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TLR4 is the signal-transducing receptor for structurally diverse microbial molecules such as bacterial LPS, respiratory syncytial virus fusion (F) protein, and chlamydial heat shock protein 60. Previous studies associated two polymorphic mutations in the extracellular domain of TLR4 (Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile) with decreased LPS responsiveness. To analyze the molecular basis for diminished responsiveness, site-specific mutations (singly or coexpressed) were introduced into untagged and epitope (Flag)-tagged wild-type (WT) TLR4 expression vectors to permit a direct comparison of WT and mutant signal transduction. Coexpression of WT TLR4, CD14, and MD-2 expression vectors in HEK293T cells was first optimized to achieve optimal LPS-induced NF-κB reporter gene expression. Surprisingly, transfection of cells with MD-2 at high input levels often used in the literature suppressed LPS-induced signaling, whereas supraoptimal CD14 levels did not. Under conditions where WT and polymorphic variants were comparably expressed, significant differences in NF-κB activation were observed in response to LPS and two structurally unrelated TLR4 agonists, chlamydial heat shock protein 60 and RSV F protein, with the double, cosegregating mutant TLR4 exhibiting the greatest deficiency. Overexpression of Flag-tagged WT and mutant vectors at input levels resulting in agonist-independent signaling led to equivalent NF-κB signaling, suggesting that these mutations in TLR4 affect appropriate interaction with agonist or coreceptor. These data provide new insights into the importance of stoichiometry among the components of the TLR4/MD-2/CD14 complex. A structural model that accounts for the diminished responsiveness of mutant TLR4 polymorphisms to structurally unrelated TLR4 agonists is proposed.


The TLRs are a family of closely related cellular receptors that initiate cellular signaling in response to a wide range of conserved pathogen-associated molecular patterns (PAMPs) followed by the differential recruitment of adapter molecules to the oligomerized receptor complex. These type I transmembrane proteins contain extracellular leucine-rich-repeat (LRR) domains and intracellular signaling domains that share similarities with the intracytoplasmic domain of the IL-1R. Both IL-1R and TLRs induce signal transduction pathways that lead to activation of transcription factors such as NF-κB, a key transcriptional regulator of the inflammatory response.

Among the 10 functional human TLR family members that have been identified to date, TLR4 has been shown to recognize structurally unrelated PAMPs such as Gram-negative enterobacterial LPS (2–4), respiratory syncytial virus (RSV) fusion (F) protein (5), and chlamydial heat shock protein (Hsp) (6, 7). In addition, endogenous mammalian molecules such as fibrinogen (8), fibrinectin (9), Hsp (10, 11), small m.w. oligosaccharide fragments of hyaluronan (12, 13), and surfactant protein A (14, 15) have also been reported to activate cells via TLR4. A secreted protein, MD-2, that binds to the extracellular domain of TLR4 (16), and a soluble or GPI-anchored glycoprotein coreceptor, CD14, are also necessary for optimal TLR4-mediated LPS responses (4, 17, 18). It is generally accepted that CD14 serves as a high-affinity receptor for LPS after catalytic transfer of LPS monomers by LPS-binding protein (LBP) and that the CD14/LPS complex (19) interacts with TLR4/MD-2 complexes on the surface of cells to initiate receptor oligomerization and downstream signaling (20).

Recently, two polymorphic point mutations at nucleotides A896G and C1196T in the cDNA of human TLR4 have been identified. These encode amino acid changes, Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile, respectively, within the extracellular domain of TLR4, and have been associated with LPS hyporesponsiveness in primary human epithelial cells and alveolar macrophages in vitro and with airway hyporesponsiveness to inhaled LPS in vivo (21). Both the Asp<sup>299</sup>Gly and the Thr<sup>399</sup>Ile mutations are encoded within the fourth exon of the TLR4 gene and have been predicted to affect a ligand-binding region and a coreceptor-binding region, respectively (22). Based on a more recent model of the extracellular domain of TLR4, Bell et al. (23) suggested that these two amino acids lie on the same face of the molecule. Transfection of wild-type (WT) TLR4 has been shown to rescue the LPS-hyporesponsive phenotype in either primary airway epithelial cells or alveolar...
macrophages obtained from individuals with these TLR4 mutations (21).

The molecular mechanisms that underlie the diminished responsiveness of individuals or cells that express the Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile TLR4 polymorphisms are largely unknown. However, impaired LPS responsiveness of individuals carrying mutant TLR4 alleles may be related to a diminished cell surface expression as demonstrated by immunohistochemistry in airway epithelial cells (21). This implies that mutations in the extracellular region of TLR4 may affect its expression, transport to the membrane, or detection. In vivo, these polymorphic forms of TLR4 have also been shown to be associated with an increased incidence of Gram-negative bacterial infections and sepsis (21, 24, 25), and more recently, with an increased incidence of severe infection with RSV (26) and an increased susceptibility to Crohn’s disease (27–29).

Conversely, individuals who express these polymorphic forms of TLR4 have been found to be at decreased risk of certain inflammatory diseases including atherosclerosis (25, 30) and rheumatoid arthritis (31, 32). Thus, the ability to signal through TLR4 may represent an immunologic balance between being able to elicit a sufficient inflammatory response to control infection vs too vigorous an inflammatory response, resulting in diseases with an inflammatory etiology (24, 33). However, a very recent study found that inheritance of these mutations is associated with increased resistance to Legionnaire’s disease (34), suggesting that the increased susceptibility to infection associated with inheritance of the Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile TLR4 mutations does not extend to all infectious diseases.

