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Trypsin-Sensitive Modulation of Intestinal Epithelial MD-2 as Mechanism of Lipopolysaccharide Tolerance

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Intestinal epithelial cells (IEC) are constantly exposed to both high concentrations of the bacterial ligand LPS and the serine protease trypsin. MD-2, which contains multiple trypsin cleavage sites, is an essential accessory glycoprotein required for LPS recognition and signaling through TLR4. The aim of this study was to characterize the expression and subcellular distribution of intestinal epithelial MD-2 and to delineate potential functional interactions with trypsin and then alteration in inflammatory bowel disease (IBD). Although MD-2 protein expression was minimal in primary IEC of normal colonic or ileal mucosa, expression was significantly increased in IEC from patients with active IBD colitis, but not in ileal areas from patients with severe Crohn’s disease. Endogenous MD-2 was predominantly retained in the calnexin-calreticulin cycle of the endoplasmic reticulum; only a small fraction was exported to the Golgi. MD-2 expression correlated inversely with trypsin activity. Biochemical evidence and in vitro experiments demonstrated that trypsin exposure resulted in extensive proteolysis of endogenous and soluble MD-2 protein, but not of TLR4 in IEC, and was associated with desensitization of IEC to LPS. In conclusion, the present study suggests that endoplasmic reticulum-associated MD-2 expression in IBD may be altered by ileal protease in inflammation, leading to impaired LPS recognition and hyporesponsiveness through MD-2 proteolysis in IEC, thus implying a physiologic mechanism that helps maintain LPS tolerance in the intestine.


Perturbed homeostasis between commensal bacteria and host immunity may serve as a critical determinant in the development of gut inflammation in the two major forms of idiopathic inflammatory bowel diseases (IBD),3 ulcerative colitis (UC) and Crohn’s disease (CD) (1). TLRs play a key role in microbial recognition, control of adaptive immune responses, and induction of antimicrobial effector pathways, contributing to efficient elimination of pathogens (2). In health, TLR2 signaling may protect intestinal epithelial barrier (3) and TLR4 may confer tolerance to commensal bacteria (4), whereas, in disease, aberrant TLR4 signaling may stimulate acute and chronic inflammatory responses (5, 6). As the frontline of the mucosal immune system, the intestinal epithelium is constantly exposed to large amounts of a variety of TLR ligands that coexist in the intestinal mucosa. Endogenous control mechanisms of intestinal epithelial tolerance include decreased expression of TRIF and high levels of Tollip leading to inhibition of proinflammatory responses in the presence of commensals (4, 7, 8). Other mucosal or luminal products may also regulate the functional activity of the TLR family (9).

TLR4 requires the coreceptor MD-2 for optimal LPS recognition and signaling (10–13). Mature MD-2 is a secreted glycoprotein of 142-aa residues that binds LPS with high affinity (14). Separate functional domains important for TLR4 binding and LPS signaling have been identified in MD-2 (15). MD-2 transfectants form disulfide-linked dimers, as well as large oligomers, which can be disrupted by disulfide reduction. However, the functional relevance of these larger forms is not fully understood (12, 16). Primary intestinal epithelial cells (IEC) constitutively lack significant amounts of TLR4 and MD-2 in healthy, normal intestinal mucosa (4, 17, 18). In contrast, TLR4 is significantly increased in primary IEC throughout the lower gastrointestinal tract in active disease of both CD and UC (17) as well as murine colitis (19, 20). Based on in vitro findings, it has recently been proposed that proinflammatory cytokines, such as TNF-α or IFN-γ, may significantly up-regulate MD-2 expression in acute intestinal inflammation of IBD (18, 21). LPS may then elicit several proinflammatory responses and trigger downstream events that may promote disease (4, 18, 21, 22).

The serine protease trypsin has previously been implicated in tissue destruction in acute small intestinal inflammation (23, 24). However, by acting as prodefensin convertase in Paneth cells, trypsin is also involved as host defense mechanism in the regulation of innate immunity in the small intestine (25). Trypsin treatment of monocytes inhibits IL-6 secretion in response to LPS, possibly through digestion of a cell surface protein distinct from membrane-bound CD14 (26) that has not yet been further identified. Because MD-2 contains multiple trypsin-cleavage sites, we speculated that trypsin may have digested MD-2 protein and this MD-2 degradation could potentially act as an innate tolerance mediator in the small intestine to limit LPS recognition and subsequent signaling.
To further understand the nature of interaction between IEC and LPS, we therefore analyzed the morphological alterations and subcellular distribution of MD-2 in IBD and subsequent signaling events in the presence and absence of trypsin.

**Materials and Methods**

**Reagents and Abs**

Highly purified (99.9% free of DNA and protein) LPS from *Escherichia coli*, serotype R515 (lots L11290; 12163), was obtained from Alexis Biochemicals. High-purity (HPLC: >99%) and LPS-free trypsin (lot B53196; sp. act., 5308 United States Pharmacopeia U/mg protein) from bovine pancreas was purchased from Calbiochem. The synthetic lipopeptide PamCys-SKKKX3H3C (PamCysSk34 (PCSK); lot F06) was obtained from EMC Microcollections. Recombinant soluble human MD-2 was synthesized in baculovirus (14) and concentrated using Microcon columns (YM-10) from Millipore. Human rCD14 and LPS binding protein (LBP) were obtained from R&D Systems. Cell culture-tested, endotoxin-free trypsin inhibitor was purchased from Sigma-Aldrich.

