Toll-like Receptor 4 Resides in the Golgi Apparatus and Colocalizes with Internalized Lipopolysaccharide in Intestinal Epithelial Cells

Mathias W. Hornef,[1] Teresa Frisan,[1] Alain Vandewalle,[2]
Staffan Normark,[1] and Agneta Richter–Dahlfors[1]

[1]*Microbiology and Tumor Biology Center, Karolinska Institutet, 17177 Stockholm, Sweden*
[2]*Institut National de la Santé et de la Recherche Médicale (INSERM) U478, Faculté de Médecine*
 Xavier Bichat, 75018 Paris Cedex 18, France

Abstract

Toll-like receptor (TLR) 4 is mainly found on cells of the myelopoietic lineage. It recognizes lipopolysaccharide (LPS) and mediates cellular activation and production of proinflammatory cytokines. Less is known about the distribution and role of TLR4 in epithelial cells that are continuously exposed to microbes and microbial products. Here we show that the murine small intestinal epithelial cell line m-IC$_{cl2}$ is highly responsive to LPS and expresses both CD14 and TLR4. Transcription and surface membrane staining for CD14 were up-regulated upon LPS exposure. Surprisingly, TLR4 immunostaining revealed a strictly cytoplasmic paranuclear distribution. This paranuclear compartment could be identified as the Golgi apparatus. LPS added to the supernatant was internalized by m-IC$_{cl2}$ cells and colocalized with TLR4. Continuous exposure to LPS led to a tolerant phenotype but did not alter TLR4 expression nor cellular distribution. Thus, intestinal epithelial cells might be able to provide the initial proinflammatory signal to attract professional immune cells to the side of infection. The cytoplasmic location of TLR4, which is identical to the final location of internalized LPS, further indicates an important role of cellular internalization and cytoplasmic traffic in the process of innate immune recognition.

Key words: toll–like receptor 4 • Golgi apparatus • lipopolysaccharide • CD14 • LPS tolerance

Introduction

Exposure to LPS results in the immediate cell activation and release of proinflammatory cytokines by macrophages. This major cell wall constituent of Gram-negative bacteria is one of the most potent inducers of an inflammatory response and is responsible for the deleterious hemodynamic dysregulation seen in patients with Gram-negative sepsis (1). The serum protein, lipopolysaccharide binding protein, catalyzes the transfer of LPS monomers from micelles to a binding site on CD14, which in turn mediates the recognition of LPS through toll-like receptor (TLR)* 4 (2–4). TLR4 belongs to a recently identified family of transmembrane proteins with a remarkably high similarity to immune recognition receptors described in the fruit fly *Drosophila melanogaster* (5). The LPS recognition complex consists of a TLR4 homodimer together with MD-2, an extracellular accessory LPS-binding protein (6). Activation initiates a cytoplasmic signaling cascade that ultimately leads to the degradation of IκB, nuclear translocation of NF-κB, and transcriptional activation of proinflammatory response genes (7). The central role of CD14 and TLR4 during this process in vivo is illustrated by the fact that deficient mice are highly resistant to septic shock and that endotoxin hyporesponsiveness in humans is associated with genetic polymorphism in the TLR4 locus (8, 9).

Less is known about TLR4-mediated LPS recognition in cells of epithelial surfaces, such as in the gastrointestinal tract. The presence of a resident bacterial microflora and high local concentrations of LPS should impede a similarly susceptible detection system as seen in macrophages. Indeed, low or absent expression of TLR4 and MD-2 was shown in human colon carcinoma cell lines and LPS responsiveness could be enhanced by TLR4–MD-2 cotrans-

fection (10). Moreover, lack of CD14 expression was recently reported in intestinal epithelial cells and isolated intestinal macrophages (11, 12). The absence of the LPS recognition complex therefore might explain the endotoxin hyporesponsiveness of the normal intestinal epithelium to microbes and microbial products (13). However, prominent enteric microbial pathogens such as *Salmonella*, specifically target the small intestine and evoke a rapid local inflammatory response.

The number of established small intestinal cell systems still remains limited. We therefore decided to use the recently developed murine intestinal epithelial cell line m-IC$_{cl2}$ derived from L-PK/Tag1 transgenic mice as a model to study LPS recognition in the small intestine (14). The surprisingly sensitive phenotype to LPS stimulation led to a careful study of the kinetic of cellular activation and the expression of molecules involved in LPS recognition, such as CD14 and TLR4. Thus, we here propose a new model for the process of TLR4-mediated LPS recognition in the small intestine. The presence of LPS-sensitive epithelial cells in the gastrointestinal tract raises important questions about protective mechanisms to prevent inadequate host-defense activation by exposure to luminal bacteria.

Materials and Methods

Antibodies and Reagents. The rabbit anti-TLR4 antiserum was raised against the synthesized polypeptide FQGIKLHELTL-RGNFNSSNIMKTC that is located in the extracytoplasmic part of the TLR4 protein (Innovagen) and affinity purified. Biotinylation of the anti-TLR4 antiserum was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) following the protocol of the manufacturer. Consequent purification was performed using Microcon YM-10 centrifugal filter devices that were purchased from Millipore. The goat anti-TLR4 peptide antibody (sc-12511) was purchased from Santa Cruz Biotechnology, Inc. This antibody was only used for immunofluorescence studies and is called SC-12511 throughout this report. The rabbit anti-CD14 antiserum (M-305) was obtained from Santa Cruz Biotechnology, Inc. The murine monoclonal anti-Golgi 58K protein (p58K) antibody was purchased from Sigma-Aldrich. The polyclonal rabbit anti-β coatomer protein (COP) anti-serum was purchased from Affinity Bioreagents, Inc. The monoclonal CTR433 was a gift from Michel Bornens (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) (15). The rat monoclonal anti-murine TLR4-MD-2 antibody, MTS510, was a gift from Kensuke Miyake (Department of Immunology, Saga Medical School, Nabeshima, Saga, Japan) (16). *Escherichia coli* K12 D31m4 (Re) LPS was purchased from List Biological Laboratories. Murine recombinant IL-1β and TNF-α as well as anti-macrophage inflammatory protein-2 (MIP-2) and anti–TNF-α antibodies were obtained from Nordic BioSite. If not otherwise stated, all reagents were purchased from Sigma-Aldrich.

Cell Culture. RAW 264.7 cells were obtained from the American Type Culture Collection and propagated in RPMI 1640 medium (GIBCO BRL) supplemented with 25 mM Hepes and L-glutamine. 10% FCS was added. m-IC$_{cl2}$ cells were grown in a modified hormonally defined FCS-supplemented (2%) medium as previously described, and incubated at 37°C in a 5% CO$_2$–95% air atmosphere (14). Growth on collagen-coated culture plates was shown to induce apical–basolateral polarization with tight junctions, which delineate the apical membrane domain as well as the apically located short microvili (14).

