Carbon Monoxide Suppresses Membrane Expression of TLR4 via Myeloid Differentiation Factor-2 in βTC3 Cells

Fredy Rocuts,*[,1] Yinghua Ma,[†,1] Xinyu Zhang,*[,1] Wenda Gao,* Yinan Yue,* Timothy Vartanian,[†] and Hongjun Wang*

Islet allografts from donor mice exposed to CO are protected from immune rejection after transplantation via the suppression of membrane trafficking/activation of TLR4 in islets/β cells. The molecular mechanisms of how CO suppresses TLR4 activation in β cells remain unclear and are the focus of this study. Cells of the insulinoma cell line, βTC3, were stably transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi plasmids and used to identify the subcellular distribution of TLR4 before and after LPS stimulation by confocal microscopy. Immunofluorescence analysis revealed that TLR4 mainly resides in the Golgi apparatus in βTC3 cells when in a quiescent state. LPS stimulation led to a rapid trafficking of TLR4 from the Golgi to the cell membrane. Physical interaction between TLR4 and myeloid differentiation factor-2 (MD-2) was confirmed by immunoprecipitation. Depleting MD-2 using small interfering RNA or blocking the N-glycosylation of cells using tunicamycin blocked membrane trafficking of TLR4. Pre-exposing cells to CO at a concentration of 250 parts per million suppressed membrane trafficking of TLR4 via inhibiting its glycosylation and the interaction between TLR4 and MD-2. In conclusion, MD-2 is required for the glycosylation of TLR4 and its consequent membrane trafficking in βTC3 cells. CO suppresses membrane activation of TLR4 via blocking its glycosylation and the physical interaction between TLR4 and MD-2. ***The Journal of Immunology*****, 2010, 185: 2134–2139.**

Toll-like receptors that recognize distinct pathogen-associated molecular patterns of diverse infectious agents play crucial roles in both innate and adaptive immunity (1). At least 10 TLRs have been identified so far (2, 3). Among them, TLR4, which binds to the lipid A moiety of LPS, a product of the outer membrane of Gram-negative bacteria, is one of the most studied TLRs (4). In the innate response, LPS is first recognized by the LPS-binding protein that forms a high-affinity complex with the lipid A moiety of LPS and then forms a ternary complex with CD14 that enables LPS to be transferred to the LPSR complex composed of TLR4 and myeloid differentiation factor-2 (MD-2) (5). This binding process induces homodimerization of TLR4. TLR4 then mediates the response to LPS via two different pathways: an early MyD88-dependent pathway and a delayed MyD88-independent pathway (6). In the MyD88-dependent pathway, TLR4 recruits MyD88 and MyD88 adaptor-like/Toll–IL-1R (TIR) domain-containing adaptor to the receptor complex, leading to the activation of NF-κB and the MAPK pathways (7–10). The MyD88-independent pathway recruits two TIR domain-containing adaptors including TIR and TIR domain containing adaptor-inducing IFN-β and is responsible for the later activation of NF-κB and the production of proinflammatory cytokines. This pathway also leads to the activation of the IFN regulatory factor-3 and the induction of IFN-β and the IFN-inducible genes (8, 9, 11, 12). A recent study indicates that TLR4 activates MyD88-dependent and independent pathways sequentially: TLR4 first induces TIR domain-containing adaptor–MyD88 signaling at the plasma membrane and then is endocytosed into the Golgi apparatus where it activates TRAM–TRIF signaling from the early endosomes (13).

Our previous studies indicate that induction of heme oxygenase-1 in or exposure of CO to islet donors protects islet allografts from immune rejection following transplantation at least in part by suppressing the activation of TLR4 in islets/β cells (14, 15). Depletion of TLR4 by using TLR4-deficient islets or infecting them with the adenovirus expressing dominant-negative TLR4 protects those islets from cytokine-induced apoptosis and leads to long-term survival of islet allografts in recipients (15). However, how TLR4 is distributed and regulated in β cells and how CO exposure suppresses TLR4 activation in those cells remain largely unknown. Two forms of TLR4 exist in the transfected human embryonic kidney 293 cells: the immature form with a molecular mass of 110 kDa and the mature form with a molecular mass of 130 kDa (16). Immature TLR4 resides in the endoplasmic reticulum (ER) and the Golgi apparatus and migrates to the cell membrane after maturation, a process that requires glycosylation at Asn^{526} or Asn^{575} (16). The molecular mechanisms by which TLR4 stays in the Golgi/ER and migrates to the cell surface are currently unknown. MD-2 has been shown not only to be essential for TLR4 binding with LPS but also indispensable for cell surface expression of TLR4 in many cell types, including HEK293 cells, bone marrow-derived dendritic cells, embryonic fibroblasts, and macrophages (17–19). Physical interaction between TLR4 and MD-2 was shown to be essential for the maturation of TLR4 and its presence in the cell membrane (17). A protein associated with TLR4 (PRAT4A) was also reported to play a role in the process of TLR4 maturation/glycosylation in bone marrow-derived

*Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; and [†]Department of Neuroscience and Neurology, Weill Medical College of Cornell University, New York, NY 10021

[1]F.R., Y.M., and X.Z. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Hongjun Wang, Department of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, SL429, Boston, MA 02215. E-mail address: hwang3@bidmc.harvard.edu

Abbreviations used in this paper: ER, endoplasmic reticulum; G, glycosylated protein; M, m.w. marker; MD-2, myeloid differentiation factor-2; ppm, parts per million; RIPA, radio immunoprecipitation assay; siRNA, small interfering RNA; TIR, Toll–IL-1R; U, unglycosylated protein; WGA, wheat germ agglutinin; YFP, yellow fluorescent protein.

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macrophages (20, 21). PRAT4A likely a component of the machinery facilitating TLR4/MD-2 trafficking to the cell surface (19, 22). Trafficking of TLR4 to the membrane of macrophages in response to LPS is also shown to be reactive oxygen species-dependent (23), and heme oxygenase-1/CO suppresses TLR4 signaling by regulating the interaction of TLR4 with caveolin-1 (24).

In this study, we designed experiments to elucidate factors that regulate membrane trafficking of TLR4 during its activation by LPS and studied the mechanisms of how CO exposure blocks this process.

Materials and Methods

Cell culture

The insulinoma cell line, βTC3 cells, were cultured in DMEM supplemented with 10% FBS in 5% CO_2 at 37˚C. Cells were exposed to 100 ng/ml LPS (*Escherichia coli* 0127:B8; Sigma-Aldrich, St. Louis, MO) and harvested at different time points. Inhibition of N-glycosylation was performed in cells by incubating them with tunicamycin (from *Streptomyces lysosuperficus*; Calbiochem, San Diego, CA) at a concentration of 40 or 80 ng/ml for 16 h. Cells were exposed to CO at a concentration of 250 parts per million (ppm) in an OxyCycler (BioSpherix, Lacona, NY). Control cells were exposed to air for the same length of time.

Small interfering RNA transfection

Small interfering RNA (siRNA) was transfected into cells by Lipofectamine 2000 solution (Invitrogen, Carlsbad, CA) as suggested by the manufacturer. Briefly, βTC3 cells (1×10^6 cells in 1 ml serum-free medium) were seeded into a 12-well plate. Lipofectamine 2000 (5 μl) was diluted in 250 μl Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) and incubated for 5 min at room temperature. siRNA (0.5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in 250 μl Opti-MEM I, mixed with Lipofectamine, and incubated for 20 min at room temperature. Lipofectamine–siRNA complex was added to each well of βTC3 cells, and the solution was mixed gently. Transfected cells were incubated at 37˚C in 5% CO_2 for 48 h before further treatment. Transfection efficiency was determined by transfecting cells with FITC-labeled control siRNA (Santa Cruz Biotechnology).

Generation of a stably transfected βTC3 cell line

pcDNA3-TLR4-YFP, which encodes human TLR4 (developed by Dr. Dough Golenbock, Addgene, Cambridge, MA), and pDsRed-Monomer-Golgi (Clontech, Palo Alto, CA) vectors were used to generate a stable cell line in which TLR4 expression could be reported by the yellow fluorescent protein (YFP) and the Golgi could be localized by red fluorescence. Cell transfection was performed using Lipofectamine 2000 as described above. Stable expression of the exogenously introduced genes was achieved by culturing the transfected cells in the presence of neomycin sulfate (1 mg/ml; Alexis, San Diego, CA) for 14 d.

