TLR4 and MD-2 expression are regulated by immune-mediated signals in human intestinal epithelial cells

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Abbreviations: TLR= toll-like receptor; IEC= intestinal epithelial cells; PAMP= pathogen-associated molecular pattern; IFN= interferon

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**Summary**

**Background:** The normal intestinal epithelium is not inflamed in spite of contact with a high density of commensal bacteria. Intestinal epithelial cells (IEC) express low levels of TLR4 and MD-2 and are LPS unresponsive. We hypothesized that immune-mediated signals regulate the expression of TLR4 and MD-2 in IEC.

**Methods:** Expression of TLR4 and MD-2 was examined in normal colonic epithelial cells or intestinal epithelial cell lines. The effect of the cytokines IFN-γ, IFN-α, and TNF-α on TLR4 and MD-2 expression was examined by RT-PCR and western blot. NF-κB transcriptional activation and IL-8 secretion were used as measures of LPS responsiveness.

**Results:** Native colonic epithelial cells and IEC lines express a low level of TLR4 and MD-2 mRNA. IFN-γ regulates MD-2 expression in both IEC lines whereas IFN-γ and TNF-α regulate TLR4 mRNA expression in IEC lines. Pre-incubation with IFN-γ and/or TNF-α sensitizes IEC to LPS-dependent IL-8 secretion. To examine MD-2 transcriptional regulation, we cloned a 1kB sequence proximal to the MD-2 gene translational start site. This promoter directed expression of a reporter gene in endothelial cells and IEC. IFN-γ positively regulated MD-2 promoter activity in IEC. Co-expression of a STAT inhibitor, SOCS3, blocked IFN-γ-mediated MD-2 promoter activation.

**Conclusion:** T cell-derived cytokines lead to increased expression of TLR4 and MD-2 and LPS-dependent pro-inflammatory cytokine secretion in IEC. IFN-γ regulates expression of the critical TLR4 co-receptor MD-2 through the JAK-STAT pathway. Th1 cytokines may initiate or perpetuate intestinal inflammation by altering TLR expression and bacterial reactivity.
Introduction

The intestinal epithelium is continually exposed to a high intraluminal concentration of diverse bacteria and bacterial products (1,2). In spite of the density of commensal bacteria and their products, the intestinal mucosa maintains a controlled state of inflammation. By contrast, invasive or toxin-producing pathogenic bacteria elicit acute inflammation and secretion of pro-inflammatory cytokines by intestinal epithelial cells and lamina propria mononuclear cells (3,4). Idiopathic inflammatory bowel disease in humans and animals is characterized by acute and chronic inflammation in the absence of a specific pathogen. Compelling evidence in genetically-susceptible animal models of inflammatory bowel disease demonstrates that Th1 cytokines and commensal bacteria are required for the induction of chronic inflammation (5-9). The recent discovery of a genetic association in inflammatory bowel disease patients with a mutation in a gene involved in LPS signaling, Nod2, supports the idea that innate immunity may be defective in patients with idiopathic inflammatory bowel disease (10,11).

We wished to understand the mechanism by which the normal intestinal epithelium guards against chronic activation in the presence of commensal flora. Commensal gut bacteria include both gram-positive and gram-negative organisms (2). The cell wall of gram-negative bacteria contains LPS, a potent pro-inflammatory pathogen-associated molecular pattern (PAMP) responsible for the systemic manifestations of septic shock (12). The response to LPS is mediated by its interaction with toll-like receptor 4 (TLR4) in conjunction with secreted MD-2 and soluble or membrane-bound CD14 and transduced via the IL-1 receptor signaling complex to activate NF-κB and pro-inflammatory cytokine secretion (13-16). We and others have previously described that intestinal epithelial cells are unresponsive to purified, protein-free LPS as measured by NF-κB activation and IL-8 secretion (17,18). To determine the reason for LPS
unresponsiveness, we assayed for the presence of TLR4 and its co-receptor MD-2 and found that intestinal epithelial cells express low levels of TLR4 and MD-2 (17). Expression of both TLR4 and MD-2 restores the ability of intestinal epithelial cells to respond to LPS suggesting that the intracellular signaling pathway leading to NF-κB is intact in these cells. These in vitro model systems are consistent with findings in normal adult human colonic biopsies, small intestinal resections and fetal intestinal epithelial cells which have demonstrated low TLR4 expression by immunohistochemistry and RT-PCR (18,19). These studies did not examine the expression of the MD-2 co-receptor which is required for LPS responsiveness nor did they measure TLR4 function.

Little is known about the regulation of TLR4 or MD-2 expression. Whereas normal intestinal epithelial cells express low levels of TLR4, colonic biopsies from patients with inflammatory bowel disease have increased TLR4 expression (19). The pro-inflammatory cytokine IL-1β can increase the level of TLR4 expression in a human fetal small intestinal epithelial cell line (18) and interferon-consensus sequences have been identified in the TLR4 promoter (20). These data support the hypothesis that dysregulated expression of TLRs in response to cytokines may contribute to the pathogenesis of idiopathic inflammatory bowel disease and inappropriate responsiveness to commensal bacteria. Because of its intimate contact with commensal bacterial products as well as potential pathogens, the intestinal epithelium must carefully regulate expression of pattern recognition receptors to avoid persistent activation. In the current study, we have examined the role of T-cell derived cytokines on the regulation of TLR4 and MD-2 expression. We have additionally cloned the promoter for MD-2. Our data demonstrate that MD-2 expression and transcriptional activity are positively regulated by interferon (IFN)-γ whereas TLR4 expression is regulated by IFN-α. Expression of a STAT
inhibitor, SOCS3, blocks IFN-γ-mediated increase in MD-2 promoter activity. The results of these studies have important implications for the understanding of host-microbial interactions in the gut.
Experimental Procedures

