TLR3-mediated NF-κB signaling in human esophageal epithelial cells

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The esophageal epithelium is continuously exposed to potential pathogens present in ingested food and saliva. Physical mechanisms that protect the esophagus against recurrent injury include efficient peristalsis, resulting in a short duration of contact with foreign antigens. Esophageal barrier function is also enhanced by epithelial tight junctions as well as a stratified squamous epithelium, which undergoes continuous cycles of desquamation and regeneration. However, the direct role of esophageal epithelial cells in host immunological surveillance and defense has not been explored.

In recent years, chronic inflammatory diseases affecting the esophagus have become increasingly recognized. Longstanding gastroesophageal reflux disease (GERD) is a risk factor for the development of Barrett’s esophagus, a premalignant condition associated with the development of esophageal adenocarcinoma (EAC). Despite the increased use and availability of acid-blocking medications, the incidence of EAC has continued to rise over the last decades (57). This observation has prompted investigators to study the relationship between chronic inflammation and carcinogenesis (12, 25, 37). Importantly, recent studies have identified NF-κB as a key transcription factor that may be involved in the transition from epithelial inflammation to cancer (30, 38, 58). In this regard, the study of innate immune responses in the gastrointestinal epithelium has become a highly relevant area of research.

Very recently, the microbial ecosystem within the esophagus has been suggested as a potential factor contributing to the pathogenesis of GERD esophagitis. Through bacterial 16S ribosomal RNA gene surveys using patient biopsy samples, Yang et al. (54) found that global alterations in the esophageal microbiome distinguish the normal esophageal phenotype from that of esophagitis and/or Barrett’s esophagus. As key participants in the host innate immune response to pathogens, Toll-like receptors (TLRs) may therefore play an important role in the interaction between the esophageal microbiome and the host inflammatory response. TLRs are pattern-recognition molecules expressed by multiple cell-types, which recognize conserved pathogen-associated molecular patterns associated with bacteria, viruses, and fungi. To date, eleven members of the human TLR family have been identified (13, 15, 21, 46, 48, 51). Once bound by their respective ligands, TLR signal transduction leads to the activation of a number of downstream pathways involved in the cellular response to stress. Common to most TLR signal transduction pathways is the activation of NF-κB, a transcription factor that is known to regulate the expression of multiple genes involved in inflammation, chemotraction, cellular proliferation, and antimicrobial activity (42). NF-κB activation is known to be tightly regulated in a cell type- and stimulus-specific fashion, through mechanisms well described by others (16, 34, 43).

In contrast to the more distal gastrointestinal tract in which TLRs have been extensively studied (1, 2, 11, 29, 32, 35), the expression and function of TLRs in the esophageal epithelium have not been well characterized. In the only previous publication regarding esophageal TLRs, Uehara et al. (47) reported that TLRs 2, 3, 4, and 7 are expressed by the squamous cell carcinoma esophageal cell line TE-1. Interestingly, whereas stimulation of TE-1 cells with synthetic TLR ligands led to the induction of antimicrobial peptide expression, TLR agonist stimulation failed to induce cytokine or chemokine expression in TE-1 cells. These findings, however, were based on transformed esophageal epithelial cell lines in which innate immune responses may already be altered (9, 28, 33). To our knowledge, the expression and function of TLRs in primary and nontransformed immortalized esophageal cell lines have never before been investigated.
In the present study, we investigated the TLR expression profile of primary esophageal keratinocytes and immortalized nontransformed human esophageal keratinocytes in vitro. We demonstrate that TLR3, which recognizes double-stranded RNA (dsRNA) produced by viral replication, is the most abundantly expressed and functional TLR in the esophageal epithelium. Using the synthetic dsRNA analog, polyinosinic polycytidylic acid [poly(I:C)], we now identify an important role for TLR3-mediated NF-κB signaling in human esophageal epithelial proinflammatory cytokine responses. Importantly, we also identify an important role for TLR3-mediated NF-κB signaling in the expression and function of TLR2, suggesting a novel innate immune regulatory role for TLR3 in the esophageal epithelium.

