A Single Amino Acid of Toll-like Receptor 4 That Is Pivotal for Its Signal Transduction and Subcellular Localization*

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Toll-like receptor 4 (TLR4) is essential for recognizing a Gram-negative bacterial component, lipopolysaccharide (LPS). A single amino acid mutation at position 712 of murine TLR4 leads to hyporesponsiveness to LPS. In this study we determined that an amino acid, a leucine at position 815 of human TLR4, is also pivotal for LPS responsiveness and subcellular distribution. By replacing the leucine with alanine, the mutant TLR4 lost responsiveness to LPS and did not localize on the plasma membrane. In addition, it does not coprecipitate with myeloid differentiation-2, an accessory protein that is necessary for TLR4 to recognize LPS. These results suggest that the leucine at position 815 is required for the normal maturation of TLR4 and for formation of the TLR4-MD-2 complex.

Toll-like receptors (TLRs)3 play essential roles in both innate and adaptive immunity (1). Thirteen members of the TLR family have been identified in mammals. TLRs have leucine-rich-repeats in their extracellular domains and a Toll/Interleukin-1 receptor (TIR) in their cytoplasmic domains, the latter of which mainly mediates intracellular signaling. Signaling pathways of TLRs, except for TLR3, depend on an adapter protein, MyD88 (myeloid differentiation factor 88), which interacts with the TIR domain of TLRs. This pathway leads to the activation of the transcription factor NF-κB and production of cytokines such as tumor necrosis factor-α and interleukin-6. Another important signaling pathway mediated by TLR3 and TLR4 that exploits the TIR domain is the MyD88-independent pathway. This pathway involves different adapter proteins, such as the TIR domain-containing adapter inducing interferon-β (TRIF) and TRIF-related adapter molecule (2–4), and is essential for production of type 1 interferon through activation of interferon regulatory factor-3.

TLRs recognize as ligands several microbial pathogen-associated molecular patterns. One such pathogen-associated molecular pattern is lipopolysaccharide (LPS), which is recognized by TLR4. LPS triggers severe immunologic reactions by the host in Gram-negative bacterial infections and has drawn attention in many clinical situations. TLR4 is the first mammalian TLR to be discovered in the context of immunology. TLR4 was identified in the search for the genes responsible for LPS hyporesponsiveness (5, 6). The defect was found to stem from a single amino acid mutation, replacement of proline with histidine at position 712, in the cytoplasmic tail of murine TLR4. The study led to the discovery of the importance of TLR4 in innate immunity.

A variety of cells are activated by LPS stimulation through TLR4. TLR4 forms a receptor complex with an accessory protein, myeloid differentiation-2 (MD-2). MD-2 first associates with TLR4 in the endoplasmic reticulum (ER) and cis-Golgi, and both proteins move together to the plasma membrane (7, 8). Upon recognition of LPS, the TLR4-MD-2 complex receives LPS on the cell surface and initiates intracellular signaling. The expression of TLR4 in the absence of MD-2 does not confer full responsiveness to LPS stimuli in experimental cell lines (9). An analysis of MD-2 knockout mice revealed that MD-2 is important not only for LPS sensing but also for cellular distribution of TLR4.

In this study we hypothesized that the cytoplasmic tail of TLR4 contains regions that control both localization and signaling. Using truncation and mutation analysis, and paying particular attention to the TIR domain, we identified a single amino acid that is pivotal for both TLR4 signaling and subcellular distribution. The site we found was on the C-terminal portion of the TIR domain for which no specific function has been yet determined.
Reagents and Other Materials—Lipopolysaccharide (LPS) from Escherichia coli O55:B5 was purchased from Sigma-Aldrich and applied without repurification. FLAG- and hexa-histidine (His6)-tagged human TLR4 expression plasmid (pEFBOS/humanTLR4flaghis) and FLAG- and His6-tagged human MD-2 expression plasmid (pEFBOS/humanMD-2flaghis) were generous gifts from Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo, Japan). Human CD14 cDNA plasmid (pCMV6-XL5/humanCD14) was purchased from OriGene (Rockville, MD). Fluorescent protein expression vector pEGFP-N3 was purchased from Clontech (Mountain View, CA). Anti-TLR4 monoclonal antibody (clone HTA125) was purchased from Abcam (Cambridge, MA). Anti-FLAG monoclonal antibody (clone M2) was purchased from Sigma-Aldrich. Anti-A. v. (GFP) monoclonal and polyclonal antibodies were purchased from Dako (Glostrup, Denmark). BlockAce (DS Pharma Biomedical, Osaka, Japan) solution was used as blocking buffer for Western blotting.