Cells and Reagents: Intestinal epithelial cell lines Caco-2, HT-29 and T84 were obtained from the ATCC (Rockville, MD). Sub-confluent monolayers of these cell lines were kept in a humidified incubator at 37°C with 5% CO₂. T84 were cultured on 12mm Transwell, Polycarbonate Membranes (Costar 3401) and maintained in DMEM/F12 (Gibco) with 5% Pen/Strep, 5% L-Glutamine, supplemented with 5% FBS as previously described (21). T84 cells were used between passage number 16 and 35 (22). Caco-2 were maintained in Minimum Essential Medium (Gibco) supplemented with 10% FBS, 2mM L-glutamine, 0.1mM non essential amino acids, 1mM sodium pyruvate and 5% Pen/Strep. HT-29 were maintained in McCoy’s 5A medium supplemented with 10% FBS and 5% Pen/Strep. The immortalized human dermal endothelial cells (HMEC) (23) (generous gift of Dr. Candal of the Center for Disease Control and Prevention, Atlanta) were cultured in MCDB-131 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM of glutamine and 100 µg/ml of penicillin and streptomycin in 24-well plates, and used between passages 10-14, as described earlier (15,23,24).

Highly purified, phenol-water-extracted Escherichia coli K235 LPS (< 0.008% protein), which was prepared according to the method of McIntire et al. (25), was obtained from Stefanie N. Vogel (Uniformed Services University of the Health Sciences, Bethesda, MD) (26,27). The purity of this LPS preparation has been previously demonstrated (25,28,29), and this preparation of E. coli LPS is active on TLR4 transfected HEK 293 cells and not on TLR2 transfectants (Stefanie N. Vogel, personal communication). Human IL-1β and TNF-α was purchased from R&D Systems (Minneapolis, MN). 5-azacytidine was purchased from Sigma. Human
Expression vectors and cDNA constructs: ELAM-NF-κB luciferase (15) and pCMV-EGFP (Clontech) (30) were used as previously described. Human IL-8 promoter-luciferase construct was kindly provided by Dr. N. Mukaida (31). A flag-tagged human TLR4 construct was obtained from Tularik (San Francisco, CA). MD-2 cDNA construct was kindly provided by Dr. Kensuke Miyake of Saga Medical School, Saga, Japan (32). IRF-1/3X-GAS-luciferase was kindly provided by Dr. Richard Jove (Moffitt Cancer Center and Research, Tampa, Florida) (33). The SOCS3 expression vector was kindly provided by Dr. Douglas Hilton (The Walter and Eliza Hall Institute of Medical Research and Cooperative Research Centre for Cellular Growth Factors, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia) (34). Plasmids were prepared with endotoxin-free Plasmid Maxi-prep kit (Qiagen, Valencia, CA).

Transient gene expression and reporter gene assays: Caco-2 cells or T84 cells were plated at a density of 150,000 cells/well or 200,000 cells/well respectively, in 12-well plates 24h prior to transfection. HMEC were plated at a concentration of 50,000 cells/well in 24-well plates. Cells were transfected the following day with Fugene 6 Transfection Reagent (Roche Molecular Biochemicals) as per manufacturer’s instructions and as described earlier (15,24). Reporter genes pCMV-β-galactosidase, ELAM-NF-κB-luciferase (0.4µg), IRF-1/3X-GAS-luciferase and pCDNA3 empty vector (0.3-0.6µg), Flag-tagged wild type human TLR4 (0.3µg), human MD-2 cDNA (0.3µg) or SOCS3 constructs were co-transfected as indicated in figure legend. After overnight transfection, cells were stimulated for 5 hours with LPS 50 ng/ml, human IL-1β 10 ng/ml, TNF-α 20 ng/ml or IFN-γ 40 ng/ml (R & D Systems). Cells were then lysed in 200 µl of...
reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured with a Promega firefly luciferase kit using a Wallac 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer). Data shown are mean ± SD of three or more independent experiments, and are reported as fold-induction over cells transfected with a control vector. Transfection efficiency was determined by assaying for β-galactosidase activity using a colorimetric method (Promega) as previously described (15).

**MD-2 Promoter Studies:** Genbank was searched for human MD-2 and yielded two accession numbers. The MD-2 gene is located on the minus strand of chromosome 8. Accession number NT_008209 (contig) was used to identify 1 kb of sequence upstream of the start site and AC009672 was used to identify 2 kb of sequence upstream of the start site. The following primers were designed to amplify a 1013bp sequence (±1kB) and a 2042 bp sequence (±2kB) upstream of the ATG start site: Primer 1 GCTTTACAATGCAAAGAGGATCAG (same primer for both ±1kB and ±2kB); ±1kB= primer 2– reverse CATGGCCTGTTAGGAATCTGGT; ±2kB = primer 3 reverse GGCTGCTAACCCTAAGCTATATCC. Human genomic DNA was used to amplify the respective sequences. PCR products were cloned into pCR 2.1 TOPO vector and inserts sequenced using M13 forward and reverse primers. After confirmation of the correct sequence, inserts were directionally cloned into the pGL3 basic luciferase reporter vector (Promega).

