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The CD14 Ligands Lipoarabinomannan and Lipopolysaccharide Differ in Their Requirement for Toll-Like Receptors

Terry K. Means,* Egil Lien, † Atsutoshi Yoshimura, † Shuyan Wang,* Douglas T. Golenbock, † and Matthew J. Fenton*

Mammalian Toll-like receptor (TLR) proteins are new members of the IL-1 receptor family that participate in activation of cells by bacteria and bacterial products. Several recent reports indicate that TLR proteins mediate cellular activation by bacterial LPS via a signaling pathway that is largely shared by the type I IL-1 receptor. We previously showed that Chinese hamster ovary (CHO) fibroblasts engineered to express CD14 (CHO/CD14) were responsive to LPS, but not to a distinct CD14 ligand, mycobacterial lipoarabinomannan (LAM). These CHO/CD14 cells were subsequently found to possess a frame-shift mutation within the TLR2 gene which resulted in their inability to express functional TLR2 protein. Thus, we hypothesized that TLR2, but not TLR4, was necessary for LAM signaling. In this paper we show that CHO/CD14 cells engineered to express functional TLR2 protein acquired the ability to be activated by LAM. Similarly, overexpression of TLR2 in murine macrophages conferred enhanced LAM responsiveness. Together, our data demonstrate that the distinct CD14 ligands LAM and LPS utilize different TLR proteins to initiate intracellular signals. These findings suggest a novel receptor signaling paradigm in which the binding of distinct ligands is mediated by a common receptor chain, but cellular activation is initiated via distinct signal-transducing chains that confer ligand specificity. This paradigm contrasts with many cytokine receptor complexes in which receptor specificity is conferred by a unique ligand-binding chain but cellular activation is initiated via shared signal-transducing chains. The Journal of Immunology, 1999, 163: 6748–6755.

The discovery of mammalian homologues of the Drosophila Toll receptor protein has elicited interest in the role of these proteins in innate immunity (reviewed in Ref. 1). Several published reports have illustrated the potential importance of Toll-like receptors (TLR) in intracellular signaling. Janeway and colleagues (2) reported a human homologue of the Drosophila Toll protein, a protein later to be designated TLR4. There were three features of TLR4 that linked this protein with innate immunity and intracellular signaling. First, because the Drosophila Toll participates in an antifungal response in the adult fly (3), it was hypothesized that mammalian homologues would participate in similar innate immune responses. Second, the Drosophila Toll participated in a signal-transduction pathway leading to the activation of the transcription factor Dorsal, the fly homologue of NF-κB. The central role played by NF-κB in signal-transduction pathways activated by cytokines, and in the regulation of cytokine genes themselves, implicated mammalian Toll proteins in cellular responses similar to those evoked by cytokines. Third, the intracellular domain of Toll shares significant sequence similarity with the type I IL-1 receptor, the known mammalian Toll-like receptors, and the cytosolic adapter protein MyD88 (4). Indeed, signaling via TLR4 has been shown to require both MyD88 and the IL-1 receptor-associated kinase (IRAK) (5). The shared use of a cytokine receptor signaling pathway suggests that Toll, and its mammalian cousins, may function as cytokine receptors. Indeed, Toll is a transmembrane receptor protein with a known protein ligand in the fly (Spatzle). Although mammalian ligands have not yet been reported, it has been speculated that the five published members of the human TLR family might recognize a family of cytokine-like ligands that participate in host immune responses (6).

An unexpected feature of TLR biology comes from reports that these proteins participate in intracellular signaling initiated by Gram-negative bacterial LPS. CD14 is the major receptor responsible for the effects of LPS on macrophages, monocytes, and neutrophils (reviewed in Ref. 7). The role of CD14 in signaling has remained unclear because it is a glycosphatidylinositol-linked protein that lacks transmembrane and intracellular domains. Several studies have suggested that CD14 acts by associating with a distinct transmembrane signal transducing protein. Recently published data support the possibility that TLR proteins may serve this function. Two groups independently reported that TLR2 could function as a signaling receptor for LPS in the presence of CD14 (8, 9). These investigators reported that HEK 293 (human 293 embryonic kidney) cells stably transfected with TLR2 could respond to LPS in the presence of CD14 and LPS binding protein, as judged by activation of a reporter gene under the control of the NF-κB-dependent endothelial cell-leukocyte adhesion molecule-1 (ELAM-1) (E-selectin) promoter. Deletion mutants of TLR2 lacking the 13 most C-terminal amino acids of the intracellular domain failed to mediate LPS responsiveness in this assay. Thus, TLR2

