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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 (Asp299Gly and Thr399Ile) 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/MDS2/CD14 complex. A structural model that accounts for the diminished responsiveness of mutant TLR4 polymorphisms to structurally unrelated TLR4 agonists is proposed.  

macrophages obtained from individuals with these TLR4 mutations (21).

The molecular mechanisms that underlie the diminished responsiveness of individuals or cells that express the Asp299Gly and Thr399Ile 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 Asp299Gly and Thr399Ile TLR4 mutations does not extend to all infectious diseases.

To assess the molecular basis for the LPS hypersensitivity of the polymorphic human TLR4 proteins in vitro, we expressed the WT or TLR4 variant 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. Collectively, 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 amoebocyte lysate (LAL) test. This was the highest concentration of LPS that was used in the luciferase assays and the amount of detectable LPS is below the TLR4 activation level. SuperFect transfection reagent was obtained from Qiagen. Anti-human TLR4 rabbit antisera was the gift of Dr. R. Medzhitov (Yale University, New Haven, CT). Monoclonal anti-human TLR4 Ab (HTA125 clone) was obtained from Imgenex. Anti-Flag epitope Abs (M2 clone) and anti-mouse FITC-conjugated secondary Abs were obtained from Sigma-Aldrich. Anti-rabbit and anti-mouse HRP-conjugated secondary Abs were purchased from Cell Signaling Technology. Purified RSV F protein was prepared as described by Roder et al. (38) with modification: RSV-infected HEp-2 cells were collected 48 h after infection, and the cell lysate was prepared. All purification steps were carried out at 4°C using columns under gravitational buffer flow, and HiTrapQ FF column (Amersham Biosciences) was used for the first purification step. All expression plasmid constructs were prepared using the EndoFree Plasmid Maxi kit (Qiagen).

Plasmid constructs and construction

In this study, we used two different wild-type human (hu) TLR4 expression vectors: Flag-CMV1–TLR4, which encodes for TLR4 with an N-terminal Flag-epitope tag, and pCDNA3–TLR4, which encodes an untagged TLR4. 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 has been described in Refs 21 and 29 was from Dr. R. Medzhitov (Yale University, New Haven, CT). Point mutations encoding an aspartic acid (Asp) to glycine (Gly) substitution at the amino acid position 299 (D299G) and a threonine (Thr) to isoleucine (Ile) substitution at aa 399 (T399I) in human TLR4 protein were created by site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions using the following primer pairs: Asp299Gly mutation (D299G) (nucleotide change, A898A; forward primer: 5′-ctgttctcaaagtgattttgggacaaTAcgcaattgct-3′; Thr399Ile mutation (T399I) (C→T, nucleotide change, C1196→T) forward primer: 5′-ctggctaaagatggtgatctgctagttcattgct-3′; reverse primer: 5′-aaaataatcaataactggataaggtagtctaa-3′; Thr399Ile mutation (T399I) (C→T, nucleotide change, C1196→T) forward primer: 5′-ctggctaaagatggtgatctgctagttcattgct-3′; reverse primer: 5′-gctcggtattgattcagttctgtctgct-3′). All expression plasmid constructs were 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 × 10^4 cells/well and incubated overnight in a CO_2 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 recovered 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 (Bgal) and luciferase (Luciferase assay system) 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 >90%, as assessed by in situ β-galactosidase staining kit (Stratagene).

For NF-κB reporter activity 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 transfected cells

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 nuclease-free distilled water and 1μl of the diluted cDNA was used.

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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 Primer Express 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 exon boundaries to exclude detection of genomic DNA. Primer sequences for the human TLR4 gene were: forward, 5’-gaccttgatccgcc-cattg-3’ and reverse, 5’-agaacaccctaccga-3’ and the primers for the housekeeping gene, human hypoxanthine-guanine phosphoribosyl transferase (HPRT), were: forward, 5’-acagacagggccagataaa-3’ and reverse, 5’-gtgcgttggtttggcagg-3’.

Real-time PCR was performed on an Applied Biosystems Prism 7900HT Sequence Detection System. Amplification was conducted in a 2.75-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 2X 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 15 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 2ΔΔCt method, where Ct is the cycle threshold (40).

Immunoprecipitation and Western blots

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 pEBOs-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 DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 0.1% BSA) and rotated 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.

For Western blotting, the immunoprecipitated samples were loaded in their entirety and resolved with 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 1/5000 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 in ice either with anti-Flag Ab (clone M2 mAb) 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 FACSort (BD Biosciences). In each sample, 10,000 cells were analyzed. FACS data was analyzed by using WinMDI Flow Cytometry Analysis version 2.8 (2).