**MATERIALS AND METHODS**

**Cell culture and reagents.** Primary human esophageal keratinocytes (EPC2) and nontransformed esophageal keratinocytes (EPC2-hTERT) were grown in monolayers at 37°C in a humidified 5% CO2 incubator and cultured in keratinocyte serum-free media supplemented with epidermal growth factor (1 ng/ml), bovine pituitary extract (50 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA). EPC2 cells used in experiments were studied between passages 10 and 20, and EPC2-hTERT cells were studied between passages 60 and 90. Cell lines were stimulated with the following TLR ligands: Pam3CSK4 (1 μg/ml), HKLM (106 cells/ml), poly (I:C) (10 μg/ml), and flagellin (100 ng/ml) (Invivogen, San Diego, CA). Peptidoglycan (10 μg/ml) was purchased from Sigma-Aldrich (St. Louis, MO). Bay11-7082 (10 μM) (Enzo Life Sciences, Plymouth Meeting, PA) or DMSO vehicle control (Sigma) were used in NF-κB inhibition studies.

RNA isolation and real-time quantitative RT-PCR. RNA was harvested from EPC2 and EPC2-hTERT cells using an RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s recommendations. RNA previously isolated from normal human esophageal biopsy samples was a kind gift from Dr. Debra Silberg (University of Pennsylvania School of Medicine). Biopsy samples were paired normal samples from the midesophagus from patients undergoing surveillance endoscopy for Barrett’s esophagus. The use of human biopsy samples was approved by the Institutional Review Board of the University of Pennsylvania (protocol no. 700399). RNA samples (0.5 μg/sample) were reverse transcribed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for the following human genes: TLR1 (Hs00413978_m1), TLR2 (Hs00610101_m1), TLR3 (Hs00152933_m1), TLR4 (Hs00152939_m1), TLR5 (Hs00152825_m1), TLR6/2 (Hs00271977_s1), TLR7 (Hs00152971_m1), TLR8 (Hs00152972_m1), TLR9 (Hs00152973_m1), TLR10 (Hs00999403_m1), IL-8 (Hs00174103_m1), and GAPDH (4352934E). Quantitative RT-PCR was performed using the TaqMan Fast Universal PCR Master Mix kit (Applied Biosystems), and reactions were performed in triplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control to normalize the samples using the ΔΔCT method of relative quantitation, where CT is the threshold cycle.

Cytokine assay. Twenty-four hours after TLR ligand stimulation, cell culture supernatants were collected and stored at −80°C until further use. IL-8 secretion was quantified using enzyme-linked immunosorbent assay (BD OptEIA human IL-8 ELISA kit; BD Biosciences, San Jose, CA) according to manufacturer’s instructions. IL-8 levels in each sample were calculated on the basis of a standard curve generated by human recombinant IL-8 provided with the kit. Results were expressed as the means + SD in pg/ml.

**Transfection and luciferase assay.** EPC2-hTERT cells were seeded in 12-well plates 1 day before transfection. The following day, cells were cotransfected with cytomegalovirus (CMV)-β-galactosidase and either the promoterless pGL2 Basic reporter plasmid (Promega, Madison, WI), a wild-type human IL-8 promoter construct ((wt)LUC), or an IL-8 promoter construct containing a site-directed mutation of its NF-κB binding element ([mNF-κB]LUC) (53) using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer’s recommendations. Twenty-four hours after transfection, cells were stimulated for an additional 24 h with poly(I:C) (10 μg/ml). Cell lysates were harvested, and luciferase activity was quantified using the Promega Luciferase Assay System according to manufacturer’s instructions. Transfection efficiency was determined by measurement of β-galactosidase activity using an ortho-nitrophenyl-β-galactoside substrate and a colorimetric assay.