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3 Abbreviations used in this paper: TLR, Toll-like receptor; LAM, lipoarabinomannan; CHO, Chinese hamster ovary; CHO/CD14, CHO cells engineered to express human CD14; FL1, channel fluorescence; RT, reverse transcriptase; LPS, LPS binding protein; ELAM-1, endothelial cell-leukocyte adhesion molecule-1.
appears to mediate LPS-induced intracellular signaling initiated by the binding of LPS to CD14. Most recently, it has been shown that LPS induces the oligomerization of TLR2 and the subsequent recruitment of the IRAK to the TLR2 complex (10).

A different facet of this story was revealed by identification of the gene responsible for the LPS hyporesponsive phenotype of the C3H/HeJ mouse (11–13). Macrophages from this mouse are markedly resistant to activation by LPS, even though they express normal amounts of CD14 on their surface. Although the genetic defect in these mice was known to arise from a single locus (lps), the gene responsible for this defect remained elusive. Positional cloning and sequencing of the lps locus mapped the defect to the tlr4 gene. In C3H/HeJ mice, a single missense mutation within the tlr4 coding sequence was identified (P712H). Evidence that this mutation is responsible for the LPS hyporesponsive phenotype of the C3H/HeJ mouse comes from the finding that the C57BL/10ScCr LPS-nonresponsive mouse does not express TLR4. More recently, both macrophages and B cells from a TLR4 knockout mouse were shown to be LPS hyporesponsive (13). Lastly, it has been reported that TLR4 can confer CD14-dependent LPS responsiveness on HEK 293 cells (14), although LPS responsiveness also depends on the concomitant expression of an additional protein, MD-2 (15). Together, these findings implicate both TLR2 and TLR4 in LPS signal transduction.

Data from our laboratory and others suggest that distinct CD14 ligands possess different requirements for CD14-associated signal transduction molecules. Savedra et al. (16) previously reported that Chinese hamster ovary (CHO) cells engineered to express human CD14 (CHO/CD14) could be activated by LPS, but not by a distinct CD14 ligand, mycobacterial lipoarabinomannan (LAM). This observation led us to hypothesize that different CD14 ligands might require distinct signal-transduction proteins to initiate intracellular signaling. In this study we tested the possibility that CD14-mediated cellular activation initiated by LPS and LAM requires distinct TLR proteins.

Materials and Methods

Cells and reagents

The CHO-K1 fibroblast, THP-1 human monocytic leukemia, and RAW264.7 murine macrophage cell lines were purchased from the American Type Culture Collection (Manassas, VA). THP-1 and RAW264.7 cells were maintained in DMEM culture medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). CHO cells were maintained in HAM F-12 culture medium (BioWhittaker) supplemented with 10% heat-inactivated FBS (HyClone), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Cells were cultured at 37°C in the presence of 5% CO2 in a humidified incubator. CHO/CD14, 3E10 (CHO/CD14/ELAM-CD25), 3E10/TLR2, and CHO/TLR4 were previously described (16, 17) and cultured as described above. Several clonal isolates of each cell line were examined, and identical results were obtained using all clones. In some experiments, cells in all medium conditions were cultured for 20–24 h before LPS treatment. To achieve maximal LPS responsiveness, cells were exposed to 10 ng/ml LPS.
was added to the DNA-medium mixture and incubated for 10 min at ambient temperature. Subsequently, 600 μl of serum-containing medium was added to the reaction mixture and added to the individual wells. Each reaction was prepared individually and each condition was performed in triplicate. Reactions were incubated with the cells for 2–3 h, whereupon the reaction was removed from the cells and fresh media-container serum was added. On the following day, individual wells were left untreated or were stimulated with either LPS or LAM as indicated in the figures. Cells were then incubated for an additional 5 h before harvesting. Luciferase assays were performed as described below. All transfection experiments were repeated at least three times using different plasmid preparations, and a single representative experiment is shown. Each single experiment represents triplicate independent transfections and data are expressed as average values ± SD.