Rabbit polyclonal Abs to human MD-2 (lot AB073003A) and human TLR4 were purchased from Immunex and eBioscience, respectively. Mouse mAb to human trypsin was obtained from Chemicon International. Mouse mAbs to phospho-cJun (JM-2), phospho-JNK2 (G-7), and p65 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal to His-Tag (27E8) and hemagglutinin (HA)-Tag mAbs to phospho-cJun (KM-1), phospho-JNK2 (G-7), and p65 were purchased from BD Transduction Laboratories. HRP-conjugated anti-rabbit and anti-mouse Abs were purchased from Amersham Biosciences. Alexa Fluor488-conjugated goat anti-mouse IgG Ab was obtained from Jackson Immunoresearch Laboratories. Alexa Fluor546-conjugated goat anti-mouse IgG Ab (highly adsorbed) was purchased from Molecular Probes. Normal rabbit IgG (Santa Cruz Biotechnology) and mouse IgG (eBioscience) were used as negative controls.

**Population and tissue samples**

Tissue samples (n = 26) were obtained from 20 patients undergoing complete colonoscopy at the Massachusetts General Hospital or South Essex Hospitals. Informed consent was obtained from all patients before the procedure, and the protocol was approved by the Human Studies Committee of the Massachusetts General Hospital or the South Essex Hospitals, respectively. In each case, the diagnosis was confirmed by standard endoscopic and histological criteria (each sample was stained with H&E (Thermo Shandon), viewed, and captured with a Leica DMLB microscope (Leica Microsystems)). Specimens were taken from all areas of the colon and the terminal ileum. Three patients (two CD and one UC) were in remission and had no clinically identifiable, active disease at the time of sample acquisition. Control (non-IBD) specimens were taken from patients with normal endoscopic findings and without macroscopic evidence for inflammatory or neoplastic disease. This group included, predominantly, patients who underwent regular colon cancer screening examinations and/or polypectomy. Thus, reported findings show representative results of all samples examined from each subgroup (non-IBD, CD, and UC). Fresh tissue samples were immediately snap frozen in OCT compound (Miles Laboratories) and stored at –80°C until further processing.

**Cell culture**

The human model IEC line SW480 constitutively expresses high mRNA levels of MD-2 and TLR4 (4, 21), but no membrane-bound CD14 (27), and thus, ideally mimics the characteristics of the intestinal epithelium in active colonic IB in vivo (17, 28). Cells (passages 1–16) were obtained from the American Type Culture Collection and maintained in a humidified incubator at 37°C without CO₂, in Leibovitz’s L-15 medium with l-glutamine (PAA Laboratories), supplemented with 10% v/v nonheat-inactivated FCS (PAA Laboratories; endotoxin free; lot A01161-245), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories). When confluent, SW480 cells were passaged by enzymatic-free, Hanks-based cell dissociation buffer (Invitrogen Life Technologies). To minimize influences of MD-2 regulators as well as protease inhibitors present in serum, cells were washed once and serum starved (0.1% v/v FCS) overnight before all experiments, if not specified otherwise. The clone of HEK-293 cells stably transfected with human HA-tagged TLR4 gene (passage 15–17; lot 2601/293H/TLR4A) was obtained from InvivoGen and maintained in a humidified incubator at 37°C with 5% CO₂ in high glucose (4.5 g/L) DMEM (PAA Laboratories), supplemented with 10% FCS and Blasticidin (10 μg/ml; InvivoGen). HEK293 cells stably transfected with full-length MD-2 or control vector (pcDNA) were maintained, as recently described (11).

**Trypsin and glycosidase digestion**

For digestion with trypsin (adapted from Ref. 29), the protease (1:14) was added to 34 μg of rMD-2 at 4°C after taking the zero time aliquot. Equal aliquots of the digestion mixture were withdrawn at different time points, transferred to a tube containing LPS-free 1× trypsin inhibitor, and separated by (MES) SDS-PAGE. Digestion of SW480-microsomal fraction was performed in the presence or absence of 0.1 μg/ml LPS with 0.3% Triton X-100 at 4°C for 60 min (30). For digestion with Endo H, microsomal fraction was denatured, as previously described (31).

For trypsin treatment of live cells (26, 32, 33), cells (0.1% FCS) were incubated in the presence or absence of low concentrations of trypsin (10–20 nM) for 30 min at 37°C, followed by LPS stimulation (250–2000 ng/ml) for the indicated time periods, and then assayed. In some experiments, trypsin (40 nM) was incubated with or without soluble MD-2 (0.85 μg) for 30 min at 37°C. The digestive reaction was stopped by adding trypsin inhibitor, and resulting complexes were then added directly to cells. Under all these conditions, cells remained adherent and viable (trypsin blue).