Western blot

The plasma membrane was separated from whole-cell lysates using the Plasma Membrane Protein Extraction Kit (Biovision, Mountain View, CA) according to the manufacturer's instructions. Whole-cell lysate or the membrane fraction of cells was lysed in SDS sample buffer (2% SDS, 0.15 M Tris, 10% 2-ME, 10% glycerol, and 2 mM PMSF) and separated in a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen). Proteins were transferred to a Hybond-P membrane (GE Healthcare, Piscataway, NJ) and blocked with 5% milk at room temperature for 60 min, followed by incubation with anti-TLR4 or anti–MD-2 Abs (Santa Cruz Biotechnology). Anti-pan cadherin Ab (plasma membrane marker; Abcam, Cambridge, MA) was used to identify the membrane proportion of cells. Blots were probed with secondary HRP Abs (Thermo Fisher Scientific, Rockford, IL) and visualized by an ECL detection kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.). The intensity of each signal was determined by ImageJ software (National Institutes of Health, Bethesda, MD).

Immunoprecipitation

Cells were washed with PBS and lysed in radio immunoprecipitation assay (RIPA) buffer containing 50 mM Tris, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and protein inhibitors. Cell lysate was incubated with the anti-TLR4 Ab (Santa Cruz Biotechnology) at 4˚C for 2 h and then incubated overnight with protein G-Sepharose (Fast Flow; GE Healthcare). Bead pellets were washed twice with RIPA buffer containing 0.1% Triton X-100 before the addition of SDS-PAGE sample buffer. Proteins were eluted from the beads using sample buffer, denatured, separated by electrophoresis, and transferred to a Hybond-P membrane. The membrane was then probed with anti–MD-2 Ab.

Separation of glycosylated and unglycosylated proteins

Wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA) was washed three times with RIPA buffer and added into cells lysate (50 μg per sample). The mixture was rotated overnight at 4˚C. Unglycosylated proteins from the supernatant were collected after spinning at low velocity. The pellet containing WGA was washed three times with RIPA buffer and filtered through a cellulose acetate polypropylene tube with filter (pore size 45 μm; Sigma-Aldrich). Glycosylated proteins bonded to the agarose were eluted using PBS supplemented with 1 N *N*-acetylglucosamine and 5% SDS. Glycosylated and unglycosylated proteins were separated by electrophoresis and analyzed through Western blot using the anti-TLR4 Ab. *N*-Acetylglucosamine was used as a competitor control to evaluate the effectiveness of WGA.

Immunofluorescence analysis

Stably transfected cells seeded in cell chambers were treated with different reagents, fixed in 2% paraformaldehyde, and stained with Hoechst 33258. Slides were analyzed by confocal or Axiovert 200M ApoTome wide-field microscopes (Zeiss, Thornwood, NY).

Flow cytometry

Cells were harvested, washed with FACS buffer (PBS, 1% BSA, and 0.1% sodium azide) and stained with PE anti-mouse TLR4 Ab or PE mouse IgG1 isotype control (eBioscience, San Diego, CA) at a concentration of 0.5 μg per 1×10^6 cells for 30 min on ice. Cells were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA) after being washed with FACS buffer. Fluorescence was acquired in logarithmic mode for visual inspection of the distributions, and the mean fluorescence intensity was measured to quantify relative expression of TLR4.

Results

TLR4 migrates from the Golgi apparatus to the cell membrane after LPS stimulation in βTC3 cells

Subcellular distribution of TLR4 before and after LPS stimulation was assessed in βTC3 cells stably transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi vectors by immunofluorescence analysis using confocal microscopy. Before LPS stimulation, most TLR4 resided in the Golgi apparatus with a small amount present in the cell membrane in βTC3 cells (Fig. 1*Aa–c*). LPS at a concentration of 100 ng/ml led to a rapid migration of TLR4 from the Golgi to the cell membrane; a dramatic amount of TLR4 was observed on the plasma membrane 5 min after adding LPS (Fig. 1*Ad–f*). However, most of the TLR4 was again observed in the Golgi 15 and 60 min after LPS stimulation (Fig. 1*Ag–l*), with only a small amount remaining on the cell membrane. The expression pattern of TLR4 on the cell membrane after LPS stimulation was confirmed by FACS analysis (Fig. 1*B*). Thus, it seems that TLR4 mainly resides in the Golgi apparatus at a quiescent state and migrates to the cell membrane to start signaling transduction after LPS stimulation in βTC3 cells.

