Toll-like Receptor 4 Region Glu\textsuperscript{24}–Lys\textsuperscript{47} Is a Site for MD-2 Binding

IMPORTANCE OF CYS\textsuperscript{29} AND CYS\textsuperscript{40}*

Toll-like receptor 4 (TLR4) is a signaling receptor for lipopolysaccharide (LPS), but its interaction with MD-2 is required for efficient responses to LPS. Previous studies with deletion mutants indicate a critical role of the amino-terminal TLR4 region in interaction with MD-2. However, it is uncertain which region in the TLR4 molecule directly binds to MD-2. The purpose of this study was to determine a critical stretch of primary sequence in the TLR4 region that directly binds MD-2 and is critical for LPS signaling. The synthetic TLR4 peptide corresponding to the TLR4 region Glu\textsuperscript{24}–Lys\textsuperscript{47} directly binds to recombinant soluble MD-2 (sMD-2). The TLR4 peptide inhibited the binding of a recombinant soluble form of the extracellular TLR4 domain (sTLR4) to sMD-2 and significantly attenuated LPS-induced NF-κB activation and IL-8 secretion in wild type TLR4-transfected cells. Reduction and S-carboxymethylation of sTLR4 abrogated its association with sMD-2. The TLR4 mutants, TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, and TLR4\textsuperscript{C29A,C40A}, were neither co-precipitated with MD-2 nor expressed on the cell surface and failed to transmit LPS signaling. These results demonstrate that the TLR4 region Glu\textsuperscript{24}–Lys\textsuperscript{47} is a site for MD-2 binding and that Cys\textsuperscript{29} and Cys\textsuperscript{40} within this region are critical residues for MD-2 binding and LPS signaling.

The innate immune system protects against invasion of microorganisms as the first line of defense and stimulates the clonal responses of adaptive immunity (1). Toll-like receptors (TLRs)\textsuperscript{2} have been implicated in recognition and signaling of pathogen-associated molecular patterns (2). Among the TLR family, TLR4 has been shown to play a critical role in recognition and signaling of lipopolysaccharides (LPS) (3). TLR4 is a type I membrane protein consisting of an extracellular domain that possesses a characteristic leucine-rich repeat (LRR) motif structure and an intracellular signaling domain. LPS-binding protein (4), CD14 (5), and MD-2 (6) are pivotal components in LPS-induced TLR4 signaling. Stimulation by LPS through TLR4 initiates an IL-1 receptor-like NF-κB signaling cascade, resulting in the production and secretion of proinflammatory cytokines (3, 7, 8). TLR4 alone cannot transmit LPS signaling, and the interaction of TLR4 with MD-2 is required for transmission of LPS signals (6). Thus, identification of a functional region of TLR4 that directly interacts with MD-2 is important to understand the mechanism of the regulation of LPS signaling. Recent studies from this and other laboratories (9, 10) have revealed that deletion of the amino-terminal TLR4 region abrogates the binding of TLR4 to MD-2 and that substitution of the amino-terminal TLR4 region for the TLR2 region confers the TLR2 chimera on the MD-2 binding activity, indicating that the amino-terminal TLR4 region is critical for the interaction with MD-2. However, it remains uncertain which region in the TLR4 molecule directly binds to MD-2. The purpose of this study was to determine the critical stretch of primary sequence of TLR4 that directly binds to MD-2 and regulates LPS signaling. We demonstrate that the TLR4 region Glu\textsuperscript{24}–Lys\textsuperscript{47} is a site for MD-2 binding and that Cys\textsuperscript{29} and Cys\textsuperscript{40} within this region are critical for interaction with MD-2 and LPS signaling.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney 293 (HEK293) cells and 293T (human embryonic kidney, SV40-transformed) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Re595 LPS was purchased from Sigma. Monoclonal antibody to a recombinant soluble form of the extracellular TLR4 domain (sTLR4), 4D9, was generated as described previously (11). Anti-FLAG polyclonal antibody, anti-FLAG antibody-conjugated agarose, and anti-V5 antibody-conjugated agarose were obtained from Sigma. Anti-V5 tag polyclonal antibody was purchased from Medical & Biological Laboratories Corp. (Nagoya, Japan).

