Th2 Cytokines Down-Regulate TLR Expression and Function in Human Intestinal Epithelial Cells

Tobias Mueller, Tomohiro Terada, Ian M. Rosenberg, Oren Shibolet and Daniel K. Podolsky

*J Immunol* 2006; 176:5805-5814; doi: 10.4049/jimmunol.176.10.5805

http://www.jimmunol.org/content/176/10/5805

---

**References**

This article cites 45 articles, 19 of which you can access for free at: [http://www.jimmunol.org/content/176/10/5805.full#ref-list-1](http://www.jimmunol.org/content/176/10/5805.full#ref-list-1)

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Th2 Cytokines Down-Regulate TLR Expression and Function in Human Intestinal Epithelial Cells

Tobias Mueller, Tomohiro Terada, Ian M. Rosenberg, Oren Shibolet, and Daniel K. Podolsky

TLRs serve important immune and nonimmune functions in human intestinal epithelial cells (IECs). Proinflammatory Th1 cytokines have been shown to promote TLR expression and function in IECs, but the effect of key Th2 cytokines (IL-4, IL-5, IL-13) on TLR signaling in IECs has not been elucidated so far. We stimulated human model IECs with Th2 cytokines and examined TLR mRNA and protein expression by Northern blotting, RT-PCR, real-time RT-PCR, Western blotting, and flow cytometry. TLR function was determined by I-κBα phosphorylation assays, ELISA for IL-8 secretion after stimulation with TLR ligands and flow cytometry for LPS uptake. IL-4 and IL-13 significantly decreased TLR3 and TLR4 mRNA and protein expression including the requisite TLR4 coreceptor MD-2. TLR4/MD-2-mediated LPS uptake and TLR ligand-induced I-κBα phosphorylation and IL-8 secretion were significantly diminished in Th2 cytokine-primed IECs. The down-regulatory effect of Th2 cytokines on TLR expression and function in IECs also counteracted enhanced TLR signaling induced by stimulation with the hallmark Th1 cytokine IFN-γ. In summary, Th2 cytokines appear to dampen TLR expression and function in resting and Th1 cytokine-primed human IECs. Diminished TLR function in IECs under the influence of Th2 cytokines may protect the host from excessive TLR signaling, but likely also impairs the host intestinal innate immune defense and increases IEC susceptibility to chronic inflammation in response to the intestinal microenvironment. Taken together, our data underscore the important role of Th2 cytokines in balancing TLR signaling in human IECs. The Journal of Immunology, 2006, 176: 5805–5814.

This appears to be particularly important for TLR signaling derived from IECs, which are constantly exposed to a vast array of TLR stimuli from the intestinal microenvironment. TLR ligands in the intestinal microflora comprise conserved structural components from both potential pathogens and from harmless commensal microbiota, and the low constitutive expression of key TLRs in IECs likely limits excessive TLR signaling (2). Moreover, IECs become hyporesponsive upon prolonged stimulation by ubiquitous TLR2 and TLR4 ligands, and inhibitory molecules such as Toll-interacting protein have been shown to further restrict TLR signaling in IECs (5).

Dysregulated TLR signaling in IECs may also be an important pathogenic factor in the development of chronic intestinal inflammation (12). Breaks within the intestinal epithelial barrier likely facilitate leakage of intraluminal Ags into the lamina propria. Subsequent nonphysiological encounters of TLR-expressing lamina propria immune cells with TLR ligands derived from the apparently normal intestinal microflora may fuel inappropriate immune responses and initiate or maintain chronic intestinal inflammation (13). Following activation by TLR ligands, immature CD4⁺ Th lymphocytes may differentiate into two functionally distinct Th cell subsets and secrete characteristic cytokines (14). Th1 cells predominantly secrete IFN-γ and TNF-α, while Th2 cells may be characterized by IL-4, IL-5, and IL-13 secretion. Imbalance of the Th cytokine profile toward a Th1 or Th2 cytokine predominance has been shown to contribute to chronic inflammatory bowel disease in humans and animal models (15) and likely also influences TLR expression in the intestinal mucosa. Recent studies associated the mucosal Th1 cytokine profile in patients with Crohn’s disease with enhanced intestinal TLR expression and signaling (16, 17). Recent in vitro findings demonstrating that the key Th1 cytokine IFN-γ promoted TLR4/MD-2-mediated signaling in model IECs (4, 18, 19), leading to increased IL-8 secretion upon stimulation with the specific TLR4 ligand LPS, underscored the important role of proinflammatory Th1 cytokines in the development and maintenance of chronic intestinal inflammation.
Primary human IECs and model IEC lines also express abundant mRNA for the IL-4R (20), which initiates signaling pathways of two key Th2 cytokines, IL-4 and IL-13. The presence of Th2 cytokine receptors on IECs suggests a regulatory role of Th2 cytokines in the innate immune responsiveness of IECs, but little is known about the influence of Th2 cytokines on TLR expression and function in human IECs. We speculated that Th2 cytokines may modulate TLR signaling in IECs in a manner distinct from the proinflammatory effects of Th1 cytokines. Therefore, we assessed the effect of the key Th2 cytokines IL-4, IL-5, and IL-13 on TLR expression and function in model IEC lines.