In transient transfection experiments, immunofluorescence was used to confirm that the transfected cells expressed similar levels of TLR proteins. Briefly, cells were cultured on Costar (Cambridge, MA) chamber slides and transiently transfected with expression plasmids encoding the Flag-TLR fusion protein. Twenty-four hours after transfection, the cells were incubated with a primary anti-FLAG mAb (1 μg/ml; Sigma), and subsequently with a secondary goat anti-mouse Ig antiserum (1:500) conjugated to rhodamine. Transfected cells that expressed the epitope-tagged TLR proteins were counted using a fluorescence microscope. Both CHO cells and RAW264.7 macrophages were found to express similar levels of TLR proteins following transient transfection with the various TLR expression plasmids (data not shown).

Luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Briefly, cells were washed and scraped on ice in cold PBS, pelleted by centrifugation, and resuspended in 100 μl of reporter lysis buffer. Samples were freeze-thawed once and centrifuged at 14000 × g for 10 min at 4°C to remove cellular debris. Supernatants were recovered and assayed for total protein using the Bio-Rad protein assay according to the manufacturer’s instructions. Fifty micrograms of total protein from each lysate was assayed for luciferase activity as measured by light emissions in a scintillation counter. In experiments using 3E10 cells, which contain a stably transduced CD25 reporter gene under the control of the NF-κB-dependent ELAM-1 (E-selectin) promoter, reporter gene expression was measured by flow cytometry as previously described (17). Data were collected using CellQuest software (Becton Dickinson) and were expressed as either mean channel fluorescence (FL1), or the ratio (fold activation) of the percent of CD25+ cells in unstimulated and stimulated cell populations (gated to exclude the lowest 5% of cells based on mean FL1).

RNA analysis by RT-PCR

Total RNA from 3E10 cells was purified using RNA-STAT (Promega) as recommended by the manufacturer. Reverse transcriptase (RT) reactions to generate cDNA were performed using avian myeloblastosis virus RT (Promega). PCR were performed using Taq polymerase (Promega), 1 μg of cDNA, 0.5 μM of each oligonucleotide primer, 2 mM MgCl₂, and 0.2 mM NTPs in a final reaction volume of 50 μl. Thirty amplification cycles were performed (1 min, 94°C denaturation; 1 min, 55°C annealing; 1.5 min, 72°C extension). As a control for contaminating genomic DNA, parallel PCR were performed in which the template nucleic acids were not reverse transcribed. Following amplification, a portion of the PCR were electrophoresed on a 1.2% agarose gel and visualized using ethidium bromide. PCR primers used in this study are as follows: sense strand IL-6 primer, 5’-TTG GGA AAT TTG CCT ACT GAA-3’; anti-sense strand IL-6 primer, 5’-AGG CAT GAC TAT TTT ATC TGG A-3’; sense-strand β-actin primer, 5’-TCA TGA AGT GTG ACC TGT ACA TCC GT-3’; and antisense strand β-actin primer, 5’-CCT AGA AGC ATT TGC GGT GCA CGA TG-3’.

Measurement of TNF protein production by stimulated macrophages

Peritoneal exudate macrophages obtained from C3H/FeJ and C3H/HeJ mice as previously described (18) were stimulated in vitro with increasing concentrations of LPS or LAM for 6 h. Supernatants were collected from unstimulated and stimulated cells, centrifuged to remove cellular debris, and TNF-α protein levels were determined using a specific ELISA (R&D Systems, Minneapolis, MN). Two mice were used per condition, cells from individual mice were cultured separately, and ELISA measurements were performed in triplicate.