Expression Vectors—The cDNAs for human TLR4 and human MD-2 were obtained as described previously (6). TLR4–3×FLAG that contains the C-terminal fusion 3×FLAG tag was generated by using PCR and subcloned into p3×FLAG-CMV\textsuperscript{TM}-14 expression vector (Sigma). MD-2–V5–His that contains the C-terminal fusion V5 tag and His\textsubscript{6} tag was generated by using PCR and subcloned into pcDNA3.1D/V5-His-TOPO (Invitrogen).
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Recombinant Proteins—sTLR4 consisting of the putative extracellular domain (Met\textsuperscript{1}–Lys\textsuperscript{631}) and a His\textsubscript{6} tag at the C-terminal end and a recombinant soluble form of MD-2 (sMD-2) containing a V5 tag and His\textsubscript{6} tag (MD-2) were expressed by a baculovirus-insect cell expression system using the method described previously (11). Schematic representations of wild type (WT) TLR4, sTLR4, and sMD-2 are shown in Fig. 1A. Analysis of the amino-terminal sequence of sTLR4 reveals that this protein starts at Glu\textsuperscript{24} (11).

Synthetic Peptides—The TLR2 peptide, ESNNQASLSCDRNKICKGSSGLSRLN5, corresponding to the amino acid residues Glu\textsuperscript{21}–Ser\textsuperscript{48} of TLR2 and the TLR4 peptide, ESWEPCVEVPNIQTYQCMELNFKYK, corresponding to the amino acid residues Glu\textsuperscript{24}–Lys\textsuperscript{47} of TLR4, were synthesized and obtained from Invitrogen.

Site-directed Mutagenesis—Cysteine to alanine substitutions were introduced into TLR4 using a QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions to express TLR4C88A, TLR4C29A, TLR4C29A,C40A, and TLR4C29A,C40A,C88A, bearing mutations at amino acids 29, 40, 88, and 29 and 40, respectively. The TLR4 mutant possessing a mutation at Pro\textsuperscript{714}→His (TLR4\textsuperscript{714H}) (8) was also constructed.

Biotinylation of TLR Peptides—The TLR2 peptide and the TLR4 peptide were biotinylated with EZ-Link-N-hydroxysulfosuccinimide-LC-Biotin (Pierce) according to the manufacturer’s instructions. In some experiments, sMD-2 (20 ng/ml) was preincubated with the indicated concentrations of purified MD-2 were incubated for 6 h in the absence or the presence of 10 ng/ml LPS with HEK293 cells, and luciferase activity was measured by the dual luciferase reporter assay system (Promega), according to the manufacturer’s instructions. In some experiments, sMD-2 (20 ng/ml) was preincubated with the indicated concentrations of the TLR2 peptide or the TLR4 peptide at 37 °C for 1 h before adding to the well.

IL-8 Secretion—HEK293 cells were plated at 1 × 10\textsuperscript{5} cells/well in 24-well plates on the day before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Applied Science) with 30 ng of an NF-κB reporter construct (pNF-κB-Luc; Stratagene, La Jolla, CA) and 10 ng of a construct directs expression of Renilla luciferase (pRL-TK; Promega, Madison, WI), together with 160 ng of cDNA for TLR4. Thirty-six hours after transfection, the indicated concentrations of purified MD-2 were incubated for 6 h in the absence or the presence of 10 ng/ml LPS with HEK293 cells, and luciferase activity was measured by the dual luciferase reporter assay system (Promega), according to the manufacturer’s instructions. In some experiments, sMD-2 (20 ng/ml) was preincubated with the indicated concentrations of the TLR2 peptide or the TLR4 peptide at 37 °C for 1 h before adding to the well.