**Protein isolation**

For preparation of whole cell lysates, cells were rinsed twice in cold PBS (without Ca²⁺/Mg²⁺) with 100 μM Na₂VO₃ and then lysed in ice-cold lysis buffer (1% Nonidet P-40 (Pierce), 50 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, containing 10 mM NaF, 10 mM DTT, 10 mM Na₂VO₃, complete Mini protease inhibitor cocktail tablet (Roche), and 2 mM PMSF ‘plus’ (Roche). For detection of MD-2 protein, cells were lysed in a different lysis buffer (1% Triton X-100 (Pierce), 137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM NaF, 10 mM Na₂VO₃, protease inhibitors (complete Mini), and 1 mM PMSF) (12). Lysates were centrifuged (12,000 × g, 15 min at 4°C), and protein concentration was determined by colorimetric Bradford protein assay (Bio-Rad). For preparation of subcellular fractions, the ProteoExtract Subcellular Proteome Extraction Kit from Calbiochem was used, according to the manufacturer’s instructions. For preparation of microsomes, SW480 cells grown subconfluent on 175-cm² flasks were detached, washed with ice-cold PBS twice, and allowed to swell on ice for 20 min in extraction buffer A (hypotonic: 10 mM HEPES (pH 7.8), 1 mM EGTA, 25 mM KCl). For preparation of whole cell lysates, trypsin (40 nM) was incubated with or without soluble MD-2 (0.85 μg) for 30 min at 37°C. The digestive reaction was stopped by adding trypsin inhibitor, and resulting complexes were then added directly to cells. Under all these conditions, cells remained adherent and viable (trypsin blue).

**Immunoprecipitation and Western blotting**

For immunoprecipitation, the supernatants were preclarified at 4°C overnight by adding 2.5 μg of mouse IgG and 50 μl of protein G-agarose (3%) v/v; Amersham Biosciences) and then incubated with primary Ab (1:200) and all of protein G-agarose (3%) overnight at 4°C. Beads were washed four times with ice-cold lysis buffer. For Western blotting, proteins were heated with or without addition of 10 mM DTT (85°C, 2 min) in LDS sample buffer (Invitrogen Life Technologies), subjected to SDS-PAGE (4–12% bis-Tris; MOPS or MES; Invitrogen Life Technologies), and transferred, followed by blocking (TBST with 5% nonfat dry milk or 1–5% BSA) for 1 h at room temperature, and immunoblotting with primary Ab (1:100–1:10,000 in 5% nonfat dry milk or 0.1–5% BSA) overnight at 4°C and then with HRP-conjugated secondary Ab (1:4,000–1:8,000; 4% nonfat dry milk in TBST) for 1 h at room temperature. Blots were developed, as previously described (34). All experiments were repeated at least three times; representative results are shown for each experiment.

**Immunohistochemistry**

To facilitate permeabilization, all biopsy specimens were pretreated with freshly made, ice-cold 2% paraformaldehyde (Electron Microscopy Sciences) and 0.1% v/v Triton X-100 (3%) overnight at 4°C, then fixed in 4,5% buffered Formalin overnight, embedded in paraffin wax, sectioned (4 μm), and mounted on polystyrene-coated glass slides (Menzel). After deparaffinization, rehydrated sections were exposed to microwave pretreatment (in 10 mM Tris/1 mM EDTA/0.05% Tween 20 (pH 9.0), at 600 W for two periods of 5 min) to enhance antigenicity. Nonspecific binding was blocked with nonfat dry milk serum (1:10–1:10,000 in PBS; Vector Laboratories) for 60 min room temperature, followed by primary Abs (1:50–1:100 in PBS) overnight at 4°C. To demonstrate tissue integrity, the section of interest or a parallel section on the same glass slide was stained for E-cadherin.
Immunocytochemistry

Serum-deprived 293hTLR4HA or 293-MD-2 cells were cultured overnight on collagen I-precoated 8-well glass slides (BD Discovery Labware) and SW480 cells on 4- or 8-well permanox slide chambers (Nalge Nunc International, respectively). After stimulation the following day, cells were washed once with PBS (without Ca\(^{2+}\)/Mg\(^{2+}\)) at 37°C, then fixed with methanol/acetone (50:50) for 5 min at −20°C, air dried, and washed three times with TBST. Cells were blocked with normal goat serum (1:100 in PBS) for 60 min at room temperature and incubated with primary Abs (1:50 –1:100) overnight at 4°C.

Confocal immunofluorescence microscopy

Alexa Fluor488-conjugated goat anti-mouse and/or CY5-conjugated goat anti-rabbit IgG Abs were used as secondary Abs (1:100, 60 min, room temperature). Samples were mounted (Vectorshield Mounting Media with anti-rabbit IgG Abs were used as secondary Abs (1:100, 60 min, room temperature and incubated with primary Abs (1:50 –1:100) overnight at 4°C.

