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Intestinal Expression of Fas and Fas Ligand Is Upregulated by Bacterial Signaling through TLR4 and TLR5, with Activation of Fas Modulating Intestinal TLR-Mediated Inflammation

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TLRs play an important role in mediating intestinal inflammation and homeostasis. Fas is best studied in terms of its function in apoptosis, but recent studies demonstrate that Fas signaling may mediate additional functions such as inflammation. The role of Fas, and the Fas ligand (FasL), in the intestine is poorly understood. The aim of this study was to evaluate potential cross-talk between TLRs and Fas/FasL system in intestinal epithelial cells (IECs). IECs were stimulated with TLR ligands, and expression of Fas and FasL was investigated. Treatment with TLR4 and TLR5 ligands, but not TLR2 and 9 ligands, increased expression of Fas and FasL in IECs in vitro. Consistent with this finding, expression of Intestinal Fas and FasL was reduced in vivo in the epithelium of TLR4 knockout (KO), SKO, and germ-free mice, but not in TLR2KO mice. Modulating Fas signaling using agonistic anti-Fas augmented TLR4- and TLR5-mediated TNF-α and IL-8 production by IECs. In addition, suppression of Fas in IECs reduced the ability of TLR4 and TLR5 ligands and the intestinal pathogens Salmonella typhimurium and Listeria monocytogenes to induce the expression of IL-8. In conclusion, this study demonstrates that extensive cross-talk in IECs occurs between the Fas and TLR signaling pathways, with the FasL/Fas system playing a role in TLR-mediated inflammatory responses in the intestine. The Journal of Immunology, 2014, 193: 000–000.

The intestinal immune system consists of multiple cell types, including intestinal epithelial cells (IECs), which constitute a single monolayer of cells found at the mucosal surface; specialized dendritic cells; and macrophages. In the gastrointestinal tract, IECs are in constant contact with luminal bacteria, their metabolites, and their various inflammatory products. The mucosal surface of the intestinal epithelium has evolved to allow the correct balance of responsiveness, being broadly unresponsive to the presence of the commensal bacteria in the gut lumen while still being able to mount an immune response to the presence of pathogenic bacteria (1). This colonic epithelial cell homeostasis is tightly regulated, as adverse effects can lead to inflammatory conditions such as inflammatory bowel disease or to neoplastic conditions such as colon cancer (2).

TLRs play an important role in mediating intestinal inflammation and homeostasis. Fas is best studied in terms of its function in apoptosis, but recent studies demonstrate that Fas signaling may mediate additional functions such as inflammation. The role of Fas, and the Fas ligand (FasL), in the intestine is poorly understood. The aim of this study was to evaluate potential cross-talk between TLRs and Fas/FasL system in intestinal epithelial cells (IECs). IECs were stimulated with TLR ligands, and expression of Fas and FasL was investigated. Treatment with TLR4 and TLR5 ligands, but not TLR2 and 9 ligands, increased expression of Fas and FasL in IECs in vitro. Consistent with this finding, expression of Intestinal Fas and FasL was reduced in vivo in the epithelium of TLR4 knockout (KO), SKO, and germ-free mice, but not in TLR2KO mice. Modulating Fas signaling using agonistic anti-Fas augmented TLR4- and TLR5-mediated TNF-α and IL-8 production by IECs. In addition, suppression of Fas in IECs reduced the ability of TLR4 and TLR5 ligands and the intestinal pathogens Salmonella typhimurium and Listeria monocytogenes to induce the expression of IL-8. In conclusion, this study demonstrates that extensive cross-talk in IECs occurs between the Fas and TLR signaling pathways, with the FasL/Fas system playing a role in TLR-mediated inflammatory responses in the intestine. The Journal of Immunology, 2014, 193: 000–000.

The online version of this article contains supplemental material.
mice, whereas blocking FasL/Fas interactions in macrophages suppresses LPS-induced (TLR4 agonist) and IL-1R1–induced inflammatory cytokine production (14). Furthermore, the Fas adaptor molecule, Fas-associated protein with death domain (FADD), has been shown to negatively regulate TLR signaling (15). Although no study has directly investigated the cross-talk between these pathways in IECs, a recent investigation into Fas signaling in alveolar epithelial cells indicated that Fas-induced inflammation occurred in a MyD88-dependent manner in these cells (16).

In the current study, we sought to determine whether Fas plays a role in the induction of TLR-induced inflammation in IECs. We found that cross-talk exists between these receptors in IECs, in terms of both the expression level of Fas and FasL, and the induction of an inflammatory response. Collectively, these results indicate a new role for Fas signaling in the intestine.

