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Surface-Expressed TLR6 Participates in the Recognition of Diacylated Lipopeptide and Peptidoglycan in Human Cells

Yoshiya Nakao,‡ Kenji Funami,*‡ Satomi Kikkawa,* Mitsue Taniguchi,* Miyuki Nishiguchi,* Yasuhiro Fukumori,† Tsukasa Seya,§§ and Misako Matsumoto†‡†

Recognition of microbial components by TLR2 requires cooperation with other TLRs. TLR6 has been shown to be required for the recognition of diacylated lipoproteins and lipopeptides derived from mycoplasma and to activate the NF-κB signaling cascade in conjunction with TLR2. Human TLR6 is expressed on the cell surface in a variety of cells, including monocytes, neutrophils, and monocyte-derived, immature dendritic cells (iDCs), whereas the expression profile of TLR6 in human cells remains obscure. In this study we produced a function-blocking mAb against human TLR6 and analyzed TLR6 expression in human blood cells and cell lines. The Journal of Immunology, 2005, 174: 1566–1573.

Toll-like receptors play an essential role in initiating the innate immune response against infectious pathogens (1–3). Ten members of the TLR family protein (TLR1 to TLR10) have been identified in humans (4–8). TLRs recognize a wide variety of pathogen-associated molecular patterns from bacteria, viruses, and fungi and elicit a wide array of antimicrobial responses. Among TLRs, TLR2 is a unique receptor that recognizes lipid- and/or carbohydrate-containing microbial components in cooperation with other TLRs (9–21). TLR1 and TLR2 are required for recognition of mycobacteria liporabinomannnan and triacylated lipoproteins/lipopeptides, such as 19-kDa mycobacterial lipoprotein and Borrelia burgdorferi outer surface protein A, in both human and mouse cells (16–19). Human TLR1 is expressed on the cell surface in monocytes, monocyte-derived immature dendritic cells (iDCs), and neutrophils (18, 22, 23). An Ab blocking study revealed that the surface-expressed human TLR1 participates in ligand recognition in conjunction with TLR2 (19). In contrast, TLR6 and TLR2 have been shown to be required for cytokine production in mouse macrophages stimulated with synthetic diacylated lipoprotein macrophage-activating lipopeptide-2 (MALP-2), a peptidoglycan derived from Staphylococcus aureus, or zymozan (13, 14). TLR1 and TLR6 are closely related to each other and are similar to TLR2 (6). In contrast to TLR1, which has been studied in both human and mouse cells, TLR6 function has been studied mainly in mouse cells. Human TLR6 is a 796-aa type I transmembrane protein that is 74% identical with mouse TLR6 and 69% identical with human TLR1 (6). It contains an N-terminal signal peptide, 19 tandemly repeated extracellular leucine-rich motifs, and a cytoplasmic domain called the Toll/IL-1R homology domain, as seen in other TLRs (24). RT-PCR analysis detected human TLR6 mRNA in monocytes, monocyte-derived iDCs, and neutrophils (22, 25–28). However, the protein expression level of human TLR6 in innate immune cells remains unknown. In this study we raised mAbs against human TLR1 and TLR6 and analyzed the expression of TLRs in peripheral blood cells and cell lines. In addition, we performed an Ab blocking study that clearly showed an important role for surface-expressed TLR2 and TLR6 in recognizing their ligands, diacylated lipopeptide and peptidoglycan (PGN).