FIGURE 1. Expression of TLR2 by CHO/CD14 cells confers responsiveness to LAM. CHO/CD14 and CHO/CD14/TLR2 were stimulated with LAM (1 μg/ml), LPS (100 ng/ml), or IL-1β (5 ng/ml) for 1 h. Nuclear extracts from stimulated and unstimulated cells were prepared as described in Materials and Methods and then assessed for the presence of NF-κB using an EMSA. The migration of the DNA-protein complex containing NF-κB is indicated. This complex was found to be specific as judged using supershifting Abs and unlabeled NF-κB competitor oligonucleotides (data not shown).

Results

TLR2 confers LAM responsiveness on CHO/CD14 cells

We previously proposed that CHO/CD14 cells failed to be activated by LAM because these cells lacked a CD14-associated signaling protein that was specifically required to confer LAM responsiveness, but that was not necessary for LPS responsiveness (16). More recently, CHO cells were found to not express a functional TLR2 protein (20). A single nucleotide deletion within the tlr2 gene of Chinese hamsters, and therefore in CHO cells derived from these animals, introduced a premature stop codon at aa 520. This mutant gene encodes a putative protein lacking both transmembrane and intracellular domains of TLR2. This finding supported the possibility that the missing LAM-responsive signaling protein was TLR2. To test this hypothesis, a CHO cell line was engineered that was stably cotransfected with a CD14 expression plasmid and an ELAM-CD25 reporter plasmid (17). A clone of this line (designated 3E10), which constitutively expressed high levels of CD14 on its surface, was subsequently stably transfected with a Flag-TLR2 expression plasmid. A clone of this line (designated 3E10/TLR2), which constitutively expressed high levels of Flag on its surface, was selected for further study. These 3E10/TLR2 cells were stimulated in vitro for 1 h with LPS, LAM, and IL-1β in the presence of FBS. Cell activation was then assessed by measuring the translocation of NF-κB to the nucleus. Following stimulation, the cells were harvested and nuclear extracts were prepared. EMSA analysis was used to measure the levels of NF-κB in the nuclear extracts. As shown in Fig. 1, both 3E10 and 3E10/TLR2 cells were strongly activated by LPS and IL-1β. In contrast, only the 3E10/TLR2 cells were strongly activated by LAM. These data demonstrate that expression of functional TLR2 by the 3E10 cells is sufficient to render these cells responsive to LAM.

We further characterized the requirements for CD14 and LPS binding protein (LBP) in the TLR2-dependent activation of CHO cells by LAM. We found that only CHO cells that express TLR2 on their surface (CHO/TLR2) were responsive to LAM in the absence of coexpressed CD14. Furthermore, this LAM responsiveness required the presence of serum, a source of LBP, and soluble
CD14 (data not shown). LAM responsiveness is most likely due to the presence of soluble CD14 and LBP in the serum, as well as to the expression of functional TLR2. Together, our data suggest that both membrane-bound and soluble CD14 can confer LAM responsiveness in the presence of TLR2. This finding is also consistent with our earlier studies using the monocyte-like THP-1 cell line where LAM responsiveness, as with LPS, was markedly augmented in the presence of LBP (16). As noted above, CHO cells do not express functional endogenous TLR2, but do express functional endogenous TLR4. Thus, expression of membrane CD14 on CHO cells renders them LPS responsive, and this responsiveness is mediated by endogenous functional TLR4.

We subsequently sought to determine whether the TLR2-dependent activation of 3E10 cells by LAM could confer functional responsiveness on these cells. These cells contain an integrated reporter gene consisting of the CD25 reporter gene under the NF-kB promoter. The capacity of LAM to activate CD25 expression was assessed by flow cytometry. As shown in Fig. 2A, LAM-activated CD25 is expressed in a dose-dependent manner in 3E10/TLR2 cells, whereas 3E10 cells were not activated by LAM to express CD25. In contrast, both 3E10/TLR2 and 3E10 cells were responsive to LPS, as judged by CD25 expression (Fig. 2B). Expression of TLR2 only modestly increased the capacity of these cells to respond to LPS. Together, these data suggest that TLR2 is an absolute requirement for LAM responsiveness, whereas TLR2 does not substantially augment LPS responsiveness in these cells.