MD-2 is essential for membrane expression of TLR4 in βTC3 cells

We evaluated whether MD-2 is required for membrane trafficking of TLR4 before and after LPS stimulation in βTC3 cells by knocking down the expression of MD-2 via transfection with a siRNA specific for MD-2. Transfection efficiency of siRNA was evaluated by transfecting cells with FITC-labeled control siRNA and analyzed by fluorescent microscopy and flow cytometry. Our data indicate that 75–80% of cells were transfected when 50 μM siRNA was used (data not shown). Transfecting cells with MD-2 siRNA efficiently knocked down MD-2 expression as analyzed by immunohistochemistry: MD-2 was undetectable in cells treated with MD-2 siRNA (Fig. 2*Ad*) as compared with those treated with the control siRNA in which most cells expressed

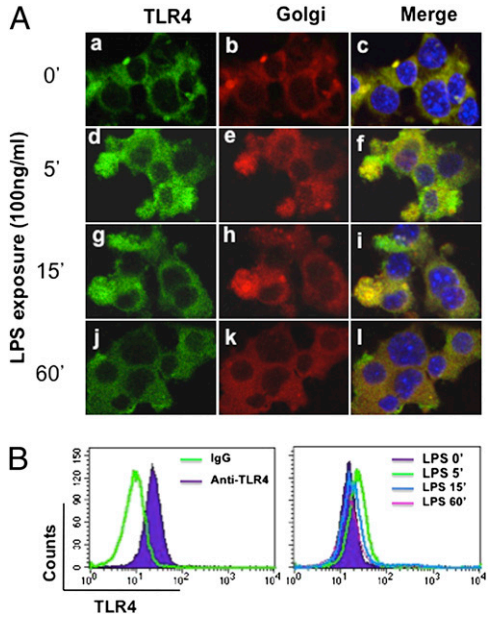

FIGURE 1. Trafficking of TLR4 from the Golgi apparatus to the plasma membrane in βTC3 cells after LPS stimulation. *A*, βTC3 cells stably transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi plasmids were exposed to LPS (100 ng/ml) for 0 (*a–c*), 5 (*d–f*), 15 (*g–i*), and 60 min (*j–l*). TLR4 expression was identified by confocal microscopy. Red identifies the Golgi apparatus, green represents the fluorescence of YFP (TLR4), and blue represents the nuclei (original magnification ×200). *B*, Membrane expression of TLR4 after LPS stimulation was analyzed by flow cytometry. Each sample was stained with IgG control or the anti-TLR4 Ab individually (*left panel*). Membrane expression of TLR4 was analyzed at 0, 5, 15, and 60 min after LPS stimulation. Images are representative of at least three independent experiments.

MD-2 (Fig. 2*Aa*). Similarly, TLR4 expression was completely absent on the plasma membranes of MD-2–deficient cells before and after LPS treatment (100 ng/ml) as compared with the control cells in which most cells expressed TLR4 (Fig. 2*Ab*, 2*Ae*). These results were confirmed by Western blot in which TLR4 expression on the membrane of MD-2–deficient cells was completely absent with or without LPS (Fig. 2*B*), indicating that MD-2 is required for the membrane expression of TLR4 in βTC3 cells.

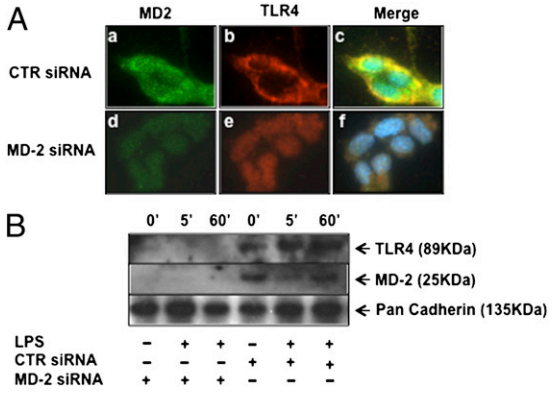

FIGURE 2. TLR4 expression in the membrane of βTC3 cells in which MD-2 was depleted by siRNA. *A*, TLR4 expression in the membrane of cells transfected with control (*a–c*) or MD-2 siRNA (*d–f*) was analyzed by immunohistochemistry using anti-TLR4 and anti–MD-2 Abs 5 min after LPS stimulation. Green represents TLR4, and red represents MD-2 (original magnification ×200). *B*, Western blot analysis of membrane expression of TLR4 in cells transfected with MD-2 siRNA or control siRNA at 0, 5, and 60 min after LPS stimulation. Membrane protein was identified by immunoblotting with the anti-pan cadherin Ab.