Expression Vector Subcloning and Mutagenesis—Wild-type TLR4 cDNA was excised from pEFBOS/humanTLR4flaghis and subcloned into pEGFP-N3 so that when expressed enhanced green fluorescent protein (EGFP) would be fused at the C terminus of TLR4 (pEGFP-N3/humanTLR4). All mutations were introduced into pEFBOS/humanTLR4flaghis and pEGFP-N3/humanTLR4 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions and were confirmed by sequencing. For the truncation analysis, two identical unique restriction sites were prepared in the TLR4-coding region of pEFBOS/humanTLR4 using a QuikChange kit, and the DNA fragment to be removed, which was also a part of the C terminus of TLR4, was excised enzymatically. After agarose gel purification, the linear double-stranded DNA was ligated to re-form a circular plasmid. Restriction sites were designed so as not to cause a frameshift between TLR4 and EGFP.

Confocal Laser Scanning Microscopy of Cells—Samples were fixed in 3% paraformaldehyde-phosphate-buffered saline at 37 °C for 10 min. Fluorescence images of fixed samples were recorded using a FluoView FV1000 Confocal Microscope (an inverted confocal laser scanning microscope, Olympus, Tokyo, Japan).

EXPERIMENTAL PROCEDURES

Immunoprecipitation—Transfected cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 1 mM 1,4-dithiothreitol, and proteinase inhibitor mixture), sonicated, and centrifuged at 4 °C. Antibody was added to the supernatant, and the sample was rotated 1 h at 4 °C followed by the addition of protein G-Sepharose (GE Healthcare Life Sciences, Piscataway, NJ) and an additional 8-h incubation at 4 °C. Bound protein was washed three times in lysis buffer. Proteins were eluted by boiling in SDS sample buffer.

Biotinylation and Purification of Cell Surface Proteins—Prior to surface biotinylation, HEK 293T cells plated in a 100-mm dish were transiently transfected as described above. Surface biotinylation and subsequent purification of biotinylated proteins were performed using a Cell Surface Protein Biotinylation and Purification Kit (Pierce) following the manufacturer’s instructions. Briefly, membrane-impermeable sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate ( Sulfo-NHS-SS-Biotin) was added to cell monolayers in the culture dishes and covalently bound to amines in proteins exposed on the cell surface. The affinity resin that binds to the biotin end of Sulfo-NHS-SS-Biotin was used to collect the biotinylated proteins. Reduction by 1,4-dithiothreitol causes cleavage of the disulfide bond in Sulfo-NHS-SS-Biotin, and the elute contains the biotinylated cell surface proteins. Each final sample obtained was considered to contain proteins from an equal amount of cells, because all culture plates were treated equally and grown to full confluence. All samples were sonicated and subjected to SDS-PAGE and Western blotting. The membrane to which protein was transferred was blocked in blocking buffer for 1 h. Then the membrane was incubated with a primary antibody, followed by incubation with horseradish peroxidase-labeled anti-immunoglobulin antibodies. The protein bands were then visualized by using a chemiluminescence reagent, Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), according to the manufacturer’s instructions.

Cell Stimulation Assays—HEK293T cells were plated and transiently transfected for assays. Thirty-six hours after the transfection, LPS was added to fresh culture medium in each well of the culture plates at the stated concentration. The duration of LPS stimulation was 7 h.