To assess the molecular basis for the LPS hyporesponsiveness of the polymorphic human TLR4 proteins in vitro, we expressed the WT or TLR4 mutant variants transiently in the LPS-unresponsive HEK293T cells (4, 35) and studied the relative effects of these mutations on TLR4-mediated signaling. Under conditions where MD-2, CD14, and WT TLR4 constructs were optimized for LPS-dependent signaling in HEK293T cells, LPS-induced NF-κB reporter gene expression was reduced in cells transfected with mutant constructs, a pattern that was recapitulated with structurally unrelated TLR4 agonists, chlamydial Hsp 60 and RSV fusion (F) protein. However, equivalent levels of signaling were observed when N-terminally Flag-tagged polymorphic variants were overexpressed at levels leading to agonist-independent signaling (36). We propose a structural model of the ectodomain of TLR4 in which both D299 and T399 residues lie on the same face of scaffold and form a saddle-like structure that may provide a docking site for either ligand or coreceptor. In the double mutant, D299G/T399I, the saddle-like structure is perturbed, possibly resulting in a reduced affinity of binding for ligand, coreceptor, or both. Collecularly, these data provide new insights into TLR4 signaling, and the potential for disease in individuals with these extracellular TLR4 mutations.

**Materials and Methods**

**Cells and reagents**

HEK293T cells were cultured in DMEM (BioWhittaker) supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Protein-free, phenol/water-extracted *Escherichia coli* K235 LPS and recombinant chlamydial Hsp 60 were prepared as described elsewhere (6, 37). The level of contaminating LPS in the Hsp 60 protein was <0.0012 ng/10 μg of protein as assessed by *Limulus* amebocyte lysate (LAL) test. This was the highest concentration of LPS used in the luciferase assays and the amount of detectable LPS is below the TLR4 activation level. SuperFect transfection reagent was obtained from Qiagen. The wild-type Flag-CMV1-TLR4 expression vector was provided by Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA) and the pCDNA3-TLR4 wild-type construct was described in Refs. 21 and 29. The pCMV1-β-gal reporter plasmid was also provided by Dr. D. Golenbock and have been described elsewhere (4, 35). The coding sequences of all plasmid constructs were confirmed by automated sequencing analysis.

**Reporter assay**

HEK293T cells were seeded into 12-well Costar plates (Corning) at 2 × 10<sup>4</sup> cells/well and incubated overnight in a CO<sub>2</sub> incubator. The next morning, cells were cotransfected for 3 h with varying amounts of Flag-CMV1-TLR4 or pCDNA3-TLR4, pCDNA3-huCD14 and pEFBOS-HA-huMD-2 (as indicated in Results) together with pELAM-luc (500 ng/well) and pCMV1-β-gal (100 ng/well) reporter constructs. The final DNA quantity was adjusted to 1.5 μg/well with the pCDNA3 vector (Invitrogen Life Technologies). The transfection was conducted with SuperFect transfection reagent (Qiagen). Cells were harvested for 20 h, washed with 1× PBS, and stimulated with TLR4 agonists for 5 h. Cells were lysed in 1× reporter assay lysis buffer (Promega), and β-galactosidase (Tropix; Galacto-Light) and luciferase (Biozym Diagnostik) activities were analyzed using a Berthold LB 9507 Luminometer (Berthold Technologies). Relative luciferase activity (RLU) was calculated by normalizing each sample’s luciferase activity for constitutive β-galactosidase activity measured within the same sample. Based on expression of β-galactosidase activity in individual cells, the transfection efficiency was consistently 70–90%, as assessed by in situ β-galactosidase staining kit (Strategene).

For NF-κB autoregulation studies, Flag-TLR4 WT and mutant constructs were used at different input quantities to transfect HEK293T cells along with the NF-κB and β-gal reporter plasmids, with or without MD-2 and CD14 constructs, as indicated. Cells were recovered for 20 h, washed with 1× PBS and lysed in 1× reporter assay lysis buffer. The samples were assayed for luciferase and β-galactosidase activity.

**Total RNA extraction from HEK293T transfecants**

Total RNA was extracted using RNA STAT60 (Tel-Test), followed by isopropanol precipitation at −20°C. After washing twice with 80% ethanol, the RNA pellet was resuspended in nuclelease-free distilled water and RNA was quantified and treated with thermostable amplification grade DNase I (Invitrogen Life Technologies). One microgram of RNA was used for oligo(dT)-primed cDNA synthesis (Promega Reverse Transcription System), according to the manufacturer’s instructions. In brief, the reaction was conducted on Applied Biosystems GeneAmp PCR System 9700 at 94°C for 60 min and 95°C for 5 min and the completed reaction was diluted 1/5 with water. For real-time PCR analysis, 2 μl of the diluted cDNA was used.
Real-time PCR for steady state mRNA quantification of untagged and Flag-tagged TLR4 WT