**Immunofluorescence.** EPC2-hTERT cells were seeded in glass chamber slides and stimulated with poly(I:C) (10 μg/ml). At various time points after stimulation, cells were washed with PBS and fixed in 10% neutral buffered formalin for 5 min. After being washed in PBS, cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature, washed with PBS, and blocked for 5 min using 5% donkey serum. Cells were incubated in rabbit anti-NF-κB p65/RelA (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. After being washed with PBS, Cy3-conjugated donkey anti-rabbit secondary antibody was applied for 2 h at room temperature (1:800). Cells were washed in PBS, stained with 4,6-diamidino-2-phenylindole (Invitrogen), and photographed using a Nikon E600 microscope using iVision software (Omaha, NE).

**Western blot analysis.** EPC2-hTERT cells were washed with ice-cold PBS and lysed with RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.9% NaCl, 25 mM Tris, and 1 mM EDTA) containing a protease inhibitor cocktail (Sigma). Cells were scraped and incubated on ice for 1 h. Lysates were cleared by centrifugation for 10 min at 4°C, and supernatants were stored at −80°C until later use. Protein concentrations were determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL). Proteins were separated by electrophoresis using NuPage 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 2.5% nonfat dry milk and 2.5% bovine serum albumin overnight at 4°C. Membranes were incubated with primary antibody for 1 h at room temperature, washed in Tris-buffered saline-Tween (TBST), incubated with secondary antibody for 1 h at room temperature, and washed again in TBST. The signal was developed using an ECL Western blotting detection kit (GE Healthcare, Piscataway, NJ) according to manufacturer’s recommendations. Rabbit anti-human IκBα (Santa Cruz Biotechnology) was used at a concentration of 1:1,000, and mouse anti-actin (Cell Signaling Technology, Beverly, MA) was used at a concentration of 1:2,000. Secondary antibodies included horseradish peroxidase-conjugated anti-rabbit (1:2,000) and anti-mouse (1:5,000) (GE Healthcare).

RNA interference knockdown. Silencer Select short inhibitory RNAs (siRNAs), TLR3 (ABI no. 4390824), and control (ABI no. 4390843) were purchased from Applied Biosystems. One day before transfection, EPC2-hTERT cells were seeded at a concentration of 1 × 105 cells per well in 12-well plates. The following day, cells were transfected with 10 nM of the designated siRNA using X-tremeGENE siRNA transfection reagent (Roche Applied Science) according to the manufacturer’s directions. The media was changed at 24 h after transfection. Seventy-two hours posttransfection, cells were either stimulated with poly(I:C) (10 μg/ml) or with untreated cell culture media. Twenty-four hours after stimulation, cells were harvested for RNA isolation.

**Statistical analysis.** The two-tailed Student’s t-test was used. A P value of <0.05 was considered to be statistically significant.
RESULTS

Human esophageal epithelial cells express TLRs 1, 2, 3, and 5. We used quantitative RT-PCR to determine the constitutive expression of human TLRs 1–10 using RNA isolated from primary esophageal epithelial cells (EPC2) and the immortalized, nontransformed esophageal epithelial cell line, EPC2-hTERT. Both EPC2 and EPC2-hTERT cell lines have been previously characterized (6, 19, 36, 44, 45). In contrast to the parental cell line EPC2, which undergoes senescence by 40–44 population doublings, EPC2-hTERT cells overcome replicative senescence and are immortalized by constitutively active telomerase without genetic or epigenetic abnormalities in the p53 and pRb pathways (6), making this specific immortalized cell line a useful in vitro model to study normal esophageal epithelial cell physiology. Indeed, both the EPC2 and the EPC2-hTERT cell lines share the morphological, cytogenetic, and biochemical characteristics of normal esophageal basal cells. Both cell lines have normal diploid status, express cytokeratins 5 and 14 found in basal cells, and can differentiate in the postconfluent state or in the presence of high extracellular calcium concentration (6).

As shown in Fig. 1A, TLRs 1, 2, 3, and 5 were expressed by both EPC2 and EPC2-hTERT cell lines. TLR4 mRNA was undetectable in both cell lines, and there was minimal mRNA expression of TLRs 6–10 in both cell lines. Importantly, the similarities in TLR profiles between the two cell lines demonstrated that telomerase immortalization of the EPC2 cell line did not have a significant impact on the overall pattern of esophageal epithelial TLR expression.