NF-κB Reporter Assay—Activation of NF-κB was measured as previously described (12, 13). HEK293 cells were plated at 1 × 10\textsuperscript{5} cells/well in 24-well plates on the day before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Applied Science) with 30 ng of an NF-κB reporter construct (pNF-κB-Luc; Stratagene, La Jolla, CA) and 10 ng of a construct directs expression of Renilla luciferase (pRL-TK; Promega, Madison, WI), together with 160 ng of cDNA for TLR4. Thirty-six hours after transfection, the indicated concentrations of purified MD-2 were incubated for 6 h in the absence or the presence of 10 ng/ml LPS with HEK293 cells, and luciferase activity was measured by the dual luciferase reporter assay system (Promega), according to the manufacturer’s instructions. In some experiments, sMD-2 (20 ng/ml) was preincubated with the indicated concentrations of the TLR2 peptide or the TLR4 peptide at 37 °C for 1 h before adding to the well.

Binding of sMD-2 to TLR Peptides Coated onto Microtiter Wells—The TLR2 peptide or the TLR4 peptide (1 μg/ml, 50 μl/well) was coated into microtiter wells (Immulon 1B; Dynex). After nonspecific binding was blocked with phosphate-buffered saline containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100 (blocking buffer), the indicated concentrations of sMD-2 (50 μl/well) in the blocking buffer were added onto the wells and were incubated at 37 °C for 1 h. MD-2 binding to the solid phase TLR peptide was detected by anti-V5 polyclonal antibody, followed by incubation with horseradish peroxidase (HRP)-labeled anti-rabbit IgG. A peroxidase reaction was carried out by using o-phenylenediamine as a substrate, and the absorbance at 492 nm was measured.

Pull-down Assay of Biotinylated TLR Peptide—The biotinylated peptide (1 μg) of TLR2 or TLR4 was incubated with or without sMD-2 (2.5 μg) in phosphate-buffered saline containing 10% fetal calf serum for 1 h at 37 °C. Streptavidin-agarose beads were then added, and the mixture (500 μl) was further incubated for 1 h at 4 °C. After the incubation, the agarose beads were washed with phosphate-buffered saline containing 0.1% Triton X-100, and the final pellets obtained were subjected to SDS-PAGE. Western blot analysis was performed to detect sMD-2 that had co-precipitated with the biotinylated peptide by using anti-V5 polyclonal antibody. The proteins that reacted with the antibodies were visualized by using a Super Signal West Pico chemiluminescence substrate (Pierce) according to the manufacturer’s instructions.

Immunoprecipitation of sMD-2—sMD-2 (1 μg) possessing the V5 tag was mixed with or without the biotinylated peptide of TLR4 or TLR2 (1 μg) and was incubated at 37 °C for 1 h. Anti-V5 antibody-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4 °C for 12 h. After the incubation, the agarose beads were washed, and the final pellets obtained were subjected to SDS-PAGE. Western blot analysis was performed to detect the TLR peptides and MD-2 by using HRP-conjugated streptavidin and anti-V5 polyclonal antibody, respectively.

Competition of the TLR Peptide with sTLR4 for sMD-2 Binding—sMD-2 (20 ng) was preincubated with or without the peptide of TLR4 or TLR2 (0.4, 2, and 10 μg), and the mixture of sMD-2 and the TLR peptide was further incubated with the sTLR4 (100 ng) at 37 °C for 1 h. Anti-V5 antibody-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4 °C for 2 h. After the incubation, the agarose beads were washed, and the final pellets obtained were subjected to SDS-PAGE. Western blot analysis was performed to detect sTLR4 and MD-2 by using anti-sTLR4 monoclonal antibody (4D9) (11) and anti-V5 polyclonal antibody, respectively.
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chemically modified sTLR4 at 37 °C for 30 min. Anti-V5 antibody-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4 °C for 2 h. After the incubation, agarose beads were washed, and the final pellets obtained were subjected to SDS-PAGE. Western blot analysis was performed to detect sTLR4 and MD-2 by using anti-sTLR4 polyclonal antibody and anti-V5 polyclonal antibody, respectively.

