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Human Toll-like receptor 2 (TLR2) is a signaling receptor that responds to LPS and activates NF-κB. Here, we investigate further the events triggered by TLR2 in response to LPS. We show that TLR2 associates with the high-affinity LPS binding protein membrane CD14 to serve as an LPS receptor complex, and that LPS treatment enhances the oligomerization of TLR2. Concomitant with receptor oligomerization, the IL-1R-associated kinase (IRAK) is recruited to the TLR2 complex. Intracellular deletion variants of TLR2 lacking C-terminal 13 or 141 aa fail to recruit IRAK, which is consistent with the inability of these mutants to transmit LPS cellular signaling. Moreover, both deletion mutants could still form complexes with wild-type TLR2 and act in a dominant-negative (DN) fashion to block TLR2-mediated signal transduction. DN constructs of myeloid differentiation protein, IRAK, TNF receptor-associated factor 6, and NF-κB-inducing kinase, when coexpressed with TLR2, abrogate TLR2-mediated NF-κB activation. These results reveal a conserved signaling pathway for TLR2 and IL-1Rs and suggest a molecular mechanism for the inhibition of TLR2 by DN variants. The Journal of Immunology, 1999, 163: 639–643.

Members of the human Toll-like receptor (TLR) family share a similar topography with an extracellular domain (ECD) containing multiple leucine rich-repeats, a single membrane-spanning domain, and an intracellular domain with significant sequence similarity to the IL-1R family (1). Toll was originally identified in Drosophila and is essential for the establishment of dorsoventral polarity in the embryo (2). Toll also plays an important role in innate immunity, inducing an antimicrobial response in the adult fly (3, 4).

Human sepsis is responsible for >20,000–50,000 deaths per year in the United States (5). In the case of sepsis induced by Gram-negative bacteria, host cells sense the presence of LPS (endotoxin), which is a major cell wall component of the invading pathogen, and mount a dysregulated innate immune response resulting in pathophysiological consequences (6). Macrophages and monocytes respond to LPS by inducing the expression of cytokines, cell adhesion molecules, and low-m.w. proinflammatory molecules. The activation of monocytes/macrophages by LPS requires a serum protein known as LPS-binding protein (LBP) and a GPI-anchored cell-surface protein, CD14 (7, 8). However, until recently, little was known about how the LPS signal is transduced across the plasma membrane (9). A role for TLRs in this process was initially suggested by the observation that a constitutively active form of one human TLR (TLR4) is capable of inducing NF-κB activation as well as the expression of several proinflammatory cytokines (10). Thus, it was proposed that human TLRs may regulate innate immunity and signal the activation of adapted immunity (10, 11). Nevertheless, the precise functions of these TLRs remain unclear.

We and others showed recently that the expression of human TLR2 renders LPS responsiveness to otherwise LPS-unresponsive cells (12, 13). The LPS response mediated by TLR2 requires the plasma protein LBP and is greatly enhanced by the presence of cell surface CD14 (membrane CD14 (mCD14)). Moreover, LPS treatment of TLR2-expressing cells results in the activation of NF-κB and in a subsequent induction of genes that initiate adaptive immunity, such as IL-8. Therefore, TLR2 appears to be a signaling molecule for LPS (12). However, the proximal signaling events leading to the activation of NF-κB by TLR2 remain to be elucidated.

Here we provide evidence that TLR2 can interact with mCD14 to form the LPS receptor complex. LPS treatment leads to receptor oligomerization and to subsequent recruitment of IL-1R-associated kinase (IRAK). In addition, our results implicate myeloid differentiation protein (MyD88), TNF receptor-associated factor 6 (TRAF6), and NF-κB-inducing kinase (NIK) in the TLR2-mediated activation of NF-κB. We demonstrate that C-terminal deletion variants act as dominant-negative (DN) receptors in that they can form complexes with wild-type (WT) TLR2 but fail to recruit IRAK in response to LPS.

Materials and Methods

Antisera

The entire TLR2 ECD fused with human IgG Fc was used as immunogen to raise anti-TLR2 polyclonal Ab. Anti-Flag M2 monoclonal and anti-IRAK antiserum were purchased from Eastman Kodak (New Haven, CT) and Transduction Laboratories (Lexington, KY), respectively.

FACS analysis

Human PBLs were isolated by Ficoll-diатriasitze density gradient centrifugation from normal volunteer donors. PBLs (1 × 10⁶) were suspended in PBS/2% BSA in a volume of 0.25 ml containing PE-conjugated CD14 mAb (Dako, 1:250 dilution) for 30 min on ice. A total of 1 μg of purified anti-TLR2 IgG and FITC-conjugated goat anti-rabbit secondary Ab (1/100 dilution; Caltag, Burlingame, CA) were added sequentially; each were incubated for 40 min on ice. FACS analyses were performed with a FACSscan (Becton Dickinson, Mountain View, CA).

