Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes

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Toll-like receptors (TLRs) are a family of mammalian proteins homologous to Drosophila Toll. Human TLR2 was shown to mediate the responsiveness to lipopolysaccharide (LPS). On the other hand, gene mutations of mouse TLR4 (mTLR4) in LPS-hyporesponsive strains have suggested that mTLR4 is essential for LPS-signaling in mice, but the role of mTLR2 has not been explored. This report describes molecular cloning of the mTLR2 cDNA. Overexpression of mTLR2 and mouse CD14 conferred LPS-inducibility of c-Jun N-terminal kinase phosphorylation and nuclear factor-κB activation to COS7 cells, suggesting that mTLR2 is a signaling receptor for LPS. Both mTLR2 and mTLR4 genes were expressed in T cells. Treatment with anti-CD3ε, PMA plus ionomycin, or interleukin-2 (IL-2)/IL-15 increased mTLR2 but not mTLR4 messenger RNA (mRNA) in some T cell lines. Specific inhibitors of mitogen-activated extracellular signal-regulated kinase and fusion protein 38 (p38) kinase inhibited mTLR2 mRNA up-regulation by PMA plus ionomycin. This suggests that extracellular signal-regulated kinase and p38 kinase pathways were involved. Additionally, LPS treatment of EL-4 cell line decreased IL-4 gene expression. Our results indicate that both mTLR2 and mTLR4 are involved in LPS signaling, but their expressions are regulated differently in T cells, and that LPS may directly affect T-cell functions by binding to TLRs.

Introduction

Gram-negative bacteria represent a major group of pathogens causing serious infection, especially among the elderly and immunocompromised humans. A glycolipid known as endotoxin or lipopolysaccharide (LPS) is the principal bacterial constituent recognized by the innate immune system. LPS is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A.1 The conserved lipid A has been identified as responsible for LPS-induced biological effects.1 LPS stimulates host cells such as monocytes, macrophages, and B cells through the activation of transcription factors and protein kinases including NF-κB,2,3 AP-1,3 extracellular signal-regulated kinases (ERKs),4,5 c-Jun N-terminal kinases (JNKs),6,7 and fusion protein 38 (p38) kinases.8,9 Although there are some lines of evidence that a significant fraction of T cells respond to LPS,10,11 its direct effects on T cells have not been fully elucidated.

CD4 is involved in mediating LPS responses by binding LPS with high affinity.12 This binding requires a serum factor, LPS binding protein (LBP),13,14 which is a plasma lipid transfer protein that transfers LPS from the bacterial membrane to a binding site on CD4.15 In cells lacking CD4, the soluble form of CD14 (sCD14) in serum functionally replaces membrane-bound CD14.16,17 However, since CD14 is a glycosylphosphatidylinositol-anchored (GPI-anchored) protein, the existence of a signaling component was presumed in the LPS receptor complex.

Toll, first identified as a protein-controlling dorsoventral pattern formation in the early development of Drosophila,18 was shown to participate in antimicrobial immune responses.19 Toll has been shown to be conserved in various species, and it encodes a transmembrane protein with an intracellular portion that is homologous to that of the interleukin-1 (IL-1) receptor family proteins.18 Recently, several mammalian Toll homologues have been identified.20 In addition to their cytoplasmic portion, they share repeating leucine-rich motifs (LRRs) in their extracellular region. One of the human Toll homologues, Toll-like receptor 2 (TLR2), has been shown to be involved in LPS signaling by 2 groups.21,22 Cells transfected with the human TLR2 (hTLR2) complementary DNA (cDNA) acquired the capability for LPS-mediated signaling. There are 2 strains in mice (C3H/HeJ and C57BL10ScCr) that exhibit an impaired ability to respond to LPS. Two recent studies found that the TLR4 gene in the chromosomal region was responsible for this defect (lps).23,24 These studies also found a missense mutation in the cytoplasmic domain of TLR4 in C3H/HeJ, which strongly suggests that TLR4 is the dominant receptor for at least some types of LPS. On the other hand, the role of mouse TLR2 (mTLR2) in LPS signaling has not been explored.

In this paper we report the molecular cloning of mTLR2 cDNA. Exogenous expression of mTLR2 in cells mediates LPS-induced intracellular signals, which suggests that mTLR2 is also involved in LPS responsiveness in mice. Although both mTLR2 and mTLR4 genes are expressed in T cells, the gene expression of mTLR2 but not that of mTLR4 is significantly increased by stimuli such as TCR stimulation and cytokine treatment. This suggests that the
expressions of LPS receptors, mTLR2 and mTLR4, are differently regulated in T cells.