**Immunoprecipitation and Immunoblotting of Membrane-bound TLR**—The 293T cells were transfected with FLAG-tagged TLR2, TLR4, or TLR4 mutants (12 µg of cDNA) along with V5-tagged MD-2 (12 µg of cDNA) by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The total amount of transfected DNA was kept constant with an empty vector. Forty hours after transfection, the cells were lysed with 20 mM Hepes buffer (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100, 20 mM EGTA, 50 mM NaF, and 2 mM Na\textsubscript{2}VO\textsubscript{4} (lysis buffer) on ice for 15 min. The cell lysates were clarified by centrifugation and then subjected to immunoprecipitation with anti-FLAG antibody-conjugated agarose or anti-V5 antibody-conjugated agarose. Immunoprecipitates were washed and released by boiling in SDS-PAGE sample buffer under reducing conditions. The protein samples were resolved by 7.5–15% SDS-PAGE and were transferred to a polyvinylidene difluoride membrane (Millipore Corp.). The membrane was then incubated with anti-FLAG polyclonal antibody or anti-V5 polyclonal antibody, followed by incubation with HRP-labeled anti-rabbit antibody.

**Flow Cytometry**—The 293T cells were transfected with TLR4 or its mutants (TLR4\textsubscript{C29A}, TLR4\textsubscript{C40A}, TLR4\textsubscript{C88A}, or TLR4\textsubscript{C29A,C40C88A}) (2 µg of cDNA) with or without MD-2 (2 µg of cDNA) by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The total amount of transfected DNA was kept constant with an empty vector. Forty hours after transfection, the cells were harvested, washed with phosphate-buffered saline containing 0.5% bovine serum albumin, and incubated with a phycoerythrin-conjugated anti-human TLR4 monoclonal antibody (eBioscience, San Diego, CA). Expression of cell surface TLR4 was analyzed by using FACSCalibur and CellQuest software (BD Biosciences).

**RESULTS**

**Electrophoretic Analysis of Recombinant Proteins**—sTLR4 and sMD-2 were produced in insect cells, and recombinant proteins were purified from the media by a column of nickel-nitrilotriacetic acid beads. The electrophoresis of purified proteins was performed under reducing and nonreducing conditions. The purified proteins of sTLR4 and sMD-2 exhibited apparent molecular masses of 80 and 23–30 kDa, respectively, under reducing conditions (Fig. 1B). sMD-2 migrated as monomeric and polymeric forms under nonreducing conditions. sTLR4 starts at Glu\textsuperscript{24} (11). sTLR4, and sMD-2 are shown. Analysis of the amino-terminal sequence has revealed that sTLR4 starts at Glu\textsuperscript{24} (11). B, electrophoretic analysis of recombinant proteins. Recombinant proteins of sTLR4 and sMD-2 produced by insect cells were subjected to SDS-PAGE (7.5–15% polyacrylamide gel) under reducing or nonreducing conditions. The proteins were visualized by Coomassie Brilliant Blue staining. Five micrograms/lane of sTLR4 and MD-2 were loaded. β-ME (+), β-mercaptoethanol.

The synthetic peptide corresponding to the amino-terminal TLR4 region of Glu\textsuperscript{24}–Lys\textsuperscript{47} and the TLR2 region of Glu\textsuperscript{21}–Ser\textsuperscript{45} (TLR4 peptide and TLR2 peptide, respectively) (Fig. 2A). We first investigated the direct interactions of sMD-2 with the synthetic peptides. When various concentrations of sMD-2 were incubated with the peptide coated onto the microtiter wells, sMD-2 bound to the solid phase TLR4 peptide in a concentration-dependent manner (Fig. 2B). However, sMD-2 did not exhibit any significant binding to the TLR2 peptide. The results suggest that the amino-terminal TLR4 region of Glu\textsuperscript{24}–Lys\textsuperscript{47} directly binds to sMD-2. The interactions of the TLR peptides with sMD-2 were also examined by a solution-phase assay. When biotinylated TLR peptide and sMD-2 were co-incubated and the biotinylated peptide was precipitated by pull-down assay with streptavidin-agarose beads, the biotinylated TLR4 peptide but not the TLR2 peptide co-precipitated sMD-2 (Fig. 2C). Conversely, when sMD-2 was immunoprecipitated after incubation with or without biotinylated TLR peptides, sMD-2 co-precipitated the TLR4 peptide but not the TLR2 peptide (Fig. 2D). Taken together, these results demonstrate that the synthetic peptide corresponding to the TLR4 region Glu\textsuperscript{24}–Lys\textsuperscript{47} binds to sMD-2.