Materials and Methods

Reagents

Agonistic human anti-Fas Ab (CH-11) was obtained from Merck Millipore (Billerica, MA), and Jo-2 (murine) was obtained from BD Biosciences (San Jose, CA). LPS, flagellin, PGN-SA, Pam3CSK4, CpG DNA (ODN 2006), and polyinosinic-polycytidylic acid were purchased from Invivogen (San Diego, CA). Recombinant TNF-α was obtained from PeproTech (Rocky Hill, NJ). The following Abs were obtained from Santa Cruz Biotechnology (Dallas, TX): anti–Fas C-20, Fas X-20, TNFR-1, IGF-IRβ C-20, TLR4 H-80 and TLR5 H-127, and normal rabbit IgG, sc-2027. Anti-FasL Ab (ab15285) was obtained from Abcam (Cambridge, U.K.). Anti-β-actin Ab, TPCA-1, and LY294002 were obtained from Sigma-Aldrich (St Louis, MO). Anti-SIGIRR Ab was obtained from Pro-Sci (Poway, CA), anti-IRAK-m from AbboMax (San Jose, CA), anti-TLR2, and anti-TLR9 Ab from Novus (Littleton, CO).

Cell lines and tissue

HT29, HCT116, SW480, and CT26 colon epithelial cells and Jurkat T cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% FCS and penicillin–streptomycin. Cells were seeded at 1 × 10^5 cells per milliliter unless otherwise stated, cultured overnight, and then treated as specified in the figure legends.

Tissue from Swiss Webster wild-type and germ-free mice and from C57BL/6 TLR2, TLR4, and TLR5 knockout (KO) and wild-type mice was kindly provided by the Alimentary Pharmabiotic Centre, University College Cork. Germ-free mice were colonized for 49 d, and tissue was obtained. Tissue from C57BL/6 MyD88 and TRIF KO mice was obtained from Prof. P. Fallon, Trinity College Dublin (Dublin, Ireland).

Generation of Fas low/negative IECs

Cells were transfected with lentiviral particles containing target-specific short hairpin RNAs (shRNAs) against Fas (sc-29311-V) or control lentiviral particles containing scrambled shRNA (sc-108080) (Santa Cruz Biotechnology), according to the manufacturer’s instructions. Briefly, cells were seeded in 12-well plates at a concentration of 7.5 × 10^3 cells per milliliter. Cells were infected 24 h later with lentiviral particles in the presence of 4 μg/ml polybrene, and cultured in selection medium containing 6–8 μg puromycin until resistant clones could be identified. Resistant clones were selected by limiting dilution. Knockdown of Fas expression was determined by Western blotting and functional analysis.

FIGURE 1. Stimulation with LPS and flagellin, but not Pam3CSK4 or CpG DNA, upregulates Fas and FasL expression in SW480 cells. SW480 cells were seeded at a concentration of 1 × 10^5 cells per milliliter. Cells were treated with increasing concentrations of TLR agonists for 24 h, and Fas, FasL, and β-actin were detected by RT-PCR (A and D). Changes in protein expression were detected by Western blotting (B, E, G, and I), with results shown representative of three separate experiments. Changes in protein expression were quantified by densitometry (C, F, H, and J), with analysis performed on four independent experiments. Values are shown as mean ± SEM (n = 4). Statistical analysis was performed, and statistical change was determined relative to untreated control. *p < 0.05, **p < 0.01.
FIGURE 2. Fas and Fasl induction by flagellin occurs in an NF-κB- and PI3K-dependent manner. SW480 cells were seeded at a concentration of $1 \times 10^5$ cells per milliliter. Cells were pretreated with either 10 μM TPCA1 or 25 μM LY294002, followed by flagellin (100 ng/ml) for 24 h, and Fas, Fasl, and β-actin were detected by Western blotting (A and C), with results shown representative of three separate experiments.

Changes in protein expression were determined comparing flagellin stimulation alone with flagellin + TPCA1 or flagellin + LY294002. *$p < 0.05$, ***$p < 0.001$.

Bacterial cell culture and infection

The Listeria monocytogenes strain EGD (serotype 1/2a) was a gift from Prof. C. Hill (University College Cork, Cork, Ireland). The Salmonella typhimurium strain SW1103 (wild-type) was a gift from Prof. P. O’Toole (University College Cork). L. monocytogenes was grown to the logarithmic growth phase in brain–heart infusion broth (Sigma-Aldrich) at 37°C, shaking at 200 rpm, whereas S. typhimurium was grown in Luria–Bertani broth (Sigma-Aldrich). Bacteria were diluted in PBS for infection at multiplicity of infection of 10:1. IECs were seeded overnight at $5 \times 10^5$ cells per milliliter and cultured with L. monocytogenes or S. typhimurium for 3 h. Supernatant was removed and replaced with gentamicin-containing media (50 ng/ml), and 24 h later cell culture supernatant was harvested.