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**TLR2 can confer the capacity of LAM to activate endogenous CHO cell gene expression**

The data presented above demonstrate that expression of functional TLR2 in the CHO cells can confer LAM responsiveness on these cells, as judged by the ability to activate NF-kB and a NF-kB-dependent reporter gene. We also assessed the capacity of TLR2 to mediate the activation of an endogenous CHO cell gene in response to LAM treatment. One of the endogenous CHO genes which can be activated by LPS in CHO/CD14 cells is IL-6 (21). We used RT-PCR analysis to determine whether LAM could activate endogenous IL-6 expression in the 3E10/TLR2 cells. As shown in Fig. 3, IL-6 mRNA levels were up-regulated in 3E10 and in 3E10/TLR2 cells stimulated with either LPS or IL-1β. In contrast, LAM only up-regulated IL-6 mRNA levels in the 3E10/TLR2 cells. The overexpression of functional TLR2 in the 3E10/TLR2 did not confer an enhanced ability for LPS to induce higher levels of IL-6 transcripts compared with levels that were induced in the 3E10 cells. These findings demonstrate that TLR2 can specifically mediate the activation of an endogenous gene in CHO cells by LAM. Also, expression of functional TLR2 does not appear to confer an enhanced capacity of these cells to respond to LPS.

**TLR2, but not TLR1 or TLR4, confers LAM responsiveness**

A recent study compared the capacities of different TLR proteins to confer LPS responsiveness on HEK 293 cells. These investigators reported that TLR2, but not TLR1 or TLR4, could confer LPS responsiveness on stably transfected HEK 293 cells (9). This finding contrasted with the conclusion that TLR4 plays the major role in LPS signaling, based on the demonstration that LPS-hyporesponsive C3H/HeJ mice possess a point mutation within the tlr4 gene (11) and that cells from TLR4 knockout mice are also hyporesponsive to LPS (13). To compare the functional capacities of different TLR proteins, we utilized a model system in which cells were transiently cotransfected with expression plasmids encoding the TLR proteins and the ELAM-luc reporter plasmid. As shown in Fig. 4A, transient cotransfection of the TLR2 expression plasmid conferred LAM responsiveness on CHO/CD14 cells. In contrast, transiently transfected cells that expressed TLR1 or TLR4 could not be activated by LAM. The responses of these cells to LPS were qualitatively different from their responses to LAM. In the CHO/CD14 cells, the TLR4 protein mediated the highest level of CD14 (data not shown). LAM responsiveness is most likely due to the presence of soluble CD14 and LBP in the serum, as well as to the expression of functional TLR2. Together, our data suggest that both membrane-bound and soluble CD14 can confer LAM responsiveness in the presence of TLR2. This finding is also consistent with our earlier studies using the monocyte-like THP-1 cell line where LAM responsiveness, as with LPS, was markedly augmented in the presence of LBP (16). As noted above, CHO cells do not express functional endogenous TLR2, but do express functional endogenous TLR4. Thus, expression of membrane CD14 on CHO cells renders them LPS responsive, and this responsiveness is mediated by endogenous functional TLR4.

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of LPS responsiveness, whereas TLR2 could only modestly enhance the responsiveness of these cells to LPS (Fig. 4B). Expression of TLR1 did not enhance the responsiveness of these cells to LPS. Thus, LAM and LPS appear to activate these cells predominantly through different TLR proteins, specifically TLR2 and TLR4, respectively.