Dual Luciferase Reporter Assays for NF-κB Activation—HEK293T cells were plated in 12-well culture plates (4 × 10⁴ cells/well), and experimental cDNA plasmids were transiently transfected 36 h later using the FuGENE 6 transfection reagent. LPS stimulation was used to drive the NF-κB-reporter plasmid expressing firefly luciferase (pNF-κB-Luc, Stratagene) and 0.05 μg of constitutively active Renilla luciferase reporter plasmid (pRL-TK, Promega, Madison, WI) in addition to 0.5 μg each of TLR4-EGFP plasmid and MD-2 plasmid. Stimulation experiments were performed 36 h later. Firefly luciferase and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and the Genelight55 luminometer (Microtech, Chiba, Japan). Relative luciferase activity (RLA) was obtained as the ratio of firefly luciferase activity to Renilla luciferase activity. Results are expressed as the ratio of RLA with LPS stimulation to RLA without LPS stimulation (RLA LPS−/RLA LPS+).
An Important Amino Acid of TLR4 for Its Function

Truncation Analysis of TLR4—To identify amino acid sequences in the cytoplasmic tail of TLR4 that are involved in both signal transduction and subcellular distribution, first we generated five truncation mutants of TLR4 with a fluorescent protein (EGFP) at the C terminus of TLR4.

Although there are no known definite sorting signal motifs in the cytoplasmic tail of TLR4, some amino acid sequences are similar or identical to known general sorting signal motifs as shown in Fig. 1. YXX0, a form of tyrosine-based sorting signal, and EXXXLL, a form of dileucine (LL)-based sorting signal, both control protein internalization, lysosomal targeting, and basolateral targeting (10), where “X” represents any amino acid, “Ø” stands for an amino acid residue with a bulky hydrophobic side chain, and other letters are single-letter abbreviations for the amino acids. “Diacidic” signals such as DXE mediate export from the ER (11). RR or RXXR is another example of an ER export signal (12).

Truncation sites were chosen so that some of these amino acid sequences were deleted in each mutant. Because the TIR domain, which is essential in TLR4 signaling and possibly subcellular localization (13), spans most of the cytoplasmic domain of TLR4, four out of five mutants have involvement in the TIR domain, which we hypothesized could result in impaired signal transduction and a change in subcellular distribution. Part of the cytoplasmic portion of the amino acid sequence of the truncation mutants is shown in Fig. 1. The five truncation mutant proteins lost their C-terminal tails at positions 826, 815, 802, 788, and 766, respectively, and were conjugated with EGFP at the C terminus of TLR4.

HEK293T cells were transfected with plasmids containing the gene for wild-type TLR4 or a truncated human TLR4-EGFP fusion protein, in addition to a luciferase reporter and human MD-2 plasmid (A) or unmodified plasmids (control) (B). After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. All results were expressed as the ratio of relative luciferase activity with LPS stimulation to that without stimulation. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), p = 0.002; TLR4 (826tr)-EGFP/MD-2 (+), p = 0.016).

**RESULTS**

**Truncation Analysis of TLR4**—To identify amino acid sequences in the cytoplasmic tail of TLR4 that are involved in

**FIGURE 1. Alignment of the cytoplasmic domains of EGFP fusion TLR4 truncation mutants used in this study.** TLR4 (766tr) signifies the mutant truncated at position 766. Others are named in the same manner. The amino acids are colored based on their physicochemical properties: pink, basic; blue, acidic; green, polar and neutral; and orange, hydrophobic. The black overline represents the TIR domain. Colored overlines indicate amino acid sequences identical to known sorting signal motifs except for two LLs, which are dileucine motif-like sequences in that they consist of solely two consecutive leucines without preceding aspartate or glutamate. Capital letters on the line signify the single-letter code for amino acids: E, glutamic acid; L, leucine; R, arginine; and Y, tyrosine. X signifies any amino acid, and Ø signifies an amino acid residue with a bulky hydrophobic side chain.

**FIGURE 2. LPS responsiveness measured by NF-κB luciferase assay.** HEK293T cells were transfected with plasmids containing the gene for wild-type TLR4 or a truncated human TLR4-EGFP fusion protein, in addition to a luciferase reporter and human MD-2 plasmid (A) or unmodified plasmids (control) (B). After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. All results were expressed as the ratio of relative luciferase activity with LPS stimulation to that without stimulation. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), p = 0.002; TLR4 (826tr)-EGFP/MD-2 (+), p = 0.016).