The Enterichia coli K-12 strain was obtained from the Alimentary Pharmabiotic Centre, University College Cork. IECs were seeded overnight at $5 \times 10^5$ cells per milliliter, stimulated for 1 h with CH-11 (100 ng/ml), and cultured with E. coli K-12 strain (10:1, bacteria/cells) for 8 h. Cells were lysed for subsequent RT-PCR analysis.

Proliferation assay

Cell proliferation was measured by resazurin reduction (17). Cells were seeded at $2 \times 10^5$ cells per milliliter in 96-well plates. After incubation for 24 h, medium supplemented with 44 μM resazurin was added, and resazurin reduction to resorufin was measured fluorometrically using a GENios plate reader (Tecan, Grodig, Austria) and Xfluor spreadsheet software. Results obtained were expressed in fluorescence units (FU) and percentage viability was calculated as follows: (FU treated/ FU control) $\times 100$. Values were normalized relative to the untreated controls.

Western blotting

Cells were lysed for 1 h on ice in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100, supplemented with complete protease inhibitors (Roche Diagnostics). The protein content of each sample was analyzed using the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were probed overnight at 4°C with primary Ab. As an internal control, all membranes were subsequently stripped of the first Ab and reprobed with anti-β-actin–specific Ab (Sigma-Aldrich). Protein bands were analyzed using ImageJ (National Institutes of Health, Bethesda, MD; http://image.nih.gov/ij/, 1997-2012). Changes in protein expression were determined after normalizing the band intensity of each lane to that of β-actin.

RT-PCR

Total cellular RNA was isolated using the GenElute Mammalian Total RNA Mini Kit (Sigma-Aldrich) according to the manufacturer’s instructions. cDNA was synthesized using the Bioline kit (London, U.K.). RT-PCR was performed using an Applied Biosystems PRISM 7500 PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA) and TaqMan Gene Expression Master Mix and the following gene expression Taqman primer-probe sets (Applied Biosystems): Fas, Hs00236330_m1; Fasl, Hs00181225_m1; IL-8, Hs99999034_ml; TNF-α, Hs00174128_m1; GAPDH, 4352934E. RT-PCR for Tollip, SIGIRR, IRAK-M, PPAR γ, 14-3-3 ε, and CXCL-1 was performed using the LightCycler480 System (Roche, West Sussex, U.K.). Individual PCR primer pairs and probes were designed using the Roche Universal ProbeLibrary Assay Design Centre (www.roche-applied-science.com/sis/tpcr/upl/ad.jsp). Primer sequences and probe combinations are provided in Table I. All samples were run in triplicate, and relative quantitation was calculated using the $2^{-ΔΔCt}$ method. Transcript levels were normalized to the amount of GAPDH/β-actin mRNA, and expression levels shown as fold induction relative to untreated.

Caspase 3/7 assay

Cells were seeded overnight in black flat-bottom 96-well plates at a density of 20,000 cells per well, treated with CH-11 for 1 h and subsequently with 100 ng/ml flagellin or 100 ng/ml LPS for 24 h, or were treated with each agonist separately. ApoONE caspase-3/7 reagent was added, and following 1 h incubation, fluorescence (485 excitation, 530 emission) was measured using a GENios Microplate Reader (Tecan Group, Männedorf, Switzerland). Changes in caspase 3/7 activation were normalized relative to untreated cells.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were deparaffinized in xylene and rehydrated prior to analysis. Ag retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 10 min. Slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum. Nonspecific binding was blocked using 5% normal goat serum in wash buffer for 1 h. Sections were incubated overnight at 4°C with primary Ab or normal rabbit IgG (sc-2027). Ab binding was localized using a biotinylated secondary Ab contained within the VECTASTAIN Elite ABC detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. A parallel negative control was also performed, using rabbit IgG instead of the primary Ab.

IL-8 ELISA

Supernatants were harvested after 24 h, and IL-8 levels were determined in triplicate by ELISA (eBioscience, San Diego, CA) according to the manufacturer’s protocol.
Statistics

Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using one-way Anova with a Tukey posttest, or by the Student paired t test. The $p$ values < 0.001 are indicated by three asterisks (***) . The $p$ values < 0.01 are indicated by two asterisks (**). The $p$ values < 0.05 are indicated by one asterisk (*).

