cells was assessed by flow cytometry with anti-Mac-1 and anti-CD3 Ab or assessed by immunocytoplasmic staining with anti-cytokeratin mAb (27). Final isolated cells contained >95% IECs.

Epithelial cells were cultured in epithelial cell medium (ECM) containing equal volumes of phenol-red-free DMEM and Ham’s F-12 medium (Invitrogen Life Technologies) with the following additives: 5 μg/ml insulin (Sigma-Aldrich), 5 × 10−5 M dexamethasone (Sigma-Aldrich), 60 mM selenium (Sigma-Aldrich), 5 μg/ml transferrin (Sigma-Aldrich), 10 μg of epidermal growth factor (Sigma-Aldrich) per ml, 20 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2% n-glucose, and 2% FCS. ECM was used within 2 wk of preparation to ensure that the activity of growth factors was maintained. Cells were cultured in 5% CO2 at 37°C. After cultured for 36 h, the viability of IECs was confirmed by flow cytometry. The forward and side scatter analysis showed that the percentage of apoptotic and necrotic cells (exhibiting increased side scatter compared with viable cells) was always <5% (data not shown). Cell viability was also assessed by trypan blue exclusion and light microscopy: >95% IECs were viable.

Virus culture and purification
The MA104 cell line (purchased from CCTCC) was used for virus cultivation. Monolayers were grown to confluence in flat-bottom plastic flasks in the presence of Eagle’s MEM supplemented with 10% FCS. Virus suspension (sminnotated with the SIMA11 strain) was pretreated with trypsin (10 μg/ml) for 30 min at 37°C to activate the virus inoculum before cultivation. MA104 monolayers were washed twice with serum-free Eagle’s MEM before adsorption of trypsin-activated rotavirus for 1 h at 37°C. After the adsorption step, the medium was removed and replaced by serum-free Eagle’s MEM containing 1 μg/ml trypsin. When cytopathogenic effects were observed, the cell cultures were harvested and then frozen and thawed three times. The viruses were concentrated by precipitation with polyethylene glycol 8000 from the supernatants and used for RNA extraction.

Viral RNA extraction
RNA was extracted from purified rotavirus using TRIzol reagent (Invitrogen Life Technologies). RNA concentration and purity were assessed by OD spectrophotometry.

Rotavirus infection on IECs
IECs were placed in wells of 24-well plates coated with rat tail collagen (Sigma-Aldrich) and incubated for 24 h in the IEC culture medium. Before infection, cells were washed three times with serum-free ECM, and the desired amount (5 PFU/cell) of rotavirus (pretreated with 10 μg/ml trypsin) was added to the cells. Cells were incubated for 1 h at 37°C in 5% CO2, followed by removal of the virus containing medium. Cells were washed three times with serum-free ECM, and the infection was continued for the indicated times in a 37°C incubator with 5% CO2.

51Cr release assay
Cytotoxicity was assessed by a 51Cr release assay as described previously (28). YAC-1 cells (Cell Bank of Chinese Science Academy), which are lymphoma cells generally used to test the natural cytotoxicity of NK cells, were used as target cells. Then, YAC-1 cells were labeled with Na251CrO4 and 1 h and placed in wells of 96-well round-bottom microtitre plates. As IECs were used as the target cells, IECs (105) isolated from B6 mice were placed in well plates of 96-well microtitre plates coated with rat tail collagen (Sigma-Aldrich) and incubated for 24 h in the IEC culture medium. Then, IECs were washed twice gently and labeled with Na251CrO4 for 1 h. Effector cells were used at a concentration of 106/100 μl and 3-fold serial dilution was made to provide E:T ratios of 1:5, 1:25, and 1:50. After 4 h incubation, the supernatant was harvested, and the amount of radioactivity released from the cells was measured with a gamma counter. The percentage of target cell lysis was calculated by using the following equation:

\[ \text{percentage of cytotoxicity} = \left( \frac{\text{experimental release cpm} - \text{spontaneous release cpm}}{\text{maximal release cpm} - \text{spontaneous release cpm}} \right) \times 100 \]