Materials and Methods

Abs and reagents

LPS (Escherichia coli serotype O55:B5) was purchased from Sigma-Aldrich, and Alexa Fluor 488-labeled LPS (E. coli serotype O55:B5) from Molecular Probes. The TLR ligands Pam3CSK4, poly(I:C), and flagellin (Salmonella typhimurium) were obtained from InvivoGen. The human cytokines IL-4, IL-5, IL-13, and INF-γ were obtained from R&D Systems. Polyclonal Abs against IL-1β and phosphorylated IL-1β were obtained from Cell Signaling. Monoclonal mouse anti-human TLR4 Ab (HTA-125), PE-labeled anti-human CD8α against TLR3 (TLR3.7), TLR4 (HTA-125), CD14, and PE-labeled isotype controls (mouse IgG1,κ and mouse IgG2a,κ) were obtained from Biorad.

Cell culture and cytokine stimulation assay

The human model IEC lines HT29, Colo205, SW480, T84, and the human monocytic cell line U937 were obtained from the American Type Culture Collection. U937 and Colo205 cells were grown in RPMI 1640 (Cellgro Mediatech). HT29 cells were grown in DMEM (Cellgro Mediatech). T84 and SW480 cells were grown in a 1:1 mixture of DMEM and Ham’s F-12 medium (Cellgro Mediatech). Media were supplemented with 50 U of penicillin/ml and 50 μg of streptomycin/ml (Invitrogen Life Technologies) and 10% heat-inactivated FCS (Atlanta Biologicals). T84 cells were grown to confluence on Transwells (0.4 μm pore diameter) and SW480 cells were grown to confluence on Transwells (0.4 μm pore diameter). For some experiments, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer’s protocol (BD Biosciences). For other experiments, cells were fixed and stained by propidium iodide (1 mg/ml) to eliminate dead cells.

RNA extraction and Northern blotting

Total RNA was isolated from IECs using TRizol reagent (Invitrogen Life Technologies) following the manufacturer’s instruction. For Northern blot analysis, 20 μg of total RNA was subjected to electrophoresis using 1% agarose-formaldehyde gels, followed by transfer by Nytran SuPerCharge nylon membranes (Amersham Biosciences), followed by spin column removal of unincorporated nucleotides. Membranes were pre-treated, hybridized in QuikHyb hybridization solution (Stratagene), washed with 2 × SSC (0.3 M NaCl plus 0.03 M sodium citrate) containing 0.1% SDS at room temperature for 5 min and then washed thrice with 0.5 × SSC containing 0.1% SDS at 42°C for 10 min. Membranes were exposed to film for 8–48 h at −80°C with intensifying screens.

RT-PCR and real-time RT-PCR

For reverse transcription, 2 μg of total RNA were transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies) following the manufacturer’s instruction. For Northern blot analysis, 20 μg of total RNA was subjected to electrophoresis using 1% agarose-formaldehyde gels, followed by transfer by Nytran SuPerCharge nylon membranes (Schliecher & Schuell). Human cDNA probes were generated from PCR products. Probes were labeled with [α-32P]dCTP using Ready-To-Go DNA labeling beads (Amersham Biosciences), followed by spin column removal of unincorporated nucleotides. Membranes were pre-treated, hybridized in QuikHyb hybridization solution (Stratagene), washed with 2 × SSC (0.3 M NaCl plus 0.03 M sodium citrate) containing 0.1% SDS at room temperature for 5 min and then washed thrice with 0.5 × SSC containing 0.1% SDS at 42°C for 10 min. Membranes were exposed to film for 8–48 h at −80°C with intensifying screens.

