The Asp<sup>299</sup>Gly Polymorphism Alters TLR4 Signaling by Interfering with Recruitment of MyD88 and TRIF

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The Asp<sup>299</sup>Gly Polymorphism Alters TLR4 Signaling by Interfering with Recruitment of MyD88 and TRIF

Leandra Figueroa,*†,1 Yanbao Xiong, † Chang Song, †,2 Wenji Piao, † Stefanie N. Vogel, † and Andrei E. Medvedev †

Asp<sup>299</sup>Gly (D299G) and, to a lesser extent, Thr<sup>399</sup>Ile (T399I) TLR4 polymorphisms have been associated with Gram-negative sepsis and other infectious diseases, but the mechanisms by which they affect TLR4 signaling are unclear. In this study, we determined the impact of the D299G and T399I polymorphisms on TLR4 expression, interactions with myeloid differentiation factor 2 (MD2), LPS binding, and LPS-mediated activation of the MyD88- and Toll/IL-1R resistance domain-containing adapter inducing IFN-β (TRIF) signaling pathways. Complementation of human embryonic kidney 293/CD14/MD2 transfectants with wild-type (WT) or mutant yellow fluorescent protein-tagged TLR4 variants revealed comparable total TLR4 expression, TLR4–MD2 interactions, and LPS binding. FACS analyses with anti-TLR4 Ab showed only minimal changes in the cell-surface levels of the D299G TLR4. Cells transfected with D299G TLR4 exhibited impaired LPS-induced phosphorylation of p38 and TANK-binding kinase 1; activation of NF-κB and IFN regulatory factor 3, and induction of IL-8 and IFN-β mRNA, whereas T399I TLR4 did not cause statistically significant inhibition. In contrast to WT TLR4, expression of the D299G mutants in TLR4

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expression of costimulatory molecules and type I IFNs via activation of TANK-binding kinase 1 (TBK1) and IFNs regulatory factors (IRF) 3 and IRF7 (2, 20). TLR2 and TLR4 require the bridging adapter TIR domain-containing adapter protein (TIRAP) (21), also called MyD88 adapter-like (Mal) (22), to recruit MyD88 to TLRS, whereas TLR4 also requires a bridging adapter TRIF-related adapter molecule (TRAM) that recruits TRIF to the TLR4 receptor complex (23, 24).

Two single nucleotide polymorphisms (SNPs) that encode mutations in the ectodomain of TLR4, Asp299Gly (D299G) and Thr399Ile (T399I), occur in humans with a frequency of ~6–10% and have been associated with blunted responsiveness to inhaled LPS, decreased LPS-mediated production of cytokines by airway epithelial cells and macrophages, Gram-negative bacteremia, and sepsis (25, 26). The D299G polymorphism has also been linked to Boutonneuse fever (27), RSV infections in infants and young children (28, 29), HIV-associated tuberculosis (30, 31), Crohn’s disease (32), ulcerative colitis (33), endometriosis (34), and tonsillitis (35). Human subjects carrying the T399I polymorphism either exhibit a milder LPS-hyporesponsive phenotype or do not manifest it at all (36). In many instances, associations between these TLR4 SNPs and predisposition to disease have been shown to depend on ethnic backgrounds, gender, and/or prior immune deficiencies (reviewed in Ref. 36). Although the D299G and T399I TLR4 polymorphisms have been associated with an increased risk of infectious diseases, they have been found to protect against inflammatory diseases such as atherosclerosis and rheumatoid arthritis (36, 37).

The molecular mechanisms by which the TLR4 SNPs affect receptor functions are largely unknown. Compromised LPS responses elicited by the D299G variant were originally associated with decreased expression of the mutant TLR4 species in human airway epithelial cells (25). In contrast, others have reported that D299G or T399I TLR4 mediate diminished NF-κB activation and cytokine gene expression in response to LPS, RSV F protein, or chlamydia Hsp60 under conditions of equal total and cell-surface expression of transfected TLR4 species in human embryonic kidney (HEK) 293T cells (29, 38). In this study, we sought to determine the impact of the D299G and T399I polymorphisms on TLR4 expression, interactions with myeloid differentiation factor 2 (MD2), LPS binding to the TLR4/CD14/MD2 complex, recruitment of signaling adapter proteins, and activation of the MyD88 and TRIF pathways. Our data indicate that the D299G polymorphism compromises LPS-inducible recruitment of MyD88 and TRIF to TLR4 and activation of MyD88- and TRIF-dependent signaling pathways while not significantly affecting total and cell-surface TLR4 expression, its interaction with MD2, or LPS binding to the TLR4–MD2–CD14 receptor complex.