We next sought to determine whether the observed TLR expression pattern in vitro was also present in normal human esophageal mucosa. We performed quantitative RT-PCR for TLRs 1–10 using RNA isolated from normal esophageal mucosal biopsy samples from four individuals and found that, consistent with our findings in vitro, TLRs 1, 2, 3, and 5 were clearly expressed in normal human esophageal mucosa (Fig. 1B).

Stimulation of esophageal epithelial cells with the TLR3 ligand poly(I:C) leads to the induction of IL-8 expression through an NF-κB-dependent mechanism. Activation of most TLRs by their specific ligands induces the differential expression of multiple genes involved in innate immune defense. We...
determined the functional significance of the expressed esophageal TLRs by quantifying the expression of the chemokine IL-8 following stimulation of EPC2 and EPC2-hTERT cells with ligands for TLRs that were most highly expressed by our cell lines. IL-8 was chosen as a physiologically relevant read out in this model system because its esophageal mucosal expression has been shown to be increased in patients with GERD-related esophagitis (24, 56). As shown in Fig. 2A, stimulation of both EPC2 and EPC2-hTERT cells with ligands for the most highly expressed esophageal TLRs (TLRs 1, 2, 3, and 5) resulted in similar patterns of IL-8 mRNA induction. Stimulation of both cell lines with peptidoglycan (PGN) (TLR2 ligand) led to modest increases in IL-8 expression, whereas stimulation with other TLR2 ligands including Pam3CSK4 (TLR1/2 ligand) and HKLM (TLR2 ligand) did not significantly alter IL-8 expression. TLR5 stimulation by its ligand flagellin led to a modest increase in IL-8 expression over controls in both EPC2 and EPC2-hTERT cells. Unexpectedly, stimulation of both EPC2 and EPC2-hTERT cells with the ligand for TLR3, the synthetic double-stranded RNA analog, poly(I:C), resulted in the highest levels of IL-8 induction in both EPC2 and EPC2-hTERT cell lines. Secretion of IL-8 by stimulated EPC2 and EPC2-hTERT cells was also quantified by ELISA analysis (Fig. 2B), confirming not only that TLR3 ligand stimulation resulted in the most robust IL-8 protein expression but also that this induction was similar in both the parental EPC2 cell line and the hTERT-immortalized EPC2-hTERT cell line. On the basis of similar constitutive TLR expression profiles and parallel IL-8 responses by both the primary EPC2 and EPC2-hTERT cell line at both the transcriptional and translational levels, we focused our subsequent studies upon TLR3 signaling in the immortalized EPC2-hTERT cell line.

Like other TLRs, the activation of TLR3 is known to initiate downstream signaling pathways, including NF-κB (4). On the basis of the degree of IL-8 mRNA induction in response to poly(I:C) stimulation, we investigated the transcriptional mechanisms of poly(I:C)-stimulated induction of IL-8 in esophageal epithelial cells. EPC2-hTERT cells were stimulated with poly(I:C) (24 h, 10 μg/ml) following transient transfection with either a luciferase reporter construct containing the −135 to +46 portion of the human IL-8 gene [(wt)LUC] or an IL-8 promoter construct containing a site-directed mutation of its NF-κB binding element [(mNF-κB)LUC] (53) (Fig. 2C). In the absence of poly(I:C) stimulation, constitutive activation of the IL-8 promoter was observed in cells transfected with both the WT and mutant construct, consistent with the notion that basal activation of the human IL-8 promoter can occur independently of NF-κB signaling (53). In contrast, poly(I:C) stimulation led to a 25-fold increase in transactivation of the IL-8 promoter over unstimulated controls. Mutation of the NF-κB binding site completely eliminated this effect, indicating that poly(I:C)-induced IL-8 promoter activation was dependent on the interaction of NF-κB with the IL-8 promoter.