Materials and methods

Reagents and antibodies

We used the following for the study: recombinant mouse IL-2 and human IL-15 (PeproTech, Seattle, WA); PD98 059, a specific inhibitor of mitogen-activated ERK kinase (MEK), and SB203 540, a specific inhibitor of p38 kinase (Calbiochem, San Diego, CA); LPS from Escherichia coli serotype B6: 026 and PMA (Sigma, St. Louis, MO); RPMI 1640 medium (Gibco BRL, Rockville, MD); and fetal calf serum (FCS) (Sigma).

Antibodies included antismouse CD14 monoclonal antibody (mAb) (Pharmingen, San Diego, CA); anti-Flag M2 mAb (Sigma); anti–phospho-JNK mAb (New England Biolabs, Beverly, MA); and anti-JNK1 polyclonal antibody (pAb) (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes JNK1, 2, and 3 isoforms.

Cell lines

All cell lines were grown in tissue culture flasks at 37°C in 5% carbon dioxide/95% air and passed every 2 or 3 days to maintain logarithmic growth. Mouse T cell lines, EL-4 and S49.1 (RIKEN Cell Bank, Tsukuba, Japan), was maintained in RPMI 1640 medium with 10% FCS. A mouse T cell line, CTLL-2 (RIKEN Cell Bank, Tsukuba, Japan), was maintained in RPMI 1640 medium with 10% FCS and 10 ng/mL mouse IL-2. A Simian virus (SV)-40-transformed kidney cell line, COS7 (ATCC), was maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FCS.

Enrichment of splenic T cells

T-cell enrichment was performed using nylon wool columns as previously described. Brieﬂy, packed sterile nylon wool in a 10 mL syringe was packed in a 10 mL syringe and then packed into a 10 mL syringe. The obtained enriched cells that were more than 95% positive for CD3 by flow cytometry were used for the further study.

Isolation of the full-length cDNA encoding mTLR2

In an effort to clone mTLR2 cDNA, 2 primers (TGCTGGAGGCCCATTTGAGAGG and GGACCATTTACTGAGTCTTACAG) were prepared based on the sequence information of the 2 mouse expressed sequence tag (EST) clones (accession numbers AI020960 and AA863729) homologous to hTLR2 cDNA. A partial cDNA was prepared by reverse transcriptase–polymerase chain reaction (RT-PCR) using these primers and the total RNA prepared from a mouse macrophage cell line, J774.1. The synthesized 0.9-kilobase (kb) cDNA fragment was 32P-labeled by random priming and used for screening a J774.1 cDNA library that was constructed in a cloning vector, pcDNA3.1(+) (Invitrogen, Carlsbad, CA). For the Flag-tagged mTLR2 expression plasmid, the coding region of mTLR2 was amplified by PCR from the isolated mTLR2 cDNA and cloned into the AscI site of the expression plasmid pEFBOS-Flag, which encodes a C-terminal Flag epitope.

The coding region of a constitutively active MEK1, a dominant negative JNK1, and a dominant negative H-Ras were cloned into the pEGFP-C1 vector (Clontech, Palo Alto, CA). The structure of the constructs was conﬁrmed by restriction enzyme mapping and DNA sequence analysis.

Transient transfection

COS7 cells were plated onto 60-mm plates at 1 × 106 cells/plate on the day before transfection. Combinations of expression plasmid DNAs (6 µg total/plate) were transfected (Lipofectamine, Gibco BRL) according to the manufacturer’s instructions. Cells were harvested 48 hours later with PBS and used for further analyses.

Flow cytometry

COS7 cells were incubated with anti-mCD14 mAb. After washes with staining buffer, goat antirat immunoglobulin G ﬂuorescein isothiocyanate (IgG-FITC) (Pharmingen) was added. Cells were analyzed by a ﬂuorescence-activated cell sorter (FACS Calibur; Becton Dickinson, San Jose, CA).