Macrophages are cells that naturally respond to both LAM and LPS. These cells also express a variety of different TLR mRNA (6). Using the transient transfection approach described above, we sought to determine whether transient overexpression of these TLR proteins could confer selective responsiveness to LAM and LPS in RAW264.7 murine macrophages. As shown in Fig. 4, C and D, these cells were capable of activating the ELAM-luc reporter plasmid in response to LAM and LPS without the overexpression of exogenous TLR proteins. Furthermore, transient co-transfection of the TLR2 expression plasmid markedly enhanced the responsiveness of macrophages to LAM. In contrast, transient transfection with the TLR4 expression plasmid did not enhance the responsiveness of these cells to LAM. Like CHO/CD14 cells, the responses of macrophages to LPS were qualitatively different from their responses to LAM. In the RAW264.7 cells, overexpression of the TLR4 protein substantially enhanced LPS responsiveness, whereas TLR2 could only modestly enhance the responsiveness of these cells to LPS relative to control cells. Expression of TLR1 did not enhance the responsiveness of these cells to LPS or LAM. Thus, like CHO/CD14 cells, LAM and LPS appear to activate these cells predominantly through TLR2 and TLR4, respectively. Our data provide the direct evidence for the role of TLR4 in LPS signaling in hamster and murine cells, and are consistent with the phenotype of the tlr4 mutation in C3H/HeJ mice.

**FIGURE 4.** LAM activation is selectively mediated by TLR2 in CHO/CD14 cells and murine macrophages. CHO/CD14 cells were transiently cotransfected with the ELAM-luc reporter plasmid and expression plasmids encoding human TLR1, TLR2, or TLR4. A portion of the cells were then stimulated with either 1 μg/ml LAM (A) or 100 ng/ml LPS (B) for 5 h. Cells were then harvested and luciferase activity was measured as described in Materials and Methods. All transfection experiments were repeated at least three times using different plasmid preparations, and a single representative experiment is shown. Each single experiment represents triplicate independent transfections, and data are expressed as average luciferase values (subtracted for background) ± SD. RAW264.7 cells were transiently cotransfected with the ELAM-luc reporter plasmid and expression plasmids encoding human TLR1, TLR2 or TLR4. A portion of the cells were then stimulated with either 1 μg/ml LAM (C) or 100 ng/ml LPS (D) for 5 h. Cells were then harvested and luciferase activity was measured. All transfection experiments were repeated at least three times using different plasmid preparations, and a single representative experiment is shown. Each single experiment represents triplicate independent transfections and data are expressed as average luciferase values (subtracted for background) ± SD.

The conserved C-terminal domain of TLR2 is required for cellular activation by LAM

Both Yang et al. (8) and Kirschning et al. (9) reported that the conserved C-terminal intracellular domain of TLR2 was required for LPS signaling through TLR2. The C-terminal 13 amino acids of TLR2 share homology with other members of the Toll and IL-1 receptor families (6). We used a similar C-terminal TLR2 mutant to determine whether LAM responsiveness also required the presence of the C-terminal 13 amino acids. CHO/CD14 cells were transiently cotransfected with the ELAM-luc reporter plasmid and a TLR2 expression plasmid, a C-terminal TLR2 mutant expression plasmid (TLR2Δ13), or both expression plasmids. As shown in Fig. 5A, the full-length TLR2 was capable of conferring LAM responsiveness, whereas the TLR2Δ13 mutant was not. Furthermore, expressing both the full-length and TLR2Δ13 mutant proteins in the same cells almost completely blocked the ability of these cells to be activated by LAM. This finding suggests that the TLR2Δ13 mutant can function as a dominant-negative protein in LAM signaling and is consistent with an earlier demonstration that a different C-terminal mutant (TLR2Δ141) can function as a dominant-negative protein in LPS signaling (8). We subsequently performed these same experiments using transiently transfected RAW264.7 macrophages, and similar results were obtained (Fig. 5B).

We noted that expressing both the human full-length and TLR2Δ13 mutant proteins in murine RAW264.7 cells did not reduce LAM responsiveness to levels lower that those of control cells. This finding differs quantitatively from data obtained using the CHO/CD14 cells, which showed that LAM responsiveness was completely blocked in cells expressing the TLR2Δ13 mutant. These differences can be explained by the lack of endogenous
TLR2 expression in the CHO/CD14 cells. Furthermore, it appears that the TLR2Δ13 mutant can function as a dominant-negative protein in LAM signaling, but only when blocking signaling through TLR2 from the same species as the mutant. To test this possibility, we transiently transfected the human TLR2Δ13 mutant into the LPS- and LAM-responsive human THP-1 macrophage-like cell line. As shown in Fig. 5C, we found that TLR2Δ13 suppressed the endogenous capacity of these cells to respond to LAM, but not to LPS.