Results

Ligands for TLR4 and TLR5, but not TLR2 or TLR9, increase the expression of Fas and FasL in IECs in an NF-κB- and PI3K-dependent manner

Because TLRs are well characterized as key innate immune sensors in the intestine, to investigate cross-talk between Fas and TLR signaling, we first sought to determine whether TLR stimulation upregulated Fas or FasL expression in IECs. SW480 human IECs were selected, as they have been previously shown to express TLRs 1–9 (18). We confirmed protein expression of TLR2, TLR4, TLR5, and TLR9 by Western blotting (Supplemental Fig. 1). Cells were stimulated with the TLR4 ligand, LPS, which upregulated Fas and FasL expression in a dose-dependent manner, at both the mRNA (Fig. 1A) and the protein levels (Fig. 1B, 1C). The TLR5 ligand, flagellin, also upregulated Fas and FasL in a dose-dependent manner in SW480 cells (Fig. 1D–F). In contrast, despite expressing TLR2 and TLR9, neither stimulation with Pam3CSK4, the TLR1/2 ligand (Fig. 1G, 1H), nor transfection with CpG DNA, the TLR9 ligand (Fig. 1I, 1J), altered Fas or FasL expression. We confirmed that SW480s could indeed respond to stimulation with Pam3CSK4 and CpG DNA. Although IL-8 expression did not change in response to stimulation with either ligand, we observed an increase in the gap-junction protein Connexin-43 upon TLR2 ligation and an increase in the Wnt-signaling protein Frizzled5 in response to TLR9 stimulation (Supplemental Fig. 2A). Both these genes have been previously shown to be activated by Pam3CSK4 and CpG DNA. Although IL-8 expression did not change in response to stimulation with either ligand, we observed an increase in the gap-junction protein Connexin-43 upon TLR2 ligation and an increase in the Wnt-signaling protein Frizzled5 in response to TLR9 stimulation (Supplemental Fig. 2A).

CROSS-TALK BETWEEN Fas/FasL AND TLRs IN IECs

FIGURE 3. Fas and FasL expression is reduced in the distal colons of germ-free, TLR4KO, TLR5KO, TRIFKO, and MyD88KO mice. Immunoperoxidase staining for Fas and FasL was performed on paraffin-embedded colonic tissue sections obtained from wild-type, germ-free, and germ-free mice colonized for 49 d (A); wildtype, TLR2KO, TLR4KO, and TLR5KO mice (C); and MyD88KO and TRIFKO mice (E). Scale bars, 100 μM. Data shown are representative of colonic tissue obtained from five mice per group. Protein was extracted from colonic tissue, and changes in Fas, FasL, and β-actin were detected by Western blotting. (B, D, and F). Data shown are representative of tissue from five mice. Changes in protein expression were quantified by densitometry. Values are shown as mean ± SEM (n = 5). ***$p$ < 0.001.
duction of IL-8 was observed following stimulation of SW480 cells with PGN, indicating that these cells are capable of responding to PGN (Supplemental Fig. 2C).

TLR4 and TLR5 activate downstream signaling pathways, such as the NF-κB signaling pathway, the MAPK, and the PI3K/Akt pathway in response to stimulation, and both the FasL and Fas promoter regions contain NF-κB binding sites (20–22). To elucidate the signaling pathway downstream of TLR4-induced expression of Fas and FasL, SW480 cells were pretreated with inhibitors of either the NF-κB or the PI3K pathway prior to stimulation with flagellin. We found that TPCA-1, an IKKβ inhibitor, reduced flagellin-induced upregulation of both Fas and FasL expression (p < 0.05) (Fig. 2A, 2B), albeit to a low level. Pretreatment of cells with LY294002, a PI3K inhibitor, also significantly prevented flagellin-induced Fas and FasL expression (p < 0.001) (Fig. 2C, 2D).

Fas and FasL expression is reduced in the colons of germ-free, TLR4, and TLR5 KO mice

To determine the in vivo relevance of the induction of Fas and FasL expression by TLR4 and TLR5 ligands, colonic tissue from conventionally reared mice was assessed for Fas and FasL expression. Fas and FasL were found to be expressed in a uniform fashion extending from the base to the surface of the colonic crypts (Fig. 3A). In contrast, expression was reduced in the colonic epithelium of germ-free mice, as assessed by both immunohistochemistry (Fig. 3A) and Western blotting (Fig. 3B), consistent with the lack of exposure of the IECs to commensal flora and thus TLR ligation. Colonization of germ-free mice, however, restored Fas and FasL expression to levels seen in conventionally reared animals, suggesting that Fas and FasL expression is, at least, partially dependent on colonization of the colon by commensal bacteria (Fig. 3A).

As we had observed that stimulation through TLR4 and TLR5 induced the expression of both Fas and FasL in SW480 cells in vitro, we next examined colonic tissue taken from TLR4KO and TLR5KO mice. In contrast to the immunohistochemical staining pattern observed in wild-type mice, colonic tissue from both TLR4KO and TLR5KO mice demonstrated a marked reduction in expression of both Fas and FasL (Fig. 3C). This reduction in expression of Fas and FasL in TLR4KO colonic tissue was confirmed by Western blotting (Fig. 3D). Consistent with our in vitro findings, Fas and FasL expression was unchanged in the epithelial colonic cells of TLR2KO mice, although it appeared reduced in the Western blot (Fig. 3C, 3D). This difference in expression may be due to the mixed population of cells represented in the Western blot compared with the specific IEC immunohistochemical staining. Expression of TNF receptor 1 (another member of the death receptor family) or the insulin growth receptor 1β, however, was unaltered in TLR4KO and TLR5KO mice (Supplemental Fig. 3A).