Northern blot analysis

Total cellular RNA was extracted (TRIZOL reagent; Gibco BRL, Rockville, MD) according to the manufacturer’s instructions. Messenger RNA (mRNA) was extracted (QuickPrep Micro mRNA Purification Kit; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. We fractionated 10-µg aliquots of the total RNA or 5-µg aliquots of the total mRNA on a 1% agarose gel containing 20 mmol/L morpholinopropene sulfonic acid (MOPS), 5 mmol/L sodium acetate, 1 mmol/L EDTA (ethylenediaminetetraacetic acid, pH 7.0), and 6% (vol/vol) formaldehyde. The aliquots were then transferred to a nylon membrane. After ultraviolet-crosslinking (UV-crosslinking), membranes were soaked in prehybridization solution (6 × SSC [standard saline citrate], 5 × Denhardt’s reagent, 0.5% SDS [sodium dodecyl sulfate], 100 mg/mL denatured salmon sperm DNA, and 50% formamide) for 3 hours at 42°C followed by incubation with a 32P-labeled probe in hybridization solution (6 × SSC, 0.5% SDS, 100 mg/mL denatured salmon sperm DNA, and 50% formamide) for 14 hours at 42°C. The membranes were washed twice in 2 × SSC and 0.1% SDS for 10 minutes at room temperature, washed twice in 0.1 × SSC and 0.1% SDS for 10 minutes at 50°C, and then exposed to film (Fuji RX-U films; Fuji Film, Tokyo, Japan).

Reverse transcriptase–polymerase chain reaction

Total cellular RNA was prepared (TRIZOL reagent, Gibco BRL) cDNA was synthesized from 2 µg of the total RNA by extension of random primers with 200 units (Superscript II, Gibco BRL). PCR of the cDNA was performed in a final volume of 50 µL containing 2.5 mmol/L magnesium chloride (MgCl2), 2.5 units (AmpliTaq; Perkin-Elmer, Norwalk, CT), and 1 µmol/L speciﬁc primers (geneAmp 2400 PCR system, Perkin-Elmer). The synthesized PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The primers were: mouse (m) β-actin sense, TGGACTACGGCACTTCAGGAAAC; β-actin antisense, TAAAAGCCGCTGCTCACCTCC; mTLR2 sense, CACGTTAAGGGCGGTCAGAG; mTLR2 antisense, TGGAGAGGCGCAGCTGCCTGCTCA; mTLR4 sense, AGTGGTCAAGGAACAGAC; mTLR4 antisense, CTTTACCAGCTATCTTACC; MLN-4 sense, CTAGGCCTAGTCTCTTGG; MLN-4 antisense, GAGTACACTCTGCCTTAC; mouse interferon γ (mIFNγ) sense, CCTCTAAGTACACTGCC; and mIFNγ antisense, TTCGGAATACGGTACAGATCC.

Isolation of S49.1 stable transfectants

S49.1 cells were transfected with an electroporator using 20 µg plasmid (Pharmingen). After washing with staining buffer, goat antirat immunoglobulin G ﬂuorescein isothiocyanate (IgG-FITC) (Pharmingen) was added. Cells were analyzed by a ﬂuorescence-activated cell sorter (FACS Calibur; Becton Dickinson, San Jose, CA).
Extract preparation and immunoblotting

Cells were lysed in the following phospholipase C (PLC) lysis buffer at 10^6 cells/mL: 50 mmol/L HEPES (pH 7.0), 150 mmol/L sodium chloride (NaCl), 1% Triton X-100, 1.5 mmol/L MgCl_2, 1 mmol/L EGTA, 100 mmol/L sodium fluoride (NaF), 10 mmol/L NaPi, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, and 10 mg/mL leupeptin. The lysates were separated on SDS-polyacrylamide gels, then electrophoresed to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Bedford, MA). The membranes were blocked for 2 hours in 2% bovine serum albumin (BSA) TBST and 20 mmol/L Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl [pH 7.6], 0.15 mol/L sodium chloride, 0.1% Tween 20), incubated with primary antibodies in TBST for 1 hour, washed 3 times with TBST, and incubated for 1 hour with horseradish peroxidase-conjugated antinoise or antirabbit Ig (Amersham Pharmacia Biotech) diluted 1:10,000 in TBST. After 3 washes in TBST, the blot was developed with the enhanced chemiluminescence system (Amerham Pharmacia Biotech) according to the manufacturer’s instructions.