Construction of TLR4 ectodomain model

The TLR4 ectodomain sequence (1–633) was analyzed by the 3DJigSaw program (www.bmm.icet.nctu.edu.tw/~3d jig saw/). 3DJigSaw 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 were predicted by 3DJigSaw were fit by least-squared mean method. By sequence similarity in LRRs, decorin (I1XKU) (41), and nogo receptor (18PT) (42) were fit onto the CD42b scaffold. This modified scaffold was input into GeneMiner ((www.bioinformatics.ucla.edu/gen emine/)) and the TLR4 ectodomain sequence (18–627) applied to the scaffold where 3DJigSaw had predicted homology. Areas such as insertions were modeled via the software package. GeneMiner internal energy minimization and Crystallography and NMR Systems (CNS) version 1.1 (43) model minimization were used to refine the model. Figures were generated in SPock (http://quorum.tamu.edu/Manual/Manual/Manual.html).

Statistical analysis

Quantitative data of NF-κB activation are presented as mean ± SE and were analyzed using a one-way ANOVA with repeated measures, followed by post hoc comparisons using Tukey’s multiple paired comparison test. GraphPad PRISM 4 program for Windows was used for these analyses.

Results

Optimization of transient transfection of WT untagged- and Flag-tagged TLR4 constructs for agonist-dependent signaling

Transient transfection of HEK293T cells with expression vectors that encode the individual components of the TLR4 receptor complex has been used extensively as an approach for studying the molecular interactions that accompany TLR4-induced activation. We initiated our studies by optimizing NF-κB signal transduction for untagged and epitope (Flag)-tagged, WT TLR4 constructs before studying the responsiveness of the respective polymorphic constructs. We initially cotransfected HEK293T cells with various concentrations of the untagged and Flag-tagged WT expression vectors in the presence of fixed input concentrations of huCD14 and huMD-2 (300 ng/well each), in addition to the NF-κB luciferase and β-gal reporter constructs. Transient transfection of either untagged (Fig. 1A, top graph) or Flag-tagged (Fig. 1A, middle graph) TLR4 vectors resulted in a dose-dependent increase in NF-κB-driven luciferase reporter activity upon LPS stimulation. In multiple side by side experiments, maximum achievable LPS-inducible luciferase activity (i.e., RLU) was typically greater in cells transfected with the Flag-tagged TLR4 construct than with the untagged construct, although the LPS-inducible dose-response curves were quite comparable, showing statistically significant activity with as little as 0.05 ng/mL LPS and maximal activity at 5 ng/mL LPS. Also, an increase in the amount of input TLR4 vector (either untagged or Flag-tagged) increased sensitivity to LPS (Fig. 1A, top and middle graphs). Therefore, we sought to determine whether TLR4 surface expression changed in response to the increase in the input vector concentration. We analyzed the surface expression of Flag-tagged TLR4 at various input concentrations by FACS. Despite the relatively low level of surface expression of Flag-tagged TLR4 on HEK293T transfectants, we were able to detect an increase in the surface expression upon increasing input levels over the range that resulted in agonist-dependent signaling (Fig. 1A, bottom panel). Importantly, a comparison of the input concentrations of vectors revealed that ~300–1000 ng/well of the untagged vector was required to achieve the same degree of responsiveness as achieved by ~7.5–10 ng/well of the Flag-tagged vector. In addition, input concentrations of >10 ng/well Flag-tagged vector resulted in LPS-independent signaling, as indicated by an increase in the basal level of activity, while even at 1000 ng/well, the untagged-TLR4 vector rarely induced agonist-independent signaling (data not shown). The increase in the basal activity by Flag-tagged TLR4 at its higher input concentrations is likely attributable to Flag-tag-induced oligomerization of the cytoplasmic domains of TLR4 molecules that mimics the agonist-induced receptor aggregation, as has been observed previously (4, 36).
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 $1 \times$ PBS, lysed in $1 \times$ 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 MD-2 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 affect 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 Asp299Gly mutant was reproducibly somewhat less sensitive to LPS than the Thr399Ile 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 LPS responsive than either of the singly mutated vectors, particularly at lower LPS concentrations, with consistently significant differences 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 using each of the doubly mutated vector input quantities had no effect on the levels of 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-stimulated signaling in WT transfecants. 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 activation among transfectants expressing the WT and mutated TLR4 species (Fig. 4E).