RT-PCR
Gene expression was determined by RT-PCR as previously described (29), and total RNA was extracted from the purified IECs or tissue using TRIzol (Invitrogen Life Technologies) reagent, according to the protocol provided by the manufacturer. A total of 5 μg of RNA was reverse transcribed using random primers and 200 U of Moloney murine leukemia virus (Invitrogen Life Technologies) incubated together for 1 h at 37°C. A total of 10 μl of cDNA was then subjected to PCR. Samples were initially denatured for 5 min at 95°C, and then 2 μl of polymerase was added for a total reaction volume of 100 μl. The mixture were subsequently subjected to 30 cycles of the PCR amplification program: denaturation for 1 min at 94°C, followed by 1 min of annealing at 58°C for TNF-α, IFN-γ, and IL-15, 55°C for IL-12p40, 50°C for IFN-α, 60°C for IFN-β, then elongation for 1 min at 72°C, and a 7-min final extension interval after the last cycle. The primer sequences used were as follows: IL-15 sense, 5′-AGGAATACATC CACCTCGTGCTA-3′, and IL-15 antisense, 5′-GGAGAAAGAGCTTC ATTCGAGA-3′; β-actin sense, 5′-GGAGCCTCATATGGGTTTGGGC GAGG-3′, and β-actin antisense, 5′-GGAGCCTCATATGGGTTTGGGC GAGG-3′; TNF-α sense, 5′-ACTGGCGAGAAGGCGACTC-3′, and TNF-α antisense, 5′-TCTGGCAACATGGTTGTTG-3′; IFN-γ sense, 5′-ACGCG GACCTATGCAT-3′, and IFN-γ antisense, 5′-AACGTCACTATGGATA GCTCGG-3′; IL-12 sense, 5′-AGTTGGTCTCCTGAGTACA-3′, and IL-12 antisense, 5′-AAGCCCAACAGCAGAAGCAG-3′; IFN-α sense, 5′-ATGGCTAGGCCCGTCTGCTCC-3′, and IFN-α antisense, 5′-CTCGTAY CAACCTCCAGCACA-3′, and IFN-β sense, 5′-AACAAGTCTTAAC CACCATGCAACAG-3′, and IFN-β antisense, 5′-TGTGAAGATTCCTGTTGATGAAGCTTG-3′.

Quantitative PCR
RNA extraction and cDNA synthesis was similar with the protocol shown in RT-PCR assay. Quantitative PCR was conducted according to the manufacturer’s instructions using a SYBR Premix Ex Taq (Takara). Reactions were conducted in reaction tubes (Takara Bio) in a sequence detector (ABI PRISM 7000; Applied Biosystems), beginning with a 30-s hot-start activation of the Taq polymerase at 95°C, followed by 45 cycles of amplification. Each reaction was performed in two steps (denaturation at 95°C for 10 s, followed by a 30-s annealing/extension at 68°C for TLR3 or at 60°C for actin). For analysis, all expression levels of target genes were normalized to the housekeeping gene β-actin (ΔCt). Gene expression values were then calculated based on
For histology, tissue from the small intestine was fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized, and stained with H&E. Morphological changes in the stained sections were examined under light microscopy.

Flow cytometry analysis

Cellular phenotypes were analyzed by incubating cells with mAbs conjugated to fluorescent labels. Double and triple immunofluorescence analyses were conducted. The mAbs used included FITC-, PE-, Cy5-, or biotin-conjugated anti-CD3 (145-2C11), anti-NK1.1 (PK136), anti-Mac-1 (M1/70), anti-DX5 (DX5), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD8β (CT-CD8b), anti-αβTCR (H57-597), anti-γδTCR (GL-3), anti-IL-15Rα (R&D Systems), and anti-cytokeratin (PCK-26; Sigma-Aldrich). To prevent nonspecific binding, respective isotype Abs were used as controls. Images of labeled cells were acquired by FACSCalibur and analyzed with WinMDI2.8 software.

ELISA

Serum samples were kept frozen at −80°C until ready for cytokine measurement. Levels of cytokines were measured using commercially available ELISA kits from R&D Systems.

Statistical analysis

Data are expressed as means ± SEM. To compare values obtained from three or more groups, one-way ANOVA was used, followed by Tukey’s post hoc test. To compare values obtained from two groups, Student’s t tests were performed. Results were considered statistically significant when p ≤ 0.05.
Results

Poly(I:C) or viral dsRNA induces severe mucosal injury of the small intestine

Intraperitoneal injection of poly(I:C) induced severe small intestinal injury, including intestinal wall attenuation (Fig. 1A), weight loss (Fig. 1B), villous atrophy (Fig. 1C), and mucosal erosion (Fig. 1D) but low mortality rates even at the doses of 30 μg/g wt (Fig. 1E). Poly(I:C) administration through the tail vein or intraintestinal injection also induced small intestinal injury similarly but oral administration did not (data not shown). The little effect of poly(I:C) via oral administration might be caused by the acidic environment in stomach. Results also show that the pathogenic effect of poly(I:C) was dose dependent (data not shown). To determine whether poly(I:C)-induced injury was specific and limited to the small intestine, other vital organs were examined. Poly(I:C) was found to have no or very mild
effect on the colon, liver, lung, kidney, brain, heart, or spleen in treated mice (data not shown).