Cell Stimulation Assays. Cells were grown on collagen-coated culture plates using rat tail collagen type 1 (2 mg/ml), diluted 1:100 in ethanol/water (60:40 vol/vol). Culture plates or flasks were rinsed and dried overnight. Cells were seeded and incubated for 6 d with medium changes every second day. LPS was vortexed, sonified for 15 min, and added to the cells at the appropriate concentration. Cell supernatant was harvested after the indicated incubation period and stored at −20°C. Serum-free cultivation of m-IC$_{cl2}$ cells was performed in Episerf medium (GIBCO BRL) for 12 d. As controls, cells were cultured in Episerf medium supplemented with 2% FCS or in the original medium (also containing 2% FCS). Cells were stimulated with LPS at a concentration of 10 ng/ml with or without the addition of 2 μg/ml recombinant CD14 (R&D Systems). The polarized secretion of MIP-2 by m-IC$_{cl2}$ cells was examined using transwell filters (Costar) as previously described (14). LPS stimulation was performed selectively either on the apical (upper chamber) or the basolateral (lower chamber) side of the cell layer. Restimulation experiments were performed as follows: cells grown on collagen-coated plates for 6 d were first exposed to LPS for 6 h, and then washed and incubated for an additional 36 h in LPS-free medium. Consequently, restimulation was performed with the indicated concentrations of LPS, IL-1β (100 ng/ml), or TNF-α (50 ng/ml). Supernatants were harvested after 6 h and stored at −20°C.

ELISA. IL-6 concentration in cell culture supernatant was determined using the IL-6 Eli-pair ELISA kit obtained from Diaclone. MIP-2 and TNF-α were measured using Nunc-Immuno 96-well plates (Nunc) coated with either polyclonal anti–MIP-2 (500 ng/ml) or monoclonal anti-TNFα (3 μg/ml) capture antibody. Samples and serial dilutions of recombinant standard proteins were coincubated with biotinylated polyclonal anti–MIP-2 (250 ng/ml) or monoclonal anti–TNF-α (500 ng/ml) for 3 h at room temperature. Consequently, plates were washed and incubated with Streptavidin-HRP (Nordic Biosite). Substrate was added and color development was quantified using a SpectraMax Plus ELISA reader (Molecular Devices) at 450 nm.

Immunoblotting. Cells were grown on collagen-coated 24-well plates and stimulated with 10 ng/ml LPS. After 0, 20, and 40 min, the supernatant was removed and 200 μl lysis buffer (3:1 WB/SB vol/vol; SB: 250 mM Tris, pH 6.5, 8% SDS, 40% glycerol; and WB: 50 mM Tris, pH 7.4, 120 mM NaCl) supplemented with protein-inhibitor cocktail (Roche Diagnostics) was added. Cells were sonified for 10 min and lysates were frozen at −80°C until further use. Protein concentration was determined (DC protein assay; Bio-Rad Laboratories) and 5-μg protein per lane was separated on a 10% acrylamide gel. Proteins were blotted on nitrocellulose and stained for phosphorylated IκB-α (Ser32/36) using a monoclonal anti–mouse antibody, and for the total IκB-α using a rabbit-polyclonal antibody (Cell Signaling Technology). For the detection of TLR4, m-IC$_{cl2}$ cells were solubilized in lysis buffer (3:1 WB/SB). Cell lysate was then separated on an 8% SDS acrylamide gel. After the transfer on nitrocellulose, filters were incubated with the anti-TLR4 antibody for 2 h at room temperature followed by incubation with the peroxidase-conjugated secondary antibody. To detect lectin binding, nitrocellulose filters were incubated with peroxidase-conjugated Con A (Sigma-Aldrich) at a concentration of 1 μg/ml for 2 h at room temperature. Staining was detected using the Renaissance chemiluminescence kit (NEN Life Science Products) in combination with Hyperfilm ECL (Amersham Pharmacia Biotech). To re-

move N-linked carbohydrates, protein extracts were treated with 100 U/ml peptide *N*-glycosidase F (PNGase F) derived from *Flavobacterium meningosepticum* or endoglycosidase H (Endo H) derived from *Streptomyces plicatus* (New England Biolabs, Inc.) for 1.5 h at 37°C as previously described (17).

Ribonuclease Protection Assay. Total RNA was isolated using TRIzol reagent (GIBCO BRL) and digested with DNase I (Amersham Pharmacia Biotech). Fragments of the genes *tlr4* (nucleotide [nt.] 967–1286), *tlr2* (nt. 1291–1501), *md-2* (nt.12–289), *cd14* (nt. 101–441), *mip-2* (nt. 629–764), *slpi* (nt. 19–199), *gapdh* (nt 535–625), and *l32* (nt. 1331–1442) were amplified from murine cDNA (Expand Long Template PCR System; Roche Diagnostics) and cloned in PCR-Script SK (+) using the PCR-Script Amp cloning kit according to the manufacturer's protocol (Stratagene). The L32- and GAPDH-encoding RNA probes were used as internal controls. Plasmids were linearized and ^{32}P-labeled anti-sense RNA probes were synthesized using the MAXIscript in vitro transcription kit (Ambion). Hybridization and RNA digest were performed according to the instruction manual of the Riboquant RPA kit (BD PharMingen). RNA was purified and separated on a 5% acrylamide/8 M urea gel and exposed overnight to a PhosphorImager screen (Molecular Dynamics).

LPS Uptake Assay. The Alexa Fluor 488–conjugated LPS from *E. coli* O55:B5 (Molecular Probes) was preincubated in FCS for 1 h and added to cells grown on collagen-coated Lab-Tek chamber slides (Nunc) at a concentration of 100 ng/ml. After the indicated incubation time, cells were fixed and stained for TLR4. Alexa 594–conjugated anti–rabbit antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Narrow-band pass filters were used to avoid overlap in fluorescence of fluorophores.

Immunohistochemistry. Cells were cultured on collagen-coated Lab-Tek chamber slides (Nunc) for the indicated period, washed with PBS, and air-dried. Slides were fixed in 50% ice-cold acetone for 30 s and 100% ice-cold acetone for 5 min. Consequently, an incubation in 0.3% H_2O_2 for 15 min in the dark was performed. After washing, three blocking steps with 10% normal goat serum, avidin, and biotin (Vector Laboratories) were performed. The primary antibody was preincubated in 25% normal mouse serum for 1 h at room temperature. Cells were then incubated for 45 min with the primary antibody at a 1:100 dilution, washed, and incubated with the biotinylated goat secondary antibody (Vector Laboratories) at a dilution of 1:200. After washing, ABC ELITE reagent (Vector Laboratories) was added and incubated for 30 min. Slides were then incubated in AEC substrate (Vector Laboratories) until color development was sufficient. Counterstaining was performed using Mayer's hematoxylin and slides were mounted in Kaiser's gelatin solution.

Immunofluorescence Double Staining Procedure. Cells were acetone fixed and blocked with 10% normal serum derived from the same species as the secondary antibody as described above. The anti-TLR4 antiserum was preincubated in 25% normal mouse serum and added to the cells for 45 min. Slides were washed and incubated for 45 min with a Cy3-conjugated donkey anti–rabbit secondary antibody (Jackson ImmunoResearch Laboratories). Counterstaining was performed using Hoechst 33258 (DAPI) nuclear stain purchased from Pierce Chemical Co. Finally, slides were mounted in Vectastain (Vector Laboratories) and analyzed under a Nikon Eclipse E400 microscope (Global Medical Instrumentation) connected to a Hamamatsu C4742–98 digital camera (Hamamatsu). Double immunofluorescence staining was performed in four consecutive incubation steps using FITC-conjugated donkey anti–mouse and TxR-

conjugated donkey anti–rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). The biotinylated anti-TLR4 antiserum was detected using TxR-conjugated streptavidin (Jackson ImmunoResearch Laboratories). For the chase experiment, cells were incubated with the anti-TLR4 antibody at 4°C for 60 min. Cells were washed with ice-cold PBS and consequently incubated at 37°C for 60 min. After fixation, the staining procedure was completed with a secondary TxR-conjugated anti–rabbit antibody as described above. Control cells were incubated with biotin-conjugated anti–H-2K^b antibodies (BD PharMingen) at 4°C. TxR-conjugated streptavidin (Jackson ImmunoResearch Laboratories) was added after 30 min to initiate cross-linking of surface MHC molecules. Cells were washed in ice-cold PBS and incubated at 37°C to facilitate internalization. To deglycosylate cellular proteins, m-IC$_{cl2}$ cells were incubated with 100 U/ml PNGaseF or Endo H in the appropriate buffer for 1 h at 37°C before and after acetone fixation. Successful deglycosylation of cellular proteins was verified by consequent incubation with 0.2-μg/ml FITC-conjugated Con A (Sigma-Aldrich) for 45 min at room temperature.