Materials and Methods

Cells and reagents

A human monocytic cell line, THP-1, and B cell lines Raji, P30, and Ball were obtained from Riken Cell Bank and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (JRH Biosciences) and antibiotics. HEK293 cells (a human embryonic kidney cell line) were maintained in DMEM supplemented with 10% FCS and antibiotics. The IL-3-dependent murine cell line BaF3 was cultured in RPMI 1640 medium containing 10% FCS, 5 ng/ml murine IL-3, 100 μM 2-ME, and antibiotics. CD14+ monocytes were isolated from human PBMCs using the MACS system (Miltenyi Biotec). Immature DCs were generated from monocytes (1 × 106...
Human IgG (10 mg/ml) was dialyzed against 0.1 M NaHCO3 at 4°C overnight. Biotin Biotinylation of anti-TLR2 and -TLR6 mAbs

Production of mAbs against human TLR6 and TLR1

Reporter gene assay

Immunofluorescence staining and confocal microscopy

Monocytes were stained with mouse IgG1, TLR2,45, or TLR1,136 (5 µg/ml) for 30 min at 4°C in FACS buffer. After the cells were washed twice with the above buffer, FITC-labeled secondary Ab (American Qualex) was added, and the cells were incubated for an additional 30 min at 4°C. For intracellular staining, cells were pretreated with the permeabilizing solution (BD Biosciences) for 10 min at room temperature, washed once with the FACS buffer, and then incubated with the indicated mAbs (0.1–0.5 µg) for 30 min at room temperature. Cells were then washed and treated with FITC-labeled secondary Ab for 30 min at room temperature. Ten percent goat serum was added to each reaction mixture to prevent nonspecific binding. Cells were analyzed on a FACScalibur (BD Biosciences).

Ab blocking and cytokine assays

THP-1 cells, monocytes, or monocyte-derived iDCs (1 x 10^6/ml) in 96-well plates were pretreated with 10 µg/ml mouse IgG1, anti-TLR1 mAb (TLR1,136), anti-TLR2 mAb (TLR2,45), or anti-TLR6 mAb (TLR6,127) for 1 h at 37°C, then stimulated with LPS (100 ng/ml), polymyxin B-treated Pam3 (100 ng/ml), or control PG (10 µg/ml) for 24 h. Concentrations of IL-8 and TNF-α in culture supernatants were measured by ELISA (Amersham Biosciences).

Production of mAbs against human TLR6 and TLR1

BALB/c mice were immunized with Ba/F3 cells that stably expressed human TLR1 or TLR6 together with pSV2neo plasmid (RIKEN Gene Bank) by electroporation. The transfectants were selected with 750 µg/ml G418 for 10 days. Expression of each TLR was confirmed by intracellular staining for the Myc epitope, which had been attached to the COOH terminus of each TLR. To obtain the stable transfectants expressing the Flag-tagged TLR2 or its mutated constructs, HEK293 cells were transfected with anti-Flag M2 mAb or anti-Myc mAb for 2 h at 4°C. The immune complexes were precipitated with protein G-Sepharose and washed thoroughly. Immunoprecipitated proteins were eluted by adding Dulbecco’s PBS containing 1% SDS, 2% 2ME and boiling, then subjected to SDS-PAGE (7.5%) under reducing conditions, followed by immunoblotting with anti-Myc Ab. The blot was reprobed with anti-Flag mAb to detect TLR2.

RT-PCR analysis

Total RNA was isolated from Raji, P30, and Ball using the RNeasy Mini kit (Qiagen). DNase I-treated total RNA (3 µg) was reverse transcribed using random primers with RNeasy H-free reverse transcriptase (Invitrogen Life Technologies). TLR1, TLR2, TLR6, and GAPDH were amplified using the specific primers described below: TLR1, 5'-CAAGTTCTCTAAA GACCTATCCC-3'; TLR6, 5'-GTC-3'; TLR2, 5'-GGCTTTGTGTGCGATACCT-3'; C5'-CAACCTCTATATTGACGT TGC-3'; TLR6, 5'-ATATCGGCCATTACTATG TG-3'; and GAPDH, 5'-CAACGTCATACGCTCAGT G-3'/5'-TATTTCCCAG TG-3'.

Biotinylation of anti-TLR2 and -TLR6 mAbs

Labeling of mAbs with biotin was performed as follows. Protein solution (1.0 mg/ml) was dialyzed against 0.1 M NaHCO3 at 4°C overnight. Biotin (Long Arm) NHS-water soluble (Vector Laboratories) was dissolved in 0.1 M NaHCO3 (1 mg/ml) immediately before use and was added to the protein solution with mixing. The mixture was kept at room temperature for 2 h, then dialyzed against PBS at pH 7.4.