Electrophoretic mobility shift assay

A 5' oligonucleotide probe (Promega, Madison, WI) was phosphorylated with [32P]ATP using T4 polynucleotide kinase (Takara Biochemicals, Tokyo, Japan). Nuclear extracts were prepared as previously described.\(^{31}\) Specific binding of extract proteins to the probe was assessed by incubation for 30 minutes at room temperature in a solution containing 10 mmol/L HEPES (pH 7.9), 50 mmol/L potassium chloride (KCl), 0.2 mmol/L dithiothreitol (DTT), 10% glycerol, 0.05% NP-40, and 0.5 µg poly (dI-dC). The lysates were separated on SDS-polyacrylamide gels, then electrotransferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Bedford, MA). The membranes were blocked for 2 hours in 2% bovine serum albumin (BSA) TBST and 20 mmol/L Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl [pH 7.6], 0.15 mol/L sodium chloride, 0.1% Tween 20), incubated with primary antibodies in TBST for 1 hour, washed 3 times with TBST, and incubated for 1 hour with horseradish peroxidase-conjugated antinoise or antirabbit Ig (Amersham Pharmacia Biotech) diluted 1:10,000 in TBST. After 3 washes in TBST, the blot was developed with the enhanced chemiluminescence system (Amerham Pharmacia Biotech) according to the manufacturer’s instructions.

Results

Isolation of the full-length cDNA clone encoding the mTLR2

To clone mTLR2 cDNA, 2 primers were prepared based on the sequence information of the 2 mouse est clones (accession numbers AI020 960 and AA863 729) homologous to hTLR2 cDNA. A mouse TLR2 mediator LPS signals in the presence of CD14

To determine if mTLR2 mediates LPS signals the same as hTLR2, we inserted the coding region of mTLR2 cDNA into a mammalian expression plasmid, pEFBOS, with a C-terminal Flag tag. The Flag-tagged mTLR2 was transiently expressed in a monkey kidney cell line, COS7, by itself or in combination with mouse CD14 (mCD14). The expression of the Flag-tagged mTLR2 and mCD14 was confirmed by Western blot analysis and flow cytometry, respectively (Figure 2A and B). Because LPS is known to induce JNK phosphorylation,\(^{6,7}\) we monitored LPS signals using an antibody specific to phosphorylated forms of JNK. Phosphorylation of p46JNK could not be analyzed due to the comigrating nonspecific band recognized by this antibody; therefore we examined the LPS-induced phosphorylation of p54JNK. Transfection of either the control vector or the CD14 expression plasmid did not mediate LPS-induced p54JNK phosphorylation (Figure 2C, lanes 1, 2, 5, and 6), which indicates that the parental COS7 cells were hyporesponsive to LPS.

Mouse TLR2 mediates LPS signals in the presence of CD14

To determine if mTLR2 mediates LPS signals the same as hTLR2, we inserted the coding region of mTLR2 cDNA into a mammalian expression plasmid, pEFBOS, with a C-terminal Flag tag. The Flag-tagged mTLR2 was transiently expressed in a monkey kidney cell line, COS7, by itself or in combination with mouse CD14 (mCD14). The expression of the Flag-tagged mTLR2 and mCD14 was confirmed by Western blot analysis and flow cytometry, respectively (Figure 2A and B). Because LPS is known to induce JNK phosphorylation,\(^{6,7}\) we monitored LPS signals using an antibody specific to phosphorylated forms of JNK. Phosphorylation of p46JNK could not be analyzed due to the comigrating nonspecific band recognized by this antibody; therefore we examined the LPS-induced phosphorylation of p54JNK. Transfection of either the control vector or the CD14 expression plasmid did not mediate LPS-induced p54JNK phosphorylation (Figure 2C, lanes 1, 2, 5, and 6), which indicates that the parental COS7 cells were hyporesponsive to LPS.

Overexpression of mTLR2 constitutively mediated slight phosphorylation of JNK, which was not strengthened further by LPS stimulation (Figure 2C, lanes 3 and 4). When mTLR2 was coexpressed with CD14, LPS significantly increased JNK
were observed in the spleen and the lung. Mouse Northern blot analysis was performed on total RNA isolated from various mouse tissues (Figure 3). The gene expression of mTLR2 constitutively showed slightly increased NF-κB DNA-binding activity. Coexpression of mCD14 with mTLR4 conferred LPS inducibility of NF-κB gene expression (Figure 4A). We therefore investigated the regulation of mTLR2 gene expression in T cells.