Isolation of Murine Intestinal Crypts. C57BL/10ScSn and C57BL/10ScN mice were purchased from Harlan Laboratories. Animal experiments were approved by the Regional Animal Ethical Committee (Stockholm, Sweden). Murine intestinal crypts were isolated following a previously described protocol (18). In brief, the small intestine of adult mice was removed and rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free PBS. Segments of intestinal tissue were then everted and incubated in 30 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS for 20 min at 37°C. Consequently, the tissue was carefully shaken to release the epithelial cell layer. Crypts were then washed and purified by centrifugation at 700 *g*.

Results

Secretion of Proinflammatory Mediators in Response to LPS. Epithelial cells have long been considered to mainly act as a physical barrier to protect the integrity of epidermal or mucosal surfaces. A growing body of evidence now suggests a more active role in host defense and a complementary function to professional immune cells such as macrophages. To analyze the LPS responsiveness of the small intestinal epithelial cell line m-IC$_{cl2}$, secretion of proinflammatory cytokine in response to endotoxin exposure was determined and compared with the macrophage cell line RAW 264.7. Whereas RAW 264.7 cells readily responded to small amounts of LPS with secretion of IL-6 and TNF-α, no such response was seen in epithelial cells (unpublished data). However, evaluation of the production of the chemokine MIP-2 revealed a fast and highly sensitive response at concentrations as low as 0.01 ng/ml LPS (Fig. 1 A). Although macrophages reached significantly higher levels of total chemokine production, both the maximal stimulatory LPS concentration and the time kinetic of MIP-2 secretion in m-IC$_{cl2}$ cells and RAW 264.7 cells were similar. Both cell types produced maximal MIP-2 secretion at 1 ng/ml LPS (Fig. 1, A and C), and highest chemokine productions were achieved after 6 h of stimulation with LPS (Fig. 1, B and D). The rapid kinetic of cell activation was further illustrated by an increase of phosphorylated IκB-α and consequent decrease of total IκB-α after
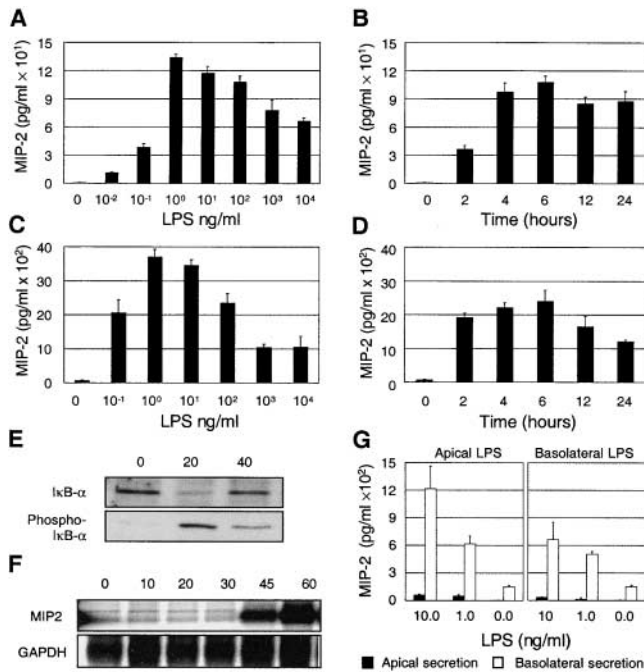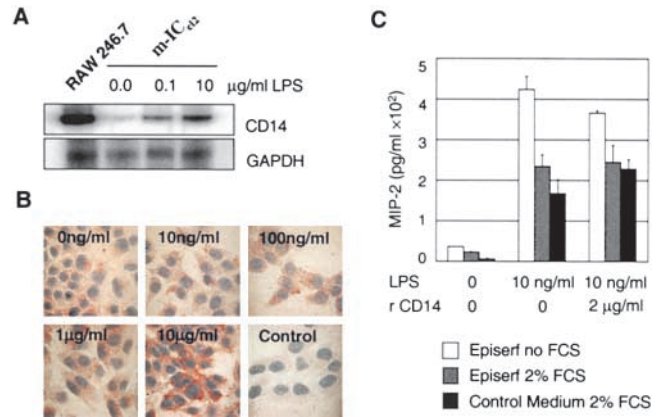
Figure 1. Comparison of MIP-2 secretion by m-IC$_{cl2}$ cells and RAW 264.7 cells after LPS exposure. (A) Dose response after 6 h of incubation for m-IC$_{cl2}$ cells and (C) RAW 264.7 cells. (B) Time kinetic at 100 ng/ml LPS concentration in cell supernatant of m-IC$_{cl2}$ cells and (D) RAW 264.7 cells. All data are expressed as the mean of triplicate samples ± SD and are representative of three separate experiments. (E) Quantitative analysis of total and phosphorylated (Ser32/36) IκB-α in cell lysate 0, 20, and 40 min after stimulation with 10 ng/ml LPS. (F) Transcriptional expression of *mip-2* mRNA upon LPS exposure. m-IC$_{cl2}$ cells were exposed to 100 ng/ml LPS for the indicated time period (min) before cell lysis and RNA extraction. Radioactively labeled *mip-2*– and *gapdh*-specific antisense RNA probes were used in a ribonuclease protection assay. (G) Comparison of apical versus basolateral MIP-2 secretion of LPS stimulated m-IC$_{cl2}$ cells cultured on transwell filters. LPS was added either at the apical (upper chamber) or basolateral (lower chamber) side of the cell layer. Results are expressed as the mean of triplicate samples ± SD and are representative of three separate experiments.


Figure 2. Transcriptional expression and immunostaining of m-IC$_{cl2}$ cells for CD14. (A) Ribonuclease protection assay using specific antisense probes for murine mRNA encoding CD14 and GAPDH. Before RNA isolation, m-IC$_{cl2}$ cells were exposed to 0.0, 0.1, or 10.0 μg/ml LPS for 12 h. RAW 264.7 cells were kept unstimulated. (B) Immunostaining for murine CD14 on m-IC$_{cl2}$ cells. Cells were incubated for 12 h in the presence of various concentrations of LPS before fixation, as indicated. As control, immunostaining was performed on untreated cells by omitting the primary antibody. Immunostaining was detected using the horseradish peroxidase reaction and cells were counterstained with Mayer's hematoxylin. ×1,000. (C) Comparison of MIP-2 secretion in response to LPS stimulation by m-IC$_{cl2}$ cells cultured in the presence or absence of serum. As control, 2 μg/ml recombinant CD14 was added before LPS stimulation.

20 min, followed by transcriptional up-regulation of MIP-2 after 45 min LPS stimulation (Fig. 1, E and F).