Poly(I:C) is a TLR3 ligand. To determine whether other TLR ligands induce small intestinal injury, we also injected mice with CpG (TLR9 ligand), LPS (TLR4 ligand), and lipoteichoic acid (LTA) (TLR2 ligand) and found that these ligands had little effect (Fig. 1 or data not shown). The small intestinal injury caused by poly(I:C) was also similarly replicated by viral dsRNA extracted from the rotavirus strain SA11 (Fig. 1, F–H), suggesting that mucosal injury may be attributed to genomic dsRNA during rotavirus infection.

**IL-15 plays an important role in poly(I:C)-induced small intestinal injury**

To explore the mechanisms underlying dsRNA-induced small intestinal injury, we examined the expression levels of cytokines in serum, liver, small intestine, and colon after poly(I:C) or CpG injection. Our findings revealed that treatment with poly(I:C) increased IL-15 production in serum (Fig. 2A) and up-regulated IL-15 mRNA levels in small intestine tissue (Fig. 2B) but administration of CpG did not. Furthermore, the role of IL-15 in poly(I:C)-induced small intestinal injury was evaluated using anti-IL-15 receptor-neutralizing Ab. As shown in Fig. 2, blocking IL-15Ra prevented mice from poly(I:C)-induced small intestinal injury partially, including weight loss (Fig. 2C), villous atrophy (Fig. 2D), and mucosal erosion (Fig. 2E). In contrast, neutralization of IL-12, IFN-α, IFN-β, and TNF-α with the appropriate Abs provided minimal protective effects, and surprisingly, neutralization of IFN-γ instead aggravated injury induced by poly(I:C). These results suggest that IL-15 is an important mediator of poly(I:C)-induced small intestinal injury.

**Treatment with poly(I:C) or viral dsRNA enhances the NK-like cytotoxicity of IELs and augments the proportion of CD3^+ NK1.1^+ IELs**

The role of mucosal lymphocytes in small intestinal injury was explored. First, we examined NK-like cytotoxicity of dsRNA-activated mucosal lymphocytes. Intraepithelial lymphocytes isolated from poly(I:C) or viral dsRNA-treated mice exhibited much stronger cytotoxicity against YAC-1 cells or IECs than IELs isolated from PBS or CpG-treated mice (Fig. 3A). However, poly(I:C)-treated intestinal LPLs exhibited minimal cytotoxicity against YAC-1 cells or IECs (Fig. 3A). These findings suggest that small intestinal injury is induced primarily by IELs. Second, we examined the surface expression of CD3 and NK1.1 on IELs by flow cytometry. As a high dose of poly(I:C) (30 µg/g wt) can potentially damage the structure of the intestinal wall and induce IEL release from the mucosa at 12 and 24 h, we used a lower dose of poly(I:C) or CpG (5 µg/g wt) to examine changes in IEL subpopulations. Our results revealed that the percentage (Fig. 3B) and number (Fig. 3C) of CD3^+ NK1.1^+ IELs increased significantly 6 h after injection of poly(I:C) or dsRNA, whereas CpG injection failed to induce any effect.

We next investigated whether CD3^+ NK1.1^+ IELs are derived from CD3^+ NK1.1^+ IELs. The sorted CD3^+ NK1.1^+ IELs isolated from nontreated B6 mice were cultured with poly(I:C) or CpG in vitro for 24 h. Results showed that poly(I:C) induced CD3^+ NK1.1^+ IELs to acquire the NK1.1 marker in vitro in the presence of IECs but CpG did not. The effect of poly(I:C) on CD3^+ NK1.1^+ IELs was lost when IECs were absent from the culture (Fig. 4). Taken together, these results suggest that dsRNA can induce CD3^+ NK1.1^+ IELs to acquire NK1.1 markers and exert NK-like cytotoxicity with the help of IECs.