Several investigators have demonstrated that C3H/HeJ mice possess a mutant tlr4 gene in which a single conserved amino acid is altered (11–13). These investigators proposed that this mutation and the mutant protein that is ultimately encoded by this gene are responsible for the LPS hyporesponsive phenotype of macrophages obtained from these mice. If this is true, then our data would predict that the tlr4 mutation in the C3H/HeJ macrophages would have no effect on the responsiveness of these cells to LAM. Peritoneal exudate macrophages were obtained from both C3H/HeJ and C3H/FeJ LPS-responsive mice as described above and then stimulated with LAM or LPS in vitro. The capacity of the cells to respond to these CD14 ligands was then assessed by measuring TNF-α secretion. As shown in Fig. 6, both LAM- and LPS-induced TNF-α secretion in the C3H/HeJ macrophages, whereas only LAM could induce substantial TNF-α release from the C3H/HeJ macrophages. This finding agrees with our hypothesis that LAM signaling requires TLR2, but not TLR4.

**Discussion**

Recent studies have implicated TLR proteins in the activation of macrophages via the LPS receptor CD14. Our previous data suggested that different CD14 ligands might require distinct signal transduction proteins to initiate intracellular signaling. Specifically, we found that CHO/CD14 cells could be activated by LPS, but not by LAM (16). These cells were subsequently found to possess a frame-shift mutation within the tlr2 gene which results in the failure to express a functional TLR2 protein (20). Furthermore,
we found that mycobacterial LAM could activate macrophages isolated from LPS-unresponsive C3H/HeJ mice. Defective LPS signaling in C3H/HeJ macrophages has been attributed to a missense mutation in the tlr4 gene which renders the encoded protein nonfunctional (11–13). Thus, we hypothesized that TLR2, but not TLR4, was necessary for LAM signaling. Here we showed that CHO/CD14 cells that were engineered to express functional TLR2 protein acquired the ability to be activated by LAM, as judged by the activation of an exogenous NF-κB-dependent promoter and the activation of endogenous IL-6 gene expression. In contrast to LAM, LPS responsiveness was only modestly enhanced by over-expression of TLR2 in these cells. Overexpression of TLR4 did not confer LAM responsiveness on CHO/CD14 cells and did not augment basal LAM responsiveness in RAW264.7 macrophages. Moreover, we provide direct evidence that TLR4 can play a major role in LPS signaling in both hamster and mouse cells. In contrast, TLR1 did not appear to mediate cellular activation by either LPS or LAM. Lastly, we found that macrophages from both LPS-responsive C3H/FeJ and LPS-unresponsive C3H/HeJ mice were similarly activated by LAM. Taken together, our data demonstrate that CD14-dependent signaling induced by LAM and LPS requires distinct TLR proteins.

Insights into the mechanism by which TLR proteins generate an intracellular signal in response to these distinct CD14 ligands have come from studying the effects of deletions of the intracellular domain of TLR2 on ligand responsiveness. We found that LAM could not activate CHO/CD14 cells which expressed a mutant TLR2 protein lacking the C-terminal 13 amino acids of the intracellular domain. This domain contains sequences homologous to sequences within the type 1 IL-1 receptor that are required for signal transduction. Furthermore, transient overexpression of this mutant TLR2 protein in either CHO/CD14 cells or the RAW264.7 macrophage cell line did not confer or enhance LAM responsiveness. In the RAW264.7 macrophages, overexpression of the human C-terminal TLR2 mutant did not affect the capacity of endogenous murine TLR2 to mediate LAM responsiveness. In contrast, the C-terminal human TLR2 mutant could function as a dominant-negative mutant when coexpressed with wild-type human TLR2 in both cell lines. Similarly, expression of this human TLR2 mutant in a human monocytic cell line that expresses functional endogenous human TLR2 blocked the activation of these cells by LAM, but not by LPS. This suggests that the ability of TLR2 to form dimers and the ability of mutants to express a dominant-negative phenotype are not shared by TLR proteins from different species. The capacity of C-terminal TLR2 deletion mutants to serve as dominant-negative mutants via their capacity to form complexes with wild-type TLR2 has been recently reported (10), although our findings demonstrate a species-specific feature to this phenotype.