The differentiated and polarized phenotype of m-IC$_{cl2}$ cells was recently demonstrated (14, 19). The functional apical-basolateral polarization was further illustrated by a predominantly basolateral-directed secretion of MIP-2 by m-IC$_{cl2}$ cells grown on transwell filters (Fig. 1 G). These data confirm the polarized phenotype of m-IC$_{cl2}$ cells and demonstrate the presence of a highly sensitive and rapid mechanism of LPS recognition. However, macrophages and epithelial cells seem to show distinct patterns of cytokine response upon challenge.

CD14 Expression and Up-Regulation in Response to LPS. The absence of CD14 expression in gastrointestinal tissue was recently suggested to explain the unresponsive phenotype in respect to the normal intestinal flora (12, 13). Surprisingly, CD14 expression was detected by ribonuclease protection assay in m-IC$_{cl2}$ cells, although at a significantly lower level as RAW 264.7 cells (Fig. 2 A). Furthermore,

preincubation of epithelial cells with increasing amounts of LPS significantly enhanced the level of CD14 mRNA expression. The relative amount of CD14 mRNA in m-IC$_{cl2}$ cells compared with RAW 264.7 cells was <1% in untreated cells, but increased to 14% at 100 ng/ml LPS and 21% at 10 μg/ml LPS for 24 h. CD14 expression was confirmed by immunohistochemistry, demonstrating a weak surface staining on untreated m-IC$_{cl2}$ cells and an increasingly intense staining signal after LPS exposure (Fig. 2 B). The functional relevance of this autonomous CD14 production was demonstrated by the preservation of the highly LPS-susceptible phenotype of m-IC$_{cl2}$ cells grown in the absence of serum (Fig. 2 C). Thus, m-IC$_{cl2}$ cells synthesize CD14 and seem to enhance rather than diminish their LPS-binding capacity in response to LPS exposure.

Analysis of TLR4 Expression in m-IC$_{cl2}$ Cells and Isolated Intestinal Crypt Epithelium. Although TLR4 might not directly bind LPS, it represents the sensor portion of the LPS recognition complex indicated by chimeric transfection experiments (20, 21). Expression of TLR4 as well as the accessory protein MD-2 was detected in epithelial m-IC$_{cl2}$ cells by ribonuclease protection assay (Fig. 3 A). Interestingly, the transcriptional levels of TLR4 and MD-2 did not significantly differ from those found in the macrophage RAW 264.7 cells. TLR2 transcription could also be demonstrated in m-IC$_{cl2}$ cells, suggesting that these cells recognize a broader spectrum of microbial pattern molecules.

Immunohistochemistry using a polyclonal anti-TLR4 antibody revealed an intense cytoplasmic staining in both m-IC$_{cl2}$ and RAW 264.7 cells, which was readily blocked by the addition of 10 μg/ml of the peptide used for immu-
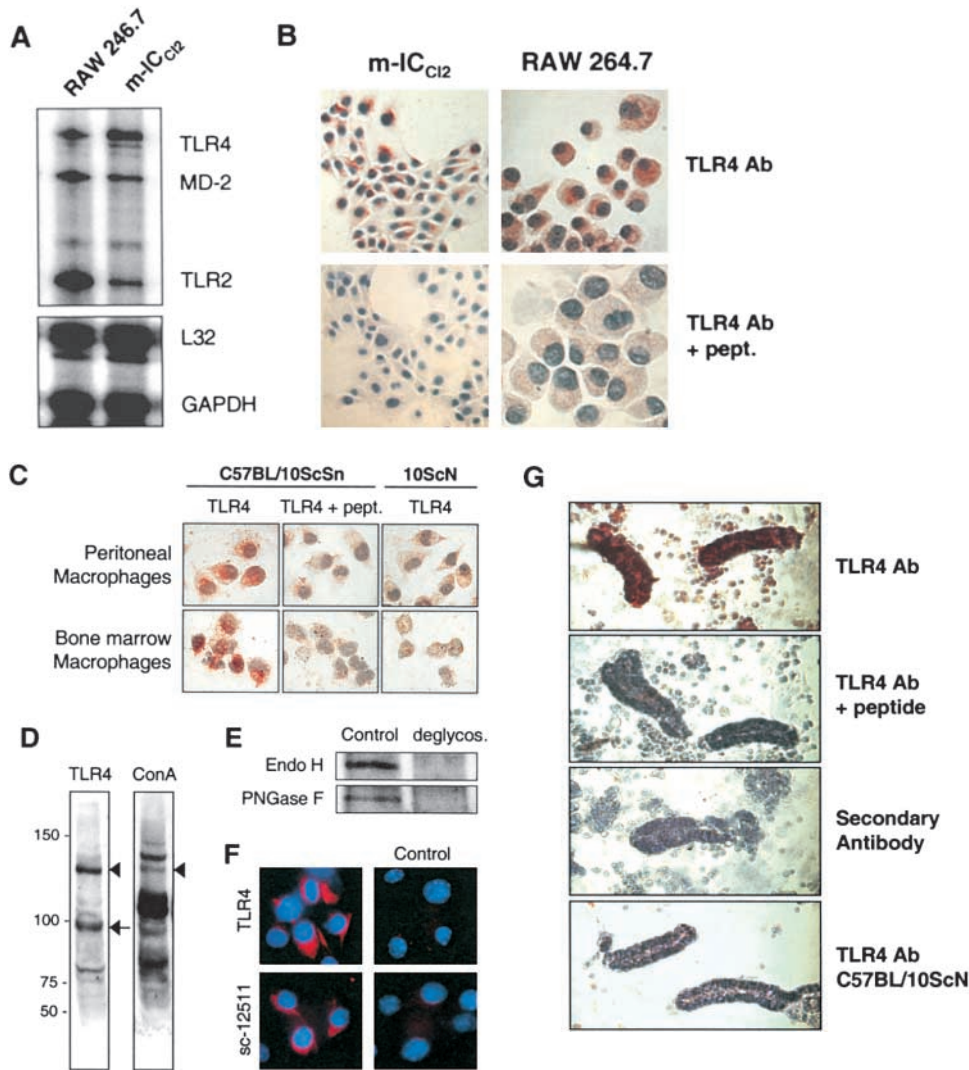
Figure 3. Analysis of TLR4 expression in m-IC$_{cl2}$ cells and isolated intestinal crypts. (A) Ribonuclease protection assay using radioactively labeled antisense RNA probes specifically hybridizing with murine *tlr4*, *md-2*, *tlr2*, *l32*, and *gapdh* mRNA. (B) Immunostaining for murine TLR4 on RAW 264.7 cells and m-IC$_{cl2}$ cells. As control, the primary antibody was incubated with 10 μg/ml of the peptide before use for immunization. ×1,000. (C) Immunostaining of peritoneal macrophages and bone marrow–derived macrophages isolated from wild-type C57BL/10ScSn and TLR4-deficient C57BL/10ScN mice. ×1,000. (D) Western blot of m-IC$_{cl2}$ cell lysate revealing a band of the predicted protein size of TLR4 at ∼96 kD (arrow). In addition, a larger band at ∼120 kD was found representing the glycosylated form of TLR4 (arrowhead). Overlay with Con A showing binding to the larger band (arrowhead). (E) The 120-kD band of m-IC^{cl2} cell lysate stained with TLR4 after treatment with Endo H or PNGase F. (F) Comparison of TLR4 staining in m-IC$_{cl2}$ cells using our TLR4 antibody and the TLR4 sc-12511 antibody. As control, the peptide-blocked primary antibody, or only the secondary antibody, was used for TLR4 and sc-12511, respectively. (G) TLR4 staining of isolated murine small intestinal crypts. Crypts were isolated from the small intestine of C57BL/10ScSn and TLR4-deficient C57BL/10ScN mice. ×400.

nization (Fig. 3 B). The overall signal intensity was more pronounced in RAW 264.7 macrophages as compared with m-IC$_{cl2}$ cells. However, an exclusively perinuclear staining pattern was seen in epithelial cells in contrast to the somewhat more diffuse cytoplasmic staining seen in the macrophage cell line.