**CD3^+ NK1.1^+ IELs play a critical role in poly(I:C)-induced small intestinal injury**

Because poly(I:C) injection augmented the percentage of CD3^+ NK1.1^+ IELs, we next examined the effect of PK136 mAb (to deplete NK1.1^+ cells) or anti-AsGM1 Ab (to deplete NK cells) to study the role of this subpopulation of IELs on poly(I:C)-enhanced NK-like cytotoxicity. The results showed that depletion of NK1.1^+ IELs using PK136 mAb inhibited the enhanced cytotoxicity of IELs, whereas depletion of CD3^+ NK1.1^+ IELs using the anti-AsGM1 Ab had no effect, suggesting that CD3^+ NK1.1^+ IELs are the major effectors in NK-like cytotoxicity (data not shown). We then depleted NK1.1^+ IELs and CD3^+ NK1.1^+ IELs in vivo
From three independent experiments. Loss (referring to the weight at 0 h) is shown (n in injection, the weight of mice was measured, and the percentage of weight height is intestinal injury. To further investigate the CD3+ intestinal injury effectors. Values in added to the cocultures of IELs and equal numbers of IECs in the presence of indicated Ab. After 24 h of incubation, IELs were isolated and used as effectors. Values in A–E are shown as means ± SEM from three independent experiments.

**FIGURE 6.** The role of CD8αα+ IELs in small intestinal injury caused by poly(I:C). A, IELs were prepared for flow cytometry analysis at 6 h post-poly(I:C) (5 μg/g wt) injection. CD3+ NK1.1+ IELs were gated, and the surface expression of DX5, CD4, CD8α, CD8β, αβTCR, and γδTCR are shown. B and C, B6 mice were pretreated with indicated Ab 24 h before poly(I:C) (30 μg/g wt) injection. B, Thirty-six hours following poly(I:C) injection, the weight of mice was measured, and the percentage of weight loss (referring to the weight at 0 h) is shown (n = 5). C, Twelve hours following poly(I:C) and PBS injection, morphometric analyses of villus height (n = 3) are shown. Values in B and C are shown in means ± SEM from three independent experiments.

before poly(I:C) injection. Our findings revealed that depletion of NK1.1+ IELs using PK136 mAb protected mice from poly(I:C)-induced weight loss (Fig. 5A), villous atrophy (Fig. 5B), and mucosal erosion (Fig. 5C), but depletion of CD3+ NK1.1+ IELs using the anti-AsGM1 Ab had minimal effect. These results show that CD3+ NK1.1+ IELs play a critical role in poly(I:C)-induced small intestinal injury.

**CD8αα+ IELs play a critical role in poly(I:C)-induced small intestinal injury**

To further investigate the CD3+ NK1.1+ IEL subpopulation, we analyzed the surface markers of these cells. As shown in Fig. 6A, these cells were CD4−, DX5−, CD8α+, and CD8β+, suggesting that these cells are CD8αα+ IELs. These results were confirmed by in vivo experiments, whereby CD8 depletion prevented the injury caused by poly(I:C) but CD4 depletion did not (Fig. 6, B and C). These results suggest that poly(I:C)-induced small intestinal injury is caused by CD8αα+ IELs.

**Poly(I:C) or viral dsRNA-enhanced cytotoxicity of IELs depends on IEC-derived IL-15**

As the effect of poly(I:C) on IELs is IEC dependent (Fig. 7A), we examined whether cell-cell interactions are necessary for IEL activation. Experiments were conducted using Transwell chambers. As shown in Fig. 7B, when IELs were placed in the lower compartment and separated from IECs, which were placed in the upper compartment, the cytotoxicity of IELs was similar to when IELs were placed in the same well with IECs. This finding suggests that IEL activation does not require direct cell-cell interactions with IECs and that soluble molecules may be involved. Because IL-15 is an important cytokine in poly(I:C)-induced injury (Fig. 2), we believe that IL-15 is involved in the IEL activation. First, poly(I:C), purified viral dsRNA, and live rotavirus up-regulated the IL-15 production of IECs, whereas CpG, LTA, and LPS had little effect (Fig. 7C). Second, IL-15 enhanced the NK-like cytotoxicity of IELs in the presence of IECs (Fig. 7E). These results demonstrate that IEL activation depends on the presence of IL-15 derived from poly(I:C)-treated IECs.