Flow cytometry

Cells were incubated with the indicated mAbs (0.5–1 µg) together with human IgG (10 µg) for 30 min at 4°C in FACS buffer (Dulbecco’s PBS containing 1% BSA and 0.1% sodium azide). After the cells were washed twice with the above buffer, FITC-labeled secondary Ab (American Qualex) was added, and the cells were incubated for an additional 30 min at 4°C. For intracellular staining, cells were pretreated with the permeabilizing solution (BD Biosciences) for 10 min at room temperature, washed once with the FACS buffer, and then incubated with the indicated mAbs (0.1–0.5 µg) for 30 min at room temperature. Cells were then washed and treated with FITC-labeled secondary Ab for 30 min at room temperature. Ten percent goat serum was added to each reaction mixture to prevent nonspecific binding. Cells were analyzed on a FACScalibur (BD Biosciences).

Immunoprecipitation

HEK293 cells in six-well plates were transfected with pFlag-CMV-ITL2 (1 µg) together with pEFBOS-HTL6-Myc (4 µg). The total amount of DNA (5 µg) was kept constant by adding empty vector. After 24 h, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 25 mM iodoacetamide, and 2 mM PMSF) for 20 min at room temperature. After centrifugation, the cell lysates were incubated with anti-Flag M2 mAb or anti-Myc mAb for 2 h at 4°C. The immune complexes were precipitated with protein G-Sepharose and washed thoroughly. Immunoprecipitated proteins were eluted by adding Dulbecco’s PBS containing 1% SDS, 0.2% Nonidet P-40, and 5% 2ME and boiling, then subjected to SDS-PAGE (7.5%) under reducing conditions, followed by immunoblotting with anti-Myc Ab. The blot was reprobed with anti-Flag mAb to detect TLR2.

cDNA expression vectors

Human TLR1 and TLR2 cDNAs were generated by PCR from cDNA derived from human monocytes cultured with recombinant human GM-CSF (PeproTech EC) for 9 days and cloned into pEFBOS, a gift from Dr. S. Nagata (Osaka University, Osaka, Japan). The Flag-TLR2 expression vector was constructed by inserting the coding region of human TLR2 minus the respective NH2-terminal signal sequence into the mammalian expression vector pFlag-CMV-1 (Sigma-Aldrich) at the HindIII and KpnI sites (15). The expression vectors for TLR2 mutants C30S, C36S, and C75S were made with a QuickChange Site-Directed Mutagenesis kit (Stratagene) using a pFlag-CMV-TLR2 expression vector as a template. The C-terminal Myc-tagged human TLR6 expression vector was a gift from Dr. S. Akira (Osaka University, Osaka, Japan) (14). The plasmids were prepared with an endotoxin-free Plasmid Maxi kit (Qiagen).

Stable transfectants

Ba/F3 cells were transfected with pEFBOS expression vectors encoding human TLR1 or TLR6 together with pSV2neo plasmid (RIKEN Gene Bank) by electroporation. The transfectants were selected with 750 µg/ml G418 for 10 days. Expression of each TLR was confirmed by intracellular staining for the Myc epitope, which had been attached to the COOH terminus of each TLR. To obtain the stable transfectants expressing the Flag-tagged TLR2 or its mutated constructs, HEK293 cells were transfected with the indicated expression vector together with pSV2neo plasmid using Lipofectamine 2000 reagent (Invitrogen Life Technologies). The transfectants were selected with 4418 (Sigma-Aldrich; final concentration, 600 µg/ml) for 2–3 wk.