The specificity of the antibody was confirmed using bone marrow– and peritoneal-derived macrophages from C57BL/10ScN mice, carrying a deletion of the T*lr4* locus. Staining was only detected in control cells derived from wild-type C57BL/10ScSn mice (Fig. 3 C). Western blot revealed a band of the predicted size of ∼96 kD (Fig. 3 D). In addition, a larger band of ∼120 kD was identified similar to the recently described glycosylated form of human TLR4 (17). The identity of this larger band could be confirmed by the binding of the lectin Con A and degradation after enzymatic deglycosylation, using PNGase F or Endo H (Fig. 3, D and E). The exclusively perinuclear localization of TLR4 in m-IC$_{cl2}$ cells was verified using another antibody (sc12511), recognizing murine TLR4 (Fig. 3 F).

To further assess the relevance of TLR4 expression found in m-IC$_{cl2}$ cells, isolated crypt tissue from wild-type C57BL/10ScSn and TLR4-deficient C57BL/10ScN mice was examined (Fig. 3 G). Immunostaining revealed strong expression of TLR4 by the crypt epithelium, whereas tissue derived from C57BL/10ScN mice remained negative. These data confirmed the presence of TLR4 expression in murine small intestinal crypts in vivo and suggested that the crypt epithelium may play a role in the detection of bacterial microorganisms.

Identification of the Cytoplasmic TLR4-harboring Compartment. The sharply defined perinuclear staining pattern suggested that TLR4 is localized close to the Golgi apparatus. To better identify the cytoplasmic compartment harboring TLR4, double immunofluorescence studies were performed using: (a) the monoclonal anti-Golgi 58K protein (p58K) antibody directed against the microtubule-binding Golgi membrane protein 58K; (b) an anti–β-COP antibody recognizing one subunit of the COP I complex involved in the regulation of retrograde transport between the endoplasmic reticulum and the Golgi complex, as well

as intra–Golgi transport; and (c) the CTR433 antibody binding to a yet uncharacterized antigen of the medium compartment of the Golgi apparatus (22). All three markers of the Golgi complex p58K, β-COP, and CTR433 showed a clear colocalization with TLR4 staining, suggesting a widespread distribution of TLR4 in the Golgi cisternae and the trans-Golgi network (Fig. 4 A). To confirm that TLR4 is structurally connected to the Golgi apparatus, we aimed to pharmacologically interfere with the structural integrity of this compartment. Whereas brefeldin A induces reversible translocation of the cis- to trans-Golgi complex membranes to the endoplasmic reticulum, treatment with nocodazole results in the vascularization and dispersion of the Golgi complex because of disruption of the microtubules. Pretreatment of cells with either nocodazole or brefeldin A clearly affected the perinuclear pattern of TLR4

immunostaining (Fig. 4 B). A cytoplasmic punctuate staining morphology was found to be similar to what has been described for TGN38 after brefeldin A treatment (23).

To exclude only temporary presence of TLR4 at the identified location, pretreatment of epithelial cells with cycloheximide, a potent inhibitor of eukaryotic protein synthesis, was used. Temporary presence of TLR4 in the Golgi apparatus would result in a continuously decreasing staining intensity, because of a lack of newly synthesized protein and an ongoing release of presynthesized molecules from the Golgi apparatus. Protein inhibition could disclose a possible final location of TLR4, visually obscured by the strong perinuclear signal. However, pretreatment of cells with cycloheximide at 50 μg/ml for 1.5 or 3 h did not result in a decreased staining signal (Fig. 4 C). In contrast, staining of cyclin B1, IκBα, or p53 almost
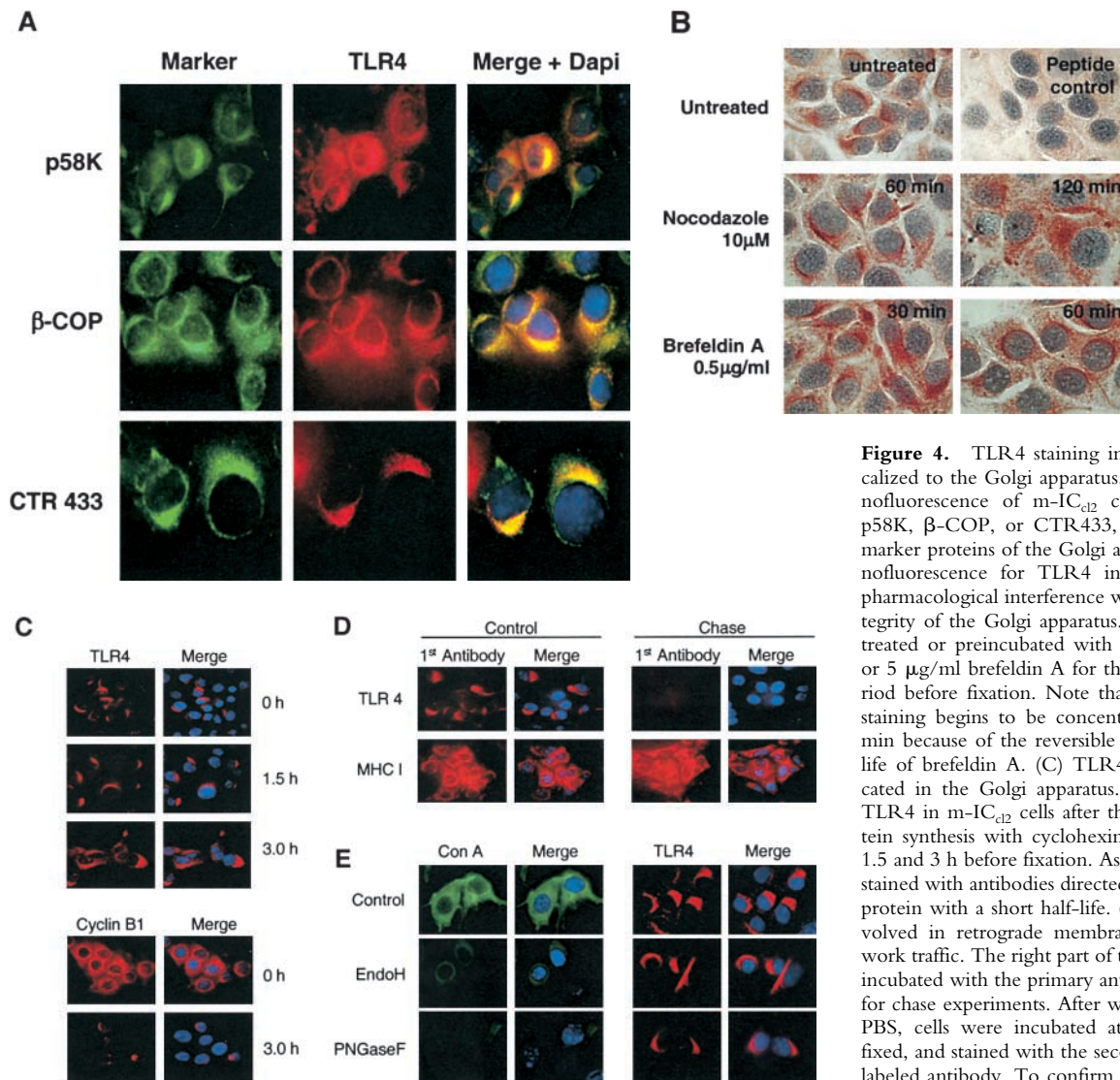
Figure 4. TLR4 staining in m-IC$_{cl2}$ cells is localized to the Golgi apparatus. (A) Double immunofluorescence of m-IC$_{cl2}$ cells for TLR4 and p58K, β-COP, or CTR433, which are all three marker proteins of the Golgi apparatus. (B) Immunofluorescence for TLR4 in m-IC$_{cl2}$ cells after pharmacological interference with the structural integrity of the Golgi apparatus. Cells were left untreated or preincubated with 10 μM nocodazole, or 5 μg/ml brefeldin A for the indicated time period before fixation. Note that perinuclear TLR4 staining begins to be concentrated again after 60 min because of the reversible effect and short half life of brefeldin A. (C) TLR4 is permanently located in the Golgi apparatus. Immunostaining of TLR4 in m-IC$_{cl2}$ cells after the inhibition of protein synthesis with cycloheximide (50 μg/ml) for 1.5 and 3 h before fixation. As a control, cells were stained with antibodies directed against cyclin B1, a protein with a short half-life. (D) TLR4 is not involved in retrograde membrane trans-Golgi network traffic. The right part of the figure shows cells incubated with the primary antibody for 2 h on ice for chase experiments. After washing with ice-cold PBS, cells were incubated at 37°C for 60 min, fixed, and stained with the secondary fluorescence-labeled antibody. To confirm cell viability, biotin-labeled anti-MHC class I (H-2K^b) antibodies and TxR–labeled streptavidin were used during primary incubation, inducing cross-linking and internalization of labeled MHC class I molecules. The left part of the figure demonstrates complete staining of acetone-fixed cells as a control. ×1,000. (E) Deglycosylation of m-IC$_{cl2}$ cells in situ. Before staining with TLR4 or Con A, cells were treated with Endo H or PNGase F. ×1,000.