**Mouse TLR2 gene expression in T cells**

To elucidate the regulatory mechanisms for mTLR2 gene expression in T cells, purified mouse T cells from the thymus and the spleen were stimulated with immobilized anti-CD3ε mAb for 2 hours, and total RNA was isolated. Although the mTLR2 gene was constantly expressed in unstimulated T cells, as assessed by semiquantitative RT-PCR, triggering by anti-CD3ε mAb resulted in a substantial increase of the mTLR2 gene expression (Figure 4A). In contrast, mTLR4 gene expression, which was also detected in unstimulated T cells, remained constant after anti-CD3ε treatment (Figure 4A).

To exclude the possibility that the positive RT-PCR products were due to a small number of contaminating macrophages or B cells, using Northern blot analysis, we tested TLR gene expressions in several mouse T cell lines (EL4, S49.1, and CTLL-2). Gene expression of mTLR4 was easily detected in these 3 cell lines (Figure 4B and C). When compared with mTLR4, mTLR2 gene expression was relatively lower in S49.1 cells (Figure 4B). When this T cell line was stimulated with the combination of PMA and ionomycin, which mimics TCR stimulation, mTLR2 gene expression increased. On the other hand, mTLR4 expression remained constant after PMA plus ionomycin treatment in S49.1 cells (Figure 4B). Next, we examined the effect of cytokine stimulation on mTLR2 gene expression in CTLL-2 cells that are responsive to IL-2 and IL-15. The 2 cytokines share IL-2Rα and γc for their signal transduction.33 Both IL-2 and IL-15 treatment significantly increased mTLR2 gene expression, whereas no remarkable change was detected in the mTLR4 mRNA level (Figure 4C).

**ERK and p38 kinase pathways are involved in mTLR2 mRNA induction in mouse T cells**

Both cytokine stimulation and anti-CD3ε mAb treatment of T cells are known to activate MAP kinase pathways including ERK, JNK, and p38 kinase.34 To investigate whether ERK and p38 kinase pathways are involved in mTLR2 mRNA up-regulation, we pretreated S49.1 cells with specific inhibitors of ERK (PD98059) and p38 kinase. The mTLR2 expression remained constant after anti-CD3ε treatment in S49.1 cells (Figure 4B). Next, we examined the effect of cytokine stimulation on mTLR2 gene expression in CTLL-2 cells that are responsive to IL-2 and IL-15. The 2 cytokines share IL-2Rα and γc for their signal transduction.33 Both IL-2 and IL-15 treatment significantly increased mTLR2 gene expression, whereas no remarkable change was detected in the mTLR4 mRNA level (Figure 4C).

**Tissue distribution of mTLR2 gene expression**

Northern blot analysis was performed on total RNA isolated from various mouse tissues (Figure 3). The gene expression of mTLR2 was detected in every tissue examined, and the highest expressions were observed in the spleen and the lung. Mouse TLR2 mRNA expression was also enriched in the brain and the thymus. We were particularly interested in mTLR2 expression in the thymus because the role of LPS signaling in T cells has not been fully established.

![Diagram](image.png)
and p38 kinase (SB208 530) pathways followed by stimulation with PMA plus ionomycin. Pretreatment with each of these MAP kinase inhibitors abrogated the increase of mTLR2 mRNA at 10 µmol/L, as assessed by semiquantitative RT-PCR (Figure 5A). We next examined S49.1 cells that constitutively expressed a dominant negative form of JNK1 (JNKDN), which has 2 amino acid substitutions of the phosphorylation sites of JNK1. Although JNK activation by PMA plus ionomycin was markedly inhibited by the expression of JNKDN in this transfectant (data not shown), mRNA up-regulation of mTLR2 by PMA plus ionomycin treatment was comparable to that of parental S49.1 cells (Figure 5A). Additionally, UV treatment, which is a potent activator of JNK, did not induce significant increase of mTLR2 gene expression (Figure 5A). Therefore, JNK activation may not be necessary for mTLR2 mRNA up-regulation by PMA plus ionomycin in S49.1 cells.

On the other hand, a dominant negative form of H-Ras (N17) only partially blocked mTLR2 mRNA increase (Figure 5A), which suggests that either the stimulation by PMA plus ionomycin works on downstream molecules of Ras or there is an alternative Ras-independent pathway for the induction of mTLR2 mRNA. Furthermore, when a series of concentrations of PD98 059 and SB208530 was tested, both of them effectively inhibited mTLR2 mRNA up-regulation at concentrations as low as 0.1 µmol/L, which suggests that the inhibition by these chemicals was specific (Figure 5B).