**The TLR4 Peptide Inhibits the Binding of sTLR4 to sMD-2**—We next examined whether the TLR4 peptide competed with sTLR4 for sMD-2 binding. When sTLR4 and sMD-2 were co-incubated and sMD-2 was immunoprecipitated, sTLR4 was co-precipitated (Fig. 3, peptide, –), indicating direct binding of
sTLR4 to sMD-2. The addition of the TLR4 peptide but not the TLR2 peptide decreased the amounts of sTLR4 co-precipitated with sMD-2 in a manner dependent upon the amounts of the TLR4 peptide added (Fig. 3, peptide, TLR2 and TLR4).

**FIGURE 3. The TLR4 peptide inhibits the binding of sTLR4 to sMD-2 in a concentration-dependent manner.** sMD-2 (20 ng) was preincubated with or without the synthetic peptide (0.4, 2, and 10 μg/mL) of TLR4 or TLR2, and the mixture of sMD-2 and the TLR peptide was further incubated with sTLR4 (100 ng) at 37 °C for 1 h. Anti-V5 antibody-conjugated agarose beads were then added into the reaction mixture, and the suspension was further incubated at 4 °C for 2 h. After the incubation, agarose beads were washed, and the final pellets obtained were subjected to SDS-PAGE. Western blot analysis was performed to detect sTLR4 and sMD-2 by using anti-sTLR4 monoclonal antibody (4D9) and anti-V5 polyclonal antibody, respectively. IP, immunoprecipitation; WB, Western blot.

Taken together with the results obtained from the direct peptide binding, these results support the conclusion that the TLR4 region of Glu^{24–Lys^{47}} is a site for sMD-2 binding.

The TLR4 Peptide Attenuates LPS-induced NF-κB Activation and IL-8 Secretion in TLR4-expressing Cells—The addition of sMD-2 elicited NF-κB activation and IL-8 secretion in response to 10 ng/ml LPS in TLR4-transfected HEK293 cells in a manner dependent upon the sMD-2 concentrations (Fig. 4, A and C). We next examined whether the TLR4 peptide affected LPS-induced NF-κB activation in TLR4-transfected HEK293 cells. The indicated concentrations of the TLR4 peptide or the TLR2 peptide that had been preincubated with sMD-2 (20 ng/ml) were further incubated with the cells in the presence of 10 ng/ml LPS. The TLR4 peptide but not the TLR2 peptide significantly attenuated LPS-elicted NF-κB activation by ~45%. The addition of the TLR4 peptide that had been preincubated with sMD-2 (100 ng/ml) also significantly decreased IL-8 secretion from TLR4-transfected cells in a concentration-dependent manner (Fig. 4D). These data indicate that the TLR4 peptide can down-regulate LPS-induced inflammation in TLR4-expressing cells.