RT-PCR and real-time RT-PCR

For reverse transcription, 2 μg of total RNA were transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies). Aliquots of 2 μl of cDNA were subjected to 45 cycles of PCR amplification with Taq polymerase (Invitrogen Life Technologies). As an internal control, PCR for GAPDH was performed at 95°C for 120 s, followed by 35 cycles at 95°C for 60 s, 58°C for 45 s, and 72°C for 60 s. Primers for GAPDH (440-bp fragment) were forward 5′-tctgtgctggcctctctc-3′, reverse 5′-gcagcctctgccacact-3′. Negative controls included the amplification of samples without prior RT reaction. The primer sequences used for the different TLR family members and TLR4 coroceptor MD-2 have been published elsewhere (5).

Real-time RT-PCR was performed in the DNA Engine Opticon 2 System (MJ Research) using the DynaNa SYBR Green qPCR kit (Finnzymes; New England Biolabs). Briefly, 100 ng of reverse transcribed cDNA was used for each PCR with 250 nM forward and reverse primers. The thermal cycling conditions comprised 4 min at 95°C, followed by 45 cycles at 94°C for 30 s, 58°C for 5 s (GAPDH), and 72°C for 45 s. Annealing temperatures were optimized for each TLR primer pair used and ranged between 59°C and 62°C. The threshold cycle (Ct) values were obtained for the reactions reflecting the quantity of the template in the sample. TLR or MD-2 ΔCt (ΔCt) was calculated by subtracting the calibrator gene GAPDH Ct value from the TLR or MD-2 Ct value and thus represented the relative quantity of the target mRNA normalized to the level of the internal standard GAPDH mRNA level. The TLR or MD-2 mRNA level in untreated IECs was defined as 1 arbitrary unit.

TLR protein immunoprecipitation (IP) and immunoblotting

IECs were grown in 10-cm plates and stimulated with Th2 and/or Th1 cytokines for 18 h. Medium was removed, and 500 μl of lysis buffer (1% Triton X-100, 0.1 M NaCl, 10 mM HEPES (pH 5.6), 2 mM EDTA, 4 mM Na3VO4, and 40 mM NaF) supplemented with protease inhibitor mixture (Complete Mini; Roche Diagnostics) was added. Supernatants of cell lysates were obtained by centrifugation at 12,000 × g for 10 min. Protein concentration was determined using the DC protein assay kit (Bio-Rad). For TLR IP, 4 μg of total protein were pre-cleared for 2 h with 50 μl of Htrip protein A/G-Sepharose beads (Calbiochem) at 4°C. Supernatants were incubated overnight with 2 μg of primary Ab at 4°C and immunoprecipitated protein were separated on 4–15% Tris-glycine gels (Invitrogen Life Technologies). TLR protein was blotted onto polyvinylidene difluoride membranes followed by blocking with 5% dry milk in TBS containing 0.1% Tween 20 (TBST). Membranes were incubated overnight with primary Abs (diluted 1/250 in blocking buffer, 4°C), washed for 60 min and stained with secondary Ab (1/5000 in blocking buffer) for 60 min at room temperature. After extensive washing of the membranes, hybridized bands were detected using an ECL kit (PerkinElmer Life Sciences).

Flow cytometry and immunocytochemistry

IECs grown in 6-well plates were plated using enzyme-free cell dissociation buffer (Invitrogen Life Technologies), washed twice with FACS buffer (PBS containing 1% FCS and 0.1% NaN3), and blocked for 20 min with FACS buffer containing 10% horse serum. For direct immunofluorescence staining, single-cell suspensions of IECs and monocytess (5 × 10^6 cells/100 μl) were incubated for 4°C in FACS buffer containing PE-labeled anti-human Abs (diluted 1/100) for 30 min. Control experiments were performed with PE-conjugated isotype-matched controls. Flow cytometry analysis was performed using a FACS Calibur cytometer (BD Biosciences). For some experiments, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer’s protocol (BD Biosciences).

Alexa Fluor 488-labeled LPS was preincubated in cell culture medium containing 10% FCS for 1 h at 37°C and added to IECs primed with medium in the absence or presence of Th cytokines in 6-well plates before the experiments. After the indicated incubation time, IECs were detached with ice-cold PBS and fixed for 20 min with ice-cold methanol at −20°C. After three washes with ice-cold PBS, IECs were saturated for 30 min with PBS containing 5% donkey serum. IECs were then incubated for 1 h with primary Abs. After washing, IECs were incubated with FITC-conjugated anti-rabbit IgG (Vector Laboratories) secondary Abs and 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear staining.