Reporter gene assay

HEK293 cells were transiently transfected in 96-well plates using Lipofectamine 2000 reagent (Invitrogen Life Technologies) with pEFBOS/TLR2 (0.15 ng), pEFBOS/TLR6-Myc (15 ng), or empty vector together with a luciferase-linked E-selectin (ELAM) promoter plasmid (40 ng) in which the promoter region of human ELAM (~241 to ~54) was ligated between the KpnI-HindIII sites of pGv-E2 (Toyo). The total amount of transfected DNA (0.2 µg) was kept constant by adding empty vector. The plasmid pHRL-TK (1 ng) was used as an internal control. Twenty-four hours after transfection, cells were stimulated with medium alone or poly-lysine B-treated MALP-2 (100 nM), or PGN (10 µg/ml) for 24 h. Concentrations of IL-8 and TNF-α in culture supernatants were measured by ELISA (Amersham Biosciences).

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The Journal of Immunology
**Results**

*Specific recognition of TLR1 and TLR6 by mAbs*

First we examined whether anti-TLR1 mAb (TLR1.136) and anti-TLR6 mAb (TLR6.127) could immunoprecipitate human TLR1 or TLR6 expressed in a variety of cells. These mAbs recognized the extracellular portion of native TLR1 and TLR6, respectively, but failed to react with the denatured form of TLR1 and TLR6 on blots. Myc-tagged TLR1 or TLR6 in lysates of HEK293 transfectants was pulled down with isotype control mouse IgG1, anti-Myc, TLR1.136, or TLR6.127; resolved by SDS-PAGE; and subjected to immunoblotting. The blots were probed with anti-Myc mAb (Fig. 1A). TLR1 and TLR6 proteins were detected in the lanes using the specific mAbs TLR1.136 and TLR6.127, respectively, indicating that these mAbs can specifically bind to TLR1 and TLR6 in cell lysates. Next, the reactivities of TLR1.136 and TLR6.127 with BaF3 cells stably expressing human TLR1 or TLR6 were examined by flow cytometry. As shown in Fig. 1B, TLR1.136 and TLR6.127 recognized distinct surface-expressed Ags. Cross-reactivity of these mAbs against human TLR2 was not observed (data not shown).

![Image](https://www.jimmunol.org/)  

**FIGURE 1.** Characterization of mAbs against human TLR1 and TLR6.  
A. Immunoprecipitation of TLR1 and TLR6 proteins in lysates of HEK293 transfectants with anti-TLR1 and anti-TLR6 mAbs. HEK293 cells expressing Myc-tagged human TLR1 or TLR6 were lysed in lysis buffer. TLR1 or TLR6 protein was immunoprecipitated with nonimmune mouse IgG3, anti-Myc mAb, anti-TLR1 mAb (TLR1.136), or anti-TLR6 mAb (TLR6.127), then subjected to Western blotting with anti-Myc mAb. Arrowheads indicate TLR1 and TLR6.  
B. Flow cytometric analysis of anti-TLR1 and -TLR6 mAbs. BaF3 cells stably expressing Flag-tagged TLR1 or TLR6 were stained with anti-TLR1 mAb (TKR1.136) or anti-TLR6 mAb (TLR6.127) and FITC-labeled secondary Ab (solid line). Shaded histograms represent cells labeled with isotype-matched control Ab. Ba/F3 cells were used for control staining.

**Expression profiles of TLR1 and TLR6 in human blood cells and cell lines**

The protein expression levels of TLR1 and TLR6 in human peripheral blood cells and cell lines were analyzed by flow cytometry using anti-TLR1 mAb (TLR1.136) and anti-TLR6 mAb (TLR6.127). TLR6 was expressed on the cell surface in monocytes, monocyte cell line THP-1, and neutrophils, but not on B, T, or NK cells. Monocyte-derived iDCs express a low level of TLR6 on the cell surface. TLR6 was not intracellularly expressed in monocytes, THP-1 cells, or iDCs (data not shown). Staining these cells with TLR1.136 (anti-TLR1 mAb) resulted in a profile similar to those previously reported (18, 22, 23). The expression profile of TLR6 most resembled that of TLR1 compared with those of TLR2, -3, and -4, and the surface levels of TLR1 and TLR6 were lower than that of TLR2 (Fig. 2A). Interestingly, TLR1 and TLR6 were barely detected in freshly isolated B cells, but were slightly expressed on the cell surface in B cell lines Raji, Ball, and P30, whereas TLR2 was not expressed in these cells at either the protein or mRNA level (Fig. 2B). In addition, NK cells isolated from peripheral blood by a positive selection method expressed neither TLR6 nor the other TLRs we tested (data not shown).