Reduction and S-Carboxymethylation of sTLR4 Abrogates Its Association with sMD-2—The extracellular TLR4 domain contains 16 cysteine residues, two (Cys^{29} and Cys^{40}) of which exist in the amino-terminal region of Glu^{24–Lys^{47}}. To determine the importance of disulfide bonding in the binding of sTLR4 to sMD-2, sTLR4 was reduced with DTT, followed by S-carboxymethylation with iodoacetate. We then examined, by immunoprecipitation with sMD-2, whether the reduced and S-carboxymethylated sTLR4 was able to bind to sMD-2. Untreated sTLR4 co-precipitated with sMD-2 (Fig. 5). However, the reduced and S-carboxymethylated sTLR4 did not co-precipitate with sMD-2, indicating that the chemically modified sTLR4 fails to bind to sMD-2. Because analysis of sTLR4 with 5,5′-dithiobis(2-nitrobenzoic acid) has revealed that there is no free thiol group in the extracellular TLR4 domain (data not shown), the present results suggest that the disulfide bonding in the TLR4 molecule is critical for its binding to MD-2.
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**FIGURE 4.** The TLR4 peptide down-regulates LPS-induced cell responses in TLR4-transfected cells. A, sMD-2-dependent LPS-elicited NF-κB activation in TLR4-transfected cells. The indicated concentrations of purified sMD-2 were incubated for 6 h in the absence (open squares) or the presence (closed squares) of 10 ng/ml LPS with HEK293 cells that had been transfected with the expression plasmid containing WT TLR4 cDNA (160 ng) together with an NF-κB reporter plasmid (pNF-κB-Luc, 30 ng) and Renilla luciferase control reporter plasmid (pRL-TK, 10 ng). NF-κB activities were determined, as described under “Experimental Procedures.” The data shown are the means ± S.E. of three experiments. *p < 0.05, **p < 0.01, when compared with the experiments in absence of LPS. B, the TLR4 peptide significantly attenuates LPS-induced NF-κB activation. HEK293 cells were transfected with WT TLR4 cDNA together with an NF-κB reporter plasmid and control reporter plasmid as described above. The indicated concentrations of the TLR4 peptide were preincubated with sMD-2 (20 ng/ml) at 37 °C for 1 h. Thirty-six hours after transfection, the cells were incubated for 6 h with 10 ng/ml LPS in the medium containing the mixture of sMD-2 and the TLR2 peptide (open circles) or the TLR4 peptide (closed circles). The data shown are the means ± S.E. of three experiments. *p < 0.01, when compared with the experiments in the presence of the TLR2 peptide. C, sMD-2-dependent LPS-induced IL-8 secretion from TLR4-transfected cells. The indicated concentrations of purified sMD-2 were incubated for 15 h in the absence (open squares) or the presence (closed squares) of 10 ng/ml LPS with HEK293 cells that had been transfected with the WT TLR4 cDNA (200 ng). After the LPS stimulation, the concentrations of IL-8 secreted into the media were determined by an enzyme-linked immunosorbent assay, as described under “Experimental Procedures.” The data shown are the means ± S.E. of three experiments. *p < 0.01, when compared with the experiments in the absence of LPS. D, the TLR4 peptide down-regulates LPS-induced IL-8 secretion from TLR4-transfected cells. HEK293 cells were transfected with WT TLR4 cDNA (200 ng). The indicated concentration of the TLR4 peptide was preincubated with sMD-2 (100 ng/ml) at 37 °C for 1 h. Thirty-six hours after transfection, the cells were incubated for 15 h with 10 ng/ml LPS in the medium containing the mixture of sMD-2 and the TLR2 peptide (open circle) or the TLR4 peptide (closed circle). The data shown are the means ± S.E. of three experiments. *p < 0.05; **p < 0.01, when compared with the experiments in the presence of the TLR4 peptide.

TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, and TLR4\textsuperscript{C29A,C40A} Fail to Associate with MD-2 and to Transmit LPS Signaling—Since reduction and S-carboxymethylation of sTLR4 abrogated its binding to sMD-2, we generated TLR4 mutants in which a cysteine residue was replaced with alanine and examined whether Cys → Ala mutation in TLR4 altered its ability to associate with MD-2. MD-2 cDNA and cDNA for TLR2, TLR4, or TLR4 mutant were co-transfected with 293T cells, and V5-tagged MD-2 was immunoprecipitated. The proteins of TLR2, TLR4, and TLR4 mutants as well as MD-2 protein were sufficiently expressed in all transfectants (Fig. 6A, IP: α-V5/ WB: α-V5 and IP: α-FLAG/ WB: α-FLAG). A significant amount of the WT TLR4 protein co-precipitated with MD-2 (Fig. 6A, IP: α-V5/ WB: α-FLAG). However, MD-2 co-precipitated no protein of TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, or TLR4\textsuperscript{C29A,C40A}. The amount of TLR4\textsuperscript{C88A} protein that co-precipitated with MD-2 was decreased, indicating that TLR4\textsuperscript{C88A} impaired but retained some MD-2-binding activity. These results clearly demonstrate that cysteine residues of TLR4 at amino acids 29 and 40 are critical for MD-2 binding.