Quantitative real-time PCR primer sets were designed using Applied Biosystems PrimerExpress version 2.0 software and were synthesized by the Biopolymer and Genomics Core Facility (UMB). Primers common to both untagged and Flag-tagged PCR products were designed to overlap adjacent exons to exclude detection of genomic DNA. Primers specific to the human TLR4 gene were: forward, 5′-gacctgtatgcacagtgattgc-3′ and reverse, 5′-aggacaacctcagcagctg-3′ and the primers for the housekeeping gene, human hypoxanthine-guanine phosphoribosyl transferase (HPRT), were: forward, 5′-acacagtcaacaggggacataaaag-3′ and reverse, 5′-gtgcctgtggtttcgccgtctg-3′. Real-time PCR was performed on an Applied Biosystems Prism 7900HT Sequence Detection System. Amplification was conducted in a 2-μl reaction volume that contained ~20 ng of cDNA from the reverse-transcribed reaction, 0.3 μM each of sense and antisense primers, and 12.5 μl of 2× SYBR Green Master Mix (Applied Biosystems) under the following thermal conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 1 s at 95°C and 1 min at 60°C, followed by a dissociation stage. The mRNA levels of the genes of interest were normalized to HPRT gene, and relative gene expression levels were determined using the 2−ΔΔCt method, where Ct is the cycle threshold (40).

Immunoprecipitation and Western blotting

HEK293T cells were plated in 100-mm tissue culture dishes (3 × 106 cells/dish), grown overnight in a CO2 incubator, and cotransfected for 3 h with pcDNA3-TLR4 WT (4.5 μg/dish, corresponding to 300 ng/well in 12-well plates) or Flag-CMV-1-huTLR4 WT (112.5 ng/dish, corresponding to 7.5 ng/well in 12-well plates), and pcDNA3-huCD14 (450 ng/dish) and pEFBOS-HA-huMD-2 (45 ng/dish) expression vectors using the SuperFect Transfection Reagent. Cells were incubated for a further 72-h posttransfection before lysing the cells and preparation of cellular extracts for the immunoprecipitations. The whole process was conducted at 4°C. In brief, the cells were collected, washed twice with cold 1× PBS and resuspended in 1 ml of ice-cold lysis buffer (20 mM HEPES, 0.5% Triton X-100, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA, 10 mM NaF, 2 mM Na3VO4, 1 mM PMSF, 1 mM peptidase inhibitor mixture), transferred to 1.5-ml Eppendorf tubes and mixed by rotation at 4°C for 1 h to completely lyse the cells. Lysates were centrifuged at maximum speed for 10 min at 4°C, transferred the supernatant to another Eppendorf tube and estimated the protein concentration. Cell lysates containing 5 μg of total protein each were preclarified with 40 μl of 1 × PBS washed EZview Red Protein A affinity gel (Sigma-Aldrich). The preclarified supernatant was then mixed with 1 μl of anti-human TLR4 polyclonal Ab, incubated at 4°C for 2–3 h and then 40 μl of red affinity beads were added and rotated overnight at 4°C. The next morning, samples were centrifuged at 3600 rpm at 4°C for 1 min, the supernatant removed, and continued washing the red affinity beads twice with cold lysis buffer and two more times with cold 1× PBS. The final red bead pellet was resuspended in 40 μl of 2× SDS-sample buffer, vortexed, and boiled for 10 min.

For Western blotting, the immunoprecipitated samples were loaded in their entirety and resolved on 4–20% Tris-Glycine SDS-PAGE gel (Invitrogen Life Technologies), transferred onto Immobilon P membrane, and developed by Western blot analysis as described elsewhere (35). The HTA125 clone of human TLR4 monoclonal primary Ab was used at 1:500 dilution. HRP-conjugated secondary Abs were used at appropriate dilutions and conditions as suggested by the manufacturer.

FACS analysis of the HEK293T transfectants

HEK293T cells were seeded in 100-mm tissue culture dishes (2.5 × 106 cells/dish), and cultured overnight. After transfection, cells were incubated for 48 h after transfection, harvested, and washed twice with 1× PBS. One million (1 × 106) cells were resuspended in 200 μl of PBS-containing 0.1% BSA, and were incubated for 1 h on ice either with anti-Flag Ab (clone 7F10, Cell Signaling Technology) or isotype control IgG1 (BD Biosciences) at a final concentration of 5 μg/ml. The cells were then washed twice with PBS + 0.1% BSA and incubated for 1 h in the dark with FITC-conjugated anti-mouse IgG (Sigma-Aldrich) (1/200 final dilution), washed twice more with PBS + 0.1% BSA, and resuspended in 200 μl of the same buffer. Cells were sorted by using FACSsort (BD Biosciences). In each sample, 10,000 cells were analyzed. FACS data was analyzed by using WinMDI Flow Cytometry Application (version 2.8).