To further determine the role of NF-κB in the TLR3-mediated induction of IL-8, EPC2-hTERT cells were stimulated with poly(I:C) in the presence or the absence of Bay11-7082, an irreversible inhibitor of NF-κB (17). Bay11-7082 inhibits NF-κB nuclear translocation and activation by selectively inhibiting the phosphorylation and degradation of its repressor, IκBα. In the presence of Bay11-7082, there was a significant inhibition in poly(I:C)-mediated IL-8 activation (Fig. 2D) compared with vehicle control. Additional control experiments using vehicle alone (DMSO) or Bay11-7082 alone did not lead to statistically significant changes in IL-8 expres-

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**Fig. 2.** Preferential induction of IL-8 by the synthetic TLR3 ligand polyinosinic polycytidylic acid [poly(I:C)] through an NF-κB-dependent mechanism in human esophageal epithelial cell lines. A: mRNA expression of IL-8 following stimulation of EPC2 and EPC2-hTERT cells with the following TLR ligands: peptidoglycan (PGN) (TLR2), Pam3CSK4 (TLR1/2), HKLM (TLR2), poly(I:C) (TLR3), and flagellin (TLR5). B: ELISA quantification of secreted IL-8 (pg/ml) by EPC2 and EPC2-hTERT cells stimulated with TLR ligands: peptidoglycan (PGN) (TLR2), Pam3CSK4 (TLR1/2), HKLM (TLR2), poly(I:C) (TLR3), and flagellin (TLR5). C: NF-κB-dependent transactivation of the human IL-8 promoter. EPC2-hTERT cells were transfected with the promoterless pGL2Basic vector, a wild-type human IL-8 promoter construct [wt(LUC)], and an IL-8 promoter construct containing a site-directed mutation of its NF-κB binding element [mNF-κB(LUC)], followed by stimulation with poly(I:C) at 10 μg/ml for 24 h. D: poly(I:C)-mediated IL-8 mRNA induction in the presence of the NF-κB inhibitor Bay11-7082 vs. vehicle control (DMSO); **p < 0.005; ***p < 0.001. All results shown are representative of three individual experiments. NS, nonsignificant.
sion over controls (data not shown). Together, these results confirmed an important role for NF-κB in the TLR3-mediated induction of IL-8 expression in human esophageal epithelial cells.

Poly(I:C) stimulation of EPC2-hTERT cells leads to the nuclear translocation of NF-κB. We next investigated the early NF-κB signaling events initiated by poly(I:C) stimulation of EPC2-hTERT cells. Immunoblots were performed using protein isolated from stimulated EPC2-hTERT cells at various time points following exposure to poly(I:C) to determine the kinetics of IκBα expression and degradation. IκBα is a cytoplasmic inhibitor of NF-κB, which, when bound to the NF-κB heterodimer, prevents its nuclear translocation (7). Following immune stimulation, IκBα is rapidly degraded, allowing the nuclear translocation and activation of NF-κB. IκBα is then resynthesized under the regulatory control of NF-κB, thus providing autoregulation of this signaling pathway (7, 16, 43). As shown in Fig. 3A, poly(I:C) stimulation of EPC2-hTERT cells led to the degradation of IκBα within 30 to 60 min after exposure. Consistent with known NF-κB-autoregulatory pathways, this was followed by the resynthesis of IκBα at the 2-h time point.

To confirm the nuclear localization of NF-κB, immunofluorescence was used to localize the p65/RelA subunit of NF-κB in unstimulated and poly(I:C) (10 μg/ml) stimulated EPC2-hTERT cells following poly(I:C) stimulation for various time points. As demonstrated in Fig. 3B, the p65/RelA subunit of NF-κB was retained in the cytoplasm in unstimulated EPC2-hTERT cells, consistent with its cytoplasmic inhibition by IκBα. Sixty minutes following poly(I:C) stimulation, p65/RelA was localized to the nucleus, in accordance with the kinetics of IκBα degradation.