We next examined whether the TLR4 mutants were able to induce NF-κB activation in response to LPS. HEK293 cells that had been transfected with MD-2 and WT TLR4 responded well to LPS (Fig. 6B). When the empty plasmid and TLR4\textsuperscript{P714H} cDNA were transfected, no response to LPS was observed. LPS did not elicit significant NF-κB activation in the cells that had been transfected with WT TLR2, TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, or TLR4\textsuperscript{C29A,C40A}. TLR4\textsuperscript{C88A} that showed weak association with MD-2 exhibited reduced but significant activation of NF-κB in response to LPS. These results correlate well with those obtained from co-precipitation with MD-2 (Fig. 6A).

Neither TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, nor TLR4\textsuperscript{C29A,C40A} Is Expressed on Cell Surface—Since transport of TLR4 to the cell surface is closely related to the presence of MD-2 and their interactions (14), we next examined the cell surface expression of TLR4 and its mutants (Fig. 7). The 293T cells had been transfected with TLR4 or its mutant with or without MD-2, and cell surface TLR4 was examined by flow cytometry. When WT TLR4 was transfected with or without MD-2, a significant amount of TLR4 was expressed on the cell surface. Co-expression with MD-2 slightly increased cell surface expression of TLR4 (Fig. 7B), albeit not significantly. However, neither TLR4\textsuperscript{C29A}, TLR4\textsuperscript{C40A}, nor TLR4\textsuperscript{C29A,C40A} appeared on the cell surface even when co-transfected with MD-2. TLR4\textsuperscript{C88A} was expressed on the cell surface only when co-transfected with MD-2. These results are consistent with those obtained from the MD-2 binding and LPS signaling (Fig. 6, A and B).
together, the results showing that TLR4<sup>C29A</sup>, TLR4<sup>C40A</sup>, or TLR4<sup>C29A,C40A</sup> is not localized on the cell surface support the conclusion that these mutants fail to bind to MD-2.

**DISCUSSION**

Since previous studies with deletion mutants of TLR4 from this and other laboratories (9, 10) have shown that the amino-terminal region of TLR4 is essential for its binding to MD-2, in this study, we attempted to identify an essential stretch of primary sequence in the extracellular TLR4 domain that directly binds to MD-2. In addition, the TLR4/2 chimera consisting of the TLR4 region Met<sup>1</sup>–Phe<sup>54</sup> and the TLR2 region Ala<sup>53</sup>–Ser<sup>784</sup> possesses the MD-2 binding property (10). MD-2 does not bind to TLR2 or CD14, both of which contain LRR structure like TLR4. Thus, we focused on the amino-terminal region containing no LRRs and generated the synthetic peptide corresponding to the amino-terminal TLR4 region Glu<sup>24</sup>–Lys<sup>47</sup> and examined whether this peptide directly binds to MD-2. The solid phase binding study (see Fig. 2B) and the pull-down and immunoprecipitation studies (see Fig. 2, C and D) demonstrate the direct binding of the TLR4 peptide to MD-2. We have further shown that the TLR4 peptide competes with the extracellular TLR4 domain for MD-2 binding (see Fig. 3). Thus, it is possible to conclude that the TLR4 region of Glu<sup>24</sup>–Lys<sup>47</sup> is a site for MD-2 binding.

The TLR4 peptide but not the TLR2 peptide has been shown to down-regulate LPS-induced NF-κB activation and IL-8 secretion in the presence of sMD-2 in TLR4-transfected cells (see Fig. 4). It is likely that a complex formation of the TLR4 peptide and its interaction with sMD-2 may prevent sMD-2 from binding to WT TLR4 on the cell surface, resulting in the down-regulation of LPS signaling. A recent study (15) has shown that plasma from patients with severe sepsis and septic shock contains soluble MD-2 that elicits LPS activation in TLR4-expressing epithelial cells and human umbilical vein
endothelial cells. Depletion of endogenous soluble MD-2 from human serum, with an immobilized TLR4-Fc fusion protein, abrogates TLR4-mediated LPS responses (16). In addition, the purified TLR4-Fc fusion protein inhibits the interaction of MD-2 with TLR4, thus preventing LPS stimulation. The study proposes that the binding of TLR4-Fc to MD-2 can be utilized for drug therapy against endotoxin-induced disease. Since the TLR4 peptide is thought to inhibit TLR4-mediated LPS responses by a mechanism similar to that of TLR4-Fc fusion protein, it is possible to infer that the TLR4 peptide can also be used for dampening endotoxin-induced inflammation, with a high probability of realization, presumably due to its availability.