Construction of TLR4 ectodomain model

The TLR4 ectodomain sequence (1–633) was analyzed by the 3DJigas program (www.bmm.icnet.uk/~3djjigas/) 3DJigas can predict structural homology from a protein sequence to known three-dimensional structures. The CD42b LRRs were chosen to create a 21 LRR scaffold onto which the LRR structures predicted by 3DJigas were fit by least-squared fitting. The CD42b LRRs were chosen to create a 21 LRR scaffold onto which the CD42b LRR structures predicted by 3DJigas were fit by least-squared fitting. The CD42b LRRs were chosen to create a 21 LRR scaffold onto which the CD42b LRR structures predicted by 3DJigas were fit by least-squared fitting.
FIGURE 1. Optimal activation by TLR4 is dependent upon the concentration of input TLR4 and MD-2 vectors. A, A total of $2 \times 10^5$ cells/well HEK293T cells were cotransfected with the indicated amounts of untagged (top graph) or Flag-tagged (middle graph) TLR4 vector and fixed amounts of MD-2 and CD14 vectors (300 ng/well each). After overnight recovery, transfected cells were treated with the indicated concentrations of *E. coli* LPS for 5 h. Then, the cells were washed twice with cold 1× PBS, lysed in 1× lysis buffer, and centrifuged to remove cell debris. Luciferase and β-galactosidase activities were measured in cell lysates as described in Materials and Methods. A representative experiment was shown ($n = 6$). *, Optimum input concentration of constructs chosen for all subsequent agonist-dependent experiments. HEK 293T cells were also transfected with fixed amounts of MD-2 and CD14 (300 ng/well each), and the indicated amounts of Flag-tagged TLR4 vector constructs. Cells were stained, and FACS analysis was conducted to assess the surface expression of Flag-tagged TLR4 as described in Materials and Methods (bottom panel). A representative experiment is shown ($n = 4$). B, HEK293T cells were cotransfected with fixed amounts of untagged-TLR4 vector (300 ng/well; top graph) or Flag-tagged TLR4 vector (7.5 ng/well; middle graph), CD14 vector (300 ng/well), and the indicated amounts of MD-2 vector. After overnight recovery, transfected cells were treated with the indicated concentrations of *E. coli* LPS for 5 h and cell lysates were prepared. Luciferase and β-galactosidase activities were measured in cell lysates as described in Materials and Methods. A representative experiment was shown ($n = 6$). *, Optimum input concentration of MD-2 constructs chosen for all subsequent agonist-dependent experiments. HEK293T cells were also cotransfected with fixed amounts Flag-tagged TLR4 vector (7.5 ng/well), CD14 vector (300 ng/well), and the indicated amounts of MD-2 vector. Cells were prepared for the FACS analysis of the surface expression of Flag-tagged TLR4 (bottom panel) as described in Materials and Methods. A representative experiment is presented ($n = 4$).
MD-2 is an essential component of the TLR4-signaling complex. MD-2 is a soluble protein that has been reported to act both as a chaperone for surface expression of TLR4 (44) and as a critical protein in agonist-dependent TLR4 signaling (45, 46). MD-2 binds to the extracellular domain of TLR4 to form stable TLR4 receptor complexes (47, 48) and is essential for TLR4-mediated responses to LPS. Visentin et al. (49) identified two amino acid residues, Lys_{128} and Lys_{132}, in MD-2 that are critical for LPS binding.

Using the input concentrations of 300 and 7.5 ng/well of the untagged and Flag-tagged TLR4 vectors, respectively, we kept the input concentration of the CD14 expression vector fixed and now varied the input concentration of the CD14 expression vector. Input concentrations for the pEFBOS-HA-huMD-2 vector from 300 to 3.0 ng/well led to a significant increase in NF-κB luciferase reporter activity in response to LPS, which was optimal for both untagged and Flag-tagged TLR4 vectors with no change in the LPS dose dependency, while 0.3 ng/well was insufficient (Fig. 1B, top and middle graphs). The absence of MD-2 resulted in no detectable luciferase activity (data not shown). Therefore, 3 ng/well pEFBOS-HA-huMD-2 was used in all subsequent studies. We also sought to determine whether different input levels of MD-2 vector would have any effect on the surface expression of TLR4 when TLR4 vector was transfected at the input level determined to be optimal in Fig. 1A. Therefore, we transfected HEK293T cells with 7.5 ng/well Flag-Tagged TLR4 WT and 30 ng/well CD14 vector, and varying amounts of MD-2 vector. The cells were analyzed by FACS analysis for the surface-expressed Flag-tagged TLR4. As shown in Fig. 1B (bottom panel), the input levels of MD-2 vector did not affect the surface expression of TLR4 unlike the NF-κB activation, which was substantially diminished at higher input levels of MD-2 vector (Fig. 1B, top and middle graph).

Using the optimized input concentrations of TLR4 and MD-2 expression vectors, we now sought to determine the optimal input requirement for CD14 expression. In the complete absence of CD14, no signaling was observed (Fig. 2). This could be overcome partially by the addition of 10% FCS, but only at the highest LPS concentrations. Unlike MD-2, however, supraoptimal expression of CD14 was not inhibitory. Therefore, for all subsequent experiments, an input concentration of 30 ng/well pCDNA3-huCD14 was used. Collectively, these data clearly demonstrate that optimal LPS-induced TLR4 signaling is profoundly affected by the stoichiometry of the key components of the TLR4-signaling complex: not only are TLR4 levels important, but also the coreceptor levels, especially MD-2, to obtain optimal, LPS dose-dependent cellular activation that simulates agonist-dependent responsiveness in primary macrophages (50).