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Abbreviations used in this paper: TLR, Toll-like receptor; MyD88, myeloid differentiation protein; IRAK, IL-1R-associated kinase; TRAF, TNF receptor-associated factor; CNTFR, ciliary neurotrophic factor receptor; WT, wild type; LBP, LPS-binding protein; ECD, extracellular domain; NIK, NF-κB-inducing kinase; DN, dominant-negative; mCD14, membrane CD14; RIP, receptor-interacting protein; gD, HSV type 1 glycoprotein D.
Construction of expression plasmids

The amino terminal epitope tag version of mCD14 (gD.mCD14) was constructed by adding an XhoI restriction site immediately upstream of thrreonine at position 21 (the first amino acid of the predicted mature form of mCD14) and linking this to amino acids 1–53 of HSV type 1 glycoprotein D (gD) as described previously (14). Mammalian expression vectors encoding DN forms of MyD88-DN (152–296), TRAF2-DN (87–501), TRAF6-DN (289–522), IRAK-DN (1–96), IRAK2-DN (1–96), receptor interacting protein (RIP)-DN (559–671), and NIK-DN (KK429–430AA) have been described elsewhere (15–20). The Flag epitope (DYKDDDDK) was positioned at amino acid 1 of the mature form of TLR2 by oligonucleotide-directed mutagenesis.

Cell culture and transfection

Human embryonic kidney 293 cells were maintained in low glucose DMEM/HAM’s F12 (30:50) medium supplemented with 10% FBS, 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin. The 293 stable cell line expressing an epitope-tagged version of TLR2, as well as transfection conditions, have been described previously (12). The total amount of DNA was kept constant in all transfections by supplementing pRK5 vector DNA.

Luciferase reporter assay

Cells were transfected with the indicated expression plasmids together with 0.5 µg of the luciferase reporter plasmid pGL3-ELAM.tK and 0.05 µg of the Renilla luciferase reporter vector as an internal control (12). After 20 h, cells were treated with Escherichia coli LCD25 LPS (50 ng/ml; List Biological Labs, Campbell, CA) supplemented with LBP in serum-free medium for 6 h. Luciferase activity was measured by using reagents from Promega (Madison, WI) and was expressed as relative luciferase activity by dividing firefly luciferase activity by that of Renilla luciferase.

Immunoprecipitation and Western blot analysis

Transfected cells were incubated with LPS in the presence of LBP, washed once with PBS (pH 7.5), and lysed for 15 min on ice in 0.5 ml of lysis buffer (25 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 20% glycerol). Lysates were clarified by centrifugation at 4°C for 15 min at 100,000 × g. Cell lysates were incubated with 1 µg of anti-gD mAb and 20 µl of 50% (v/v) protein A-agarose (Pierce, Rockford, IL) overnight at 4°C with gentle rocking. After three washings with lysis buffer, precipitated complexes were solubilized by boiling in SDS sample buffer, fractionated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS (pH 7.5) containing 5% nonfat dry milk and 0.05% Tween 20 and were subsequently analyzed by flow cytometry.

Results and Discussion

Coexpression of mCD14 and TLR2 in a subset of leukocytes

We have shown previously that TLR2 serves as a signal transducer to mediate transmembrane LPS signaling (12). In addition, mCD14, when coexpressed with TLR2, can dramatically increase LPS responsiveness in 293 cells. To examine the biological significance of this observation, we determined whether mCD14 and TLR2 are coexpressed on the cell surface of leukocytes by using FACS analysis. Freshly isolated human PBLs were incubated sequentially with a PE-conjugated anti-CD14 mAb and an unconjugated TLR2 Ab followed by FITC-conjugated goat anti-rabbit IgG secondary Ab and were subsequently analyzed by flow cytometry. mCD14 is a marker for monocytes, and we observed that nearly all mCD14+ cells also expressed TLR2. In addition, a population of cells was detected that expressed TLR2 but not mCD14 (Fig. 1).

We have not yet characterized these cells. However, the identification of two subsets of leukocytes, either expressing TLR2 alone or coexpressing mCD14 and TLR2, is consistent with the CD14-independent and CD14-dependent signaling pathways of LPS activation observed in vivo as well as in vitro (21, 22). Alternatively, LPS-responsive cells may use soluble CD14 for signaling.