We subsequently examined whether the observed change in Fas and FasL expression in the colonic tissue of TLR4 and TLR5 KO mice was present in other tissues. The expression pattern in the lung was similar to that observed in the colon; that is, expression of Fas and FasL was downregulated in the lung epithelium (see arrows) of both TLR4KO and TLR5KO mice (Supplemental Fig. 3B). In contrast, expression of Fas and FasL was unchanged in the cerebellum or kidneys (Supplemental Fig. 3C, 3D, respectively). This observation suggests that the regulation of Fas and FasL by TLR4 and TLR5 is tissue specific and may be dependent on the presence of the commensal flora.

These findings indicate that IECs show specificity in their ability to upregulate Fas and FasL expression in response to TLR ligation. One possible explanation for this specificity may be differences in the signaling pathways activated by the TLRs. Although all four TLRs examined in this study (TLR2, TLR4, TLR5, and TLR9) use the TLR adaptor protein MyD88, only TLRs 4 (23) and 5 (24) use the adaptor molecule TRIF. This utilization of TRIF by TLR5 was shown specifically in IECs (24). Therefore we next examined Fas and FasL expression in TRIF and MyD88 KO tissue. Expression of both Fas and FasL protein was significantly reduced in TRIF KO tissue, as assessed by both immunohistochemistry (Fig. 3E) and Western blotting (Fig. 3F). Expression was also reduced, albeit to a much lower level, in MyD88 KO tissue (Fig. 3E, 3F). These findings indicate that signaling through TRIF by TLR4 and TLR5, but not TLR2 and TLR9, agonists may be responsible for the induction of Fas and FasL in IECs.

Stimulation of IECs with agonistic anti-Fas Ab and TLR4 or TLR5 ligands results in augmented cytokine production

As TLR and Fas signaling can induce the production of inflammatory cytokines, we subsequently examined whether stimulation of both receptors in IECs resulted in an altered cytokine profile. To investigate this, SW480 cells were treated with the agonistic anti-Fas Ab, CH-11, followed by stimulation with either LPS or flagellin. Cytokine gene expression was initially analyzed by quantitative RT-PCR (Table I). Stimulation with CH-11, LPS, or flagellin alone induced the expression of TNF-α and IL-8, whereas stimulation with a combination of CH-11 and either TLR agonist resulted in augmented transcription of these cytokines (Fig. 4A, 4B). Similar findings were observed upon stimulation of SW480 cells with CH-11 and the Gram-negative bacterium E.coli, strain K-12, which would be expected to stimulate cells through TLR4 (Fig. 4C, 4D).

The augmented production of IL-8 in SW480 cells was confirmed at the protein level by ELISA, with stimulation of the cells with both CH-11 and LPS or flagellin resulting in a statistically significant increase in IL-8, relative to either treatment alone (Fig. 4E). We also confirmed that the augmented cytokine production observed upon stimulation of SW480 cells was not specific to SW480 cells. HT29 and HCT116 IECs, which express TLR4 and TLR5 (Supplemental Fig. 1), were treated in the same fashion as the SW480 cells. IL-8 protein secretion was significantly augmented following stimulation of HT29 and HCT116 cells with flagellin and CH-11.

Table 1. qRT-PCR primers and corresponding UPL probe numbers

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<th>Gene</th>
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<th>DNA Sequence (Antisense 5′−3′)</th>
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UPL, Universal ProbeLibrary.
The augmented cytokine production observed following stimulation of IECs with CH-11 and TLR4 or TLR5 ligands is independent of cell death

The Fas/FasL system is best characterized in terms of its role in apoptosis, with some reports showing that cytokine expression occurs concomitantly with apoptotic cell death (25). Moreover, TLRs have been shown to induce apoptosis in certain cell types; for instance, TLR5 stimulation can induce apoptosis in IECs (20).

To determine whether the augmented cytokine production observed occurred concomitantly with, or was independent of, IEC apoptosis, cells were stimulated with CH-11 and/or LPS or flagellin, and cell viability and caspase 3/7 activation were assessed. Treatment of cells with LPS, flagellin, or agonistic anti-Fas CH-11 alone or in combination did not alter cell viability in either SW480 or HT29 cells. In contrast, treatment of cells with staurosporine resulted in substantially reduced cell viability (Fig. 5A). Moreover, no increase in caspase 3/7 activity was observed in either cell line with either treatment (Fig. 5B), whereas a 4-fold induction in caspase 3/7 activity was noted in Jurkat T cells, cells known to be sensitive to Fas-mediated apoptosis, following CH-11 stimulation relative to untreated control (Fig. 5C).