**Statistical Analyses**—All quantitative experiments were repeated three times, and each experiment was done in triplicate. The ratio of relative luciferase activity of LPS+ to LPS− was calculated as the index of the responsiveness to the stimuli as explained above. When positive response is observed, the ratio should significantly exceed one. The means of the ratio were represented in bar graphs. The 95% confidence interval of the mean of the ratio was calculated and indicated on each bar in the graph, and p values were calculated using Student’s t distribution compared with the hypothetical mean, one.

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perinuclear area. These findings were consistent with observations by others (14, 15). TLR4 is reported to localize in the Golgi apparatus as well as on the plasma membrane. Our observation of TLR4-EGFP accumulation in the perinuclear area does not contradict the report that TLR4 partly localizes in the Golgi apparatus (14).

TLR4-EGFP truncation mutants, 815tr, 802tr, 788tr, and 766tr apparently did not localize at the plasma membrane. No particular fluorescence pattern that might be characteristic of localization to a specific intracellular compartment was observed. Only TLR4 (826tr)-EGFP, which has the shortest truncation, was expressed on the plasma membrane and in the perinuclear area, and the fluorescence pattern was similar to that of wild-type (Fig. 3A). No TLR4 genotypes, including wild-type TLR4-EGFP, clearly localized on the plasma membrane in the absence of MD-2 (Fig. 3A). MD-2 is reported to be necessary for localization of wild-type TLR4 at the plasma membrane (15), which is consistent with our observation. Intracellular distribution of mutant TLR4 varied depending on the genotype, but no particular cellular structure was identified as an alternative target site. Furthermore, we examined the plasma membrane expression of TLR4 (826tr)-EGFP by cell surface protein biotinylation. The expression level of TLR4 (826tr)-EGFP was markedly decreased without coexpression of MD-2 (Fig. 3B), which is compatible with the microscope observation.

Removal of the C-terminal segment of TLR4 at residue 826 does not qualitatively affect LPS responsiveness and subcellular distribution. However, when more residues, up to position 815, were removed, both signal transduction and plasma membrane localization were impaired. These results suggest that residues 815–826 of TLR4 contain at least one segment that is critical for those functions.

Amino Acid Sequence Replacement Analysis—To identify critical amino acid sequences in this region, we generated an amino acid replacement mutant of TLR4 instead of truncation mutants. As shown in Fig. 1, although it is not a canonical sequence, leucine-leucine at 815–816 partially fits a known sorting signal motif, a dileucine motif, (D/E)XXXL(L/I) or DXXL, which plays an important role in internalization of plasma membrane protein or sorting from the trans-Golgi network (10). Thus, as has been done in a similar study (16), a mutant was generated in which alanines were substituted for both leucines at positions 815 and 816.

We measured the NF-κB activity of TLR4 (L815AL816A)-EGFP, the mutant in which both leucines were replaced with alanines, under LPS stimulation (Fig. 4A). This mutant protein did not respond to LPS stimuli. Microscopic observation revealed that TLR4 (L815AL816A)-EGFP was not expressed on the plasma membrane regardless of whether MD-2 was cotransfected (Fig. 4B). The phenotype of this doubly substituted mutant appeared to be the same as that of the truncation mutants. These results imply that the leucines in positions 815 and 816 play an important role in TLR4 plasma membrane localization.

Analysis of Single Amino Acid Substitution Mutants—As previously mentioned, the amino acid sequence leucine-leucine at positions 815 and 816 does not completely match the dileucine motif, i.e. it lacks a preceding acidic amino acid. Therefore it
was reasonable to explore whether leucines 815 and 816 need to be adjacent to each other. We created five genotypes of single amino acid mutants of TLR4: TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP. We excluded the amino acid at position 814 from the analysis, because the amino acid in position 814 of wild-type TLR4 is alanine. The amino acid sequence alignment of wild-type TLR4 and the single amino acid replacement mutants is shown in Fig. 5. DNA sequences were confirmed by sequencing.

As was done with truncation mutants, we measured NF-κB activity of wild-type TLR4-EGFP, TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP in response to LPS stimulation. All mutants except TLR4 (L815A)-EGFP showed responsiveness to LPS stimulation with coexpression of MD-2 (Fig. 6A). Without MD-2, no genotype of TLR4-EGFP responded to LPS stimulation (Fig. 6B). LPS stimulation was performed in an identical manner as with truncation mutants.