N-Glycosylation of TLR4 is required for its membrane trafficking after LPS activation in βTC3 cells

To evaluate whether glycosylation of TLR4 is required for its membrane trafficking in βTC3 cells, cells were incubated with tunicamycin, an antibiotic that inhibits N-glycosylation of protein, before being stimulated with LPS. Membrane expression of TLR4 was evaluated by flow cytometry using the anti-TLR4 Ab. As evident in Fig. 3*A*, LPS stimulation led to a rapid increase of TLR4 in the plasma membrane 5 min after adding LPS and was followed by decreases at 15 and 60 min. In contrast, the membrane presence of TLR4 was blocked in cells in which N-glycosylation was inhibited by tunicamycin. Tunicamycin at a concentration of 40 ng/ml partially blocked membrane trafficking of TLR4; a much lower increase of TLR4 was observed 5 min after LPS stimulation as compared with that in the control cells (Fig. 3*A*). In addition, membrane trafficking of TLR4 was completely blocked in cells incubated with tunicamycin at a concentration of 80 ng/ml (Fig. 3*A*). These results were confirmed by immunohistochemistry in cells stably transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi vectors (i.e., TLR4 remained in the Golgi apparatus and failed to migrate to the cell membrane at 5 and 60 min after LPS stimulation when N-glycosylation was blocked by tunicamycin) (Fig. 3*Bb*, 3*Be*).

MD-2 expression is required for the N-glycosylation of TLR4 in βTC3 cells

To further understand the role of MD-2 in the membrane trafficking of TLR4, we evaluated whether MD-2 is required for the glycosylation of TLR4 in βTC3 cells. MD-2 was knocked down by transfecting cells with siRNA. Glycosylated and unglycosylated proteins from whole cells before and after LPS stimulation were separated using WGA and loaded side by side for SDS-PAGE analysis. Expression of TLR4 at 0 and 60 min after LPS stimulation was analyzed by Western blot using the anti-TLR4 Ab. The percentage of glycosylated TLR4 in total TLR4 protein was quantified by the ImageJ system. As shown in Fig. 4, 11.5 and 25% of TLR4 were glycosylated in cells transfected with the control

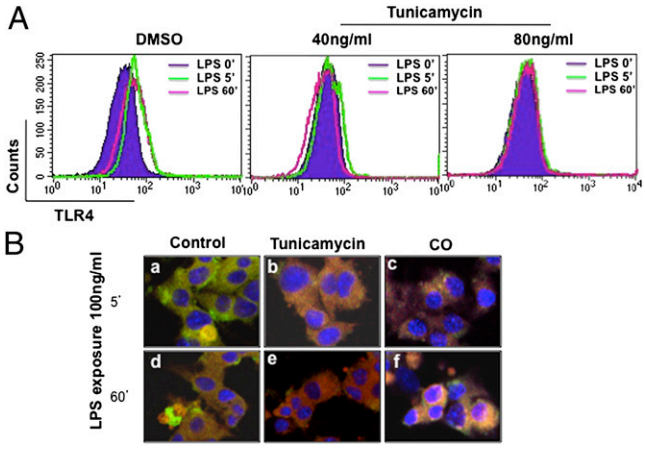

FIGURE 3. Membrane expression of TLR4. *A*, N-glycosylation is required for membrane expression of TLR4. βTC3 cells were treated with an N-glycosylation inhibitor, tunicamycin, at a concentration of 40 or 80 ng/ml for 16 h before being exposed to LPS at a concentration of 100 ng/ml. Membrane expression of TLR4 was quantified by flow cytometry at 0, 5, and 60 min after LPS stimulation. *B*, Tunicamycin treatment and CO exposure block membrane trafficking of TLR4 as analyzed by immunohistochemistry. TLR4 failed to migrate to the cell membrane in cells preincubated with tunicamycin (*b*, *e*) or exposed to CO (*c*, *f*) before LPS stimulation as compared to cells in the control group (*a*, *d*) as analyzed by confocal microscopy (original magnification ×200). Green represents TLR4, and red represents Golgi.