Qualitative and quantitative colocalization analysis

The two channels were merged in the Zeiss LSM510 v3.2 software during acquisition, allowing qualitative judgments about colocalizing of dual fluorescent dyes (Alexa Fluor488 and CY5). Two-step image analysis was then performed: 1) after computing a scattergram of raw data, a morphological filter was applied, removing the noise spikes while maintaining the original contrast ratio between specific staining and background. A subsequent filter then automatically selected the most contrasted areas, leading to a binary, threshold mask, in which the colocalized image pixels (yellow) were extracted from the rest of the images. 2) Quantification of the extent of colocalization in the sections was assessed using an algorithm in the image analysis program (Colocalizer Pro 1.2 Software) to determine the Pearson’s correlation and the Manders’ overlap coefficients, as previously described (35).

ELISA

For human IL-8 ELISA, 1–2 × 10\(^5\) SW480 cells/well were plated in 48-well plates. IL-8 concentration in cell culture supernatant was determined 4 h after stimulation using the OptiEIA IL-8 ELISA kit II (BD Biosciences), according to the manufacturer’s instructions.

Confirmation of specificity of anti-MD-2 Ab

The specificity of the commercially available anti-MD-2 Ab (36) to human MD-2 Ag was initially confirmed by Western blotting and immunocytochemistry (data not shown): multimers of the recombinant, His-tagged soluble MD-2 protein made from baculovirus were specifically detected by anti-His Ab at the approximate sizes of 42, 62, and 80 kDa, as previously described (12, 16, 37). A single doublet of apparent molecular mass of 25 kDa was detected consistent with the MD-2 monomer. Similar results were
obtained by anti-MD-2, revealing corresponding multimers at the same approximate sizes. Subsequent reprobing with isotype IgG showed no specific bands at the appropriate molecular sizes. Ab specificity was additionally confirmed by showing presence of distinct MD-2 staining in MD-2-positive HEK293 cells, but absence in MD-2-deficient HEK293-control cells.

Statistical analysis

Data are expressed as means ± SD of two or more independent experiments. Differences between means were evaluated using the two-sided t test (Microsoft Excel; Microsoft), where appropriate. Values of \( p < 0.05 \) were considered as significant.

Results

**MD-2 expression is absent in normal colonic or ileal IEC, but significantly up-regulated in active IBD colitis**

Only minimal endogenous MD-2 protein expression was detected in primary IEC and lamina propria mononuclear cells (LPMNC) in all samples of normal terminal ileum (Fig. 1B) and colon. In contrast, MD-2 was abundantly expressed by colonic epithelial cells and LPMNC in involved areas of inflammation in all patients with active UC (Fig. 1D) and CD colitis (Fig. 1F). No difference between crypts vs villi was observed. However, MD-2 expression was only minimally detectable in intact IEC and LPMNC in mucosal areas of terminal ileum exhibiting active CD despite the presence of severe inflammation with massive infiltration of inflammatory cells in the lamina propria (Fig. 1H). Moderate intestinal epithelial MD-2 staining was occasionally found in microscopically noninvolved margins of these inflamed regions. MD-2-positive IEC were also found in inactive disease in both terminal ileum and colon of two CD patients, but not in quiescent colonic disease of one UC patient (data not shown). Inflammation in corresponding intestinal biopsy samples was confirmed by conventional H&E staining (Fig. 1, A to B, C to D, E to F, and G to H). Specificity of the Ab reaction was confirmed by presence or absence of staining by: 1) omission of the primary Ab (Fig. 1J); 2) replacement of the primary Ab with isotype control IgG at equivalent concentration; and 3) absorption of anti-MD-2 Ab with MD-2 protein (Fig. 1, K (anti-MD-2 + PBS) vs L (anti-MD-2 + soluble MD-2).

**Endogenous MD-2 primarily interacts with selective endoplasmic reticulum (ER) chaperones in IEC**

To define the subcellular localization of endogenous MD-2, double immunofluorescence labeling using confocal laser-scanning microscopy was performed on tissue sections of active UC (Fig. 2). Clear discernible, mostly apico-cytoplasmic staining of MD-2 that significantly colocalized with the fine reticular cytoplasmic and perinuclear pattern of both ER chaperones, calreticulin (Fig. 2A) and calnexin (Fig. 2B), was present in primary IEC and confirmed in SW480 cells in vitro (data not shown). In contrast, significant MD-2 colocalization with the cis-Golgi matrix, GM130, was only detected in a few, peripheral areas (Fig. 2C). Comparable results