We analyzed the subcellular distribution of single amino acid mutants of TLR4-EGFP with and without MD-2 coexpression by fluorescence microscopy. TLR4 (K813A)-EGFP and TLR4 (D817A)-EGFP showed a similar fluorescence pattern to the wild-type, which localized at the plasma membrane when coexpressed with MD-2. No genotypes of TLR4-EGFP localized on the plasma membrane without MD-2 (Fig. 7). The cells transfected with TLR4 (L815A)-EGFP coexpressed with MD-2 did not show plasma membrane fluorescent pattern. Also, TLR4 (L815A)-EGFP showed comparatively weaker fluorescence than other mutants, possibly due to lower expression of the protein. Fluorescence of TLR4 (L816A)-EGFP with MD-2 was ambiguous as for the plasma membrane expression. Some kind of membranous structure was observed in the cytoplasmic area, but the intensity of the plasma membrane green fluorescence was obscure. Together with the results from the LPS stimulation experiment, the leucines at positions 815 and 816 are considered to play important roles in signal transduction and/or subcellular distribution of TLR4.

Because EGFP consists of 239 amino acids, which is about one-third the size of the complete TLR4 protein, the experimental results obtained using TLR4-EGFP could have been influenced by the presence of the EGFP fused at the C terminus of TLR4. To rule out this possibility, we tested the functional integrity of both TLR4 (L815A) and TLR4 (L816A) with and without EGFP at the C terminus. Reporter assays were performed under the same conditions except that the shorter tag, FLAG-His6, which has only 21-amino acid tags at the C terminus, was fused to TLR4 in place of EGFP. There was no difference
between EGFP-tagged proteins and FLAG-His<sub>6</sub>-tagged proteins in the relative pattern of responsiveness against LPS stimulation (Fig. 8A). Because CD14 is also important for LPS recognition by TLR4, we examined the effect of CD14 coexpression on the phenotypic changes of the mutants (17, 18). Coexpression of CD14 did not change the phenotypes of wild-type TLR4, TLR4 (L815A), and TLR4 (L816A) in terms of LSP responsiveness (data not shown).

Cell surface expressions of the wild-type, L815A mutant, and L816A mutant TLR4-FLAG-<sup>H</sup>is<sub>6</sub> fusion proteins were also examined. Live cells transfected with wild-type TLR4, the L815A mutant or the L816A mutant as well as human MD-2 and CD14 were biotinylated on the cell surface, and the biotinylated proteins were affinity-purified and subjected to Western blotting. Fig. 8B shows the marked difference in cell surface expression of wild-type and mutants L815A and L816A. Note that biotinylated proteins have additional residues on every amine of the extracellular domain, which leads to a band shift during electrophoresis. Although both mutants were detected far less than the wild-type on the cell surface, comparatively more L816A mutant was expressed on the plasma membrane than L815A mutant, and the amount of L815A mutant seemed to be negligible compared with the wild type. These results may clarify the ambiguity of the microscopic observation of TLR4 (L815A) and TLR4 (L816A). Plasma membrane expression of TLR4 was impaired when the leucine at 815 or 816 was replaced to alanine. But the leucine at 815 is more critical, and the mutant L816A seems to be somewhat faint in the mutant, whereas in the wild type the heavier band is at least as dense as the lighter one. TLR4 can be detected as two separate bands in a Western blot (19), especially under transient transfection conditions. The difference in proportion of the heavy and light bands between wild-type and mutant TLR4 may suggest that there is some difference in glycosylation. Furthermore, wild-type TLR4 was coprecipitated with MD-2-FLAG-<sup>H</sup>is<sub>6</sub>, but the mutant TLR4 could not be detected (Fig. 8C, lanes 4 and 8). Because MD-2 is
associated with TLR4 (9), it is logical to expect that immuno-
precipitating MD-2-FLAG-His<sub>6</sub> with anti-FLAG antibody
should cause TLR4 to be coprecipitated with it. It is suggested
by the result here that the association of the TLR4 mutant with
MD-2 is impaired.