disappeared during this time period, demonstrating the high turnover of these molecules (unpublished data for IκBα and p53).

To examine the possibility of retrograde plasma membrane trans-Golgi network traffic, a chase experiment with incubation of the primary antibody at 4°C, washing with ice-cold PBS, and continuous incubation at 37°C was performed. Cells were fixed and stained with the secondary antibody. In contrast to internalization of cross-linked MHC class I molecules, no staining for TLR4 was detected (Fig. 4 D). These data suggest that TLR4 permanently localizes to the Golgi apparatus of m-IC$_{cl2}$ cells. They further imply a rather long half-life of the TLR4 protein. To exclude the possibility of a reduced affinity of the TLR4 antibody to maturely glycosylated TLR4, m-IC$_{cl2}$ cells were treated with Endo H or PNGase F before immunostaining (Fig. 4 E). Deglycosylation with both enzymes resulted in a significant reduction of Con A binding. However, no alteration of the staining distribution for TLR4 was detected.

LPS Uptake by m-IC$_{d2}$ Cells. Close physical proximity between LPS and the TLR4 molecule was recently shown to be required for recognition (20, 21). However, we could not detect any membrane staining of TLR4 on m-IC$_{cl2}$ cells. Even cellular activation by LPS did not induce surface staining of TLR4. Furthermore, all efforts to inhibit LPS-induced cell activation using the polyclonal anti–TLR4 antiserum or the monoclonal anti–TLR4/MD-2 MTS510 antibody were unsuccessful (unpublished data). We therefore speculated about the possibility of LPS uptake and intracellular recognition by TLR4. Sonicated Alexa 488–conjugated LPS incubated with 10% FCS for 60 min was added to m-IC$_{cl2}$ cells at 100 ng/ml. Fluorescence could first be detected after 10–20 min of incubation and showed a somewhat punctuated cytoplasmic appearance (Fig. 5). To determine the cellular distribution of cytoplas-

mic LPS relative to the location of TLR4, Alexa 488–LPS-exposed cells were fixed after various incubation times, and stained for TLR4. Strikingly, fluorophoreconjugated LPS colocalized with TLR4 staining to a perinuclear cytoplasmic compartment after 10–30 min of incubation. These results suggested that the LPS recognition event might not occur on the cell surface, but rather in a cytoplasmic compartment.

Tolerance Induction in m-IC$_{cl2}$ Cells. Protection from continuous LPS stimulation is achieved in macrophages by down-regulation of endotoxin responsiveness after primary LPS exposure (24). This hyporesponsive phenotype has been called tolerance. m-IC$_{cl2}$ cells were exposed to 1 μg/ml LPS for 6 h, washed, and incubated for an additional 36 h in LPS-free medium. Reexposure was performed at various LPS concentrations for 6 h and the MIP-2 concentration was determined in cell supernatant (Fig. 6 A). Pretreatment at a concentration of 1 μg/ml induced a complete abrogation of the stimulatory activity of consequent LPS exposure above the residual MIP-2 level resulting from the pretreatment. Even higher LPS concentrations did not induce additional MIP-2 production. Interestingly, pretreatment with LPS concentrations as low as 0.1 ng/ml significantly inhibited subsequent cell activation. The maximal inhibitory effect was reached by pretreatment with 100 ng/ml LPS (Fig. 6 B). These data reflect the presence of a sensitive but highly suppressive regulatory mechanism to avoid LPS overstimulation in m-IC$_{cl2}$ cells.