**Reverse transcription–polymerase chain reaction (RT-PCR) analysis:** Total RNA was isolated from T84, and HT-29 using a Qiagen kit (Valencia, CA) following manufacturer’s instruction and treated with RNase free DNase I. For RT reaction, the MMLV Preamplification system (Life
Technologies, Gaithersburg, MD) was used. PCR amplification was performed with Taq polymerase (Perkin Elmer, Foster City, CA) using two distinct set of primers and conditions. The first set of primers described amplify short products of MD-2 and TLR4 (150bp) and β-actin (300bp) and the second set of primers and conditions amplifies longer products of MD-2 (422bp) and TLR4 (548bp) and GAPDH (983bp). The shorter product increases the sensitivity for detection of these transcripts. The TLR4 oligonucleotide primers used for RT-PCR were described earlier (24). The oligonucleotide primer sequences for MD-2 were kindly provided by Dr. Jesse C. Chow, (Esai Research Institute, Wilmington, MA). GAPDH primers were obtained from Clontech (Palo Alto, CA) and used as per manufacturer’s instructions. The TLR2, TLR4 and MD-2 RT-PCR fragments were purified and sequenced to confirm the identity of the fragments. To quantify the level of mRNA expression, RT-PCR products run on a 1% ethidium bromide-stained agarose gel were analyzed on an AlphaImager 2000 densitometer (Alpha Innotech Corporation). AlphaEase software (Alpha Innotech Corporation) was used to compare density of products when corrected for intensity of GAPDH or β-actin expression and fold induction of expression over unstimulated cells.

| Gene        | Forward primer | Reverse primer | Conditions                                                                 | Size of product (bp) |
|-------------|----------------|----------------|-----------------------------------------------------------------------------|----------------------|
| TLR4 (short)| GCTTCTTGCT     | GAAATGGAGG     | 32 cycles at 94°C for 45 sec., 60 °C for 45 sec., and 72°C for 60 sec         | 150                  |
|             | GGCTGCATAA     | CACCCCTTC      |                                                                           |                      |
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|            | Primer Sequence 1 | Primer Sequence 2 | PCR Conditions | Cycles |
|------------|-------------------|-------------------|----------------|--------|
| **MD-2 (short)** | GCAGAGCTCT        | GGTTGGTGTA        | 32 cycles at 94°C for 45 sec., 60 °C for 45 sec., and 72°C for 60 sec | 150    |
|            | GAAGGGAGA         | GGATGACATC        |                |        |
|            | GACT              | C                 |                |        |
| **β-actin** | GGCTACAGCT        | GCCAGACAGC        | 35 cycles at 94°C for 45 sec., 58 °C for 45 sec., and 72°C for 60 sec | 300    |
|            | TCACCACCAC        | AGTGTGGTTGG       |                |        |
|            | G                 | C                 |                |        |
| **TLR4**   | TGGATACGTT        | GAAATGGAGG        | 38 cycles at 95°C for 45 sec., 54 °C for 45 sec., and 72°C for 1 min | 548    |
|            | TCCTTATAAG        | CACCCCTTC         |                |        |
| **MD-2**   | GAAGGTCAGA        | GGTTGGTGTA        | 35 cycles at 94°C for 45 sec., 55 °C for 45 sec., and 72°C for 45 sec | 422    |
|            | AGCAGTATTG        | GGATGACAAA        |                |        |
|            | GGTC              | CTCC              |                |        |

ELISA and Western blotting: For TLR4 western blots, T84 cells were lysed in IP lysis buffer containing 50 mM Hapes, pH 7.9, 250 mM NaCl, 20 mM β-glycerophosphate, 2 mM DTT, 1 mM sodium orthovanadate, 1% NP-40, 1:100 Protease Inhibitor Set III (Calbiochem). Protein concentration was determined using a colorimetric assay Biorad DC protein assay. A total of 55 µg of protein was analyzed on a 10% Tris-HCl polyacrylamide gel (BioRad, Hercules, CA).
Proteins were transferred to nitrocellulose membranes and stained with Ponceau S to verify equal protein loading. Membranes were blocked in 5% milk, 0.1% Tween-20 in TBS for 2-3 hours at 4°C, incubated overnight at 4°C with anti-human TLR4 Ab (Santa Cruz) (1:250) followed by a 1 hour incubation at room-temperature with anti-rabbit HRP (1:2000), developed by Lumiglo (Cell Signaling) and exposed to radiographic film.

For human IL-8 ELISA, 10,000 cells were plated per well in 96 well plates. Cells were treated with LPS 50 ng/ml, IFN-γ 40 ng/ml or TNF-α 20 ng/ml for 18h and supernatants harvested for measurement of IL-8. IL-8 ELISA (Pharmingen) were performed as per manufacturer’s instructions. Fold increase in IL-8 production was derived by calculating the difference between cytokine-stimulated IL-8 production and cytokine-stimulated plus LPS-mediated IL-8 production. This difference was then divided by LPS-dependent IL-8 production alone.

**Laser capture microscopy:** Frozen sections derived from human intestinal resections were obtained under the auspices of Cedars-Sinai Medical Center IRB# 1465. The tissue used for this study included uninvolved areas of intestine from patients with inflammatory bowel disease or colon cancer. Slides were gently fixed in 100% ethanol followed by a light hematoxylin and eosin staining. An Arcturus laser capture microscope was used to microdissect the tissue. Briefly, intestinal epithelial cells were identified based on appearance and location, microdissected and captured on a microfuge cap. Lamina propria was separately microdissected and captured from each intestinal specimen. Photo documentation was obtained before and after dissection. Total RNA was made by incubating cells at -80°C overnight in lysis buffer containing Tris-HCl 50 mM (pH 8.0), NaCl 100 mM, MgCl2 5 mM, Triton X-100 0.5%, DTT 1 mM and RNase inhibitor at 1000 U/ml. After lysis and centrifugation, total RNA was followed
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directly by reverse transcription using random hexamers and Superscript II (Gibco/BRL). The cDNA generated was amplified as described above with the exception that 38 cycles were used for amplification.