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**FIGURE 2.** Intestinal epithelial MD-2 is mainly retained in the calnexin/calreticulin cycle in active IBD-associated colitis. Active UC colon cells were stained with anti-MD-2 pAb (\( A^2-C^2 \)) and subcellular markers (\( A^3 \), calreticulin; \( B^3 \), calnexin; or \( C^3 \), GM130), followed by anti-rabbit Cy5 (\( A^2-C^2 \) (red))- and anti-mouse Alexa Fluor488 (\( A^3-C^3 \) (green))-conjugated secondary Abs and visualized by confocal laser microscopy, as described in Materials and Methods. To confirm structural integrity of the colitic tissue section, an additional biopsy section (4 \( \mu M \) apart) on the same glass slide was stained in parallel with anti-E-cadherin mAb (\( A^4-C^4 \)), followed by anti-mouse Alexa Fluor488-conjugated secondary Ab (\( A^4-C^4 \); green). For subcellular orientation, nuclei were counterstained with propidium iodide (\( A^5-C^5 \); blue). Merged (\( A^2-C^2 + A^3-C^3 + A^4-C^4 \) (yellow)); extraction of colocalization areas (\( A^5-C^5 \) (yellow)). Representative samples of one patient with active UC are shown (\( A^2-C^2 \), ×40/1.3 oil; scan zoom, 1.0; all other images, ×63/1.4 oil; scan zoom, 2.0).
FIGURE 3. MD-2 resides in an Endo H-sensitive compartment and colocalizes with the ER-resident chaperone calnexin in SW480 cells. A. Microsomal fractions of SW480 monolayers (0.1% FCS; confluent on 175-cm² flask) were prepared by sequential centrifugation and treated with Endo H for 60 min at 37°C, and resulting degradation products were analyzed by SDS-PAGE and Western blotting using anti-MD-2 pAb, as described in Materials and Methods. B. SW480 monolayers (0.1% FCS) were treated with LPS (500 ng/ml) for 60 min, and cell fractions were immunoprecipitated with anti-calnexin, analyzed by SDS-PAGE under reducing conditions and Western blotting using anti-MD-2, and reprobed using anti-calnexin.

were obtained in samples of patients with CD colitis (data not shown).

To further substantiate that intestinal epithelial MD-2 indeed preferentially resides in the ER, microsomes of SW480 cells were isolated by differential centrifugation and treated with Endo H, which led to partial digestion of the multimeric complex to the monomeric doublet of MD-2 with apparent molecular mass of ~25 kDa, implying that intestinal epithelial MD-2 glycoprotein carries both Endo H-resistant and Endo H-sensitive, N-linked oligosaccharides (Fig. 3A). To quantitate the degree of colocalization of endogenous MD-2 with these organelle markers in SW480 cells, Pearson’s correlation coefficients were measured: MD-2/calreticulin, 0.614 ± 0.10; MD-2/calnexin, 0.703 ± 0.06; MD-2/GM130, 0.351 ± 0.01. The difference between colocalization of MD-2 with ER vs MD-2 with cis-Golgi was determined to be statistically significant (MD-2/calreticulin vs MD-2/GM130, p < 0.001; MD-2/calnexin vs MD-2/GM130, p < 0.001). But the quantitated intensity of the overlap of MD-2 with calnexin was significantly greater in response to LPS than compared with calreticulin (MD-2/calnexin vs MD-2/calreticulin: −LPS, p = 0.05; +LPS, p < 0.01). Mander’s overlap coefficient values were comparable to the values of the Pearson’s correlation coefficient for each experimental condition (p > 0.5). Immunoprecipitation corroborated the interaction of endogenous MD-2 with the ER marker calnexin in SW480 cells. MD-2 multimer was detected in anticalnexin immunoprecipitates in membrane fractions, but not in control nuclear fractions (Fig. 3B). Consistent with the precedent immunofluorescence coefficient finding, interaction between endogenous MD-2 and calnexin was significantly enhanced in response to LPS.

Trypsin-induced proteolytic digestion of MD-2 multimer desensitizes IEC to LPS

We then investigated the possible cause of low expression of MD-2 in ileal areas of severe inflammation in active CD. Protein analysis using ExPASy demonstrated that MD-2 contained multiple trypsin cleavage sites. High amounts of proteolytically active trypsin have recently been found predominantly at mucosal sites of acute inflammation in the small intestine (24, 38, 39). As confirmed by immunofluorescence microscopy, trypsin content was significantly increased in severely inflamed areas of ileal mucosa in active CD ileitis, whereas colitic specimens of UC or CD patients exhibited negligible amounts of trypsin (Fig. 4). Because staining intensity of trypsin correlated inversely with MD-2 expression (Fig. 4, A vs B), we investigated the potential ability and functional consequence of trypsin to cleave soluble and cellular MD-2 in IEC.

Digestion of soluble MD-2 protein by trypsin resulted in the appearance of a major polypeptide at 20 kDa after 10 s. When the proteolytic process was prolonged up to 10 min, this doublet band was further cleaved to fragments of 10 kDa (Fig. 5A). Live HEK293 cells stably transfected either with MD-2 or TLR4 were then stimulated with trypsin in vitro. Immunofluorescence demonstrated significant reduction of peripheral MD-2 staining in response to trypsin, but not TLR4 (Fig. 5B). Trypsin treatment of live SW480 cells that express endogenous MD-2 correlated with a significant decrease of LPS-induced MD-2 multimer formation of 62 kDa in whole cell lysates, as assessed by Western blotting (Fig. 5C). Trypsin also had no effect on endogenous expression of TLR4 in SW480 cells. As no digested oligomers of MD-2 were detectable by this crude cell lysate extraction method, probably buried in a membrane-associated compartment that was not broken up, microsome-enriched fractions from nonstimulated IEC were isolated and treated with trypsin ex vitro. Endogenous MD-2 multimer formation at ~90-kDa band was readily digested by trypsin in the presence of detergent to yield a broad ~25-kDa band (consistent with the known size of the MD-2 monomer) and a weak ~10-kDa species (consistent with Fig. 5A), resulting in a thin undigested band of multimeric MD-2 (~80 kDa) (Fig. 5D).