Next, to test if constitutive activation of the ERK pathway is sufficient to induce mTLR2, S49.1 cells were transfected with an expression plasmid encoding a constitutively activated form of MEK1 (MEK1A), an upstream activator of ERK. The mRNA levels of mTLR2 in 3 independently isolated S49.1 cell lines overexpressing the active mutant of MEK1 were constitutively higher than that of the parental S49.1 cells. (A typical result is shown in Figure 5C.) Thus, mTLR2 induction by PMA plus ionomycin treatment is dependent on both ERK and p38 kinase activation, whereas continuous activation of the ERK pathway seems sufficient to induce mTLR2 gene expression in S49.1 cells.

LPS treatment directly affects the cytokine expression of the EL4 cell line

It has recently been reported that LPS and lipid A directly inhibit IL-4 production by mouse T helper cell 2 (Th2) clones. To investigate the direct effects of LPS on the cytokine production of EL4 cells, we treated EL4 cells with serial dilutions of LPS and analyzed the cytokine expression profile by semiquantitative RT-PCR (Figure 6). The gene expression of IFNγ was weakly induced at 0.1 µg/mL LPS, but it was undetectable at higher concentrations of LPS. In contrast, IL-4 mRNA was constitutively detectable and decreased in the presence of 10 µg/mL of LPS. Thus, LPS directly affects the cytokine expression profile of the EL4 cell line.

**Figure 4. Mouse TLR2 and TLR4 gene expressions in mouse T cells.** (A) Mouse T cells purified from thymuses and spleens were either unstimulated or stimulated with immobilized anti-CD3 mAb for 2 hours. Total RNAs were extracted, and mTLR2 and mTLR4 expressions were analyzed by semiquantitative RT-PCR. (B) S49.1 and EL4 cells were either unstimulated or stimulated with 100 ng/mL PMA plus 1 µg/mL ionomycin for 2 hours. We isolated 5 µg mRNA from the cells and analyzed them by Northern blot analysis using mTLR2 and mTLR4 cDNA probes. (C) CTLL-2 cells were starved overnight without factors and either left unstimulated or stimulated with 10 ng/mL mIL-2 or 10 ng/mL hIL-15 for the indicated time.
Discussion

In this study, we describe the isolation and cloning of mTLR2, which mediates LPS-induced cellular signals similar to its human counterpart. We also show that mTLR2, as well as another putative LPS receptor, mTLR4, is expressed in mouse T cells, and that the gene expression of mTLR2 is differentially regulated from that of mTLR4. In addition, LPS directly inhibited IL-4 expression in a Th2-type cell line, EL-4, which expresses both mTLR2 and mTLR4. LPS may thus directly affect T-cell functions through TLR-mediated signal pathways.

The extracellular segments of TLR proteins are distinctively composed of 24-28 amino acid leucine-rich repeats (LRRs). LRRs are also found in the Drosophila homologues, Toll and 18 Wheeler (18w), Although amino acid sequence homology between hTLR2 and mTLR2 is higher in the cytoplasmic domain, the LRR structure is conserved well, suggesting that it plays a critical role in their biological functions. CD14, which binds LPS with high affinity, also contains 10 copies of the LRR motif. However, LRRs of CD14 are not necessary for LPS binding, as shown by mapping studies. Human TLR2 has been reported to bind LPS by itself, but the KD is low and may not be physiologically important. We have not tested the binding affinity of mTLR2 for LPS. However, it is unlikely that mTLR2 reacts to physiological concentrations of LPS by itself because COS7 cells expressing mTLR2 alone did not show a detectable response to 10 µg/mL LPS in either JNK or NF-kB activation (Figure 2).

It is noteworthy that the cytoplasmic domain is conserved well at the amino acid level between hTLR2 and mTLR2. TLRs 1-5 share a highly significant homology called the Toll-homology (TH) domain. The TH domain was first discovered as a resemblance of a cytoplasmic domain between Toll and IL-1 receptor type I (IL-1R). It was later shown that the TH domain was also shared by numerous proteins including plant disease-resistance proteins, an intracellular myeloid differentiation marker (MyD88), ST2, IL-1Racp, and IL-1RtP/IL-18R. The TH domain contains 10 conserved blocks consisting of 5 alternating b-strands and a-helices, and all the blocks are conserved in mTLR2 as well as in hTLR2 (Figure 1). Since both Toll and IL-1R mediate signaling by similar Rel-type transcription factors, the TH domain presumably plays an important role in the activation of the NF-kB pathway. In assays using carboxy-terminal truncations of hTLR2, deletion of carboxy-terminal 13 or 15 amino acid markedly impaired the LPS-mediated NF-kB activation by hTLR2. These deletions remove only the last block of a-helices, which suggests that the complete conformation of the TH domain is necessary for proper signaling. This finding is consistent with the fact that all 10 blocks of TH are conserved between mTLR2 and hTLR2 (Figure 1).