Localization of Reduced LPS Responsiveness in Tolerant Cells. The diminished susceptibility of cells of the myelopoietic lineage to repeated LPS exposure was recently proposed to be due to the down-regulation of TLR4 (25). Therefore, transcription of TLR4 and MD-2 in m-IC$_{cl2}$ cells was analyzed at different time points after exposure to LPS. Probes detecting MIP-2 as well as TLR2 expression were included as internal controls (Fig. 6 C). Furthermore, secretory leukocyte protease inhibitor (SLPI), a protein recently described to inhibit LPS-mediated cell activation, was included (26). The high degree of cellular activation through LPS was illustrated by an immediate up-regulation of MIP-2 transcription. Interestingly TLR2 as well as SLPI expression showed a marked temporary up-regulation similar to the situation described in macrophages (26, 27). However, no significant alteration of the transcription level of TLR4 and MD-2 was found. Moreover, immunohistochemistry revealed no significant change of the staining intensity or the cellular distribution of TLR4 upon LPS exposure (unpublished data). Therefore, down-regulation or redistribution of TLR4 seems to not be involved in the development of tolerance in m-IC$_{cl2}$ cells. TLR4 and the receptor of IL-1β (IL-1R) share the main cytoplasmic signaling pathway, whereas induction through the TNF-α receptor is mediated via a different downstream cascade (7). IL-1β and TNF-α induced significant secretion of MIP-2 in untreated m-IC$_{cl2}$ cells. LPS pretreatment diminished the susceptibility of IL-1β to a similar degree as seen for LPS ($P < 0.01$), whereas the chemokine response to TNF-α was enhanced (Fig. 6 D). Collectively, these results locate
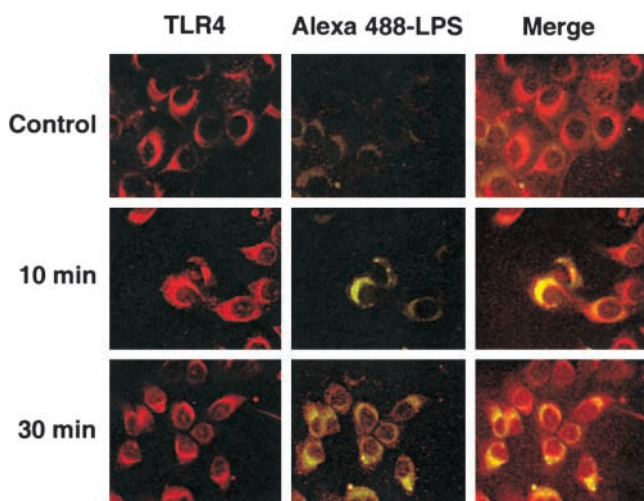
Figure 5. LPS is internalized in m-IC$_{cl2}$ cells and colocalizes with TLR4. m-IC$_{cl2}$ cells were incubated for the indicated time periods with Alexa-488–conjugated LPS, fixed, and immunostained for TLR4 using a secondary Alexa 594–conjugated anti–rabbit antibody, as described in the Materials and Methods section. ×1,000.
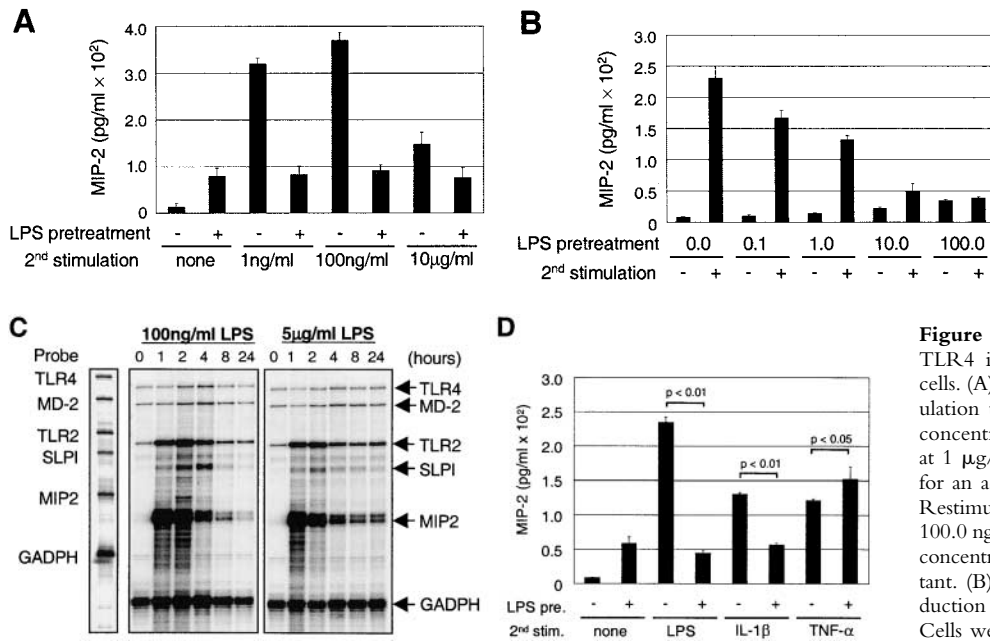
Figure 6. Transcriptional expression of TLR4 is not altered in LPS-tolerant m-IC$_{cl2}$ cells. (A) Pretreatment with LPS abrogates stimulation to second LPS exposure, even at high concentrations. m-IC$_{cl2}$ cells were preincubated at 1 μg/ml LPS for 6 h, washed, and cultured for an additional 36 h with LPS-free medium. Restimulation was performed at 0.0 ng, 1.0 ng, 100.0 ng, or 10.0 μg/ml LPS for 6 h, and MIP-2 concentration was determined in cell supernatant. (B) Minute amounts of LPS lead to a reduction of LPS susceptibility of m-IC$_{cl2}$ cells. Cells were preincubated for 6 h with 0.0, 0.1, 1.0, 10.0, or 100.0 ng/ml LPS. Restimulation was performed with 100 ng/ml LPS for 6 h. (C) Transcription is not altered by LPS exposure. Ribonuclease protection assay from m-IC$_{cl2}$ cells exposed to 100 ng/ml or 5 μg/ml of LPS for the indicated time period. Antisense RNA probes specifically hybridizing with *tlr4*, *md-2*, *tlr2*, *slpi*, *mip-2*, and *gapdh* mRNA were used. (D) LPS tolerance is located downstream of TLR4. m-IC$_{cl2}$ cells were pretreated with 1 μg/ml LPS for 6 h and restimulation was performed with 1 ng/ml LPS, 100 ng/ml IL-1β, or 50 ng/ml TNF-α. All data are expressed as the mean of triplicate samples ± SD and are representative of three separate experiments. Student's *t* test was used for statistical analysis.

the functional LPS hyporesponsiveness of tolerant m-IC$_{cl2}$ cells downstream of TLR4.

Discussion

Enteric mucosal surfaces withstand constant exposure to a great burden of bacteria and bacterial products, a prerequisite for the intimate relationship between the host and its beneficial normal microflora. Low or absent intestinal expression of molecules involved in LPS recognition intriguingly explains local endotoxin hyporesponsiveness (10–13). However, here we describe a highly LPS-responsive phenotype of the murine small intestinal cell line m-IC$_{cl2}$. Significant levels of TLR4 and MD2 expression were found, and even minute amounts of LPS led to rapid and pronounced cell activation. In fact, the maximal stimulatory LPS concentration and the kinetic of MIP-2 secretion were found to be similar in m-IC$_{cl2}$ cells and macrophage-like RAW 264.7 cells. However, the response of m-IC$_{cl2}$ cells was limited to the production of the chemokine MIP-2. No secretion of the proinflammatory cytokines TNF-α, or IL-6 was detected. Interestingly, m-IC$_{cl2}$ cells expressed the LPS coreceptor CD14. Although the basal level of CD14 expression was low, m-IC$_{cl2}$ cells maintained full LPS susceptibility under serum-free conditions. Furthermore, LPS exposure induced a dose-dependent increase leading to a significant intensity of CD14-specific surface staining. Hence, m-IC$_{cl2}$ cells seem to be well equipped to respond to LPS in order to initiate a proinflammatory response and attract professional immune cells.

How can intact recognition of LPS be present in the gastrointestinal tract without evoking proinflammatory stimulation? Fetal enterocytes were shown to express TLR4 and ex vivo–derived intestinal organ cultures from fetal tissue demonstrated significantly higher LPS susceptibility as compared with infant tissue (28, 29). Therefore, down-regulation of endotoxin recognition only seems to arise with microbial colonization of the intestine after birth. It is noteworthy that few if any resident Gram-negative microflora were found in the small intestine (with the exception of the terminal ileum) in contrast to the colon (30). Interestingly, a recent thorough investigation of the phenotypic markers of m-IC$_{cl2}$ cells identified a number of important properties and differentiated functions as found in intestinal epithelial crypt cells. m-IC$_{cl2}$ cells show a high nucleus to cytoplasma ratio, rudimentary brush border, and regular tight junctions that delineate the apical membrane domain. They express glycoconjugates, polymeric immunoglobulin receptor, the cystic fibrosis transmembrane conductance regulator, and the multidrug resistance-associated protein MRP-1, which is characteristic of intestinal crypt epithelium (14, 19). Although little is known about the exact environmental conditions in the intestinal crypts in vivo, recent work indicates that the local conditions might differ significantly from the situation in the intestinal lumen. High local concentrations of α–defensins along with a permanent secretory flow might create a largely bacteria-free environment in the enteric crypts of healthy individuals (31). The LPS-neutralizing capacity of antimicrobial peptides could further diminish the local soluble

LPS concentration (32). These exceptional conditions in enteric crypts would allow the presence of an intact endotoxin recognition system. Indeed, crypts that are isolated from murine small intestinal tissue showed a strong TLR4 expression. This is in accordance with the finding that human TLR4 expression in fetal small intestine was predominantly found in crypt enterocytes (28). The fact that prominent Gram-negative enteric pathogens specifically target the small intestine might even demand the preservation of intact LPS recognition in this part of the gastrointestinal tract. Tissue destruction by pathogenic microorganisms would expose crypt cells to LPS and consequently initiate an inflammatory response.