FIGURE 4. MD-2 staining intensity correlated inversely with amount of mucosal trypsin in intestinal inflammation. A and B. Fresh ileal and colonic specimens were costained with anti-MD-2 pAb (white) and anti-trypsin mAb (blue), followed by anti-rabbit Cy5- and anti-mouse Alexa Fluor488-conjugated secondary Abs, respectively, and visualized by confocal laser microscopy, as described in Materials and Methods. Representative images are shown: active distal UC (A); active CD ileitis (B). LP, lamina propria; E, epithelium. Blue arrows indicate trypsin-positive staining (×40/1.3 oil; scan zoom, 2.0).
We next investigated the functional consequences of trypsin-induced proteolysis of endogenous MD-2 on LPS signaling via TLR4 in IEC. In time-dependent parallel to trypsin-induced proteolysis of MD-2 (Fig. 5), pretreatment with trypsin significantly attenuated downstream LPS-induced phosphorylation of JNK2 and c-Jun (Fig. 6A). Trypsinized cells remained fully responsive to the TLR2 ligand PCSK (Fig. 6A). When adding high concentrations of serum (10% FCS), trypsin-induced LPS tolerance was completely abolished (Fig. 6B). We then examined the effects of trypsin on the subcellular localization of the p65 subunit of NF-κB in LPS-LBP-CD14-stimulated IEC by immunofluorescence analysis (Fig. 6C). As shown recently, LPS-induced NF-κB activation is limited under low serum conditions in IEC (22). Binding of CD14 with LPS is catalyzed by LBP and then binds TLR4 complexed with MD-2 to rapidly transduce LPS signals (40). To enhance ligand responsiveness under serum-deprived (0.1% FCS) conditions, we therefore added LPS together with LBP-CD14 in complex form in the following NF-κB assays. Under basal conditions in the presence or absence of trypsin, p65 was predominantly localized in the cytoplasm of IEC. Stimulation with LPS-LBP-CD14 resulted in significant translocation of p65 from the cytoplasm to the nucleus, reflecting significant activation of NF-κB, which was completely abolished when pretreating cells with trypsin.

To investigate whether soluble MD-2 was also rendered inactive by trypsin digestion and to assess the role of trypsin in intestinal epithelial LPS tolerance, rMD-2 was preincubated with or without trypsin for 30 min, and resulting digests were added together with LPS-LBP-CD14 complexes to IEC monolayers for 4 h. As measured by ELISA, IL-8 secretion was significantly induced by LPS-LBP-CD14 (Fig. 6D). IL-8 response was increased further by addition of intact MD-2, but not trypsin-digested MD-2, implying that trypsin acts directly on MD-2 to attenuate LPS-induced immune responses in IEC.

Discussion
MD-2 is an accessory protein of TLR4, essential for assembling a functional receptor complex to sense low concentrations of LPS. The present study provides evidence that ER-associated MD-2 expression is selectively altered in association with IBD, which may (at least partially) be induced by high amounts of ileal trypsin in inflammation, and that trypsin-induced proteolysis of MD-2 desensitizes IEC to LPS. These findings suggest a previously unappreciated physiologic mechanism that contributes to LPS tolerance in the small intestine.

We demonstrate that primary IEC of normal, nondiseased mucosa expressed minimal endogenous MD-2 protein. MD-2 downregulation in the healthy intestinal mucosa could thus minimize recognition of luminal LPS. In contrast, MD-2 was significantly increased in IEC throughout the colon in active IBD patients. We have demonstrated recently that TLR4 expression is significantly increased in IEC throughout the colon in active IBD patients. Based on this study and our previous findings, LPS tolerance may be broken in IBD-associated colitis as a result of combined MD-2/TLR4 up-regulation. It remains to be shown whether gain-of-function mutations of MD-2 exist that could functionally exhibit exaggerated proinflammatory effects in IEC.