Previous studies have shown that mutation of a single amino acid within the intracellular domain of TLR4 was sufficient to render macrophages from C3H/HeJ mice unresponsive to LPS (11–13). We have shown in this paper that this mutation did not affect the responsiveness of these cells to LAM, as judged by the production of TNF-α. This finding is consistent with a previous study by Chatterjee et al. (22). The LAM responsiveness of CHO/CD14 cells that overexpress TLR2 was selective, as judged by the inability of LAM isolated from M. bovis bacillus Calmette-Guérin or from Mycobacterium tuberculosis to activate the cells (23). It should be noted that the LAM used in our studies was purified from a rapidly growing avirulent mycobacterium (AraLAM), and is chemically distinct from LAM expressed by either M. bovis bacillus Calmette-Guérin or M. tuberculosis (ManLAM, reviewed in Ref. 24). Despite this chemical specificity, several distinct molecules have been reported to activate cells via TLR2. These include LPS, lipoteichoic acid, and soluble peptidoglycan (8, 25, 26). Together these data implicate TLR2 as a pattern recognition receptor for bacterial products.

Several recent publications have demonstrated roles for both TLR2 and TLR4 in LPS signaling. Kirschning et al. (9) reported that overexpression of TLR2, but not TLR4, could confer LPS responsiveness on HEK 293 cells. The same investigators also reported that overexpression of TLR1 did not confer LPS responsiveness on HEK 293 cells (9). A role for TLR2 in LPS responsiveness was also reported by Yang et al. (8) using the same cell line. Both groups of investigators showed that a C-terminal truncation mutant of TLR2 failed to confer LPS responsiveness on the HEK 293 cells. This mutant TLR2 could also function as a dominant-negative mutant in LPS-stimulated human U373 astrocytoma cells (8). In contrast to these studies, a recent paper by Chow et al. (14) reported that TLR4 could also confer LPS responsiveness on HEK 293 cells. These findings are consistent with the capacity of CHO/CD14 cells and of Chinese hamster macrophages that lack functional TLR2 but express functional TLR4 to respond to LPS (20). Moreover, both TLR4-deficient C3H/HeJ mice (11, 12) and TLR4 knockout mice (13) are hyporesponsive to LPS. Thus, while both TLR2 and TLR4 can confer LPS responsiveness when over-expressed in transfected cells, TLR4 appears to be the predominant LPS receptor in vivo. In this paper we have shown that TLR4 is capable of conferring a high level of LPS inducibility on CHO/CD14 cells and that it could further enhance LPS responsiveness in RAW264.7 macrophages. Enhanced LPS responsiveness was not conferred by TLR2. The reasons for these differences are unclear, but might reflect functional or species-specific differences between TLR2 and TLR4.

The data presented here demonstrate that the chemically distinct bacterial glycolipids LAM and LPS can interact with CD14 and activate cells via different TLR proteins. The dissimilar chemical structures of LAM and LPS underscore the notion that a variety of bacterial products can be recognized by CD14, which functions as a pattern recognition receptor (27) and subsequently activate cells in a TLR-dependent manner. Our findings suggest a novel receptor-signaling paradigm in which the binding of distinct ligands (e.g., LAM and LPS) is mediated by a common receptor chain (CD14) but cellular activation is initiated via distinct signal-transducing chains that confer ligand specificity (TLR2 and TLR4). This paradigm contrasts with many cytokine receptor complexes in which receptor specificity in conferred by a unique ligand-binding chain, but cellular activation is initiated via shared signal-transducing chains (e.g., IL-2R and IL-15R). A major question that remains to be answered is whether different TLR proteins initiate distinct signaling events or if all TLR proteins utilize a common receptor-signaling paradigm in which the binding of distinct ligands is mediated by a common receptor chain (CD14) but cellular activation is initiated via distinct signal-transducing chains that confer ligand specificity (TLR2 and TLR4).
possibility that different TLR proteins may preferentially signal through different IRAK family members.

References