Materials and Methods

Reagents and cell culture

pcDNA3-YFP-TLR4, pcDNA3-CD14, pEFBOS-Flag- or hemagglutinin (HA)-MD2, pcDNA3-AU1-MyD88, pEFBOS-Flag-TRIF, pELAM-luciferase (Luc), p125-Luc, and pTK-Renilla-Luc (10 ng), pELAM-Luc, or p125-Luc (300 ng each), with the total amount of plasmid DNA adjusted to 1 μg with pcDNA3. For gene expression studies and Western blotting, cells were plated in six-well plates and transfected with pcDNA3-CD14 (0.5 μg), pEFBOS-MD2 (0.5 μg), pcDNA3-YFP-TLR4 (1.0 μg), or pELAM-Luc (2.0 μg) (all amounts are per well). For immunoprecipitation, HEK293T cells were plated in 100-mm tissue-culture dishes and transfected with pcDNA3-CD14 (2.0 μg), pEFBOS-MD2 (2.0 μg), and pcDNA3-YFP-TLR4 (6.0 μg) (all amounts are per dish). Transient transfection of HEK293T cells was performed using SuperFect transfection reagent (Qiagen, Valencia, CA) per the manufacturer’s recommendations. iBMDMs were transfected with plasmids encoding human YFP-TLR4 species using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. To obtain stable cell lines, TLR4-transfected HEK293 cells were selected in cDMEM containing 1 ng/ml G418 (Sigma-Aldrich).

Animal procedures

Six- to 8-wk-old WT C57BL/6 mice were purchased from the Charles River Laboratories (Wilmington, MA), and TLR4+/− mice were bred and maintained within the accredited animal facility at the University of Maryland School of Medicine. All animal experiments were conducted with institutional approval. Bone marrow cells were isolated from femurs and tibiae of mice and cultured in cRPMI 1640 in tissue-culture dishes. To obtain primary BMDMs, bone marrow cells were differentiated for 7 d in cRPMI 1640 containing 25% (v/v) of L929 cell-conditioned, M-CSF− containing supernatant, as described (43). BMDMs were detached and used for nucleofection.

LPS binding and FACS

We adapted a previously reported LPS-binding protocol (44). In brief, cells were treated for 30 min at 37°C with medium or 1 μg/ml biotinylated LPS, washed with HBSS, and incubated with or without 1 μg/ml SA-allylphococyanin on ice for 30 min. To examine TLR4 cell-surface expression, HEK293/C44D14/MD2 transfectants expressing YFP–TLR4 variants were stained for 30 min on ice with HATA125-PE (Santa Cruz Biotechnology), anti–HLA-A−B−C−PE (positive control), or isotype control IgG-PE (BD Biosciences) Abs. After incubation, cells were washed with ice-cold PBS, fixed for 15 min in 2% paraformaldehyde, and analyzed on an LSR II Flow Cytometer (BD Biosciences) to measure YFP (total YFP–TLR4 expression), PE (cell-surface TLR4 expression), or allylphococyanin (LPS binding) fluorescence. The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Preparation of cell extracts, immunoprecipitation, and Western blot analyses

Cell extracts were prepared as described (45, 46) using a lysis buffer containing 20 mM HEPES (pH 7.4), 1 mM PMFS, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM EDTA, 150 mM NaCl, 6.0 mM Trition X-100, 20 mM β-glycerol phosphate, and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). For immunoprecipitation, cell lysates (0.5–1 mg total protein) were precleared by incubation for 2 h at 4°C with 20 μl 50% slurry of protein G-agarose (Roche Applied Science). Preclarced lysates were incubated for 20 h at 4°C with the respective Abs, followed by addition of protein G-agarose (45 μl 50% slurry/sample) and incubated for 4 h at 4°C. After extensive washing with ice-cold lysis buffer, beads were resuspended in Laemml sample buffer (Bio-Rad), boiled for 10 min, and supernatants were analyzed by immunoblotting, as reported previously (40). Densitometric analysis of
intensities of the corresponding bands was performed using the Quantity One software (Bio-Rad) as described previously (45).