Our results have demonstrated that overexpression of mTLR2 in combination with mCD14 confers responsiveness to LPS stimulation in COS7 cells. Although hTLR2 was shown to mediate LPS signaling, the role of mTLR2 in LPS signaling remains questionable. Two recent studies examined a single autosomal locus (lps), which is responsible for the LPS hyporesponsiveness (lps") of 2 mouse strains (C3H/HeJ and C57BL10ScCr). The lps" gene was found in the lps locus and mutations in the lps" gene in both strains were identified. This strongly suggests that a defective mTLR4 is responsible for the LPS-hyporesponsiveness of these mice. More recently, mice lacking the mTLR4 gene have been generated, and these mice show LPS-hyporesponsiveness very similar to that in the 2 lps" mouse strains. These findings are consistent with the idea that the mTLR4 gene is essential in LPS signaling.

Then what is the role of mTLR2 in LPS responsiveness? There are four possible explanations. First, it is possible that mTLR2 works as an alternative to mTLR4. Lps" mice are hyporesponsive but not unresponsive to LPS. They show LPS-induced gene expressions in the presence of a larger dose of LPS, which suggests that there is some redundancy in LPS signaling. Our present data, which confirm that mTLR2 mediates LPS signals, may indicate that mTLR2 works to back up mTLR4. Another explanation is that mTLR2 and mTLR4 may respond to different types of LPS. Cells from C3H/HeJ mice are as sensitive as their normal counterparts to certain types of LPS (eg, Porphyromonas gingivalis LPS). There is divergence in LPS structure among Gram-negative bacteria, and it is presumably that mTLR4 responds to certain types of LPS better than mTLR2, while mTLR2 responds better to others. The third possibility is that mTLR2 and mTLR4 may cooperate to respond to LPS. It has not been shown how TLRs are involved in the formation of the LPS receptor complex, and it is possible that mTLR2 associates with mTLR4 to form a dimer or oligomer. Further studies, including a study of mice lacking the mTLR2 gene, need to be done to fully understand the involvement of mTLR2 in the LPS receptor complex.

The fourth possibility is based on the inducibility of mTLR2 expression (Figures 4 and 5). In our current study we have shown that in some T cell lines, the expression level of mTLR2 is very low, but it significantly increases after stimulation with anti-CD3+ mAb, PMA plus ionomycin, and IL-2/IL-15. However, no change is detected in mTLR4 expression after such treatments (Figure 4). Also, mTLR2 is significantly up-regulated in macrophages by stimulation with LPS and IFN-γ (T. Matsuguchi, unpublished data, June 1999). Interestingly, the sensitivity of Lps" mice is restored by treatment with IFN-γ or BCG, and the expression level of mTLR2 in immune cells is likely to be higher after these treatments in Lps" mice. All these findings may suggest that immune-component cells react to LPS mainly with TLR4 at first, and TLR2 is the second LPS receptor boosting the immune response by the host. This hypothesis explains well why mTLR4 is essential in LPS responsiveness.

It is of note that the coexpression of mCD14 with mTLR2 was necessary for the proper activation of JNK and NF-κB by LPS in COS7 cells. In contrast, in 2 earlier reports using hTLR2, 293 cells transfected with only hTLR2 increased the transcription of NF-κB-dependent reporter genes in response to LPS. The exact reason for this discrepancy is unknown. It is possible that mTLR2 and hTLR2 respond differently to LPS in the absence of CD14. It is also possible that the discrepancy is due to the difference of the cell lines used for the transfection. The parental COS7 cells that we used in our experiments did not show any CD4 surface expression (Figure 2B). The 293 cells tested in the earlier reports might contain a small amount of endogenous CD14, which worked with the exogenous TLR2. It has recently been reported that hTLR4 needs a small molecule called MD-2 for proper transduction of the LPS signal. Therefore, it is also possible that COS7 and 293 may express different ways to express an unknown coactivator protein, similar to MD-2, which is necessary for proper TLR2 function.