Statistical Analysis: Student t-tests, standard deviation and standard errors were performed using the statistics package within Microsoft Excel. P values were considered statistically significant when < 0.05.
Results

Normal human colonic epithelial cells express low levels of MD-2

We have described our findings in three intestinal epithelial cell lines with respect to the expression and function of TLR4 and MD-2 (17). Our finding of low TLR4 expression by intestinal epithelial cells is corroborated by a recent study demonstrating that intestinal epithelial cells from normal human intestinal biopsies express low levels of TLR4 by immunohistochemistry (19). In order to assess the level of MD-2 expression by primary human intestinal epithelial cells, we utilized laser capture microscopy to micro-dissect intestinal epithelial cells and separate these from lamina propria cells from five distinct colonic resections (Figure 1. A.). Using RT-PCR to examine the expression of TLR4 and MD-2, we found that both colonic epithelial cells and lamina propria-derived cells express a low level of TLR4 (Figure 1. B.). By contrast, our data demonstrate that MD-2 is not expressed in normal colonic epithelial cells but is found in some samples of lamina propria-derived cells (Figure 1.B.). These data support the hypothesis that the intestinal epithelium normally down-regulates expression of TLR4 and MD-2.

Cytokines of the adaptive immune system regulate expression of TLR4 and MD-2 in intestinal epithelial cells

TLR4 and MD-2 expression is low in intestinal epithelial cells compared with human dermal endothelial cells. IFN-γ has recently been shown to stimulate TLR4 expression in endothelial cells (35) and HL-60 monocytic cells (36) but not in murine macrophages (37,38). TLR4 expression is increased in intestinal epithelial cells in patients with inflammatory bowel disease (19). Because inflammatory bowel disease is associated with increased mucosal...
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production of the Th1 cytokines IFN-γ and TNF-α, we hypothesized that these cytokines regulated expression of TLR4 and its co-receptor MD-2 in intestinal epithelial cells (39,40). We tested this hypothesis by exposing T84, a crypt-like intestinal epithelial cell line, (41) and HT-29 cells, a colonocyte-like intestinal epithelial cell line, to the cytokines IFN-γ, TNF-α or a combination of the two and evaluated expression of messenger RNA for TLR4 and MD-2 by RT-PCR (Figure 2A). With respect to TLR4 expression (Figure 2A top panels), IFN-γ and TNF-α modestly increased TLR4 expression which was highest at 24h in HT-29 cells and 6h in T84 cells. MD-2 expression by contrast (Figure 2A, middle panels) was primarily regulated by IFN-γ in both IEC lines. IL-1β which potently induces IL-8 secretion by CaCo-2 cells had no effect on TLR4 expression (data not shown). Expression of TLR4 protein was subsequently evaluated by western blotting in T84 cells (Figure 2C). As expected, resting T84 cells do not have detectable TLR4 protein. TNF-α treatment of T84 cells permits a low level of TLR4 detection. In summary, Th1 cytokines differentially regulate the expression of TLR4 and MD-2 in intestinal epithelial cells in vitro. The relative absence of TLR4 protein expression suggests that mRNA expression may not be directly correlated with protein expression.

Viral gastroenteritis is associated with increased production of IFN-α by dendritic cells in the gut-associated lymphoid tissue and results in chemokine production by intestinal epithelial cells (42-44). IFN-α sensitizes splenic and peritoneal leukocytes to LPS-mediated TNF-α production in the setting of viral infection (45). We next addressed whether IFN-α regulated TLR4 or MD-2 expression in intestinal epithelial cell lines. T84 and HT-29 cells were exposed to IFN-α and the expression of messenger mRNA for TLR4 and MD-2 was assessed by RT-PCR (Figure 2B). IFN-α led to an increase in TLR4 expression in T84 cells but not in HT-29 cells (Figure 2B). IFN-α had no effect on MD-2 expression in either cell line (data not shown).
Western blotting confirmed an increase in TLR4 protein expression in IFN-α-treated T84 cells (Figure 2C). These data demonstrate that IFN-α differentially regulates TLR4 expression in intestinal epithelial cells. We speculate that the response of intestinal epithelial cells to cytokines in vivo may be regulated differentially along the crypt-to-villus axis.

Cytokine stimulation of intestinal epithelial cells reconstitutes the response to LPS

We have shown that IFN-γ and TNF-α differentially regulate expression of TLR4 and MD-2 at the mRNA level in intestinal epithelial cells. We next wished to test whether the increase in TLR4 and MD-2 expression restored LPS responsiveness in these cells. HT-29 cells were stimulated with IFN-γ, TNF-α or a combination of these cytokines and then exposed to LPS. We measured IL-8 secretion as a relevant cytokine produced by intestinal epithelial cells in response to pathogenic bacteria or during chronic inflammatory bowel disease. We have previously described that TNF-α with or without IFN-γ stimulates IL-8 secretion in HT-29 cells (Figure 2) (46). The combination of IFN-γ and TNF-α results in apoptosis and therefore lower IL-8 secretion (21). Pre-incubation of HT-29 cells with TNF-α or IFN-γ + TNF-α followed by LPS increased IL-8 production compared with cytokines alone. The increase in IL-8 secretion attributable to LPS following TNF-α or IFN-γ + TNF-α was 47-fold and 30-fold higher, respectively, than the secretion of IL-8 in response to LPS stimulation alone (Figure 2, indicated by bracket) (calculation described in Methods). IFN-γ does not by itself stimulate IL-8 secretion. Following IFN-γ pre-incubation, HT-29 cells secreted 40% more IL-8 than with LPS stimulation alone albeit the amount of IL-8 secretion is of relatively small magnitude. IFN-γ and/or TNF-α stimulation of T84 cells did not result in LPS-dependent IL-8 secretion or NF-κB activation (data not shown). This finding was not surprising given that TLR4 protein expression
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is not substantially increased in response to these cytokines (Figure 2C). These findings of enhanced LPS-dependent IL-8 production in response to TNF-α in HT-29 cells correlates with TLR4 and MD-2 mRNA expression and support the concept that an inflammatory milieu may increase intestinal epithelial cell responsiveness to bacteria. In addition to regulation of TLR4 and MD-2, cytokines such as TNF-α may have other effects on TLR signal transduction and IL-8 secretion leading to potentiation of the LPS response.