Stimulation of cells with agonistic anti-Fas reduces the expression of TLR inhibitory proteins

Previous studies in macrophages have shown that in the absence of Fas signaling, the Fas adaptor protein, FADD, is present in the cytoplasm bound to the TLR adaptor protein, MyD88, suppressing TLR-induced cytokine production (14, 15). Engagement of Fas prevented this interaction, releasing MyD88 and promoting TLR-mediated inflammation. To address the potential mechanism whereby stimulation of Fas augments TLR4- and TLR5-induced TNF-α and IL-8 protein in IECs, we first investigated whether FADD interacts with MyD88. Tagged constructs of FADD and MyD88 were overexpressed in HCT116 cells, and

(Fig. 4F, 4G, respectively), and with LPS and CH-11 in HT29s (Fig. 4F), compared with either stimulation alone.
coimmunoprecipitation studies were performed. However, no interaction between FADD and MyD88 was observed in this cell type (data not shown).

TLR signaling is regulated by an extensive array of TLR inhibitory proteins such as SIGIRR and TOLLIP (26, 27). Thus, we investigated whether signaling through Fas altered the expression of TLR inhibitory proteins shown to be important in the intestine. Cells were stimulated with CH-11, LPS, and/or flagellin, and changes in a panel of TLR inhibitory proteins were assessed by RT-PCR (Table I). Stimulation of SW480 cells with CH-11 reduced the transcription of SIGIRR, TOLLIP, and 14-3-3ε, but not IRAK-M or PPARγ (Fig. 6A). Furthermore, costimulation with CH-11 and either LPS or flagellin resulted in a greater reduction in expression of 14-3-3ε. The change in protein levels of SIGIRR, but not IRAK-m, was confirmed by Western blotting (Fig. 6B). Together these findings suggest that the augmentation in TLR4- and TLR5-induced cytokine production by Fas may be due to its ability to downregulate the expression of several key TLR inhibitory proteins.

Suppression of Fas expression limits the ability of HT29 cells to respond to TLR4 and TLR5 ligands

Given the cross-talk seen between the Fas and TLR4 and TLR5 signaling pathways, we next sought to determine whether suppression of Fas expression altered the response of the IECs to TLR ligands. Fas was suppressed in HT29 cells, using shRNA specific against Fas (HT29Fas shRNA). Suppression was confirmed by both Western blotting (Fig. 7A) and functional analysis; CH-11 did not induce IL-8 in HT29Fas shRNA cells, in contrast to the induction seen in cells transfected with scrambled shRNA (HT29scr shRNA) (Fig. 7C). The specificity of suppression was confirmed by immunoblotting for TNFR-1 (Fig. 7B).

Both LPS- and flagellin-induced IL-8 production was reduced to the level of unstimulated control cells following suppression of Fas expression (Fig. 7C). This reduction was not due to any alteration in TLR4 or TLR5 expression in the knockdown cells (Supplemental Fig. 1). To determine whether IL-8 secretion in response to other TLR ligands, or known inducers of IL-8 such as TNF-α, was also affected by Fas suppression, cells were stimulated with Pam3CSK4, CpG DNA, and TNF-α. HT29 cells did not secrete IL-8 in response to Pam3CSK4, irrespective of Fas expression. However, HT29 cells did secrete IL-8 in response to stimulation with CpG DNA and TNF-α, and this was unaffected by Fas suppression (Fig. 7C).

These results were confirmed in CT26 murine IECs. Fas was stably suppressed in these cells by shRNA (CT26Fas shRNA) (Fig. 7D). As murine cells do not produce IL-8, the induction of CXCL-1, a murine IL-8 homolog, was determined. Consistent with suppression of Fas expression, the CT26Fas shRNA cells failed to upregulate CXCL-1 in response to Jo-2 (a murine agonistic anti-Fas Ab) (Fig. 7E). The ability of LPS to induce CXCL-1 in CT26Fas shRNA was also reduced to basal levels consistent with our findings in HT29 cells. As CT26 cells do not express TLR5 (28), we were unable to assess their response to flagellin (Fig. 7E). These findings suggest that signaling through Fas augments TLR4- and TLR5-induced cytokine and chemokine production in IECs.

Fas is required for the response of IECs to intestinal pathogens

L. monocytogenes and S. typhimurium

Given that the IEC response to TLR4 and TLR5 ligands was attenuated upon suppression of Fas expression, the ability of HT29Fas shRNA cells to respond to the LPS-expressing Gram-negative bacterium S. typhimurium or the flagellin-expressing bacterium L. monocytogenes was assessed. In contrast to HT29scr shRNA cells, the ability of HT29Fas shRNA cells to produce IL-8 in response to stimulation with either bacterium was suppressed (Fig. 8). This finding was shown in two separately derived knockdown clones. Taken together, these findings suggest that Fas signaling is likely to play a role in intestinal host defense against pathogens.