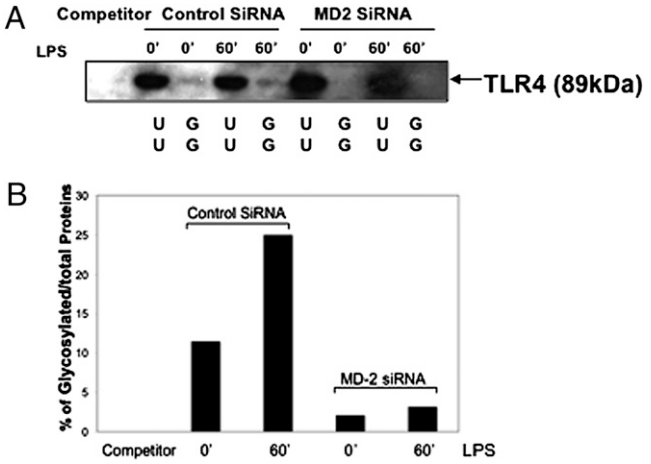

FIGURE 4. MD-2 is necessary for the glycosylation of TLR4 in the presence or absence of LPS. *A*, Expression of TLR4 analyzed in glycosylated and unglycosylated proteins in which MD-2 was knocked down by siRNA. *B*, Percentage of glycosylated TLR4 in the total TLR4 protein. G, glycosylated protein; U, unglycosylated protein.

siRNA at 0 and 60 min after LPS activation, respectively. In contrast, only 2.1 and 3.2% of glycosylated TLR4 were observed in cells transfected with the MD-2 siRNA at the same time points, suggesting that MD-2 expression is required for the glycosylation of TLR4 in βTC3 cells. We also evaluated whether knocking down MD-2 in βTC3 cells inhibits glycosylation of TLR2. Our data indicate that the absence of MD-2 did not change the glycosylation of TLR2 (data not shown).

CO suppresses LPS-induced membrane trafficking of TLR4

To assess whether CO suppresses LPS-induced membrane trafficking of TLR4, βTC3 cells stably transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi plasmids were pre-exposed to CO at a concentration of 250 ppm or air before being stimulated with LPS. Membrane presence of TLR4 was analyzed by confocal microscopy. In cells pre-exposed to CO, most TLR4 remained in the Golgi apparatus at 5 and 60 min after LPS activation, and much less TLR4 was observed in the membrane of cells as compared with cells pre-exposed to air (Fig. 3*Bc*, 3*Bf* versus Fig. 3*Ba*, 3*Bd*). These data suggest that CO exposure suppresses membrane trafficking of TLR4 induced by LPS in βTC3 cells.

CO blocks the interaction between TLR4 and MD-2

We evaluated whether pre-exposing cells to CO inhibits interaction between TLR4 and MD-2 by immunoprecipitation. Cells pre-exposed to CO at a concentration of 250 ppm or air were stimulated with LPS at a concentration of 100 ng/ml. Cells were harvested 0, 5, 15, 30, 45, and 60 min after stimulation. TLR4-positive proteins were pulled down using the anti-TLR4 Ab. The presence of MD-2 in those proteins was analyzed by Western blot using the anti–MD-2 Ab. MD-2 was found to be physically associated with TLR4 before and after LPS activation in cells pre-exposed to air (Fig. 5, *lower panel*). In contrast, pre-exposing cells to CO inhibited the interaction between TLR4 and MD-2 (i.e., no TLR4/MD-2 interaction was observed in cells pre-exposed to CO at 30–60 min after LPS stimulation as analyzed by immunoprecipitation) (Fig.5, *upper panel*). Our data indicate that CO pretreatment inhibits physical interaction between TLR4 and MD-2, a process that is required for the membrane trafficking of TLR4.

CO suppresses glycosylation of TLR4

Glycosylation of TLR4 was required for its membrane trafficking in βTC3 cells (Fig. 3). To elucidate whether CO suppresses

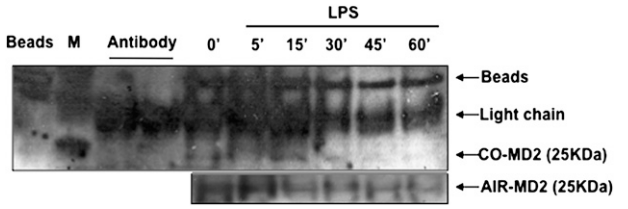

FIGURE 5. CO blocks the interaction between TLR4 and MD-2. Whole-cell lysate was immunoprecipitated with the anti-TLR4 Ab and immunoblotted with the anti–MD-2 Ab. Antibody, samples incubated only with the anti-TLR4 Ab without beads; Beads, samples incubated only with beads without precipitatiation Ab; M, m.w. marker.

membrane expression of TLR4 via inhibition of glycosylation of TLR4, cells pre-exposed to CO or air were stimulated with LPS at a concentration of 100 ng/ml. Glycosylated and unglycosylated proteins were separated with WGA, and the expression of TLR4 was analyzed by Western blot as described in the *Materials and Methods*. As evident in Fig. 6, much less glycosylated TLR4 was observed in cells pre-exposed to CO as compared with those pre-exposed to air before LPS activation [25% of TLR4 was glycosylated in air-treated cells versus only 3.2% in CO-treated cells (Fig. 6*B*)], indicating that CO exposure prevents the glycosylation of TLR4 in βTC3 cells. In addition to TLR4, CO treatment also inhibits the glycosylation of TLR2 in βTC3 cells (Fig. 6*A*).