Poly(I:C) stimulation of EPC2-hTERT cells induces the NF-κB-regulated expression of TLR2 and enhances esophageal epithelial responses to TLR2 ligand stimulation. In addition to the induction of proinflammatory genes, TLR agonist stimulation is known to modulate the expression and function of other TLRs, thereby regulating the host response to potential pathogens. In this regard, enhanced TLR expression and signaling has been observed in the setting of pathogen-associated inflammation both in vitro and in vivo. Stimulation of the mouse macrophage line RAW264.7 with the TLR4 ligand LPS, for example, induces the expression of TLR9(5). In the setting of sepsis, the leukocyte surface expression of TLR2 (20, 50) and TLR4(20) is increased compared with normal controls. To date, however, the inducible expression of TLRs in the human esophageal epithelium has not been previously reported.

To determine whether TLR expression could be modified in human esophageal epithelial cells, we stimulated EPC2-hTERT cells with poly(I:C) for various time points and quantified the expression of TLRs 1–10. TLR3 mRNA expression was up-regulated eight- to tenfold following 24-h stimulation with its ligand, poly(I:C) (data not shown). Surprisingly, TLR2 expression was markedly induced in a time-dependent fashion by poly(I:C) stimulation, achieving maximal expression by 24 h (Fig. 4A). The expression of the remaining TLRs was unaffected by poly(I:C) stimulation (data not shown). Importantly, stimulation of EPC2-hTERT cells with ligands for TLR5 and the TLR2 superfamily (TLRs 1, 2, and 6) did not modify the expression of TLRs 1–10 (data not shown), suggesting a unique and specific role for TLR3 agonist stimulation in the regulation of esophageal epithelial TLR2 expression.

TLR2 expression has been previously shown by others to be regulated by NF-κB activation in cells of nonepithelial origin (31, 41). We next determined whether NF-κB signaling played a role in the poly(I:C)-mediated induction of TLR2 gene expression in esophageal epithelial cells. EPC2-hTERT cells were stimulated with poly(I:C) in the presence of the NF-κB inhibitor, Bay11-7082 vs. vehicle control (Fig. 4B). A modest increase in poly(I:C)-mediated TLR2 expression in the presence of vehicle control was significantly suppressed in the presence of Bay11-7082, implicating NF-κB in the inducible expression of TLR2 in esophageal epithelial cells. There was no significant change in the expression of TLR2 in the presence of DMSO or Bay11-7082 alone (data not shown).

To investigate whether the poly(I:C)-induced increase in TLR2 expression was associated with enhanced TLR2 function, EPC2-hTERT cells were stimulated with various combinations of poly(I:C) and the TLR2 ligand, PGN, again using

![Fig. 3. Kinetics of NF-κB activation following poly(I:C) stimulation of EPC2-hTERT cells. A: Western blot analysis of the cytoplasmic NF-κB inhibitor IκBα, using proteins isolated from EPC2-hTERT cells following poly(I:C) (10 μg/ml) stimulation for various time points. B: immunofluorescent localization of p65/RelA subunit of NF-κB in unstimulated and poly(I:C)-stimulated EPC2-hTERT cells (10 μg/ml) at the 60-min time point; ×40 magnification.](http://ajpgi.physiology.org/)

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IL-8 expression as a physiological read out of the inflammatory response. On the basis of our observation that TLR2 expression was maximally induced by 24 h of poly(I:C) stimulation, we hypothesized that EPC2-hTERT cells might be more responsive to TLR2 ligand stimulation following 24 h of TLR3 ligand priming. EPC2-hTERT cells were sequentially stimulated with poly(I:C) for 24 h, followed by PGN stimulation for an additional 24 h. IL-8 mRNA expression in these sequentially stimulated cells were compared with that of cells that were stimulated individually with either poly(I:C) or PGN for 24 h or costimulated with poly(I:C) and PGN for 24 h, followed by recovery in untreated cell culture media for 24 h. As shown in Fig. 4C, TLR2 and TLR3 ligand costimulation [poly(I:C)/PGN] resulted in an additive increase in IL-8 expression. Importantly, we observed a synergistic effect on IL-8 expression when cells were first primed by the TLR3 agonist poly(I:C) for 24 h, followed by subsequent TLR2 ligand (PGN) stimulation. Together, this indicated that priming of EPC2-hTERT cells by poly(I:C) increased the inflammatory response to the TLR2 agonist PGN and suggested that the TLR3-mediated increase in TLR2 expression was associated with enhanced TLR2 function.