ELISA

For ELISA of secreted IL-8, 1 × 10^6 IECs were plated per well in 12-well plates, grown to 75–90% confluence and primed with cytokines for 18 h before stimulation with LPS (100 ng/ml), Pan3CSK4 (50 μg/ml), poly(I:C) (50 μg/ml) or flagellin (500 ng/ml). Supernatants were harvested after the indicated time periods and IL-8 ELISA (BD Pharmingen) was performed according to the manufacturer’s instructions.

Statistical analysis

Statistical significance between groups was assessed by Student’s t test. Data are expressed as means ± SDs. Triplicate determinations were performed in the IL-8 ELISA experiments, and all experiments were repeated.
at least three times. A \( p \) value \( \leq 0.05 \) was considered to be statistically significant.

**Results**

*The signature Th cytokines IL-4 (Th2) and IFN-\( \gamma \) (Th1) exhibit opposite effects on mRNA expression of key TLRs in IECs*

Recent data have demonstrated that differentiated T84 cells constitutively express different TLR family members and their associated signaling molecules, suggesting that they are suitable models for in vitro IEC studies. To evaluate possible effects of Th2 cytokines on TLR mRNA expression, polarized T84 cell monolayers were stimulated from the basolateral compartment with 50 ng/ml IL-4 or 50 ng/ml IFN-\( \gamma \) overnight. Northern blotting showed decreased TLR3 and TLR4 mRNA expression in T84 cells after priming with IL-4, while IFN-\( \gamma \)-enhanced TLR3 and TLR4 mRNA expression (Fig. 1A). In contrast, IL-4 stimulation did not markedly affect TLR2 and TLR5 mRNA expression in T84 cells (data not shown). Comparable results were obtained in HT29 cells, an undifferentiated colonic cancer cell line which has recently been shown to be susceptible to Th1 cytokine-mediated changes in TLR signaling (18, 19), and SW480 and Colo205 cells (data not shown).

IL-4-mediated down-regulation of constitutive TLR4 mRNA expression in T84 cells was concentration-dependent. The representative Northern blot in Fig. 1B demonstrates detectable down-regulation of TLR4 mRNA expression at 10 ng/ml IL-4 (15% decrease as determined by real-time RT-PCR, data not shown), which became more apparent after stimulation with 25 ng/ml IL-4 (25–30% decrease) and was significant after stimulation with 50 ng/ml or 100 ng/ml IL-4 (55–65% decrease). Similar data were obtained from IL-13-stimulated IECs (data not shown). The decrease in TLR4 mRNA expression was first apparent after 6 h of cytokine stimulation, was clearly marked after 12 h and was sustained throughout the 24-h observation period (Fig. 1C). In subsequent experiments, IECs were primed with 50 ng/ml IL-4 for 18 h.

**FIGURE 1.** IL-4 and IFN-\( \gamma \) exert opposite effects on TLR expression in T84 cells. A, Northern blot showing mRNA expression for TLR3 and TLR4 in unstimulated T84 cells (Medium) or T84 cells primed with 50 ng/ml IL-4 or 50 ng/ml IFN-\( \gamma \) (overnight). B, Representative Northern blot showing concentration-dependent down-regulation of TLR4 mRNA expression in T84 cells after overnight stimulation with increasing concentrations of IL-4. C, RT-PCR showing time-dependent down-regulation of TLR4 mRNA in T84 cells after stimulation with 50 ng/ml IL-4. Comparable data were generated in HT29 cells (data not shown). Representative blots from five Northern blots and four RT-PCRs are shown.

IL-4 and IL-13 decrease TLR3 and TLR4 mRNA expression in IECs without significant effect on TLR2 and TLR5 mRNA expression

Real-time RT-PCR was used to quantify the down-regulatory effect of IL-4 and IL-13 on TLR mRNA expression in differentiated T84 cells. Steady-state TLR mRNA expression (determined from the TLR:GAPDH mRNA ratio in unstimulated T84 cells) was set as 1 arbitrary unit and compared with the TLR:GAPDH mRNA ratio after cytokine stimulation (Fig. 2). IL-4-primed T84 cells exhibited significantly diminished mRNA expression of TLR3 (60% decrease; \( p = 0.03 \)) and TLR4 (54% decrease; \( p = 0.02 \)). Priming with IL-13 similarly decreased mRNA encoding for TLR3 and TLR4 (55% decrease for TLR3, \( p = 0.04 \), respectively, 50% decrease for TLR4, \( p = 0.03 \)). TLR2 mRNA expression also diminished under the influence of Th2 cytokines, but did not reach significance (23% decrease after IL-4 stimulation, \( p = 0.3 \), vs 29% decrease after priming with IL-13, \( p = 0.3 \)). IL-4 and IL-13 also minimally down-regulated TLR5 mRNA expression (15% decrease after stimulation with IL-4, \( p = 0.6 \), vs 19% decrease after stimulation with IL-13, \( p = 0.7 \)). In contrast, IFN-\( \gamma \)-priming significantly enhanced mRNA expression of TLR2 (4.2-fold increase; \( p < 0.01 \)), TLR3 (7.9-fold increase; \( p < 0.01 \)), TLR4 (5.7-fold increase; \( p < 0.01 \)) and TLR5 (6.7-fold increase; \( p = 0.03 \)). Comparable data were obtained in HT29 cells (data not shown).