**Anti-TLR6 mAb blocks cytokine production induced by MALP-2 and peptidoglycan**

Gene-disrupting studies and experiments with a dominant-negative mutant suggest that TLR2 and TLR6 are required for cytokine production by mouse macrophages in response to diacylated lipopeptide and peptidoglycan. Because TLR2 and TLR6 were expressed on the cell surface in human monocytes, monocyte cell line THP-1, and monocyte-derived iDCs, we performed Ab blocking experiments to analyze the roles of TLR2 and TLR6 in the responses of these cells to MALP-2 and peptidoglycan. Pretreatment of cells with either anti-TLR2 or -TLR6 mAb inhibited cytokine production in response to MALP-2 or peptidoglycan (Fig. 3A). In contrast, anti-TLR6 mAb failed to block cytokine secretion by THP-1 cells, monocytes, and iDCs in response to triacylated lipopeptide, Pam3, or LPS (Fig. 3A). Unexpectedly, TLR2.45 could not inhibit TNF-α production by monocytes in response to MALP-2, peptidoglycan, or Pam3. Because this mAb exhibits inhibitory activity on CD14+ cells such as THP-1 cells and iDCs, but not on CD14+ monocytes, interaction of membrane-bound CD14 with TLR2 may affect ligand recognition by TLR2. The blocking activity of anti-TLR2 and -TLR6 mAbs on MALP-2-induced NF-κB activation was also observed in reporter gene assays using HEK293 cells transiently expressing TLR2 and TLR6 (Fig. 3B). These results indicate that the extracellular portions of TLR2 and TLR6 participate in the recognition of diacylated lipopeptide and peptidoglycan at the cell surface. TLR1.136 (anti-TLR1 mAb) was not a function-blocking Ab (Fig. 3A).

**TLR6 colocalizes with TLR2 at the cell surface**

Next we examined whether TLR6 physically associates with TLR2. HEK293 cells were cotransfected with Flag-tagged TLR2 and Myc-tagged TLR6. Immunoprecipitation of Flag-tagged TLR2 resulted in coprecipitation of Myc-tagged TLR6, indicating the physical association of TLR6 with TLR2 in HEK293 transfectants. In contrast, TLR1 was hardly coprecipitated with TLR6 under the same experimental conditions (Fig. 4B).

Cell surface expression and colocalization of TLR2 and TLR6 on human monocytes were further analyzed by immunofluorescence staining and confocal microscopy. Because the expression levels of TLRs are different among individuals, we used monocytes that expressed relatively high level of TLR1 and TLR6 (Fig.
FIGURE 2. Expression profile of TLRs in human blood cells and cell lines. Freshly isolated human monocytes (n = 12), neutrophils (n = 12), B cells (n = 6), and their cell lines and monocyte-derived iDCs (n = 6) were stained with anti-TLR mAbs and FITC-labeled secondary Ab (solid line). Shaded histograms represent cells labeled with isotype-matched control Ab. RT-PCR analysis of TLR1, TLR2, and TLR6 mRNA expression in the B cell line, Raji, P30, and Ball is shown (B). +, Message positive; −, message negative. For THP-1 and B cell lines, representative data from a minimum of four separate experiments are shown. For blood samples, a representative profile from one donor is shown.