The MD-2 promoter is differentially regulated in LPS-responsive endothelial cells compared with LPS-unresponsive intestinal epithelial cells

We have previously demonstrated that MD-2 expression is low in intestinal epithelial cells but highly expressed in endothelial cells (17). We hypothesized that the level of MD-2 expression was regulated by differential transcriptional activation of the MD-2 promoter. In order to study the regulation of the MD-2 gene, we cloned a sequence that was upstream of the MD-2 gene translational start site (Figure 4, diagram). Human MD-2 is located on the reverse strand of chromosome 8. Using the sequence available from GenBank (accession numbers NT_008209 and AC009672) we cloned a -1kB and -2kB fragment 5’ upstream of the first methionine with the distal 3’ end of the fragment within the 5’ untranslated region of the gene. These fragments were sequenced and cloned upstream of a luciferase gene (empty vector pGL3) and will be referred to as −1kB-MD-2 pGL3 and −2kB-MD-2 pGL3. Intestinal epithelial cells and HMEC were transfected with −1kB-MD-2 pGL3 or −2kB-MD-2 pGL3 and basal luciferase expression was compared to that of an empty luciferase vector pGL3 (Figure 4 graph). The promoter-less luciferase vector was not expressed in T84 or HMEC cells. By contrast, both the −1kB-MD-2 pGL3 and −2kB-MD-2 pGL3 vectors directed expression of luciferase in T84
and HMEC cells. Importantly, the level of MD-2 promoter activity was significantly higher in HMEC than T84 cells (90-fold versus 20-fold), consistent with the observed differences in mRNA expression between these two cell lines. These data suggest that the sequence within 2 kB upstream of the MD-2 translational start site positively regulates MD-2 expression and contains elements that are differentially regulated in diverse tissues.

**Interferon-γ regulates MD-2 promoter activity through the JAK-STAT pathway**

The data above demonstrate that the 5’ region of the MD-2 gene can direct basal expression of a luciferase reporter gene. We have also shown that IFN-γ results in an increase in MD-2 mRNA expression suggesting that IFN-γ leads to transcriptional activation of the MD-2 gene. Recently, the promoter for TLR4 was shown to contain an interferon response factor (IRF)-binding site that regulates expression of TLR4 in myeloid cells (20). In order to determine whether the MD-2 gene promoter was regulated by interferons, we transfected T84 cells with the 1kB-MD-2 pGL3 and 2kB-MD-2 pGL3 reporter vectors and stimulated cells with IFN-γ or IFN-α. Addition of IFN-γ and to a lesser extent IFN-α resulted in transcriptional activation of the 1kB-MD-2 pGL3 and 2kB-MD-2 pGL3 vectors (Figure 5A, IFN-γ 100-fold over empty vector control and 3-fold over unstimulated 1kB-MD-2 pGL3). These changes in response to interferons were not seen with transfection of the promoter-less pGL3 luciferase vector. These data suggest that the MD-2 promoter contains an IFN response element within its -1kB fragment.

IFN-γ and transduces its signal by binding to its receptor leading to the phosphorylation and activation of Janus kinases (JAKs) and subsequently signal transducers and activators of transcription (STATs) (47,48). Translocation of STAT transcription factors to the nucleus
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regulates gene expression through binding of STATs to interferon-γ activation sites (GAS) and interferon-stimulated response elements (ISRE) in specific gene promoters. To determine if the JAK-STAT pathway plays a role in MD-2 expression, we first asked whether IFN-γ resulted in transcriptional activation of a GAS sequence in T84 cells. IFN-γ led to specific transcriptional activation of a multimerized GAS reporter derived from the IRF-1 gene promoter, supporting the idea that IFN-γ could activate STAT binding to its consensus sequence in intestinal epithelial cells (Figure 5B). These data suggest that IFN-γ is able to transduce a signal leading to transcriptional activation of a STAT-dependent reporter gene in intestinal epithelial cells.

We next reasoned that if STAT binding plays a role in activation of the MD-2 promoter then expression of an inhibitor of STATs, i.e. suppressor of cytokine signaling (SOCS)-3, should inhibit the ability of IFN-γ to activate the MD-2 promoter. Expression of SOCS3 is increased in animal models of inflammatory bowel disease and inhibition of SOCS3 function results in more severe colitis (49). To address whether STAT signaling plays a role in MD-2 transcriptional regulation, T84 cells were transfected with the −1kB-MD-2 pGL3 reporter gene and co-transfected with a SOCS3 expression vector or its empty vector control (Figure 5C). Stimulation with IFN-γ significantly increased reporter gene activation in T84 cells and this activation was blocked by 80% in cells co-transfected with SOCS3. Our findings demonstrate that transgenic expression of SOCS3 specifically blocks IFN-γ-mediated MD-2 promoter activation but has no effect on basal MD-2 promoter activity. These data suggest that IFN-γ-mediated MD-2 promoter activation depends on the STAT pathway and points to a potentially important point of cytokine-mediated regulation of innate immunity.