Because we had chosen 300 ng/well of the untagged WT TLR4 construct and 7.5 ng/well of the Flag-tagged TLR4 construct as optimal input concentrations based on comparability of NF-κB activation induced by LPS, we next sought to verify the comparability of TLR4 expression under optimal conditions of transfection with TLR4, MD-2, and CD14 expression vectors. Real-time PCR revealed comparable expression of untagged or Flag-tagged WT TLR4 mRNA levels (Fig. 3A), as well as comparable levels of total protein as assessed by immunoprecipitation followed by Western analysis (Fig. 3B). In addition, using anti-Flag Ab, we could also compare cell surface expression of each of the four Flag-tagged polymorphic constructs following transient transfection of HEK293T cells with optimized vector concentrations as determined for WT Flag-tagged TLR4 in Figs. 1 and 2. FACS analysis of HEK293T cells transfected with WT or mutated Flag-tagged TLR4 constructs indicated a low, but comparable, degree of cell surface expression for all of the Flag-tagged polymorphic TLR4 variants (Fig. 3C). Also, analysis of total TLR4 expression in whole cell lysates failed to reveal any significant difference in the level of untagged or Flag-tagged TLR4 expression among the HEK293T cells transiently transfected to express the respective individual polymorphic variants (data not shown). Unfortunately, surface expression of untagged-TLR4 on HEK293T transfectants by FACS analysis was below the limit of detection with the anti-TLR4 Abs currently available.

**TLR4 signaling is diminished in HEK293T cells expressing Asp^{299}Gly and/or Thr^{399}Ile mutations**

Now that the HEK293T transient transfection system had been optimized for LPS responsiveness with WT TLR4/MD-2/CD14 vectors, we sought to carry out a quantitative comparison of the Asp^{299}Gly and Thr^{399}Ile polymorphic TLR4 variants. In addition, because there is evidence from several studies of cosegregation of these two polymorphisms (Ref. 51, and A. Awomoyi, P. Rallabhandi, T. Pollin, E. Lorenz, M. Szlein, M. Boukhvalova, V. Hemming, J. Blanco, and S. Vogel, submitted for publication) we also constructed an additional vector that expressed both mutations. Arbour et al. (21) had shown decreased responses of both the Asp^{299}Gly and Thr^{399}Ile mutations in transiently transfected THP-1 cells when either IL-1α secretion or NF-κB transactivation were measured. Because these mutations are both contained in exon 4, and have been modeled to lie in ligand and coreceptor
interaction domains, respectively (22), on the same face of the molecule (23), we hypothesized that the double mutant may show an even greater signaling deficit than expression of either polymorphism alone.

Fig. 4, A and B, shows that the response to LPS is diminished in cells transfected with the TLR4 mutant constructs, and, as previously reported by Arbour et al. (21), the Asp<sup>299</sup>Gly mutant was reproducibly somewhat less sensitive to LPS than the Thr<sup>399</sup>Ile mutant. Cells transfected with the vector that expresses both mutations (“Double”) were consistently less LPS responsive than either of the singly mutated vectors, particularly at lower LPS concentrations (“Double”) were consistently less responsive than either of the singly mutated vectors, particularly at lower LPS concentrations, with consistently significant values <0.001.

Recognizing that we had, indeed, conducted these studies under optimal conditions for LPS-dependent signaling in cells transfected with WT TLR4, we wondered whether we could see an even larger signaling deficit in the mutants by decreasing the amount of serum in the cultures. Fig. 4, C and D, illustrates that the effect of decreasing the concentration of serum from 10% to 1% in cultures transfected with the WT untagged or Flag-tagged TLR4 vector, respectively, had a relatively minor effect on LPS-induced signaling, although at lower concentrations of LPS (e.g., 0.05 and 0.5 ng/ml), luciferase reporter activity was somewhat reduced. Similarly, in the case of the cells transfected with the vector expressing both Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile mutations, the decreased signaling in the presence of 10% serum mirrored the data shown in Fig. 3B, and reducing the serum concentration to 1% diminished signaling only slightly. However, in the complete absence of serum, where the WT TLR4 response is markedly reduced, cells that express the doubly mutated vector exhibited a disproportionately diminished response, with only minimal responsiveness at the very highest concentrations of LPS. This suggests that an interaction of TLR4 with serum component(s) may be disrupted by the presence of both mutations. In contrast to LPS stimulation, IL-1β and TNF-α treatment (in the presence of 10% serum) induced comparable NF-κB activation among transfectants expressing the WT and mutated TLR4 species (Fig. 4E).

**Agonist-independent oligomerization overcomes reduced TLR4 signaling in HEK293T cells expressing Asp<sup>299</sup>Gly and/or Thr<sup>399</sup>Ile mutations**