To determine whether IL-4 counteracted the up-regulatory effect of IFN-\( \gamma \) on TLR expression in IECs, T84 cells were concomitantly stimulated with IL-4 and IFN-\( \gamma \). Fig. 2 shows that IL-4 attenuated IFN-\( \gamma \)-dependent enhancement of TLR mRNA, but failed to completely neutralize the up-regulatory effects of IFN-\( \gamma \).

Th2 cytokines decrease mRNA of MD-2, a coreceptor molecule necessary for TLR4 signaling, and attenuate MD-2 mRNA induction in IFN-\( \gamma \)-stimulated IECs

Real-time RT-PCR in Th2 cytokine-primed T84 cells was performed to assess concomitant changes in the mRNA expression of MD-2.
MD-2, the requisite TLR4 coreceptor. As shown in Fig. 3A, left panel, priming with IL-4 markedly decreased MD-2 mRNA expression in T84 cells to 47% of MD-2 mRNA expression in unstimulated T84 cells (p = 0.02). IL-13 priming similarly downregulated MD-2 mRNA to 53% of MD-2 expression in unstimulated T84 cells (p < 0.04). Moreover, concomitant priming with IL-4 also significantly attenuated up-regulation of MD-2 mRNA expression in IFN-γ-stimulated T84 cells. Although IFN-γ stimulation alone increased MD-2 mRNA expression in T84 cells <7-fold (p < 0.01), IL-4/IFN-γ-primed T84 cells showed only 1.5-fold enhanced MD-2 mRNA expression.

Recent studies demonstrated that HT29 cells exhibit increased sensitivity to LPS after IFN-γ stimulation, which has been largely attributed to increased MD-2 expression (18, 19). We examined the effect of Th2 cytokines on MD-2 mRNA expression in HT29 cells and were able to demonstrate that IL-4 and IL-13 markedly decreased MD-2 mRNA expression in unstimulated and IFN-γ-primed HT29 cells (Fig. 3A, right panel). Moreover, real-time RT-PCR and RT-PCR (Fig. 3B) clearly depicted that concomitant IL-4 priming significantly attenuated enhanced MD-2 mRNA expression in IFN-γ-primed HT29 cells.

Taken together, these data indicated that signature Th2 cytokines appeared to exert a broad down-regulatory effect on mRNA expression for both TLR4 and its important coreceptor MD-2 in IECs.

**Diminished TLR4 protein expression reflects down-regulated TLR4 mRNA expression in Th2 cytokine-primed IECs**

IP-Western blotting was performed to determine whether decreased TLR4 mRNA expression translated into diminished TLR4 protein expression in Th2 cytokine-treated T84 cells. Fig. 4A, left panel, shows that IL-4 and IL-13, but not IL-5, markedly decreased TLR4 protein expression. Comparable data were obtained in HT29 cells (data not shown). Densitometric analysis of IP-Western blots from a series of four independent experiments showed up to 50% decreased TLR4 protein in Th2 cytokine-primed T84 cells (p = 0.01; Fig. 4B, left panel).

Moreover, the representative IP Western blot of Fig. 4A, right panel, shows that concomitant IL-4 priming partly reversed the up-regulatory effects IFN-γ on TLR4 protein expression in IECs (4, 19). Fig. 4B, right panel, summarizes a series of four separate experiments, showing a 3.7-fold increase in TLR4 protein expression after IFN-γ stimulation compared with a moderate 1.6-fold increase after concomitant IL-4- and IFN-γ-priming of T84 cells. Comparable data were obtained from HT29 cells (data not shown).

Taken together, these data demonstrated that Th2 cytokine-mediated down-regulation of TLR4 mRNA expression in resting and Th1 cytokine-primed IECs leads to a significant reduction in TLR4 protein expression.