**Poly(I:C) or viral dsRNA induces severe mucosal injury via TLR3-dependent manner**

We next investigated whether TLR3 or protein kinase R (PKR) was involved as both of these molecules are independently capable of mediating responses to dsRNA (10, 30). We used anti-TLR3 polyclonal Ab (21), inhibitors of intracellular expressed TLR3 signaling (chloroquine) (11, 31, 32), and inhibitors of PKR (2-aminopurine) (33, 34) to inhibit surface-expressed TLR3 signaling, intracellularly expressed TLR3 signaling, and PKR signaling, respectively. As shown in Fig. 7A, when surface-expressed and intracellular TLR3 signaling were both inhibited, IL-15 production induced by poly(I:C), purified viral dsRNA, or live rotavirus decreased significantly. These results indicate that IL-15 production induced by dsRNA is TLR3 dependent. To further confirm the role of TLR3 signaling, TLR3-deficient mice were used. As shown in Fig. 7B and C, poly(I:C) failed to induce small intestinal injury in TLR3−/− mice. We then found that TLR3 expression on the small
The results presented here provide direct evidence that aberrant activation of TLR3 signaling induced by dsRNA (all experiments were done using poly(I:C) with confirmation in some by purified rotavirus genomic dsRNA) causes a breakdown of the mucosal homeostasis, leading to mucosal damage. dsRNA is thought to be derived from the genome of dsRNA virus or the replication intermediate of some viruses. So, these results suggest that TLR3 may be involved in the pathogenesis of viral infection in the gastrointestinal tract since both poly(I:C) and purified genomic rotavirus dsRNA induce mucosal injury. These results are consistent with reports by Wang et al. (35). Their study found that TLR3-dependent inflammatory responses were needed to disrupt the blood-brain barrier, which enabled viral entry into the brain. Taken together, these results indicate that TLR3 signaling produces dual actions contributing not only to host defenses but also to viral pathogenesis.

The results presented here also provide a mechanism for how abnormal TLR signaling influences mucosal homeostasis. IL-15 expression is strictly regulated at multiple distinct steps to mediate appropriate levels of the cytokine expression (36–40). In human bowel diseases, including inflammatory bowel disease and celiac disease (CD), dysregulated IL-15 expression has been suggested as the cause of mucosal homeostasis disruption (41–44). However, causative factors leading to IL-15 dysregulation remains unknown. Our results provide a link between IL-15 dysregulation and abnormal TLR signaling. In this study, we found that abnormal TLR3 signaling induces IECs to produce elevated levels of IL-15. Indeed, other TLR ligands, such as LPS, lipopeptide, and flagellin, also induce production of IL-15 by macrophages (45). Because NF-κB is a transcription factor that is universally used by all TLRs and reportedly essential for transcriptional activation of the IL-15 gene (46, 47), we inhibited the activity of NF-κB by treatment with poly(I:C) for 48 h in the presence of indicated reagents, and the supernatants were collected for measuring IL-15.

Discussion

The results presented here provide direct evidence that aberrant activation of TLR3 signaling induced by dsRNA (all experiments were done using poly(I:C) with confirmation in some by purified rotavirus genomic dsRNA) causes a breakdown of the mucosal homeostasis, leading to mucosal damage. dsRNA is thought to be derived from the genome of dsRNA virus or the replication intermediate of some viruses. So, these results suggest that TLR3 may be involved in the pathogenesis of viral infection in the gastrointestinal tract since both poly(I:C) and purified genomic rotavirus dsRNA induces mucosal injury. These results are consistent with reports by Wang et al. (35). Their study found that TLR3-dependent inflammatory responses were needed to disrupt the blood-brain barrier, which enabled viral entry into the brain. Taken together, these results indicate that TLR3 signaling produces dual actions contributing not only to host defenses but also to viral pathogenesis.

The results presented here also provide a mechanism for how abnormal TLR signaling influences mucosal homeostasis. The consequence of abnormal TLR3 signaling is induction of IECs to produce IL-15, a key regulatory cytokine involved in mucosal homeostasis. IL-15 expression is strictly regulated at multiple distinct steps to mediate appropriate levels of the cytokine expression (36–40). In human bowel diseases, including inflammatory bowel disease and celiac disease (CD), dysregulated IL-15 expression has been suggested as the cause of mucosal homeostasis disruption (41–44). However, causative factors leading to IL-15 dysregulation remains unknown. Our results provide a link between IL-15 dysregulation and abnormal TLR signaling. In this study, we found that abnormal TLR3 signaling induces IECs to produce elevated levels of IL-15. Indeed, other TLR ligands, such as LPS, lipopeptide, and flagellin, also induce production of IL-15 by macrophages (45). Because NF-κB is a transcription factor that is universally used by all TLRs and reportedly essential for transcriptional activation of the IL-15 gene (46, 47), we inhibited the activity of NF-κB to determine the signaling link between TLR3 activation and IL-15 expression. We found that IL-15 production by poly(I:C)-treated IECs decreased (data not shown). These results together suggest that IL-15 dysregulation is possibly caused by inappropriate activation of TLR signaling in the disruption of mucosal homeostasis.