Discussion

The aim of this study was to investigate the cross-talk between TLRs and Fas in the intestinal epithelium. Our principal findings are that stimulation through TLRs 4 and 5, either by their cognate ligands or by commensal flora, increases expression of Fas and Fasl in vitro and in vivo, and that stimulation of Fas in intestinal
cells augments TLR4- and TLR5-induced cytokine and chemokine production. These observations are significant, as they indicate a hitherto unappreciated role for Fas in the intestinal immune response.

Although upregulation of Fas by TLR2, TLR3, TLR4, TLR7, and TLR9 ligands (29–31) has been previously reported in macrophages, to the best of our knowledge no report has demonstrated an upregulation of Fas and FasL by pathogen-activated molecular patterns in IECs. In IECs, LPS and flagellin upregulated Fas and FasL expression, whereas Pam3CSK4 and CpG DNA had no effect. Moreover, we found that expression of both Fas and FasL was reduced in the epithelial cells of the colon of TLR4 and TLR5, but not TLR2, KO mice. In addition to the obvious differences in cell lineage, another possibility for the differences observed between IECs and macrophages is that upregulation of Fas and FasL expression may occur in a TRIF-dependent manner in IECs. It is well established that TLR4 uses the adaptor molecule TRIF (23), and more recently TLR5 has been shown to use TRIF in IECs (24), whereas neither TLR2 nor TLR9 has been reported to use TRIF. Of interest, a recent study examining the respective importance of TRIF-dependent versus MyD88-dependent gene expression in IECs revealed that the number of TRIF-dependent genes by far exceeded the number of genes regulated by the adaptor protein MyD88 (32). Consistent with this finding, expression of Fas and FasL was greatly reduced in TRIF KO mice, with only a modest reduction observed in MyD88 KO mice. This observation suggests that the specificity of TLR-induced upregulation of Fas and FasL in IECs may be due to the ability of TLRs 4 and 5 to use TRIF.

In this study, we found that germ-free mice have reduced Fas and FasL expression, and colonizing germ-free mice effectively “rescues” the phenotype of reduced Fas and FasL expression. This finding suggests that signaling from the commensal microbiota in IECs, via TLRs, regulates Fas and FasL expression. Recognition of commensal bacteria through TLRs also plays a role in intestinal homeostasis, as mice deficient in TLR4 or MyD88 exhibited increased morbidity and mortality with DSS-induced colitis (33). Our data indicate that TLR4 KO mice, as used in the study of Rakoff-Nahoum et al. (34), would also have reduced Fas and FasL expression. Of note, a recent study has shown that Fas can play a cytoprotective role in the intestinal epithelium (13). Thus, failure of the commensal flora to upregulate Fas and FasL in TLR4 KO mice would further decrease the protective barrier of IECs against pathogen invasion.

**FIGURE 6.** Fas activation reduces the expression of key TLR inhibitory proteins in IECs. SW480 cells were treated with 200 ng/ml agonistic anti-Fas Ab (CH-11) for 1 h followed by stimulation with 100 ng/ml LPS or 100 ng/ml flagellin for 4 h. Changes in inhibitory proteins were detected by qRT-PCR (A). Cells were treated with CH-11, as above, for 24 h, and changes in protein expression were detected by Western blotting and quantified by densitometry (B). Results shown are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA (A) and the paired Student t test (B), and statistical change was determined compared with untreated control. Values are shown as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
mice may exacerbate the intestinal injury seen in these mice upon DSS administration. Our data would also indicate the possibility that other, as yet unknown, proteins may also be absent in TLR4 and TLR5 KO mice, resulting in roles being ascribed to TLRs that are actually due to the absence of other proteins.

Despite being best characterized as an apoptotic inducer, activation of Fas did not alter the sensitivity of the IECs to Fas-mediated apoptosis. Fas activates the extrinsic apoptotic pathway, wherein activation of Fas induces the formation of a death-inducing signaling complex, resulting in the cleavage and activation of procaspase-8.