Discussion

TLRs play critical roles in many disease conditions, including the pathogenesis of atherosclerosis (25, 26), chronic cardiac allograft rejection (27), liver and heart ischemia/reperfusion injury (28–30), and type 1 diabetes (31–33). Several groups have demonstrated that TLR4 activation is directly involved in the chronic rejection of transplanted organs (27, 34). Although the activation of innate immune cells itself is not sufficient for acute graft rejection without the participation of T cells, activation of TLRs might be essential for the development of the alloimmune response to the transplanted

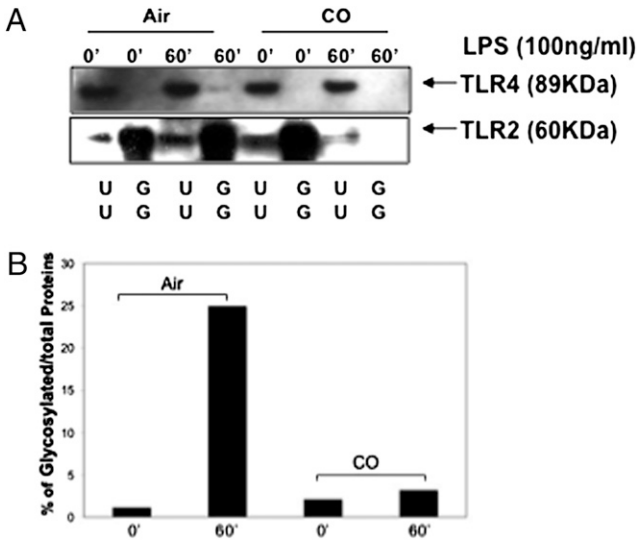

FIGURE 6. CO inhibits the glycosylation of TLR4 and TLR2. Cells were exposed to air or CO at a concentration of 250 ppm for 2 h. *A*, Expression of TLR4 and TLR2 in glycosylated and unglycosylated proteins before and after LPS stimulation as measured by Western blot. *B*, Percentage of glycosylated in total TLR4 protein. G, glycosylated protein; U, unglycosylated protein.

organ (35). Our previous study indicates that TLR4 is activated in β cells during the islet isolation procedure and its activation initiates inflammation that almost certainly contributes to islet/β cell death and graft rejection (15). CO exposure to the islet donor protects islet allografts from immune rejection at least in part via suppressing TLR4 activation in β cells. We undertook this study to elucidate the molecular mechanisms of how CO exposure suppresses TLR4 activation after LPS stimulation of βTC3 cells.

We first studied the subcellular distribution of TLR4 in βTC3 cells. Our confocal microscopy analysis of cells transfected with pcDNA3-TLR4-YFP and pDsRed-Monomer-Golgi plasmids shows that TLR4 mainly resided in the Golgi apparatus in βTC3 cells at a quiescent state. LPS activation led to a rapid recycling of TLR4 between the Golgi and the plasma membrane in a time-dependent fashion; dramatic TLR4 migration from the Golgi to the membrane occurred as early as 5 min after LPS stimulation. After binding to LPS, TLR4 was internalized into cells that led to a reduction on the cell membrane and an increase in the Golgi apparatus (Fig. 1*A*). It seems that the Golgi plays an important role in the activation of TLR4 by LPS. The major function of the Golgi in a cell is to modify and secrete newly synthesized proteins and lipids received from the ER to their final destination. In addition, the Golgi complex recycles plasma membrane components that are retrieved by endocytosis (36). Thus, we believe that the Golgi is the major cellular organelle for the storage of TLR4 in βTC3 cells.

We then evaluated factors that might regulate membrane trafficking of TLR4. We focused on the membrane activation of TLR4, because it seems that this is a critical step for its transduction of signals via both MyD88-dependent and independent pathways after LPS activation (13). Blocking membrane transport of TLR4 may block its transduction of signals and the consequent generation of proinflammatory cytokines.