Discussion
Although the individual cells that compose the intestinal mucosa and gut-associated lymphoid tissue are known, the complex relationship between the intestinal epithelium and the innate and adaptive immune systems are still being unraveled. The intestinal epithelium must on the one hand remain mute to the presence of commensal flora while on the other hand be ready to defend against invading pathogens. With the elucidation of pattern recognition receptors responsible for sensing bacteria, we can begin to understand how the intestinal epithelium copes with these dual responsibilities. Inflammatory bowel disease is characterized by uncontrolled inflammation in the absence of a recognized pathogen. Dysregulated production of Th1 cytokines in the presence of commensal bacteria have been implicated in the pathogenesis of inflammatory bowel disease (6,9,50-53). Recent identification of the IBD1 susceptibility gene on chromosome 16 as a gene involved in toll receptor signaling, Nod2, provides further rationale for pursuing a study of toll receptor signaling in the gut (10,11). The results of our studies present a mechanism by which Th1 cytokines may secondarily lead to intestinal epithelial over-reactivity in the presence of commensal bacteria by increasing TLR4 and MD-2 expression and LPS responsiveness.

Human urinary tract epithelial cells, another mucosal epithelium, express TLR4 and are LPS responsive (54,55). Because the intestinal epithelium should remain immunologically silent in response to commensal flora and LPS, it is logical that intestinal epithelial cells express low levels of TLR4 and its co-receptor MD-2. We hypothesized that expression of TLR4 and MD-2 would be carefully regulated in intestinal epithelial cells to both suppress expression of these molecules in the uninflamed state but retain the capacity to express these receptors when danger is sensed. In the current paper, we have shown that indeed intestinal epithelial cells derived from human intestinal resections express a low level of TLR4 mRNA. We are the first to examine
MD-2 expression in intestinal epithelial cells in vivo and have found a low level of MD-2 expression compared with lamina propria-derived cells in the uninflamed intestine. These data corroborate our results using intestinal epithelial cell lines and our hypothesis that suppressed expression of TLR4 and MD-2 protects against chronic activation in the presence of luminal LPS. In order to study the functional responses of intestinal epithelial cells, we have used two well-characterized intestinal epithelial cell lines, a crypt-like T84 cell line and colonocyte-like HT-29 cell line. Although T84 cells and HT-29 cells reproduce the intestinal epithelial cell phenotype in many respects, homogenous cell lines cannot replicate all stages of colonic epithelial cell differentiation. Thus, our data must be interpreted in light of the inherent limitations, as well as benefits, of in vitro systems.

If the intestinal epithelium is normally unable to respond to LPS present in the lumen, how then does it respond to potential pathogens? Enteroinvasive bacteria activate NF-kB in intestinal epithelial cells and lead to the secretion of pro-inflammatory cytokines through a process that is not solely dependent on LPS since non-invasive strains of the same bacteria do not elicit this response (56-59). M cells, specialized intestinal epithelial cells, have long been recognized to sample luminal contents and pass antigens including live pathogenic bacteria such as Salmonella to lymphoid follicles (60). A recent study in mouse intestine demonstrated that dendritic cells send projections between the intestinal epithelial cells in the gut to sample the intestinal lumen and communicate their findings to underlying lymphoid follicles (61). In this system, dendritic cells exposed to Salmonella exit the epithelium and return to the lamina propria whereas they do not in response to commensal, non-pathogenic E. coli. These data suggest that rather than risk non-specific, chronic inflammation in the gut, there is a defined sampling mechanism that may serve to activate the innate and adaptive immune systems only in response
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to pathogenic organisms.

Relatively little is known about the regulation of TLR4 expression and almost nothing about MD-2 regulation. Rehli et al. have identified a 75 base pair sequence upstream of the major transcription initiation site of TLR4 that directs expression of a reporter gene in myeloid cell lines (20). This region contains an interferon response factor site which may explain the finding of IFN-α and IFN-γ inducibility in intestinal epithelial cells. We are the first to characterize regulation of MD-2 and its promoter. Our results demonstrate that a 1kB sequence upstream of the major translation initiation site of MD-2 directs expression of MD-2 in intestinal epithelial cells and endothelial cells. The level of MD-2 promoter activity was significantly higher in endothelial cells compared with intestinal epithelial cells, consistent with the observed differences in mRNA expression between these two tissues. IFN-γ led to transcriptional activation of the MD-2 promoter and increased MD-2 mRNA expression. Future experiments will elucidate the specific regions of the MD-2 promoter involved in IFN-γ mediated transcriptional activation of MD-2. IFN-γ-dependent increase in MD-2 promoter activity could be blocked by co-expression of a specific inhibitor of the JAK/STAT pathway, SOCS3. In human and animal models of colitis, high levels of STAT1 and STAT3 have been observed in ulcerative colitis, Crohn’s disease and murine dextran sodium sulfate-induced colitis (49). Transgenic expression of a dominant-negative mutant of SOCS3 worsens colitis in animals supporting the important role of this pathway in the development of colitis. The intersection of TLR4 and MD-2 expression with STAT signaling suggests another pathway involved in the development of colitis and thus a potential point of therapeutic intervention.