TLR2 associates with CD14, and LPS induces receptor oligomerization and association of IRAK

We subsequently examined whether TLR2 and mCD14 could form a complex on the cell surface. 293 cells were transiently transfected with a version of CD14 containing the gD epitope tag alone (gD.mCD14) or with Flag-tagged versions of either TLR2 (Flag.TLR2) or, as a control, IL-1R and IL-1R accessory proteins (Flag.IL-1Rs). Lysates of these cells were immunoprecipitated with Abs specific for the gD epitope tag, and coprecipitating Flag-tagged receptors were detected on blots with an anti-Flag mAb. Under these conditions, we observed that mCD14 does indeed associate with TLR2 in the absence of LPS, and this interaction was also observed after LPS stimulation (Fig. 2A). We did not observe an association between mCD14 and IL-1Rs, suggesting specificity of the interaction between mCD14 and TLR2 (Fig. 2A).

Because ligand-induced oligomerization is a commonly used mechanism to activate cytokine receptors (23), we determined whether TLR2 formed oligomers upon LPS treatment. Flag.TLR2 was transiently expressed in a cell line that stably expressed a gD-tagged TLR2 protein (gD.TLR2). We detected some association of Flag.TLR2 with gD.TLR2 in untreated cells, and this interaction was enhanced upon LPS stimulation (Fig. 2B). The formation of the LPS receptor complex between mCD14 and TLR2 is reminiscent of a multiple chain receptor complex used by several cytokines. For example, the IL-6/leukemia inhibiting factor/ciliary neurotrophic factor receptor (CNTFR) complex is composed of a ligand-binding subunit and the signal transducer glycoprotein 130 (24, 25). The high-affinity receptors for CNTF (CNTFpRα) and for glial cell line-derived neurotrophic factor (glial cell line-derived neurotrophic factor receptor α), like CD14, are also GPI-anchored proteins (25–27).

The Ser/Thr kinase IRAK has been implicated in the induction of NF-κB by IL-1 and by a constitutively active TLR4 (28, 29). We examined whether IRAK was recruited to the TLR2 complex. IRAK coprecipitated with TLR2 in 293 cells stimulated with LPS, and this complex reached a peak at 5 min after LPS stimulation (Fig. 2B). The kinetics of association of TLR2 with IRAK recruitment closely followed the time course of TLR2 oligomerization (Fig. 2B). These results show that TLR2 recruits IRAK, and suggest that LPS-induced TLR2 oligomerization is a critical step for TLR2 signal transduction. However, it remains possible that LPS may induce other molecular events, such as the recruitment of

![FIGURE 1. Expression of mCD14 and TLR2 on PBLs. Human PBLs were prepared and stained with anti-CD14 and TLR2 Abs as described in Materials and Methods. Data are presented as double color fluorescence histograms with a log scale of fluorescence intensity. The percentage of two subsets of PBLs is indicated; one expressed only TLR2, and the other contained both mCD14 and TLR2 on the cell surface. Experiments were performed twice with similar results.](http://www.jimmunol.org/Downloadedfromhttp://jimmunol.org/2406913/fig1_1.png)
additional receptor subunits, which in turn create docking sites for more signaling proteins.

TLR2 C-terminal deletion variants form complexes with TLR2 and fail to recruit IRAK

We have reported previously that TLR2 variants with C-terminal deletions of either 13 or 141 aa (TLR2-Δ1 and TLR2-Δ2, respectively) and a variant in which the entire ECD of TLR2 is replaced with a portion of the ECD of CD4 (CD4.TLR2) are defective for the induction of an NF-κB responsive endothelial leukocyte adhesion molecule reporter gene (12). We used these variants to characterize the domains of TLR2 that are necessary for TLR2 oligomerization. gD-tagged TLR2-Δ1, TLR2-Δ2, or CD4.TLR2 were transiently coexpressed with full-length Flag-tagged TLR2 in 293 cells. Immunoprecipitation of WT gD.TLR2, gD.TLR2-Δ1, or gD.TLR2-Δ2 resulted in the coprecipitation of Flag-TLR2 (Fig. 3). Likewise, the reciprocal immunoprecipitation of Flag.TLR2 results in the coprecipitation of WTgD.TLR2, gD.TLR2-Δ1, or gD.TLR2-Δ2 (data not shown). CD4.TLR2 failed to associate with WT Flag.TLR2, suggesting that the ECD is required for receptor complex formation. We subsequently determined whether the C-terminal truncation variants of TLR2 were defective in the recruitment of IRAK. The full-length TLR2 (WT) or two deletion constructs were transiently expressed in 293 cells, and receptor complexes were immunoprecipitated and immunoblotted with anti-IRAK antiserum. Whereas the WT TLR2 showed LPS-inducible IRAK recruitment, TLR2-Δ1 or TLR2-Δ2 failed to associate with IRAK (Fig. 4). The C-terminal region of 13 aa that is deleted in TLR2-Δ1 is homologous to a region of the IL-1R that is required for association with IRAK. Likewise, this carboxyl-terminal tail of TLR2 appears essential for IRAK recruitment and subsequent signal transduction.