**Th2 cytokines reduce intracytoplasmic TLR3 and TLR4 protein expression in IECs**

Membranous TLR4 expression has been located on the cell surface and in the cytoplasm of IECs. We performed flow cytometry to determine whether Th2 cytokines changed the surface and/or the cytoplasmic TLR expression of SW480 cells. SW480 cells have been validated as a model IEC line for studying TLR4 protein expression by flow cytometry because they exhibit detectable surface TLR4 expression, while fixed and permeabilized SW480 cells do not show TLR4 surface expression. We used intact SW480 cells to evaluate changes in cytoplasmic TLR protein expression. The abundant TLR surface expression in human U937 monocytes served as a positive control. As shown in Fig. 5, IL-4 (blue graphs) and IL-13 (red graphs) only slightly decreased constitutive surface TLR4 expression (green graphs) in SW480 cells (Fig. 5B), but markedly diminished cytoplasmic TLR4 protein expression (Fig. 5C). In contrast, U937 monocytes exhibited a marked decrease in constitutive surface and cytoplasmic TLR4 protein expression after IL-4 and IL-13 stimulation (Fig. 5, D and E).

These data extended earlier studies examining the effect of Th2 cytokines on TLR expression in human PBMC (21). SW480 and U937 monocytes did not show surface TLR3 protein expression (data not shown), but priming with IL-4 (blue
and IL-13 (red graph) significantly diminished constitutive cytoplasmic TLR3 expression in both cell lines (green graph, Fig. 5, F and G). Taken together, FACS analysis corroborated our IP-Western blot findings and attributed decreased TLR4 protein expression in Th2 cytokine-primed IECs to diminished cytoplasmic TLR4 expression. Our flow cytometry data also suggested that Th2 cytokine-induced down-regulation of TLR3 mRNA expression in IECs and monocytes led to diminished cytoplasmic TLR3 protein expression in both cell types.

**Th2 cytokine-primed IECs incorporate less LPS**

Next, we attempted to determine whether our TLR expression data correlated with changes in the TLR function in IECs. We hypothesized that the simultaneous decrease of TLR4/MD-2 expression in IECs after Th2 cytokine-priming might diminish LPS signaling triggered by the TLR4/MD-2/CD14 complex. We performed functional LPS uptake studies in several IEC lines known to respond to IL-4. Among these cell lines, earlier experiments by our group had demonstrated that Colo205 cells and HT29 cells appeared to be most suitable for LPS internalization studies (19). We used flow cytometry to detect incorporated fluorescent LPS particles in Colo205 cells and evaluated the mean fluorescence intensity (MFI) of unstimulated and Th2 cytokine-primed Colo205 cells after incubation with fluorescent LPS. As shown in a representative experiment in Fig. 6A, IL-4- and IL-13-primed Colo205 cells incorporated significantly less fluorescent LPS than unstimulated cells (Fig. 6A). In a series of four independent experiments, IL-4 and IL-13 decreased LPS uptake over the complete observation period (Fig. 6B). Similar data were obtained in HT29 and SW480 cells (data not shown). Diminished LPS uptake seemed to be independent...
of CD14 expression, as flow cytometry detected no changes in CD14 expression after Th2 cytokine stimulation (data not shown).

**Th2 cytokine-primed IECs show functionally impaired TLR signaling**

Both HT29 cells and T84 cells were used for additional functional studies. HT29 cells have been reported to respond to all major TLR ligands, including the TLR5 ligand flagellin (22). Moreover, as noted above, recent studies also used HT29 cells to show increased LPS responsiveness after IFN-γ priming (4, 18, 19).

We first examined the influence of IL-4 on LPS-induced phosphorylation of inhibitory I-κBα in unstimulated and IFN-γ-primed HT29 cells, which would eventually lead to the activation of the NF-κB signaling complex. As shown in Fig. 7, concomitant IL-4 stimulation prevented the increase in I-κBα phosphorylation following LPS stimulation in IFN-γ-primed HT29 cells, suggesting that Th2 cytokines attenuated the increased LPS responsiveness in IFN-γ-primed IECs.