Our paper highlights a potential path of cooperativity between the innate and adaptive immune systems in intestinal epithelial cells. Cooperativity between the innate and adaptive
immune systems is seen in the clearance of Mycobacterium tuberculosis from macrophages which requires IFN-γ and TNF-α production by Th1 cells as well as LPS-dependent activation of TLR4 and TLR2 (62-65). Based on our findings using a variety of T-cell-derived cytokines, the intestinal epithelium may be recruited into the inflammatory response at a relatively late stage when the adaptive immune system has been activated and requires additional help to eliminate a pathogen. TLR4 and MD-2 appear to be differentially regulated by cytokines. Whereas TLR4 expression is increased by IFN-α, MD-2 expression is preferentially induced by IFN-γ. The coordinate exposure of intestinal epithelial cells to specific cytokines may be important to the generation of functional TLR4/MD-2 complexes. In addition, the response of intestinal epithelial cells to cytokines with respect to TLR4 and MD-2 expression may be distinct along the crypt-to-villus axis. Our studies lay the foundation for exploring the complex regulation of innate immunity in the gut.
Running title: Regulation of TLR4 and MD-2 in intestinal epithelial cells

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Figure Legends

Figure 1. Expression of TLR4 and MD-2 in human colonic epithelial cells compared with lamina propria mononuclear cells. A. Laser capture microscope dissection of colonic crypts and lamina propria cells. Normal human colon was obtained from surgical resections. Top row demonstrates dissection of the colonic crypts using a laser beam to remove colonic epithelial cells from the tissue (left panel-before, middle panel-after dissection) and transfer to a microfuge cap (right panel). A similar procedure was used for dissection the lamina propria (bottom row).

Figure 1. B. Expression of TLR4 and MD-2 were analyzed by PCR following reverse transcription of total RNA from colonic crypt epithelial cells (C/E) or lamina propria cells (C/L) from five different colonic resections. LPS-responsive human microvessel endothelial cells (HMEC) were used as positive control (+). β-actin was analyzed to verify similar cDNA loading (not shown). Both colonic epithelial cells and lamina propria cells express TLR4. The colonic crypts tested do not express MD-2. Samples 1 and 2 express MD-2 in the lamina propria.

Figure 2. Expression of TLR4 and MD-2 in response to cytokine stimulation of intestinal epithelial cells. A. Control (C) HT-29 cells (left) or T84 cells (right) were exposed to IFN-γ (I) 40ng/ml, TNF-α (T) 20 ng/ml singly or both (B) for the indicated times. β-actin (bottom panel) was analyzed to verify similar cDNA loading. Expression of TLR4 (150 bp) and MD-2 (150 bp) were analyzed by PCR following reverse transcription of mRNA from IEC as indicated. An AlphaImager densitometer was used to quantify intensity of PCR products corrected for expression of β-actin. Fold induction compared with unstimulated cells is shown. TLR4 mRNA is increased in HT-29 and T84 cells in response to IFN-γ and TNF-α with different kinetics.
Both cell lines demonstrate an increase in MD-2 mRNA following IFN-γ induction. This is one representative experiment of three with similar results.

2. B. T84 cells were exposed to IFN-α at indicated concentrations for 18 hours. GAPDH (top panel) was analyzed to verify similar cDNA loading. Expression of TLR4 (548 bp) was analyzed by PCR following reverse transcription of total RNA from T84 cells. TLR4 mRNA is increased in response to IFN-α in T84 cells. This is one representative experiment of three with similar results.

2. C. TLR4 protein expression in T84 intestinal epithelial cells following cytokine stimulation.

T84 cells were exposed to IFN-γ, TNF-α or IFN-α for 18h as indicated. Whole cell lysates of T84 cells were analyzed by western blot probed with an anti-human TLR4 Ab. Whereas TLR4 protein is not detectable in T84 cells, treatment with IFN-α and to a lesser extent TNF-α increased TLR4 protein expression.

Figure 3. Effect of cytokines on LPS-dependent IL-8 secretion in intestinal epithelial cells.

HT-29 cells were exposed to IFN-γ 40ng/ml, TNF-α 10ng/ml, singly or in indicated combinations for 18h and then exposed to LPS 50ng/ml for an additional 18h. Supernatants were harvested for measurement of IL-8. Graph is a representative experiment of three and was performed in triplicate. Error bars indicate standard error. Spontaneous IL-8 secretion was 975pg/ml. IL-8 secretion in response to LPS alone was 5774pg/ml, IFN-γ alone was 856pg/ml and IFN-γ followed by LPS was 8675. The increase in IL-8 secretion attributable to LPS following TNF↑α or IFN↑γ + TNF↑α was 47-fold and 30-fold higher, respectively, than the secretion of IL-8 in response to LPS stimulation alone (indicated by bracket) (difference in IL-8 production cytokine alone versus cytokine plus LPS divided by LPS alone). IFN↑γ does not by itself stimulate IL-8 secretion. Following IFN↑γ pre-incubation, HT-29 cells secreted 40%
more IL-8 than with LPS stimulation alone.

Figure 4. Cloning and characterization of the MD-2 promoter. Top panel demonstrates cloning of a −1kB and −2kB fragment of the MD-2 gene promoter upstream of luciferase in the pGL3 expression vector. Bottom panel demonstrates transfection of T84 cells and HMEC with −1kB-MD-2 pGL3, −2kB-MD-2 pGL3 or the empty pGL3 vector control and a β-galactosidase expression vector. The day following transfection, cells were lysed for luciferase and β-galactosidase measurements. Reporter gene activation was significantly higher in cells transfected with 1kB-MD-2 pGL3 or 2kB-MD-2 pGL3 vectors compared with the empty pGL3 vector control in both cell types, p<0.01. Reporter gene activation was significantly higher in HMEC compared with T84 cells, p=0.001. This graph is one experiment representative of three with similar findings and was performed in triplicate. Error bars indicate standard deviation.