FIGURE 3. TLR6 recognizes MALP-2 and peptidoglycan. A, Anti-TLR6 mAb inhibits cytokine production in THP-1 cells, monocytes, and iDCs stimulated with MALP-2 or PGN. Cells were preincubated with medium alone (without Ab), 10 μg/ml mouse IgG1, anti-TLR1 mAb (TLR1.136), anti-TLR2 mAb (TLR2.45), or anti-TLR6 mAb (TLR6.127) for 1 h at 37°C, then stimulated with MALP-2 (100 nM), PGN (10 μg/ml), Pam3 (100 ng/ml), or LPS (100 ng/ml) for 24 h. −, No stimulus. Concentrations of IL-8 and TNF-α in culture supernatants were measured by ELISA. Representative data from a minimum of three separate experiments using cells from different individuals are shown. B, Anti-TLR2 and anti-TLR6 mAbs inhibit MALP-2-induced TLR2/6-mediated NF-κB activation. HEK293 cells transiently transfected with pEFBOS-hTLR2 and pEFBOS-hTLR6-Myc together with ERAM-luciferase reporter plasmid were pretreated with medium alone or 10 μg/ml mouse IgG1, TLR2.45, or TLR6.127 for 1 h, then stimulated with medium alone or 100 nM MALP-2 for 6 h. Luciferase activities are normalized with respect to an internal control and are shown as the mean relative stimulation ± SD. Representative data from a minimum of three separate experiments are shown.
TLR6 was not coimmunoprecipitated with TLR1 (lane 4 in the upper panel). Nonspecific band (~75 kDa) was observed in all lanes. The expressions of TLR1 (middle panel) and TLR6 (lower panel) were confirmed by immunoprecipitation with respective anti-tag Ab, followed by Western blotting.

Confocal microscopic images showed that TLR1, TLR2, and TLR6 were distributed discontinuously at the plasma membrane. Notably, TLR6 or TLR6 colocalized with TLR2 at the plasma membrane of monocytes (Fig. 5B), as recently reported (19). TLR1 and TLR6 seemed to be expressed in the same regions, which were partly merged. Hence, the results can be interpreted to mean that TLR2 is accessible to either TLR1 or TLR6, but the latter two molecules are rarely coupled after ligand stimulation. There was no difference in localization of TLR2 and TLR6 between MALP-2-stimulated and -unstimulated cells (data not shown).

Discussion

In this study we first produced anti-human TLR6 and anti-human TLR1 mAbs and, using these mAbs, analyzed their expression and functions in human cells. TLR1 and TLR6 were coexpressed with TLR2 on monocytes, monocyte-derived iDCs, and neutrophils, but not on B, T, or NK cells, although their expression levels were lower than that of TLR2. Confocal microscopic analysis demonstrated the colocalization of TLR6 with TLR2 at the plasma membrane of monocytes. Furthermore, an Ab blocking study revealed that human TLR6 recognized diacylated lipopeptide and peptidoglycan at the cell surface cooperatively with human TLR2. Functional blocking was not observed with anti-TLR1 mAb.

The role of TLR6 in the innate immune system has been extensively studied using TLR6-deficient murine macrophages or the macrophage cell line RAW264.7 expressing dominant negative TLR6 (13, 14). Murine TLR6 is required for TNF-α production in response to peptidoglycan from Gram-positive bacteria and diacylated mycosomal lipopeptide, but not in response to triacylated bacterial lipopeptide. We found that the repertoire of ligands recognized by human TLR6 is similar to that recognized by murine TLR6.

Flow cytometric analysis of TLR expression on human blood cells from healthy donors revealed that the expression levels of TLRs are different among individuals. However, in all individuals we tested, the expression level of TLR2 was highest on both monocytes and neutrophils. The levels of TLR1 and TLR6 on monocytes were almost equivalent, whereas the level of TLR1 on neutrophils was higher than that of TLR6 (Fig. 2). Interestingly, TLR4 was scarcely expressed on neutrophils, whereas the expression level of TLR4 was similar to that of TLR1/6 on monocytes. The different expression patterns of TLRs in the innate immune cells may explain the different responsiveness of each cell type against microbial products.