The first factor that we studied was MD-2, a 20- to 25-kDa extracellular glycoprotein that binds to TLR4 and LPS. Although functional TLR4 can be present on the cell membrane without MD-2, MD-2 is essential for membrane trafficking of TLR4 in many cell types (17, 21, 37). Through immunoprecipitation, we found that MD-2 was physically associated with TLR4 in the presence or absence of LPS stimulation. By knocking down MD-2 expression using siRNA specific for MD-2, we showed that MD-2 is required for the membrane appearance of TLR4 in β cells.

TLR4 has nine N-linked glycosylated sites that are important to its functional integrity as a LPSR. Studies have shown that the binding of TLR4 to MD-2 is not sufficient for the translocation of TLR4 to the membrane surface; the glycosylation of Asn^{526} or Asn^{575} is necessary for translocation (16). We thus evaluated whether the glycosylation of TLR4 is required for its membrane trafficking. Through the use of an N-glycosylation inhibitor, tunicamycin, we observed that when the N-glycosylation of TLR4 is blocked most TLR4 remains in the Golgi apparatus and fails to migrate to the cell membrane after LPS stimulation (Fig. 3). It seems that TLR4 remains in an unglycosylated (immature) stage in the Golgi of β cells at the quiescent state and becomes glycosylated (mature) when stimulated by LPS. TLR4 after glycosylation migrates to the plasma membrane to start transduction of signaling, and the glycosylation process is essential for membrane trafficking in βTC3 cells.

It has been reported that TLR4 can undergo multiple glycosylations without MD-2 but the specific glycosylation essential for cell surface transport requires the presence of MD-2 (16, 38). To elucidate whether MD-2 is required for the glycosylation of TLR4 and its consequent membrane trafficking in βTC3 cells, we separated glycosylated and unglycosylated TLR4 using WGA agarose in cells in which MD-2 was knocked down by siRNA. Our data indicate that the depletion of MD-2 blocks the glycosylation of TLR4, suggesting the critical role of MD-2 in this process. It seems that TLR4 had already been associated with MD-2 in the Golgi before its glycosylation and transport to the cell membrane in βTC3 cells as well as in other cell types (39). We anticipate that the association of TLR4 and MD-2 may lead to a conformational change and expose TLR4 to glycosylation in βTC3 cells.

We also evaluated whether CO exposure or the absence of MD-2 regulates glycosylation of TLR2, another TLR that can be inhibited by CO. Our data indicate that the glycosylation of TLR2 was suppressed by CO exposure but not by the absence of MD-2. Because TLR2 and TLR4 both belong to the TLR superfamily, these observations suggest that the effect of CO on glycosylation is likely common to the TLR family members, because glycosylation is important to a variety of TLR functions, including ligand recognition (40), protein biosynthesis and secretion (41), etc.

CO at a concentration of 250 ppm blocks membrane transport of TLR4 in βTC3 cells and in isolated islets (15). We postulate that CO inhibits TLR4 signaling via different pathways at multiple levels. First, CO suppresses physical interaction between TLR4 and MD-2, which is required in the glycosylation and membrane trafficking of TLR4. Second, CO inhibits MD-2 expression and thus formation of the TLR4/MD-2 complex. Third, CO may interact with the glycans that are involved in the glycosylation process. The ultimate result of such suppression is to reduce expression levels of proinflammatory cytokines that might be deleterious to β cells. Our data demonstrate a model of how CO directly suppresses TLR4 activation in β cells. We used LPS as an activator for TLR4 throughout the study as proof of principle, although most likely TLR4 was induced in β cells during islet isolation by endogenous factors other than LPS because we have excluded the possible contamination of LPS during islet preparation (15). Although the potential role of TLR4 in recognizing endogenous ligands and its influence on the consequent development of autoimmune or other inflammatory disorders are still controversial, we believe that endogenous ligands released by dead cells are the major factors that trigger TLR4 activation during islet preparation.

Besides directly regulating TLR4 activation, we envisage that CO might suppress TLR4 via its known antiapoptotic action in β cells (i.e., CO exposure of the islet donor or isolated islets suppresses islet/β cell death, thus avoiding the "leaking" of potential endogenous TLR ligands to the extracellular environment that might lead to TLR activation in neighboring cells). These possibilities are currently being investigated.

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Disclosures

The authors have no financial conflicts of interest.

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