We then examined the effects of Th2 cytokines on IL-8 secretion from HT29 cells and T84 cells following stimulation with specific TLR ligands. To exclude direct Th2 or Th1 cytokine-mediated effects, increasing concentrations of IL-4, IL-13 and IFN-γ were tested in HT29 and T84 cells for their potential influence on spontaneous IL-8 secretion (data not shown). We found that preincubation with up to 20 ng/ml IL-4 or IL-13 and 10 ng/ml IFN-γ did not change subsequent spontaneous IL-8 secretion from HT29 cells during the observation period (Fig. 8A). HT29 cells secreted varying amounts of IL-8 after stimulation with the specific TLR ligands (Fig. 8, B–E). IL-4 and IL-13 priming significantly decreased IL-8 secretion triggered by the TLR3 ligand poly(I:C) (Fig. 8C) and the TLR4 ligand LPS (Fig. 8D), but did not affect IL-8 secretion after stimulation with the TLR2 ligand Pam3CSK4 (Fig. 8B) or the TLR5 ligand flagellin (Fig. 8E). In contrast, IFN-γ-primed HT29 cells secreted significantly more IL-8 after stimulation with these ligands (Fig. 8, B–E). In conclusion, these data suggested that the down-regulatory effect of Th2 cytokines on TLR expression in IECs led to a functional impairment of TLR3- and TLR4-mediated signaling in IECs.

**Discussion**

Human IECs express TLRs and their associated signaling molecules, which serve important immune and nonimmune functions in the intestinal mucosa. Because of the intimate contact between the IEC monolayer and adjacent intraepithelial and lamina propria lymphocytes, pro- and anti-inflammatory cytokines secreted by the latter (23, 24) may influence TLR-mediated innate immune responses of IECs. Recent in vitro studies demonstrated that the signature proinflammatory Th1 cytokine IFN-γ enhanced TLR4/MD-2 expression and LPS sensitivity of human IECs and monocytes (4, 18, 19, 25). In contrast, the key Th2 cytokines IL-4 and IL-13 have been shown to down-regulate TLR4 surface expression...
in human peripheral blood monocytes (21) and inhibited the secretion of proinflammatory cytokines after LPS stimulation (26). We speculated that Th2 cytokines may also exert broad anti-inflammatory effects on human IECs and potentially impair their capacity to initiate appropriate TLR-mediated immune responses.

In the present study, we examined whether key Th2 cytokines influenced TLR expression and function in validated model IEC lines. Incubation with IL-4 and IL-13 (but not IL-5) markedly decreased TLR3 and TLR4 mRNA and protein expression. Moreover, reduced I-κBα phosphorylation and diminished IL-8 secretion after stimulation with specific TLR ligands showed that TLR signaling in Th2 cytokine-primed IECs was functionally impaired. The lack of response to IL-5 may be explained by the absence of IL-5Rs in IECs, which contrasts the abundant expression of IL-4 and IL-13Rs in primary and model IECs (20, 27, 28).

Increased LPS responsiveness in Th1 cytokine-primed IECs has not only been attributed to increased TLR4 expression but also to the enhanced expression of MD-2, the important TLR4 coreceptor.
which might also influence TLR4 expression patterns in IECs (18, 19, 29). We were able to demonstrate that Th2 cytokines down-regulated both constitutive and IFN-γ-induced TLR4 and MD-2 expression. These data suggested that Th2 cytokines may dampen the increase in IEC immune reactivity after Th1 cytokine stimulation by counteracting both the up-regulation of TLR4 and the increase of MD-2 expression.

IL-4 and IFN-γ have been shown to activate STAT6 and STAT1, respectively, leading to the promotion of target gene transcription (30). In mouse macrophages, IL-4-induced STAT6 activation directly suppressed IFN-γ-triggered, STAT1-dependent gene transcription by competing with STAT1 for the same regulatory STAT binding element (31). Transcriptional repression of MD-2 and select TLRs by STAT6 may also explain our observations in Th2 cytokine-stimulated IECs. We were able to demonstrate STAT6 phosphorylation in HT29 cells after IL-4 stimulation (data not shown), and another study showed STAT6 phosphorylation in HT29/B6 cells after stimulation with IL-13 (28). Further analysis of the promoter regions of MD-2, TLR3 and TLR4 in IECs will clarify how these two factors may compete for regulatory regions with opposite functional consequences.

Taken together, key Th2 and Th1 cytokines appeared to have opposite regulatory effects on TLR expression and function in human IECs: IFN-γ enhanced TLR-mediated IEC immune responses by increasing key TLR signaling, while IL-4 and IL-13 appeared to impair IEC immune defense by down-regulating TLR3 and TLR4/MD-2 expression and function. A similar negative correlation between IL-4 and TLR4 expression has been described in the lungs of patients with tuberculosis, another organ with abundant TLR expression and constant exposure to a vast array of Ags (32).