Up-regulated IL-15 production induced by abnormal TLR signaling subsequently breaks down mucosal homeostasis. Our results demonstrate that IL-15 disturbs mucosal homeostasis by acting on CD8α^+ T ELs. The influence of IL-15 on IELs has been investigated extensively. It has been shown that mice deficient in the IL-15 or IL-15Rα gene had a reduced number of CD8α^+ IELs, suggesting that IL-15 is involved in the development and proliferation of CD8α^+ IELs and is needed for maintaining mucosal homeostasis (48, 49). In this study, we found that IL-15 stimulates IELs to acquire NK1.1 and exerts “NK-like” cytotoxicity against IEC. Indeed, IL-15 can effectively convert IELs to LAK cells and promote IELs killing of IECs via a NKG2D-dependent manner in human CD (43, 44). Although IELs have been
identified as the pathological cells in both human CD and our mice model, the IEL subpopulations are different. In our mice model, the pathological cells are the innate-like CD8αα- IELs, whereas in human CD, the primary pathological cells triggered by IL-15 are the conventional CD8αβ+ TCRαβ+ IELs (44). The reason we think this is that poly(I:C)-induced injury is acute and mediated by innate immune responses, but human CD is a chronic inflammation mediated by conventional CD8+ CTL.

The IL-15R is composed of IL-15Rα-chain, which uniquely binds IL-15, as well as IL-2Rβ and γ chain (50, 51). Although soluble IL-15 can bind the IL-15R complex and induce signals in a similar manner with other cytokines (52, 53), accumulating evidence suggests that IL-15 can be transpresented to opposing cells via IL-15Rα (54–56). Our results show that rhIL-15 or poly(I:C)-treated IECs can stimulate IELs without direct presentation by IECs in vitro (Fig. 7, B and D). Indeed, it has been reported that IL-15 can stimulate human IELs in vitro (44). These results suggest two possibilities: 1) IL-15 can bind to IL-15R directly and stimulate IEL activation, or 2) IL-15 can be presented to IL-15Rα+ IELs by IL-15Rα+ IECs. In fact, not only IECs but also a portion of CD8αα IELs express IL-15Rα (data not shown). Although IL-15 can stimulate IELs without the direct presentation of IECs in vitro, we still don’t know how IL-15 derived from IECs stimulates IELs in vivo. Overall, the mechanisms involved in the IEL activation induced by IEC-derived IL-15 need to be further investigated.

An interesting question is why poly(I:C)-induced injury is specific to the small intestine. To answer this question, further investigation is needed; however, we think it is associated with the high expression of TLR3 and the particularity of IELs in small intestine. First, the mRNA levels of TLR3 on small intestine is higher than that in colon before or after poly(I:C) injection (Fig. 8D). Second, CD8αα IELs, which cause the injury of small intestine, possess the majority of small intestine IELs but only a small fraction of lymphocytes from liver, colon, spleen, and lung (data not shown).

Taken together, our results demonstrate that aberrant activation of TLR3 signaling on IECs can disrupt mucosal homeostasis via IL-15-dependent mechanism. This mechanism has also been observed in human CD in which the triggering factor is gliadin (43, 44). Why both TLR3 signaling and gliadin can use a similar mechanism to damage small intestine remains to be further investigated; however, a recent study by Thomas et al. (57) provided a possible explanation. Their study found that the effect of gliadin on IECs was dependent on MyD88, a key adaptor molecule in the TLR/R scaffold. This mechanism has also been observed in the mouse gut epithelium and lamina propia and circulate in the thoracic duct lymph. J. Exp. Med. 191: 823–834.


11. Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, Y. Yamamoto, K. Fujihashi, K. Kawabata, J. R. McGhee, and H. Kiyono. 1998. Toll-like receptor (TLR) signaling pathways, but were neither TLR2 nor TLR4 dependent. These results suggest that gliadin may be recognized by TLR3 signaling pathways, but were neither TLR2 nor TLR4 dependent. These results suggest that gliadin may be recognized by Toll-like receptor 3 ligand attenuation by Toll-like receptors. J. Clin. Invest. 111: 1297–1308.