Figure 5. Interferon-γ regulates MD-2 promoter activity through the JAK-STAT pathway

A. The MD-2 promoter is activated by IFN-γ in intestinal epithelial cells.

T84 cells were transfected with 1kB-MD-2 pGL3, 2kB-MD-2 pGL3 or the empty pGL3 vector control and a β-galactosidase expression vector. The day following transfection, T84 cells were stimulated with IFN-γ for 5h as indicated and cells lysed for luciferase and β-galactosidase measurements. Stimulation of 1kB-MD-2 pGL3 or 2kB-MD-2 pGL3 with IFN-γ significantly increased reporter gene activation in T84 cells, p=0.005 and p=0.001, respectively. Stimulation of 1kB-MD-2 pGL3 with IFN-α significantly increased reporter gene activation in T84 cells, p=0.008 but not the 2kB-MD-2 pGL3 reporter gene p=0.14. This graph is one experiment representative of three with similar findings and was performed in triplicate. Error bars indicate standard deviation.
Figure 5B. IFN-γ activates a gamma interferon-activated sequence (GAS) in intestinal epithelial cells. T84 cells were transfected with a luciferase reporter gene under the control of a multimerized GAS sequence derived from the IRF-1 promoter or an empty vector control and a β-galactosidase expression vector. The day following transfection, T84 cells were stimulated with IFN-γ 40ng/ml for 5h as indicated and cells lysed for luciferase and β-galactosidase measurements. Stimulation with IFN-γ significantly increased reporter gene activation in T84 cells, p<0.001. This graph is one experiment representative of three with similar findings and was performed in triplicate. Error bars indicate standard deviation.

5. C. Expression of a STAT inhibitor blocks IFN-γ-mediated activation of the MD-2 promoter. T84 cells were transfected with 1kB-MD-2 pGL3 or the empty pGL3 vector control and a β-galactosidase expression vector and co-transfected with a SOCS3 expression vector or its empty vector control. The day following transfection, T84 cells were stimulated with IFN-γ 40ng/ml for 5h as indicated and cells lysed for luciferase and β-galactosidase measurements. Stimulation with IFN-γ significantly increased reporter gene activation in T84 cells and this activation was blocked by 80% in cells co-transfected with SOCS3, p<0.01. This graph is one experiment representative of three with similar findings and was performed in triplicate. Error bars indicate standard deviation.
Figure 1

A.

Colon Crypts

Before		After		Capture

Lamina Propria

B.

| MD-2 | TLR4 |
|------|------|
| C/E  | 150 bp |
| C/L  | 150 bp |
Figure 2

A. 

**HT-29**

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **TLR4** | C  | I   | T   | B   |
| Fold     |    | 1.2 | 1.2 | 1.8 |
|          |    | 1.2 | 1.4 | 1.2 |

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **MD-2** | C  | I   | T   | B   |
| Fold     |    | 2.0 | 2.2 | 3.4 |
|          |    | 1.5 | 1.4 | 4.0 |

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **β-actin** | C | I   | T   | B   |
|          |    | 2.0 | 1.2 | 2.0 |
|          |    | 2.0 | 2.0 | 1.2 |
|          |    | 2.0 | 1.2 | 2.0 |

**T84**

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **TLR4** | C  | I   | T   | B   |
| Fold     |    | 1.2 | 2.0 | 3.0 |
|          |    | 1.5 | 1.4 | 1.2 |
|          |    | 1.2 | 1.4 | 1.2 |

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **MD-2** | C  | I   | T   | B   |
| Fold     |    | 2.0 | 1.2 | 2.0 |
|          |    | 2.0 | 2.0 | 1.2 |
|          |    | 2.5 | 1.2 | 2.0 |

|          | 6h | 18h | 24h |
|----------|----|-----|-----|
| **β-actin** | C | I   | T   | B   |
|          |    | 2.0 | 1.2 | 2.0 |

**B.**

**T84**

|          | 0  | 100 | 1000 |
|----------|----|-----|------|
| **GAPDH** |    | 983 | bp   |
| **TLR4**  |    | 548 | bp   |
| **MD-2**  |    | 422 | bp   |

|          | 1.0 | 1.3 | 1.2 |
|----------|-----|-----|-----|
| **Fold** |     |     |     |

|          | 1.2 |
|----------|-----|

|          | 1.0 |
|----------|-----|

**C.**

**T84**

|          | UTX | IFN-γ | TNF-α | TNF-α | IFN-α |
|----------|-----|-------|-------|-------|-------|
| **kDa**  | 92.3| 69.5  | 54.4  |       |       |

![Arrow pointing to a specific band on the blot]
Figure 3

IL-8 Production (pg/ml) x 10^3

IFN-γ  
TNF-α  
LPS
Figure 4
Figure 5

A. 

![Bar graph showing fold increase for IFN-α and IFN-γ treatments with pGL3b and -1kb, -2kb promoters.](image)

B. 

![Bar graph showing fold increase for GAS and Vector treatments with IFN-γ.](image)

C. 

![Bar graph showing fold increase for SOCS and IFN-γ treatments with pGL3b and -1kb promoters.](image)
TLR4 and MD-2 expression are regulated by immune-mediated signals in human intestinal epithelial cells
Maria T. Abreu, Elizabeth T. Arnold, Lisa S. Thomas, Rivkah Gonsky, Yuehua Zhou, Bing Hu and Moshe Arditi

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