FIGURE 5. Trypsin induces MD-2 proteolysis in IEC. A, Equal aliquots of rMD-2 were treated with trypsin (14:1) for various time periods, reactions were stopped by addition of trypsin inhibitor, and resulting digests were separated by (MES) SDS-PAGE under nonreducing conditions, immunoblotted, and probed with anti-MD-2 pAb, as described in Materials and Methods. B, HEK cells stably transfected with either full-length MD-2 or HA-tagged TLR4 (0.1% FCS) were stimulated with or without trypsin (10 nM) for 30 min. Cells were fixed and stained with anti-MD-2 pAb or anti-HA mAb (white), followed by anti-rabbit CY5- or anti-mouse Alexa Fluor488-conjugated secondary Abs, respectively. Nuclei were counterstained with propidium iodide (red) (×40/1.3 oil; scan zoom: 4.0 (upper panel), 2.0 (lower panel)). C, SW480 cells (0.1% FCS) were stimulated with trypsin (20 nM) for 30 min, followed by LPS stimulation (500 ng/ml) for 60 min. Crude whole cell lysates for detection of MD-2 protein were prepared, immunoblotted, and probed with anti-MD-2 pAb, as described in Materials and Methods. Blots were reprobed with anti-TLR4 pAb. D, Microsomal fractions isolated from subconfluent SW480 cells by sequential centrifugation were treated in the presence or absence of 0.1 μg/μl trypsin with 0.3% Triton X-100 at 4°C for 60 min. Resulting digests were separated by SDS-PAGE under reducing conditions, immunoblotted, and probed with anti-MD-2 pAb, as described in Materials and Methods.
response to physiological concentrations to LPS. To our knowledge, no possible IBD predisposition region has yet been described at the MD-2 locus (chromosome 8q21.11). MD-2/TLR4 up-regulation could also result from ligands other than lumenal LPS. T cell-derived cytokines, such as IFN-γ and TNF-α, which play significant pathophysiological roles in triggering mucosal inflammation in IBD, have been found to up-regulate intestinal epithelial MD-2 and TLR4 expression in vitro (18, 21).

We further show in this study that in active CD, intestinal epithelial MD-2 expression was significantly up-regulated in colonic inflammation, but, in contrast, hardly detectable in the involved areas of severely inflamed terminal ileum. IEC of noninvolved areas close to ileal inflammation were positive for MD-2 in CD, suggesting that translational synthesis is not primarily disturbed and that reduction may rather reflect the secondary effect of some inflammatory mediator(s) present in active ileitis. Trypsin exposure directly induced significant proteolysis of soluble and endogenously expressed MD-2 multimer in IEC. This suggests that low MD-2 expression in active CD ileitis may reflect the local effect of enzymatic digestion by high mucosal levels of trypsin in the inflamed tissues. In contrast, TLR4 was resistant to digestion by trypsin. Others have previously shown that trypsin exposure did not degrade CD14 (26), suggesting that MD-2 is the sole trypsin-sensitive target of the LPS-recognition receptor complex.

Trypsin-induced proteolysis of MD-2 led to significant attenuation of downstream immune responses to LPS, which remain responsive to the TLR2 ligand PCSK (as shown in this study) or other agonists such as TNF, formyl peptide, or PMA (26). Digestion of MD-2 glyco complexes with trypsin yielded multiple proteolytic fragments that produced decreased TLR4-dependent cell activation in response to LPS, presumably by abolishing the interaction of LPS with TLR4.

![FIGURE 6. Trypsin desensitizes IEC immune responses to LPS.](http://www.jimmunol.org/)

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<tr>
<th>Condition</th>
<th>LPS (250ng/ml, 60 min)</th>
<th>PCSK (10μg/ml, 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trypsin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Presented data reflect at least two independent experiments, each performed in duplicate per condition. Data are expressed as means ± SD (LPS+LBP+CD14 vs LPS, LBP, CD14) vs negative, p < 0.001; +, LPS+LBP+CD14 vs soluble MD-2 (sMD-2) + (LPS+LBP+CD14), p < 0.032; *, trypsin/sMD-2 + (LPS+LBP+CD14) vs sMD-2 + (LPS+LBP+CD14), p < 0.023, trypsin/sMD-2 + (LPS+LBP+CD14) vs (LPS+LBP+CD14), 0.720.)

A. Trypsin desensitizes IEC immune responses to LPS. A, SW480 cells (0.1% FCS) were pretreated with or without trypsin (20 nM) for 30 min, followed by LPS stimulation (250 ng/ml) or PCSK (10 μg/ml) for 60 min. Cell lysates were immunoblotted and probed with anti-phospho-JNK2 Ab and re probed with anti-β-actin. B, SW480 cells (0.1 or 10% FCS) were pretreated with or without trypsin (20 nM) for 30 min, followed by LPS stimulation (250 ng/ml). Cell lysates were immunoblotted and probed with anti-phospho-cJun Ab and re probed with anti-cJun. C, SW480 cells (0.1% FCS) were pretreated with or without trypsin (10 nM) for 30 min, followed by stimulation with LPS-LBP-CD14 complexes containing LPS (1 μg/ml), LBP (10 ng/ml), and CD14 (10 ng/ml) for 60 min. Cells were fixed and stained with anti-p65 mAb (left panel), followed by anti-mouse Alexa Fluor488-conjugated secondary Ab. Nuclei were counterstained with propidium iodide (right panel). D, Soluble MD-2 (0.85 μg) was incubated with or without trypsin (40 nM) for 30 min at 37°C; trypsin activity was then stopped by addition of trypsin inhibitor; and resulting complexes were immediately added to SW480 cell monolayers for 4 h. Cells were costimulated with LPS-LBP-CD14 complexes containing LPS (500 ng/ml), LBP (10 ng/ml), and CD14 (10 ng/ml). Presented data reflect at least two independent experiments, each performed in duplicate per condition. Data are expressed as means ± SD (LPS+LBP+CD14 vs LPS, LBP, CD14) vs negative, p < 0.001; +, LPS+LBP+CD14 vs soluble MD-2 (sMD-2) + (LPS+LBP+CD14), p < 0.032; *, trypsin/sMD-2 + (LPS+LBP+CD14) vs sMD-2 + (LPS+LBP+CD14), p < 0.023, trypsin/sMD-2 + (LPS+LBP+CD14) vs (LPS+LBP+CD14), 0.720.)
shown to be unable to confer LPS responsiveness (37). However, through intramolecular disulfide bonds between oligomers that create an active tertiary structure, MD-2 is capable of binding with TLR4 and facilitating receptor activation to LPS (15, 41, 42). Trypsin may thus contribute to intestinal epithelial LPS tolerance through the mechanism of proteolytic disruption and modulation of MD-2 protein. We showed in initial studies (43) that LPS-induced downstream signaling of c-Jun was not inhibited by pretreatment with neutralizing antiserum against proteinase-activated receptor-2, a G protein-coupled receptor for trypsin, whereas proteinase-activated receptor-2 agonist-mediated immune response was not blocked by neutralizing antiserum against TLR4, suggesting that these two pathways function independently (data not shown).