We have also shown that both mTLR2 and mTLR4 mRNAs are expressed in mouse T cells and some T cell lines. Although we could not show the protein expressions due to the lack of...
appropriate mAbs. LPS directly inhibited IL-4 expression in a mouse Th2-type cell line, EL4, suggesting the surface expression of LPS receptors on this T cell line. In spite of great interest, the direct effects of LPS on T cells have not been fully understood. Because it is now known that both TLR2 and TLR4 are expressed of LPS receptors on this T cell line. In spite of great interest, the direct effects of LPS on T cells have not been fully understood. Because it is now known that both TLR2 and TLR4 are expressed on T cells, it is reasonable to presume that LPS can directly affect some of the T-cell functions. To support our findings, it has recently been reported that both LPS and lipid A decrease IL-4 production in purified mouse CD4⁺ T cells and Th2 clones, indicating that LPS directly modulates cytokine expressions by T cells. Additionally, an earlier report showed the activation and subsequent apoptosis of T cells in LPS-treated mice. Although T cells usually do not express CD14, a soluble form of CD14 (sCD14) in the serum has been shown to bind LPS and induce signals in cells lacking CD14. It has also recently been shown that CD11c/CD18, which is expressed in some T cells, may work as a component of the LPS receptor.

In the present study, we have shown that a low concentration (0.1 µg/mL) of LPS induces weak expression of IFNγ mRNA in EL4 cells (Figure 6). The same result was repeatedly observed in different sets of experiments (data not shown). Recent reports have shown that both JNK1 and JNK2 are involved in the development of Th1 cells producing IFNγ. Our results have revealed that mTLR2 is a potent inducer of JNK phosphorylation (Figure 2C). Therefore, it is a reasonable to presume that LPS induces signal pathways promoting Th1 cell development. Interestingly, the up-regulation of IFNγ mRNA was not observed at higher concentrations of LPS (Figure 6). The reason for this phenomenon is not clear. It was not due to the increased cell death because LPS as high as 100 µg/mL did not affect the viability of EL4 cells (data not shown). LPS treatment of this cell line might activate different downstream signals in a concentration-dependent manner. The LPS-binding affinities for TLR2 and TLR4 have not been clearly shown, and it is interesting to presume that either TLR2 or TLR4 works preferably at the low concentration of LPS, thereby mediating better IFNγ expression. EL4 cells constitutively express both TLR2 and TLR4 (Figure 4B).

The expression level of mTLR2 but not of mTLR4 increases in T cells with the stimulation by immobilized anti-CD3ε mAb, PMA plus ionomycin, IL-2, and IL-15. All of these stimulators are known to activate both ERK and p38 kinase, and our data indicate that mTLR2 mRNA up-regulation by PMA plus ionomycin is dependent on both ERK and p38 kinase pathways (Figure 5). It is becoming evident that the binding of MAP kinases to transcription factors is a critical determinant of their specificity. ERK is specifically targeted to the efs domain transcription factors, Elk-1 and Lin-1, while p38 has recently been shown to be targeted to the MADS-box transcription factors, MEF2A and MEF2C. Mouse TLR2 gene may be under the regulation of multiple transcription factors that are activated by different MAP kinases, ERK, and p38 kinase. Collaboration between ERK and p38 kinase has also been reported for the induction of cytokine and iNOS synthesis in macrophages. However, the overexpression of a constitutively active mutant of MEK1 increased mTLR2 mRNA (Figure 5C), which suggests that the continuous activation of the ERK pathway is sufficient to induce the mTLR2 gene expression.

Lastly, it is interesting that mTLR2 is induced by IL-15 in some T cells. Since LPS is a potent inducer of IL-15 production from macrophages, these findings may suggest that at least a significant fraction of T cells responds to LPS in a 2-step manner: first by using constitutively expressed mTLR4, then by using mTLR2, which is induced later by cytokines like IL-15. It is interesting to speculate that mTLR2 and mTLR4 induce different intracellular signals; as a result, the same T-cell population might respond differently to LPS during the course of Gram-negative bacterial infection.

In conclusion, the present study has shown that both mTLR2 and mTLR4 are able to mediate LPS signaling. The expression of these proteins in mouse T cells indicates that LPS may have direct effects on T-cell activity during Gram-negative bacterial infection.

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Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes

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