Since the extracellular TLR4 domain contains 16 cysteine residues, we investigated the role of cysteine residues in its association with MD-2. The reduced and S-carboxymethylated sTLR4 did not co-precipitate with sMD-2 (see Fig. 5). Titration analysis with 5,5′-dithiobis(2-nitrobenzoic acid) reveals that sTLR4 contains a free thiogroup. Thus, this study indicates that the disulfide bonding is important for the binding of TLR4 to MD-2. The amino-terminal TLR4 region Glu24–Lys47 contains Cys29 and Cys40, and the TLR4 mutants, TLR4C29A, TLR4C40A, and TLR4C29A.C40A, fail to associate with MD-2 and to transmit LPS signaling (see Fig. 6). These mutants are not expressed on the cell surface (see Fig. 7). Glycoprotein Ibα (GPIbα) is a member of LRR proteins, and its amino-terminal sequence is similar to that of TLR4 (17). This protein possesses the amino-terminal β-hairpin that has two anti-parallel strands with a disulfide bridge (Cys54–Cys17) at the base. A structure homology search indicates that GPIbα also shows greatest similarity with TLR3 (18–20). Although we have not determined whether Cys29 and Cys40 of TLR4 form a disulfide bridge, it is likely that these two cysteine residues are strongly involved in MD-2 binding, since the hairpin is a ligand binding site of GPIbα (21).

The TLR4 mutants, TLR4C29A, TLR4C40A, and TLR4C29A.C40A, were not expressed on the cell surface (see Fig. 7). This is consistent with the result showing that these mutants do not possess the ability to bind MD-2 (see Fig. 6A). TLR4CR88A retains weak ability to bind MD-2 and is expressed on the cell surface only when transfected with MD-2. Consistently, LPS signaling mediated by this mutant is quite weak but nonetheless significant. These results are reasonable, since interaction with MD-2 has been shown to be critical for correct intracellular distribution of TLR4 and LPS responsiveness (14). WT TLR4 and TLR4CR88A, which are expressed on the cell surface, exhibit highly glycosylated forms, whereas TLR4C29A, TLR4C40A, or TLR4C29A.C40A that is not expressed on the cell surface does not contain a highly glycosylated band (see Fig. 6A; IP: α-FLAG, WB: α-FLAG). A mature form of full-length TLR4 containing the upper minor band, which is an endoglycosidase H-resistant form of carbohydrate chains, is localized on cell surface when it is co-expressed with MD-2 (9). Immature TLR4 is suggested to be glycosylated in the endoplasmic reticulum but does not reach the medial Golgi compartment, where glycosylation of endoglycosidase H-resistant carbohydrate occurs. Thus, the present data suggest that TLR4C29A, TLR4C40A, and TLR4C29A.C40A are retained in the endoplasmic reticulum and are not processed to a mature form of the highly glycosylated protein.

In this study, we have shown that the amino-terminal TLR4 region of Glu24–Lys47 containing no LRRs directly binds to MD-2. Lipid A interacts with a cell surface receptor complex of TLR4 and MD-2 with higher affinity than with MD-2 alone or CD14 alone when expressed on the cell surface (22). It remains unknown how the receptor complex of TLR4 and MD-2 interacts with LPS. Although an answer to this question may have to await analysis of the receptor complex with crystal structure, it is possible to assume that the LRR motif of TLR4 is still available for interaction with LPS, since only the short segment of the amino-terminal TLR4 region is used for the MD-2 binding. The crystal structure of human TLR3 ectodomain reveals a large, horseshoe-shaped solenoid assembled from 23 LRRs (23). Two patches of positively charged residues and a TLR3-specific LRR insertion in the LRR motifs have been suggested to provide an appropriate binding site for its ligand, double-stranded RNA.

In conclusion, the TLR4 region Glu24–Lys47 is a site for MD-2 binding. Cysteine residues at amino acids 29 and 40 within this region are critical for the interaction of TLR4 with MD-2 and LPS signaling.

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