Loss of TLR3 expression reduces the inflammatory response to poly(I:C) stimulation. Although poly(I:C) is classically known as the ligand for TLR3, poly(I:C) can also activate other intracellular dsRNA signaling pathways including the Ser/Thr protein kinase R (PKR) and the RNA helicases retinoid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) (39, 52, 55). PKR, RIG-1, and MDA-5 signaling leads to the activation of several downstream transcription factors that are also activated by TLR3, including NF-κB. The redundancy of these pathways has also been demonstrated in vivo because mice with targeted deletions in TLR3 are still capable of mounting reduced inflammatory responses to systemically administered poly(I:C) compared with wild-type mice (4).

Given the existence of multiple, redundant dsRNA signaling pathways, we sought to determine the relative contribution of TLR3 signaling to the poly(I:C)-induced inflammatory response in esophageal epithelial cells. We quantified changes in mRNA expression of TLR2 and IL-8 in EPC2-hTERT cells stimulated with poly(I:C) following TLR3 RNA interference using TLR3 siRNA knockdown. As shown in Fig. 5, TLR3 knockdown was effective, reflected by a 75% reduction in poly(I:C)-stimulated TLR3 mRNA expression in TLR3 siRNA-transfected cells compared with cells transfected with control siRNA. Importantly, poly(I:C)-induced TLR2 induction was partially suppressed by TLR3 siRNA transfection, indicating that TLR3 signaling plays a role in its induced expression. Compared with control siRNA-transfected cells, TLR3 siRNA-transfected cells demonstrated abrogated inflammatory responses to poly(I:C) stimulation with a 40–50% reduction in IL-8.

Together, these results suggested that, despite the existence of redundant dsRNA signaling pathways, TLR3 contributes significantly to the poly(I:C)-induced inflammatory response in the esophageal epithelium.

**DISCUSSION**

In this study, we demonstrate that TLR3 is the most abundantly expressed and functional TLR in the esophageal epithelium. Our data reveal for the first time that primary and immortalized
nontransformed human esophageal epithelial cells can function autonomously as innate immune effector cells in response to TLR ligand stimulation. Importantly, we now show that TLR3 stimulation by its synthetic ligand poly(I:C) leads to the early activation of NF-κB, which not only induces esophageal epithelial chemokine expression but also regulates the expression and ligand responsiveness of TLR2.

Our results contrast with those of Uehara et al. (47), who previously demonstrated that stimulation of squamous cell esophageal carcinoma cell line TE-1 with poly(I:C) failed to induce the expression of cytokines including IL-8, IL-6, and monocyte chemoattractant protein-1. The immediate and robust response to synthetic dsRNA demonstrated by our primary and nontransformed esophageal epithelial cell lines may therefore point to potential differences in innate immune signaling between primary and transformed esophageal epithelial cell lines. We have found that TE-1, EPC2, and EPC2-hTERT cells express comparable levels of TLR3 mRNA (unpublished observations), making differences in TLR3 expression an unlikely explanation for this observation. A more plausible explanation for the lack of poly(I:C) responsiveness by TE-1 cells may be an inability to internalize exogenous poly(I:C). Indeed, Hirabayashi et al. (22) previously reported that poly(I:C)complexed with cationic liposomes led to a direct antiproliferative effect on tumor cells of epithelial origin, whereas nonliposomally delivered poly(I:C) had no effect on epithelial tumor cells. Whether this represents an adaptive mechanism for tumor survival in the setting of esophageal neoplasia is certainly an intriguing possibility.

Our findings may also suggest a potential role for TLR signaling in the pathogenesis of esophageal inflammation. The induction of IL-8 in our model system is consistent with previous reports by others that show enhanced IL-8 mRNA expression in esophageal biopsies of patients with both erosive and nonerosive esophagitis secondary to gastroesophageal reflux (24, 56). Isomoto et al. (23) further showed that NF-κB was activated in the esophageal epithelium of patients with GERD, primarily in IL-8-positive cells in the epithelium. Our results expand on the findings of Isomoto et al., suggesting innate immune signaling through TLR3 as a mechanism by which NF-κB-mediated IL-8 induction may be activated in the setting of esophageal inflammation.