Previous studies have shown that Flag-tagged TLR4 vectors can elicit constitutive luciferase reporter activity at high input concentrations of vector (4), presumably due to artificial aggregation of TLR4 molecules as a consequence of the cytoplasmic domain interactions induced by N-terminal Flag epitopes (36). We sought to determine whether autoactivation would still reveal differences in the capacity to signal in the mutant vectors. Therefore, each of the mutants was expressed at 1000 ng/well, a concentration found to induce a significant level of autoactivation when using the Flag-tagged WT TLR4 expression vector, even in the absence of MD-2 or CD14 (Fig. 5, “No Co-R”). In contrast to the differential response to LPS observed in the cells transfected with mutated TLR4 vectors (Fig. 4, A and B), overexpression of Flag-tagged mutant proteins leading to agonist-independent signaling resulted in responses that were not statistically different from WT. Interestingly, autoactivation of luciferase reporter activity was highly significantly MD-2-dependent (Fig. 5) and increased to levels achieved under conditions of optimal agonist-induced stimulation in WT transfectants. Thus, under conditions where coexpression of high concentrations of MD-2 (300 ng/well) and Flag-tagged WT or mutant TLR4 vectors (1000 ng/well) results in agonist-independent signaling, this high level of MD-2 expression is not inhibitory and contributes significantly to agonist-independent signaling. This is in contrast to the inhibitory effect of MD-2 when TLR4 signaling is fully agonist dependent (Fig. 1B, top and middle graphs). CD14 expression vector input quantities had no effect on the levels of agonist-independent activation (data not shown).
HEK293T cells expressing Asp299Gly and/or Thr399Ile mutations exhibit decreased responsiveness to structurally unrelated TLR4 agonists

Although the reduced sensitivity of Asp299Gly and Thr399Ile mutations in human TLR4 to LPS have been confirmed and extended in this study, the question remained as to whether TLR4 molecules expressing the two mutations would also respond less well to structurally unrelated TLR4 agonists. Recent studies have shown that chlamydial Hsp 60 (6) and RSV F protein (5) to be TLR4 agonists. Here, we show that, like the response to LPS, the response to chlamydial Hsp 60 (Fig. 6A) was reduced significantly in cells transfected with the Asp299Gly and/or Thr399Ile TLR4 constructs (p < 0.001). The recombinant chlamydial Hsp 60 preparation used in this study was found to have <0.0012 ng of LPS by LAL assay per 10 μg of protein, the highest concentration of Hsp 60 tested. This concentration of LPS is below the threshold for TLR4-dependent activation in this system. Nonetheless, to insure that the observed activity of the preparation of recombinant chlamydial Hsp 60 was not due to contaminating LPS, we treated LPS (5 ng/ml) and the chlamydial Hsp 60 (10 μg/ml) preparations with polymyxin B (PB) (150 μg/ml), and found that PB essentially eliminated the LPS response, but not the response to chlamydial Hsp 60 (Fig. 6B). Because chlamydial LPS has been shown previously to be sensitive to PB treatment (52, 53), our findings support the notion that the TLR4 agonist activity induced by chlamydial Hsp 60 in our system was not due to contaminating LPS.
NF-κB responded robustly to all concentrations of F protein with HEK293T cells transiently transfected with plasmids that encode activation. The dose dependency of LPS stimulation was essentially the same for the two constructs; however, ~100-fold more of the untagged vector was required to activate HEK293T cells optimally than in cells transfected with the Flag-tagged TLR4. Although not on identical expression vectors, both TLR4 constructs are driven by the CMV promoter, and this suggests that while we were able to drive TLR4 expression at the cell surface, there were differences in their efficiency of transfection. We have previously shown that cell surface expression levels can be different among cells transfected with Flag-tagged TLR4 versus untagged TLR4, presumably due to differences in the efficiency of transient transfection. Although these cells were transfected with plasmids that encode CD14 and MD-2, we did not observe the enhanced LPS responsiveness that has been observed by others (37). In addition, boiling the chlamydial Hsp 60 preparation for 30 min before treating the cells led to a reduction of activity, equivalent to that seen with PB-treated LPS. Finally, proteinase K treatment of chlamydial Hsp 60, but not LPS, abolished its agonist activity (data not shown).

Similarly, a structurally unrelated TLR4 agonist, RSV F protein, also showed differential NF-κB-induced luciferase activity in HEK293T cells transiently transfected with plasmids that encode WT TLR4, the Asp299Gly mutation, the Thr299Ile mutation, or both mutations. Fig. 6C illustrates that cells that expressed WT TLR4 responded robustly to all concentrations of F protein with NF-κB-induced luciferase production, while, expression of either mutation alone resulted in diminished response to F protein that was most evident at the lower concentrations of agonist. As was observed for both LPS and chlamydial Hsp 60, the presence of both mutations resulted in a significantly lowered response to F protein at all concentrations tested (p < 0.001). To ensure that this differential responsiveness was not attributable to contaminating LPS, F protein preparations were pretreated with PB, proteinase K, or anti-F Ab. In contrast to LPS, the F protein preparations were not affected by PB, but were sensitive to proteinase K or anti-F Ab (data not shown). Thus, mutations in the extracellular domain of TLR4 reduce the sensitivity of cells to both chlamydial Hsp 60 and RSV F protein, in addition to LPS.

Discussion

Optimal TLR4-mediated signal transduction reflects a highly integrated series of protein-protein interactions. Collectively, our data provide strong evidence for the hypothesis that agonist-induced TLR4 signaling is profoundly affected by the stoichiometry of the key components of the TLR4-signaling complex: both TLR4 vector and the vectors encoding the coreceptors, CD-14 and MD-2, were carefully titrated to achieve optimal, dose-dependent cellular activation that is akin to the dose-dependent LPS responsiveness exhibited by primary macrophages (21). We used both untagged and Flag-tagged TLR4 expression vectors to tease out the basis for discrepancies in the literature with respect to the sensitivity of this system and the phenomenon of “autoactivation” that has been described in cells transfected with tagged vectors (4, 36).