DISCUSSION

In this research, we performed mutagenesis analyses of par-
ticular amino acid residues in TLR4 to explore the mechanisms
of TLR4 intracellular signal transduction and subcellular dis-
tribution. We found the candidate residues by analyzing trunca-
tion mutants of TLR4 in the cytoplasmic region, in which both
signaling and normal subcellular distribution of TLR4 are dis-
turbed. Because we are focusing on a common mechanism for
the impaired signaling and distribution, we finally picked a sin-
gle amino acid mutant that does not respond to LPS stimuli, as
measured with NF-κB reporter luciferase assay, and one that
does not localize on the plasma membrane. TLR4 (L815A) is a
mutant that meets these conditions, and our results suggest
that the leucine at position 815 of TLR4 is required for both
signal transduction and plasma membrane localization.

The best known single amino acid mutant of TLR4 is TLR4
(P712H) known as the Lps<sup>d</sup> mutation in the C3H/HeJ mouse,
which corresponds to position 714 in this study of human TLR4
(5, 6, 20). Mice carrying this mutation opened up the rediscover-
y of TLR4 as a key player in innate immunity. Because this
proline residue at this position is within the TIR domain and is
conserved among TLRs or TLR4s of other species, it is assumed
that the residue plays an important role in TLR4 function. The
association of TLR4 (P712H) with its adapter proteins is
reported to be intact, and the explanation for the functional
impairment of TLR4 (P712H) is not clear (21–23).

Some single amino acid variants are found in humans, and
these are related to the incidence or prognosis of some infec-
tions and other diseases. A growing body of data suggests that
the ability of certain individuals to respond properly to TLR4
ligands may be impaired by single-nucleotide polymorphisms
within TLR4 genes (24). The D299G and T399I alleles of the
TLR4 gene have been associated with increased risk of severe
infections (25).

By clarifying the subcellular component where the mutant
protein is retained, or by clarifying to which compartment the
mutant is not delivered, the abnormal intracellular sorting that
is caused by the mutation in TLR4 (L815A) could be elucidated
more precisely. Usually a sorting signal motif is comprised of
several amino acids. In this regard, if the leucine at position 815
is a part of a motif, there should be other amino acids that are
also members of the motif. Although replacement of leucine
with alanine at position 816 did not cause an apparent signal
transduction impediment, plasma membrane expression of
TLR4 (L816A) was impaired to a certain extent. Positive

![Figure 8](image-url)

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response to LPS stimulation by TLR4(L816A) could be attributable to this small amount of expression on the plasma membrane. Mutagenesis analyses of neighboring amino acids of the leucine at 815 were not definitive, but the results could be suggestive that the adjacent leucine at 816 may work together with the leucine at 815. Leucines at position 815 and 816 could be in the same motif, and the leucine at position 816 may be less critical.

Several proteins have been reported to be involved in TLR4 cell surface expression. Heat shock protein gp96 is necessary for TLR4 association with MD-2 in the ER and for subsequent cell surface expression (26). PRAT4A and PRAT4B are associated with TLR4 and regulate TLR4 cell surface expression (27, 28). In embryonic fibroblasts of MD-2 knockout mice, TLR4 localization on the cell surface is severely impaired, and most TLR4 is retained in the ER or Golgi apparatus (15). MD-2 binds to TLR4 at its extracellular domain and is essential for LPS recognition by TLR4 (29). Although proteins such as CD14 and LPS-binding protein are reported to have important roles in LPS recognition by TLR4, in an in vitro setting HEK293T cells gain LPS responsiveness by introducing only TLR4 and MD-2 genes when measured by NF-κB reporter assay (9, 30). Without transfection, HEK293 cells do not express TLR4, MD-2, or CD14, for TLR4 function. Asparagine residues in the extracellular portion of neighboring amino acids of the leucine at 815 may cause a conformational change in the extracellular portion of the protein, which may interfere with the association between L815A mutant TLR4 and MD-2, leading to inhibition of glycosylation and cell surface expression of the mutant protein. Further investigation may reveal the mechanism involved in this phenotypic change in TLR4 (L815A), which would lead to better understanding of the mechanism of wild-type TLR4 signaling and trafficking.

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