Although direct anti-inflammatory effects of Th2 cytokines may be beneficial for the host by restricting ongoing inflammation (likely linked with a Th1 cytokine profile leading to increased TLR signaling), down-regulated TLR-expression and function can also paradoxically impair the host innate immune response. In this context, the down-regulated TLR4 expression after Th2 cytokine priming has been shown to reduce the ability of human mononuclear phagocytes to eliminate *Listeria monocytogenes* (33). Furthermore, Th2 cytokines impair IEC barrier function (28, 34), which likely facilitate nonphysiological encounters of the TLR ligand-bearing intestinal microflora with the subsequently “unprotected” IECs, rendering the host susceptible to chronic inflammation in response to the apparently normal intestinal microenvironment.

The IECs used in our study represent validated models for studying molecular mechanisms in the human intestine in vitro, and TLR down-regulation after Th2 cytokine priming was consistently found in different IEC lines, suggesting that these effects do not reflect idiosyncratic features of a single cell line. However, model IEC lines also have inherent limitations and may not entirely address the functional relevance of Th2 cytokine-mediated changes in TLR signaling in vivo. Models IECs may fail to address the complex interaction between primary IECs and the host intestinal microenvironment with its heterogeneous mucosal cytokine milieu including the presence of abundant commensals and potential pathogens which likely stimulate multiple TLRs. It will be important to define how Th2 cytokine-mediated effects on TLR signaling may be modulated by products of the luminal flora, which have been shown to induce selective TLR trafficking (35) and may also directly regulate TLR expression in IECs (36). In our experiments, highly purified TLR ligands were used to characterize TLR function in IECs in a homogenous Th2 and/or Th1 cytokine environment.

The influence of the intestinal microenvironment may also explain the discrepancy between our present findings and earlier immuno-histochemical studies showing down-regulated TLR3 expression in the mucosa of patients with Crohn’s disease (17), usually considered a prototypic Th1 cytokine disease. This study also showed up-regulated mucosal TLR4 expression in patients with ulcerative colitis, which has recently been linked to atypical...
mucosal IL-13 expression (28, 37). However, other studies described different mucosal Th cytokine profiles in patients with ulcerative colitis, ranging from diminished mucosal IL-13 expression (38, 39) to increased IL-5 expression (40).

Murine models of chronic intestinal inflammation underscore the alternative proinflammatory potential of Th2 cytokines within the intestinal microflora, exemplified by the induction of potentially fatal colitis in healthy mice after colonic (but not systemic) adenoviral IL-4 overexpression (41). A mucosal IL-4 predominance within the intestinal microenvironment may play an immunopathogenic role in the development and/or maintenance of both hapten-induced (42) and spontaneous chronic colonic inflammation in mice (43). Moreover, IL-4 producing Th2-type T cells appear to mediate the development of chronic inflammation and epithelial damage in a recently described murine model showing spontaneous Crohn’s-like ileitis, where immunobacterial interactions may be an important trigger for the onset of chronic inflammation (44, 45). Our study may be helpful in understanding increased IEC damage in these models, as down-regulated TLR signaling in IECs likely weakens the host innate immune defense against the apparently normal microflora.

In conclusion, the present study showed that the key Th2 cytokines IL-4 and IL-13 down-regulated TLR3 and TLR4/MD-2 expression and function in validated model IECs. Further in vivo studies are needed to determine whether the Th2 cytokine-induced attenuation of TLR-mediated innate immune signaling in IECs protects the host from excessive innate immune responses or whether impaired TLR signaling in IECs may render the host more susceptible to chronic intestinal inflammation.

**FIGURE 8.** Th2 cytokines impair TLR ligand-induced IL-8 secretion in HT29 cells. HT29 cells were primed with medium alone, medium containing 20 ng/ml IL-4 or 20 ng/ml IL-13 or 10 ng/ml IFN-γ before stimulation with specific TLR ligands. Supernatants were assayed for IL-8 secretion. A, Spontaneous IL-8 secretion does not change after stimulation with Th cytokines. B–D, Comparison of IL-8 secretion from unstimulated or Th2 or Th1 cytokine-primed HT29 cells after stimulation with TLR2 ligand Pam3CSK4 (B), TLR3 ligand poly(I:C) (C), TLR4 ligand LPS (D), and TLR5 ligand flagellin (E). Comparable data were obtained in T84 cells (data not shown). Data are mean ± SE of three separate experiments. * p < 0.05.
suscipible for the development of chronic intestinal inflammation in response to the intestinal microenvironment.

Acknowledgments
We are grateful to Dr. Hans-Christian Reinecker, Dr. Nicolas Barnich, Dr. Jiri Kalabis, Dr. Jesus Yamamoto-Furusho, Dr. Emiko Mizoguchi, and Kathryn Devaney for helpful discussions and critical reading of the manuscript.

The authors have no financial conflict of interest.

References