In this study, we determined that input concentrations of 300 and 7.5 ng/well of the untagged and Flag-tagged TLR4 vectors (Fig. 1A), respectively, resulted in robust, LPS-dependent cellular activation. The dose dependency of LPS stimulation was essentially the same for the two constructs; however, ~100-fold more of the untagged vector was required to activate HEK293T cells optimally than in cells transfected with the Flag-tagged TLR4. Although not on identical expression vectors, both TLR4 constructs are driven by the CMV promoter, and this suggests that while we lowered the input threshold for autoactivation, Flag-tagged molecules may be preassociated such that signaling is more readily
triggered upon addition of agonist. Although, the surface expression of TLR4 is relatively low on HEK293T transfectants, we observed increased expression of Flag-tagged WT TLR4 when the cells were transfected with increasing input concentrations of the TLR4 vector, and this correlated with the capacity for cellular activation (Fig. 1A, bottom panel). Specifically, a suboptimal input concentration of TLR4 vector (5 ng) resulted in a FACS profile that was slightly greater than seen in cells transfected with control vector, but less than that seen with 7.5 ng of TLR4 vector, the dose chosen as optimal with respect to NF-κB activation. Increasing the input concentration to 10 ng resulted in no further increase in surface expression and occasionally resulted in low levels of “autoactivation.”

Varying the concentration of CD14 vector input from 3 to 300 ng/well did not significantly alter LPS responsiveness; however, at 3 ng/well, a marked drop in the magnitude of the LPS-induced response was observed. In the complete absence of CD14 (i.e., no input vector and no serum as a source of soluble CD14), LPS-inducible signaling was not detectable (Fig. 2). This contrasts to a loss of NF-κB-dependent gene expression in CD14 knockout macrophages cultured in autologous serum, which could be overcome by high concentrations of LPS (18, 50). This implies that in macrophages, in contrast to HEK293T cells, there are alternative molecules that can substitute to some extent for the lack of CD14 to enable TLR4-dependent signaling, e.g., MAC-1 (50, 54).

In contrast to the titration of CD14 vector, reducing the input concentration of MD-2 from 300 to 3 ng/well resulted in significantly higher LPS-inducible, NF-κB-driven luciferase activity (Fig. 1B, top and middle graphs) that was completely abrogated when MD-2 was omitted during the cotransfection (Ref. 35; data not shown). Despite the loss of TLR4-mediated signaling at high concentrations of MD-2, surface expression of Flag-tagged TLR4 molecules was similar at all input concentrations of MD-2 (Fig. 1B, bottom panel). The inhibition observed at higher MD-2 input vector concentrations may be due to sequestration of LPS by binding to excess MD-2, and hence, a reduced availability and/or transfer of LPS to TLR4 by MD-2 (55, 56), and cannot be attributed to a decreased level of surface TLR4 expression (Fig. 1B, bottom panel). These findings may account for a previous observation by Viriyakosol et al. (47) that TLR4-mediated signaling was inhibited by exogenous MD-2 in U373 cells, which express relatively low levels of TLR4 compared with CHO-TLR4 stable transfectants, while signaling in CHO-TLR4 stables was not. FACS analysis of our transiently transfected HEK293T cells revealed a much lower level of TLR4 expression than CHO-TLR4 stable transfectants (data not shown). These data further suggest that excess MD-2 may compete for LPS or some other component that amplifies TLR4-mediated signaling when expression of TLR4 is more physiologic.

Overexpression of Flag-tagged TLR4 constructs elicited robust, agonist-independent, NF-κB-driven luciferase activity. This has been attributed to epitope-mediated oligomerization of the cytoplasmic domains of N-terminal Flag-tagged TLR4 proteins such that downstream signaling molecules can be recruited to the receptor complex and signaling initiated in the absence of agonist (36). We found that autoactivation of Flag-tagged TLR4 was enhanced by higher input concentrations of MD-2 vector, while increasing the CD14 vector input had no appreciable effect (data not shown). This may be explained partially by the earlier suggestions that MD-2 acts as a chaperone (in addition to its role as a coreceptor for TLR4) in transporting TLR4 to the cell surface (44). Therefore, higher MD-2 vector expression might facilitate increased density of Flag-tagged TLR4 at the cell surface, and hence, a higher probability for interactions among Flag-tagged TLR4 molecules leading to agonist-independent cell activation (Fig. 4). In support of this, we observed that overexpression of Flag-tagged TLR4 at concentrations known to “autoactivate” cells results in relatively higher surface expression and mean fluorescent intensity of TLR4 by FACS analysis than when cells were transfected under conditions found to be optimal for agonist-driven responses (data not shown). In addition, overexpression of MD-2 may facilitate interactions among TLR4 molecules when they are expressed at a supraoptimal density by stabilizing the interactions among Flag-tagged molecules.