Agonist-independent oligomerization overcomes reduced TLR4 signaling in HEK293T cells expressing Asp299Gly and/or Thr399Ile 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-stimulated signaling in WT transfecants. 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. Nonetheless, to insure that the observed activity of the preparation of recombinant chlamydial Hsp 60 did not arise from contaminating LPS, we treated LPS (5 ng/ml) and the chlamydial Hsp 60 (10 ng/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 is not due to contaminating LPS.

**FIGURE 4.** TLR4 mutants show deficiency in signaling that is serum dependent. A, HEK293T cells cotransfected with optimized amounts of untagged-TLR4 vector and its mutants (300 ng/well) or B, Flag-tagged TLR4 vector and its mutants (7.5 ng/well), MD-2 vector (3 ng/well), and CD14 vector (30 ng/well), were treated with the indicated concentrations of E. coli LPS for 5 h and prepared cell lysates. Luciferase and β-galactosidase activities were measured as described in Materials and Methods. The symbols represent the p values as follows: +, <0.05; #, <0.01; *, <0.001. C, Untagged TLR4 WT and double mutant, and similarly, D, Flag-Tagged TLR4 WT and double mutant were transfected into HEK293T cells along with MD-2 and CD14 vectors. After an overnight recovery, the cells were treated with the indicated concentrations of LPS in the presence of 10%, 1%, or no serum (0%) for 5 h and cell lysates prepared. Luciferase and β-galactosidase activities were measured in cell lysates as described in Materials and Methods (+, p < 0.001) and E, HEK293T cell transfected with either Flag-tagged TLR4 WT or mutant TLR4 constructs along with MD-2 and CD14 constructs. After an overnight recovery, the cells were treated with TNF-α (50 ng/ml) or IL-1β (25 ng/ml) for 5 h in DMEM containing 10% serum, and cell lysates prepared. Luciferase and β-galactosidase activities were measured as described in Materials and Methods. Shown is a representative experiment (n = 5). Similar results were seen using untagged TLR4 constructs (data not shown).
NF-κB responded robustly to all concentrations of F protein with WT TLR4, the Asp299Gly mutation, the Thr399Ile mutation, or 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 were 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 observed for both LPS and chlamydial Hsp 60, the presence of 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.

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 Thr399Ile 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 insure 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 “auto-activation.”

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 trans-vector concentrations may be due to sequestration of LPS by binding agonist-independent, NF-κB (data not shown). These data further suggest that excess MD-2 level of TLR4 expression than CHO-TLR4 stable transfectants our transiently transfected HEK293T cells revealed a much lower 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).

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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 Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile TLR4 polymorphisms are unknown, although Arbour et al. (21) suggested that the Asp<sup>299</sup>Gly/Thr<sup>399</sup>Ile 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 Asp<sup>299</sup>Gly/Thr<sup>399</sup>Ile 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), Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile 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 biological data regarding the two TLR4 polymorphisms, Asp<sup>299</sup>Gly and Thr<sup>399</sup>Ile. 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
that express either the Asp299Gly or Thr399Ile mutation. This tentatively responds more poorly to stimulation than TLR4 molecules.

Our observation that the doubly mutated TLR4 molecule consis-
ting with the remaining interactions, consistent with the molecular surface of TLR4 ectodomain colored by electrostatic potential. Blue shading indicates basic residues and red indicates acidic residues. Asp299 and Thr399 are highlighted to show the areas of interest. B, Role of Asp299 and Thr399 residues. Worm cartoon (rotated 90° out of the plane of the paper) 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 D299Gly/T399Ile double mutant exhibits an alteration in secondary structure as described in the text. C, Molecular surface colored by electrostatic potential as in Fig. 7B (rotated 90° out of the plane of the paper) of LRRs 10–15 that encompass the two mutation sites of interest, D299 and T399 and their change to G299 and I399.

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 unrelated TLR4 agonists show the same pattern of sensitivity to the polymorphic molecules and our observation that agonist-independent 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 mechanism by which these polymorphisms mediate their synergistic effects will have to await atomic resolution of the structure, the model provides us with a testable interpretation of the structure from which to hypothesize.

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 residues and red indicates acidic residues. Asp299 and Thr399 are highlighted to show the areas of interest. B, Role of Asp299 and Thr399 residues. Worm cartoon (rotated 90° out of the plane of the paper) 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 D299Gly/T399Ile double mutant exhibits an alteration in secondary structure as described in the text. C, Molecular surface colored by electrostatic potential as in Fig. 7B (rotated 90° out of the plane of the paper) 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