Unexpectedly, we found that TLR3-mediated NF-κB activation was not only important in the regulation of IL-8 expression but was also critical for the regulation of poly(I:C)-induced TLR2 expression. Despite the presence of several potential binding sites for NF-κB in the human TLR2 promoter (14, 18), the involvement of NF-κB in the transcriptional regulation of the human TLR2 gene has remained controversial. Whereas some studies have demonstrated that NF-κB binding is required for the inducible regulation of TLR2 expression in human endothelial cells (41) and murine hepatocytes (31), others have shown that NF-κB does not play a direct role in the activation of the TLR2 promoter in human monocytes and macrophages (18). Our findings now highlight an important role for NF-κB signaling in the inducible expression of TLR2 in human esophageal epithelial cells, suggesting that the NF-κB regulation of the human TLR2 gene may occur in a tissue-specific fashion.

In addition to the enhanced expression of TLR2, the “priming” of EPC2-hTERT cells by TLR3 ligand stimulation also...
enhanced the responsiveness of TLR2 to its own ligands. The notion that TLR ligands can alter immune responsiveness of other TLRs has been previously reported by others (8, 40); our results are now the first to identify these innate immune regulatory pathways in the esophageal epithelium. Although signaling through either TLR2 or TLR3 can independently lead to the activation of NF-κB, TLR2 and TLR3 signal transduction pathways are mediated by distinct adaptor molecules. Whereas TLR2, like most other members of the TLR family, signals exclusively through the adaptor molecule MyD88, TLR3 is unique among TLRs in its exclusive signaling through the adaptor molecule TLR3 interacting adaptor protein inducing IFN-β (TRIF). Both signaling pathways converge through the common downstream signaling molecule TNF receptor-associated factor 6, leading to the activation of NF-κB (26, 31, 49). Evidence for synergistic TLR3 and TLR2 signaling has been previously reported by Zhu et al. (59), who reported that TLR3/TRIF signaling enhanced TLR2/MyD88 signaling in dendritic cells; however, a mechanism by which TLR3 enhances TLR2/MyD88 signaling has not been proposed. Hence, our observation that TLR3 signaling induces the NF-κB-dependent expression of TLR2 may suggest a mechanistic explanation for enhanced TLR2 responsiveness in esophageal epithelial cells.

Although we used the synthetic TLR3 ligand poly(I:C) in our study, the naturally occurring ligand for TLR3 is dsRNA produced during the replicative cycle of viruses. Although viral esophagitis secondary to Herpes simplex virus or CMV infection can occur in up to 10% of patients following liver or kidney transplantation (3), such infections are rare in immunocompetent individuals. Therefore, the phylogenetic role for TLR3 signaling in the esophageal epithelium may not seem intuitive. Despite its longstanding association with TLR3, recent evidence now suggests that virus-derived dsRNA may not be the sole physiological ligand for TLR3. Endogenous cellular mRNA was recently identified as a TLR3 ligand by several groups. Kariko and colleagues (27) not only showed that exposure of human embryonic kidney 293 cells to in vitro transcribed RNA led to an NF-κB-dependent activation of TLR3 but also demonstrated that endogenous mRNA released from necrotic cells stimulated dendritic cells to secrete IFN-α. Similarly, Brentano et al. (10) showed that human fibroblasts exposed to necrotic synovial fluid cells produced high levels of IL-6 through a TLR3-dependent mechanism. Together, these key studies suggest that, in addition to viral pathogen stimulation, TLR3 can be activated in response to cellular injury, potentially linking TLR3 signaling to clinically relevant diseases, including reflux esophagitis in which epithelial damage by low pH or bile acids plays an inciting role. Most importantly, these observations help to distinguish TLR3 from other dsRNA signaling molecules, such as PKR, RIG-1, and MDA-5, which have exclusive roles in viral defense, and suggest a unique niche for TLR3 in the immune response to epithelial injury in the esophagus.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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