Importantly, the distributions of TLR1, TLR2, and TLR6 were not uniform at the plasma membrane (Fig. 5B), suggesting that there may be TLR-rich microdomains on the monocyte surface. Confocal microscopic images showed the colocalization of TLR6 with TLR2 at the plasma membrane of monocytes as well as TLR1 and TLR2. Thus, TLR2 and TLR6 or TLR1 readily associate with each other in a ligand-dependent manner and transmit signals to
activate NF-κB. Although TLR1 and TLR6 appeared to be expressed in the same region, these two molecules were rarely coupled (Figs. 4 and 5).

It was intriguing that the TLR2 mutants, C30S and C36S, when stably expressed in HEK293 cells, did not appear on the cell surface and failed to respond to MALP-2 (Fig. 6A) or peptidoglycan (data not shown). Cys30 and Cys36 are located at the N-terminal sequences that flank the LRRs in the TLR2 ectodomain (N-terminal flanking region of LRR (LRR-NT)); this region structurally resembles the N-terminal cap of CD42b (25). Because these mutants were expressed to similar extents as wild-type TLR2 in HEK293 cells, mutations of Cys30 and Cys36 probably affect the membrane trafficking of TLR2, rather than the stability of these proteins. Indeed, both TLR2 mutants resided inside the cell. Furthermore, they lost the epitope for TLR2.45, but retained the overall structure required for interaction with TLR6. Remarkably, TLR1, TLR6, and TLR10 do not contain LRR-NT (25). Sandor et al. (19) reported that TLR1 was distributed inside the cell when expressed alone in HEK293 cells. TLR6, however, was predominantly expressed on the cell surface of monocytes, iDCs, and HEK293 transfectants.

The essential residues in the TLR2 ectodomain for recognition of MALP-2 and peptidoglycan were recently identified (33–35). Leu107, Leu112, and Leu115 in the third LRR and Ser40-Ile64 in the N-terminal region of TLR2 (LRR-NT and the first LRR) are involved in the recognition of MALP-2 and peptidoglycan. The deletion mutant TLR2delS40-I64 was expressed on the cell surface and completely lost activity (33, 35). In addition, it was reported that a TLR2 deletion mutant, TLR2delC30-S39 induced NF-κB reporter activity, but the level of activity was significantly reduced compared with that induced by wild-type TLR2 (33, 35). One possible interpretation of this finding is that a deletion of the region of Cys30 to Ser39 may affect cell surface expression of TLR2delC30-S39, similar to our TLR2 mutations. Interestingly, the residues 30cys and 36cys in TLR2 are conserved across human, mouse, and chicken, but 75cys is not conserved (36). Although the mechanism of cell surface trafficking of TLR2 is currently unknown, the LRR-NT of TLR2 may play a crucial role in the cell surface expression of TLR2 as well as ligand recognition.

Previously, Ozinsky et al. (13, 37) reported that hemagglutinin epitope-tagged TLR2 and V5-epitope-tagged TLR6 were expressed at the cell surface, and both were recruited to macrophage phagosomes after phagocytosis of zymozan particles in CHO or RAW cells. They proposed that TLR2 and TLR6 sample the contents of the vacuole and trigger an inflammatory response against the specific organism. Our results showed that, like TLR4-mediated signaling, TLR2/6-mediated signaling did not require endosomal maturation. Furthermore, mAbs against TLR2 and TLR6 blocked MALP-2-induced TLR2/6-mediated NF-κB activation. Although it is still unknown where TLR2/6-mediated signaling occurs, our results indicate that surface expression of TLR2 and TLR6 is crucial for ligand recognition and signaling. Thus, the receptor localization and the nidus for signaling mediated by
TLR2/6 and TLR4, both of which are expressed on the cell surface and recognize lipids and/or carbohydrate-containing, pathogen-associated molecular patterns, are completely different from those of TLR3, -7, -8, and -9, which are intracellularly expressed and recognize pathogen-derived nucleic acids (38–40).

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