TLR2-Δ1 and TLR2-Δ2 abrogate WT TLR2-induced NF-κB activity in 293 cells

The ability of Δ1 or Δ2 to form heteromers with WT TLR2 (Fig. 3) coupled with their failure to recruit IRAK suggests that these
and the IL-1R family, we examined whether TLR2-mediated
(28, 29).
relays a signal through the NIK to I-
other cytoplasmic adapter protein named TRAF6, which in turn
the receptor complexes. IRAK and IRAK2 then interact with an-
associated adapter protein, MyD88, recruits IRAK and IRAK2 to
mediated NF-
variants might act as DN proteins. To test this possibility, we co-
transfected Δ1 or Δ2 with WT TLR2 and examined their effects on
LPS-induced NF-κB activity. Whereas expression of WT TLR2
results in LPS responsiveness, coexpression of Δ1 or Δ2 results in
a significant blocking of TLR2-mediated NF-κB activity (Fig.
5). These results demonstrated that these intracellular deletion
variants can serve as DN receptors and are in agreement with the
observation that TLR2 is capable of forming oligomers in the over-
expressing 293 cells (Fig. 3). In addition, the expression of WT
TLR2, as determined by immunoblots, remained unchanged in the
presence of Δ2 mutant (Fig. 5). The residual NF-κB-dependent
reporter gene activity seen during the coexpression of WT and the
deletion variants of TLR2 is probably mediated by a portion of
homomeric WT TLR2.

**MyD88, IRAK, TRAF6, and NIK participate in TLR2-mediated
NF-κB induction**

Recent studies have revealed the proximal signaling events leading
to IL-1-induced NF-κB activation (30, 31), in which the receptor-
associated adapter protein, MyD88, recruits IRAK and IRAK2 to
the receptor complexes. IRAK and IRAK2 then interact with an-
other cytoplasmic adapter protein named TRAF6, which in turn
relays a signal through the NIK to I-κB kinases 1 and 2, leading to
NF-κB activation. Similar results have been reported for the activation of NF-κB by a constitutively active version of TLR4
(28, 29).

Due to the conservation of the cytoplasmic domains of TLR2
and the IL-1R family, we examined whether TLR2-mediated
NF-κB activation requires the signaling components that are
known to be used in the IL-1 pathway. We observed that DN
versions of MyD88, IRAK, TRAF6, or NIK attenuated TLR2-
mediated NF-κB activation (Fig. 6). Activation was not blocked by
DN versions of IRAK2, TRAF2, or RIP. TRAF2 and RIP are
signaling molecules involved in the activation of NF-κB by TNF,
whereas IRAK2 binds to IL-1R1. These results implicate MyD88,
IRAK, TRAF6, and NIK in signaling the activation of NF-κB by
TLR2. Similarly, a recent report showed that DN versions of
MyD88, IRAK, TRAF6, and NIK but not TRAF2 inhibited LPS-
induced NF-κB activity in human dermal microvessel endothelial
and monocytic leukemia cells (32). We reproducibly observed that
NIK-DN was a more potent inhibitor of TLR2 activity than
IRAK-DN or TRAF6-DN. One interpretation of these results that
is currently being investigated is that there are IRAK- and TRAF6-
independent pathways that lead to the activation of NIK; thus, DN
versions of IRAK or TRAF6 only partially block NF-κB activation.

In summary, we have dissected the early signaling events in the
activation of TLR2 by LPS. We observed that TLR2 forms a com-
plex with CD14 before LPS treatment, and that LPS treatment
results in TLR2 oligomerization and concomitant recruitment of
IRAK to the signaling complex, at least in overexpressing 293
cells. It is now of interest to verify whether such signaling events
occur in native lymphoid cells. In addition, TLR2 shares a com-
mon signaling pathway leading to NF-κB activation with the IL-
1R. The involvement of IRAK, MyD88, TRAF6, and NIK in
TLR2 signaling appears to extend to other members of the TLR
family, such as TLR4 (28, 29). Recent molecular genetic data
suggested that TLR4 also participates in the LPS response, although
this remains to be demonstrated at cellular or biochemical levels
(33–35). TLRs and IL-1Rs appear to define a receptor superfamily
that uses overlapping signaling components to orchestrate the reg-
ulation of the immune response and inflammation.
References