The molecular mechanisms involved in the diminished LPS responsiveness of individuals who express the Asp299Gly and Thr399Ile TLR4 polymorphisms are unknown, although Arbour et al. (21) suggested that the Asp299Gly/Thr399Ile mutant protein may not be as well expressed as WT TLR4 molecule based on immunohistochemical staining. However, these differences were observed using a polyclonal anti-TLR4 Ab. An alternative interpretation might be that the Asp299Gly/Thr399Ile double mutant alters an immunodominant epitope that, in turn, results in diminished detection of mutant TLR4 molecules. By using an anti-Flag Ab to detect a common, N-terminal epitope of TLR4 on the surface of HEK293T cells, we were able to show by FACS analysis that our transfectants expressed comparable levels of surface TLR4 (Fig. 3C). Expression of WT TLR4 was confirmed to be equivalent in Flag-tagged and untagged transfectants at the mRNA level by real-time PCR (Fig. 3A) and at the level of total protein level as detected by immunoprecipitation and Western blotting (Fig. 3B). We have also consistently observed that the total WT and mutant TLR4 proteins were expressed equivalently within each respective set of transfectants (i.e., untagged or Flag-tagged) as assessed by immunoprecipitation and Western blotting (data not shown). Thus, under conditions of comparable TLR4 surface expression (Fig. 3C), Asp299Gly and Thr399Ile mutants showed decreased sensitivity to Gram-negative LPS, with the double mutant showing the most significant loss of responsiveness (Fig. 4, A and B). Similar hyporesponsiveness was observed in response to two structurally unrelated TLR4 agonists, chlamydial Hsp 60 (Fig. 6A) and the RSV F protein (Fig. 6C). That WT and mutant TLR4 HEK293T transfectants responded comparably to IL-1β or TNF-α for induction of NF-κB luciferase reporter activation (Fig. 4E) indicates that mutant TLR4 molecules do not act by sequestering shared signaling molecules such as MyD88. Reducing the serum concentration in the culture medium from 10 to 0%, however, has a much more profound effect on LPS responsiveness of HEK293T cells expressing the doubly mutated TLR4 vs WT TLR4 (Fig. 4, C and D). This suggests that an interaction between the mutant TLR4 and a serum component (e.g., CD14, LBP, or MD-2) may be disrupted by the presence of the two TLR4 mutations. Studies are currently underway to examine whether the interactions of MD-2 and/or CD14 with the polymorphic TLR4 molecules differ.

Although the structure of TLR3 ectodomain is now known (57, 58), the TLR4 structure has not yet been elucidated. Nonetheless, the predictable confirmation of LRRs has allowed us to construct a hypothetical model of the TLR4 ectodomain (Fig. 7A). Although the actual structure will most likely differ in subtle ways, such as small portions of secondary structure, the overall size and threading of sequence provides a good estimate of the receptor structure. We have, therefore, used the following model to interpret our biologic data regarding the two TLR4 polymorphisms, Asp299Gly and Thr399Ile. In the model, these two residues are predicted to lie on the same face of the scaffold. Interestingly, these residues are found in LRRs (10 and 14) that contain insertions and are predicted in the “worm” model (Fig. 7B) to be elongations of the LRR. Fig. 7C represents the “space filling” model with surface
potentially responds more poorly to stimulation than TLR4 molecules. Our observation that the doubly mutated TLR4 molecule consis-
ting with the remaining interactions, consistent with
Removal of both “tips” of the saddle in the Asp299Gly/Thr399 double mutant (Fig. 7B) removes both a potential negative charge (indicated in red by the surface potential in Fig. 7C, left), and increases rotational freedom about the peptide bond, which may affect secondary structure immediately adjacent to this residue. The Thr to Ile mutation at amino acid 399 (Fig. 7B) conserves the branched side chain but increases the overall steric bulk in this region, possibly precluding ligand/cofactor docking. Removal of both “tips” of the saddle in the Asp299Gly/Thr399Ile double mutant (Fig. 7B, right) may result in loss of docking that cannot be overcome by the remaining interactions, consistent with our observation that the doubly mutated TLR4 molecule consistently responds more poorly to stimulation than TLR4 molecules that express either the Asp299Gly or Thr399Ile mutation. This model also accommodates both the fact that three structurally un-
related TLR4 agonists show the same pattern of sensitivity to the polymorphic molecules and our observation that agonist-indepen-
dent activation achieved by TLR4 oligomerization through the cytoplasmic domains induced by Flag epitopes overcomes the deficit seen with the mutant TLR4 molecules. Although the precise mecha-

FIGURE 7. TLR4 ectodomain model. A, Worm cartoon of TLR4 ectodomain model. LRRs, N and C termini are labeled. Asp299 and Thr399 are shown in ball and stick to highlight the two single nucleotide polymorphism (SNP) sites of interest and also the molecular surface of TLR4 ectodomain colored by electrostatic potential. Blue shading indicates basic resi-
dues and red indicates acidic residues. Asp299 and Thr399 are labeled to highlight the areas of interest. B, Role of Asp299 and Thr399 residues. Worm cartoon (rotated 90° out of the plane of the paper from Fig. 7A) of LRRs 10–15 encompassing the two mutation sites of interest, D299 and T399. Both D299 and T399 lie on the same face of the LRR scaffold and the D299G/ T399I double mutant exhibits an alteration in secondary

C, Molecular surface colored by electrostatic potential as in Fig. 7B (rotated 90° out of the plane of the paper from Fig. 7A) of LRRs 10–15 that encompass the two mutation sites of interest, D299 and T399 and their change to G299 and I399.

Disclosures
The authors have no financial conflict of interest.

References


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