Surprisingly, immunostaining of m-IC$_{cl2}$ cells revealed a dense cytoplasmic perinuclear localization of TLR4. This cytoplasmic compartment could be identified as the Golgi apparatus using three independent markers. Pharmacological disruption of the Golgi structure significantly altered the TLR4 staining morphology, confirming the identified location. No secondary cellular location or cycling between the cell surface and the Golgi complex could be demonstrated as it was reported for TGN38 or the convertase furin (23, 33). Thus, TLR4 of m-IC$_{cl2}$ cells seems to be permanently located in the Golgi apparatus. Interestingly, the Golgi apparatus has previously been associated with LPS-mediated cell activation (34, 35). In contrast to m-IC$_{cl2}$ cells, surface staining of TLR4 was detected on nonpermeabilized murine peritoneal macrophages, using a monoclonal anti-TLR4 antibody and FACS® analysis (16). Moreover, TLR2 was demonstrated on the surface of cells using transient transfection and a flag-based detection system (36). Consequently, internalization of zymosan particles was accompanied by the concentration of TLR2 to phagosomes. Since peritoneal macrophages in the healthy individual reside in a microbe-free environment, the localization of TLR4 on the plasma membrane exposed to the surrounding environment might ensure the highest LPS sensitivity. In contrast, the intracellular location of TLR4 in epithelial cells would add an additional regulatory barrier to prevent uncontrolled stimulation. Similarly, an intracellular system of LPS recognition has recently been suggested in intestinal epithelial cells (37). However, further analysis of cellular TLR distribution in intestinal tissue in vivo is clearly needed to validate the importance of this finding.

Close physical contact between LPS and TLR4 was clearly demonstrated to be necessary to mediate cell activation (20, 21). However, aside from the prominent paranuclear location, membrane staining of TLR4 was not detected in m-IC$_{cl2}$ cells. Chase treatment, protein synthesis inhibition, and cell activation with LPS also did not reveal any detectable surface membrane staining. Moreover, all efforts to block TLR4-mediated signaling using an antibody directed against the TLR4–MD-2 complex failed, although this strategy was successful in macrophages using the same antibody (16). These results indicate that TLR4 is not on the plasma membrane of m-IC$_{cl2}$ cells during LPS-induced cell activation, but rather in an intracellular

compartment. The recent finding that oligomers of MD-2 associate with TLR4 in the endoplasmic reticulum–cis Golgi network strengthens the hypothesis that the complete LPS recognition complex might be present in the Golgi compartment (38). Considering the highly LPS-responsive phenotype, this exclusively intracellular location of the LPS recognition complex strongly suggests that internalization and transport of endotoxin is needed to facilitate close proximity between ligand and receptor at the Golgi complex.

Indeed, uptake of fluorophore-conjugated LPS and cytoplasmic transport could be observed in m-IC$_{cl2}$ cells. Most importantly, endocytosed cytoplasmic LPS colocalized to the TLR4-positive cellular compartment. Our data therefore give an explanation for the recently described transport of LPS to the Golgi compartment (34, 35). Internalization of LPS in the cytoplasm of several cell types has been demonstrated in a number of studies (34, 35, 39–43). Colocalization strategies could identify several intracellular compartments. LPS was found in early endosomes, lysosomes, sarcomeres, and the Golgi apparatus (34, 35, 39, 40). Moreover, it seems that more than one way of LPS uptake exists and the kind of internalization determines both the consequences and the fate of the LPS (39, 41–44). It was suggested that large aggregates of LPS are internalized along with CD14 and deacylated via the lysosomal pathway, whereas monomeric LPS is transported to the Golgi apparatus and initiates cell activation (34, 43). In addition, the structure of LPS and the functional integrity of TLR4 is at least indirectly implicated in this internalization process (45, 46). The importance of LPS uptake for the process of cell activation still remains controversial (40, 47). However, an increasing body of evidence suggests that LPS internalization is an obligatory event directly linked to recognition and cell activation in several cell types (38, 45, 46, 48, 49). We are currently evaluating the possible routes involved in LPS internalization and their importance for subsequent cell activation in our cell model.

After the initial signal that directs professional immune cells to the site of infection, the release of proinflammatory mediators needs to be controlled to avoid ongoing phagocyte infiltration and tissue damage. A negative regulator of endotoxin-mediated cell activation is the serine protease inhibitor SLPI, which inhibits LPS transfer to CD14, internalization, and prostaglandin synthesis (50, 51). Indeed, intestinal m-IC$_{cl2}$ cells showed a marked up-regulation of SLPI upon LPS stimulation. Another mechanism to limit the proinflammatory response might be the induction of tolerance. Epithelial m-IC$_{cl2}$ cells abrogated MIP-2 secretion in response to LPS after endotoxin pretreatment. Reduction of LPS responsiveness was achieved by very low amounts of LPS and could not be overcome even with high concentrations of LPS. This reduced LPS susceptibility was recently found to coincide with the temporary down-regulation of TLR4 after endotoxin exposure, which suggests a regulatory role of TLR4 expression (25). However, in m-IC$_{cl2}$ cells neither the transcription of TLR4 or MD-2, nor the total signal intensity or cytoplas-

mic distribution of TLR4 staining, was significantly altered after LPS exposure. The preservation of susceptibility to TNF-α, but not to IL-1β, in tolerant cells suggested that the cause of LPS hyporesponsiveness is located downstream of TLR4. These findings are in accordance with recent reports describing induction of cross-tolerance by ligands using different members of the TLR family, and overexpression studies on tolerant Chinese hamster ovary cells (52, 53).

In conclusion, we propose a model of intact LPS recognition in small intestinal crypts to ensure immediate host-defense activation upon microbial infection. Murine crypt intestinal m-IC$_{cl2}$ cells seem to be well equipped to recognize LPS and show a highly sensitive phenotype. Continuous LPS stimulation is inhibited by a sensitive and tight state of tolerance, induced by inhibition of the cytoplasmic TLR4 signaling pathway. TLR4 does not seem to be present on the cell surface membrane, but instead resides at high concentration in a cytoplasmic compartment, the Golgi apparatus. LPS internalization and colocalization with TLR4 indicates that LPS uptake represents an obligatory step in the process of endotoxin recognition. Thus, we present a novel model of the process of TLR4-mediated LPS recognition in intestinal epithelial cells. The results from this study provide, for the first time, an explanation to the functional importance of LPS internalization and transport to the Golgi complex. Membrane dynamics and cell transport might therefore represent a novel level of regulation of innate immune recognition, cell activation, and host defense.

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