**FIGURE 7.** Suppression of Fas expression in HT29 cells prevents TLR4/5-induced IL-8 production. HT29 cells were lentivirally transfected with either scrambled control shRNA or shRNA against Fas. Expression levels of Fas and TNFR-1 in HT29 scrambled control transfected (HT29scr shRNA) and HT29 Fas shRNA clones were determined by Western blotting (A and B). HT29scr shRNA and HT29 Fas shRNA clones were treated with either 200 ng/ml agonistic anti-Fas Ab (CH-11), 100 ng/ml flagellin, 100 ng/ml LPS, 5 μM CPG, 10 μM Pam3CSK4, or 100 ng/ml TNF-α for 24 h. Cell supernatants were collected, and IL-8 protein concentration was determined by ELISA (C). CT26 cells were lentivirally transfected as above, and expression levels of Fas were determined by Western blot (D). CT26 cells were treated with either 100 ng/ml agonistic anti-Fas Ab (JO-2), 100 ng/ml LPS, 100 ng/ml flagellin, 1 mg/ml polyinosinic-polycytidylic acid, or 100 ng/ml TNF-α for 4 h. Changes in mCXCL-1 were detected by qRT-PCR (E). Statistical analysis was performed, and statistical change was determined between the scrambled and knockdown clones. Values are shown as mean ± SEM (n = 3). **p < 0.01, ***p < 0.001.
Caspase-8, in turn, activates a caspase cascade, culminating in the apoptotic death of the cell. Numerous proteins, including c-FLIP, have been shown to suppress Fas-mediated apoptosis. IECs express high levels of c-FLIP, which suppresses Fas-mediated apoptosis through its ability to prevent processing of procaspase-8 to its mature active form (35, 36). Thus, despite coexpressing Fas and FasL, IECs are protected against Fas-mediated apoptosis under homeostatic conditions. Given that Fas has been shown to mediate several nonapoptotic functions, such as inflammation (11), our data suggest that in IECs, these nonapoptotic functions of Fas may predominate.

The findings of this study also demonstrate extensive cross-talk between the Fas and TLR signaling pathways in IECs in terms of cytokine induction and response to bacterial infection. One of the first indications of a cross-talk between the Fas and TLR signaling pathways came from studies in macrophages. LPS-activated macrophages produce a large amount of IL-1β upon Fas stimulation (14, 31), and TLR4 signaling is reduced in lpr/lpr and gld/gld peritoneal macrophages, which have mutations in Fas and FasL, respectively (37). The proposed mechanism for this cross-talk was that MyD88 was inhibited by FADD via a direct interaction between the adaptor proteins (14), and that activation of the Fas signaling pathway prevented this interaction. We were, however, unable to detect this interaction in IECs, suggesting that the cross-talk seen in IECs occurs via a different mechanism. We subsequently determined that in IECs, Fas signaling alters the expression levels of TLR inhibitory proteins. TLR signaling is tightly regulated by a range of TLR inhibitory proteins, several of which have been shown to be important in intestinal homeostasis (38, 39). Stimulation of Fas in IECs reduced the expression of three of the five inhibitory proteins examined by 20–40%. This level of reduction is in line with that seen in other studies, and has been shown to be sufficient to alleviate the suppression of TLR signaling (40). Of those reduced, both SIGIRR and Tollip have been previously shown to be essential negative TLR regulators in IECs (38, 41), with a reduction in expression of SIGIRR, in particular, being associated with exacerbated colitis in murine models (40, 42). We also observed a reduction in 14-3-3 ε, which has recently emerged as a TLR inhibitory protein (43). It is worth noting that we did not observe a reduction in CpG DNA–induced IL-8 in our HT29(Fas shRNA)-knockdown clones. This finding may be attributed to the fact that the expression of several inhibitory proteins examined remained unchanged upon Fas activation and that these may be more important in suppressing TLR9-mediated inflammation in IECs. Thus the augmentation of TLR4- and TLR5-induced cytokine production seen upon Fas stimulation may be, at least in part, due to the reduced expression of 14-3-3 ε, Tollip, and SIGIRR.

In summary, we have shown that extensive cross-talk exists between Fas and TLRs 4 and 5 in IECs in vitro and in vivo. Our data further advance the evidence in favor of nonapoptotic functions of Fas and reveal a hitherto unknown link between Fas and FasL expression and the presence of commensal flora in IECs. The alterations in TLR-mediated cytokine production shown in this study, upon manipulation of both the expression and the activation of Fas, suggest that targeting Fas has potential therapeutic applications and warrants further investigation.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure S1. Intestinal epithelial Cells express TLRs 2, 4, 5 and 9. Cells were seeded at a concentration of 1 x 10^6 cells per ml. Protein expression was detected by Western blotting.
Supplementary Figure S2. SW480 cells respond to TLR2 and TLR9 agonists.
SW480 cells were seeded at a concentration of $1 \times 10^5$ cells per ml. Cells were treated with CpGDNA or Pam3Csk4 as specified and fold change of IL-8, Frizzled5 and Connexin43 determined by RT-PCR (a). Cells were treated with PGN for 24 hours and changes in Fas detected by Western blotting (b) and changes in IL-8 detected by ELISA (c).
Supplementary Figure S3: Fas and FasL expression is reduced in the lung but not the cerebellum or kidney of TLR4 and TLR5 knockout mice. Immunoperoxidase staining for TNFR-1, IGF-IRβ, Fas, FasL was performed on paraffin embedded a) colonic b) lung c) cerebellum and d) kidney tissue sections obtained from wildtype, TLR4KO and TLR5KO C57BL/6 mice. Scale bar = 100μM where not otherwise specified.