Trypsin-induced intestinal epithelial LPS tolerance was significantly abrogated by supplementation of high concentrations of serum in vitro. Serum is known to contain several different types of polypeptides that are able to inhibit the catalytic function of trypsin (44), such as α2-macroglobulin (45), which are elevated in acute inflammation and could therefore unmask innate immune responses to LPS in IEC (and other cells) via the intact and up-regulated TLR4/MD-2 complex.

Based on biochemical evidence, it has recently been suggested that augmented amounts of soluble MD-2 may reside in the ER/cis-Golgi at steady state (12). In this study, we confirm this finding in IEC and show that endogenous MD-2 predominantly resides in the ER. We also demonstrate that trypsin induced direct proteolysis of microsomal (= ER-associated) MD-2. Intracellular trypsin has recently been localized to perinuclear secretory granules in IEC (46), potentially as part of the ER/Golgi (47), i.e., in close proximity to intracellular MD-2. Taken together, these findings imply that the ER may represent the main organelle site of interaction between MD-2 and intracellular trypsin in IEC.

We provide further evidence that endogenous MD-2 is retained in the chaperone system for proofreading of newly synthesized proteins, the so-called calnexin-calreticulin cycle, of IEC in acute inflammation in vivo. MD-2 undergoes N-linked glycosylations at Asn194 and Asn114 that are essential for TLR4-mediated activation of NF-κB by LPS (48). The calnexin-calreticulin cycle ensures correct folding of proteins that carry monoglycosylated N-linked glycans and retains misfolded conformers in the ER, regulating ER to Golgi transport (49). Interestingly, LPS stimulation increased MD-2 binding to calnexin in IEC, which seems to be more important than calreticulin as folding assistant (50).

LPS-TLR4 complexes redistribute between cell surface and endosomal structures that may be part of the Golgi apparatus (34, 51, 52). GM130 is a peripheral protein strongly associated with Golgi membranes on the cis side, which receive the entire output of newly synthesized proteins from the ER (53). We show that in acute inflammation only a limited portion of intestinal epithelial MD-2 glycoprotein was allowed to proceed to the Golgi, the last quality control step, and then subsequently to the cell surface. It remains to be shown in colitis whether such minimal amounts of surface MD-2 may be sufficient to confer cellular responsiveness to low doses of LPS (10).

Our findings imply that endogenous MD-2 aggregates as large multimers in intestinal epithelial ER-associated microsomes. The conformational stability of the folded protein is an important factor that determines the efficiency of secretion. Tertiary stability of MD-2 is very low at physiological temperatures (54), which could explain why a large fraction of the protein may be misfolded and retained in the ER in vivo. MD-2 buildup in the ER lumen as a primary defect or secondary to the inflammatory milieu could contribute to the ER stress response in acute inflammation. Conversely, alterations in intestinal epithelial homeostasis caused by cytokines, hypoxia, or free radicals in inflammation may induce ER stress (55) and subsequent failure of the ER to properly fold large amounts of newly produced MD-2. Future studies will need to investigate whether epithelial ER dysfunction is present in acute IBD, which could contribute to intracellular MD-2 accumulation in IEC. Aberrant retention of MD-2 in the ER and failure of transport to the Golgi could provide means for limiting host reactivity via up-regulated MD-2/TLR4 to prevent overwhelming inflammatory responses to the resident microflora.

Based on the results of this study, we conclude that trypsin-induced proteolysis of MD-2 may act as a mechanism of intestinal epithelial LPS tolerance, helping limit exaggerated immune responses to commensal-derived ligands. Acute intestinal inflammation is associated with trypsin-mediated changes in selective expression and distribution of ER-associated MD-2 in the intestinal epithelium. Further studies are needed to investigate whether other serine proteases also contribute to reduced MD-2 in ileitis and to clarify how immune imbalance in IBD may either lead to and/or result from MD-2 dysregulation in intestinal mcosa.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