**RNA isolation, reverse transcription, and real-time quantitative PCR**

RNA was isolated with TRIzol (Invitrogen), treated for 1 h at 37°C with DNase (Promega), repurified, and cDNA was prepared using reverse transcription system (Promega) and analyzed by real-time quantitative PCR (RT-qPCR) using 5 μl cDNA, 0.3 μM gene-specific primers, and SYBR Green Supermix (Bio-Rad) on a MyQqReal-Time PCR machine (Bio-Rad). The following primers were used: human hypoxanthine phosphoribosyltransferase (HPRT), forward, 5'-GCTGACCTGCTGATTACATT-3' and reverse, 5'-GAGAGATCATCTCCACCA-3'; human IL-8, forward, 5'-CACCAGAGAAACATCCTC ACT-3' and reverse, 5'-TGACACCTTCACAGAGCTGC-3'; human IFN-β, forward, 5'-ACTGCTCAGGACAGGATTG-3' and reverse, 5'-AGCCAGAGGTTTCTCACAACATG-3'; and mouse HPRT, forward, 5'-CCGAGAGGTCATAGATCTCTC-3' and reverse, 5'-GCTGAGGTTGGTGTCTCAACATG-3'; and mouse IFN-β, forward, 5'-CCGAGCTGCTGATTACATT-3' and reverse, 5'-GAGAGATCATCTCCACCA-3'. Melting curve analysis was performed to ensure specific amplification, and data were processed using 2-ΔΔCT method (47).

**Nucleofection**

pcDNA3-enhanced GFP and pcDNA3-YFP-TLR4 vectors encoding WT or D299G variants were introduced into WT and TLR4−/− BMDMs by nucleofection using the Nucleofector I device and the mouse macrophage nucleofection kit (Lonza, Basel, Switzerland), as recommended by the manufacturer. After recovery for 20 h, cells were treated with medium, LPS, and TNF-α, and RNA was isolated, reverse transcribed, and analyzed by RT-qPCR.

**Reporter assays**

Stably (HEK293) or transiently transfected (HEK293T) cells expressing WT, D299G, or T399I YFP-TLR4, along with CD14 and Flag-MD2, were treated for 5 h at 37°C with medium, LPS, or TNF-α (100 ng/ml each) in a 5% CO₂ atmosphere. After treatment, cells were lysed with passive lysis buffer (Promega), and firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) on a MyQ Real-Time PCR machine (Bio-Rad). The following primer sets were used: human hypoxanthine phosphoribosyltransferase (HPRT), forward, 5'-GCTGACCTGCTGATTACATT-3' and reverse, 5'-GAGAGATCATCTCCACCA-3'; human IL-8, forward, 5'-CACCAGAGAAACATCCTC ACT-3' and reverse, 5'-TGACACCTTCACAGAGCTGC-3'; human IFN-β, forward, 5'-ACTGCTCAGGACAGGATTG-3' and reverse, 5'-AGCCAGAGGTTTCTCACAACATG-3'; and mouse HPRT, forward, 5'-CCGAGAGGTCATAGATCTCTC-3' and reverse, 5'-GCTGAGGTTGGTGTCTCAACATG-3'; and mouse IFN-β, forward, 5'-CCGAGCTGCTGATTACATT-3' and reverse, 5'-GAGAGATCATCTCCACCA-3'. Melting curve analysis was performed to ensure specific amplification, and data were processed using 2-ΔΔCT method (47).

**Confocal microscopy**

Cells were seeded (4 × 10⁵ cells) on poly-L-lysine–coated coverslips (Fisher Scientific, Pittsburgh, PA) in phenol-free MEM/DMEM and cultured for 20 h. Transfection was performed using SuperFect transfection reagent (Qiagen), followed by gentle washing and recovery for 48 h. Cells were fixed with 2% paraformaldehyde and mounted onto glass slides using a DABCO-based anti-fade fluorescent mounting media (Sigma-Aldrich). Images were acquired using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus, Melville, NY) at excitation wavelength 515 nm and standard emission filters for YFP detection (YFP-TLR4).

**Statistical analysis**

Data were analyzed using the GraphPad Prism 5 program for Windows (GraphPad), and statistical differences were evaluated by the Student t test with the level of significance set at p < 0.05. Values are expressed as mean ± SD.

**Results**

The D299G polymorphism impairs LPS-induced p38 and NF-κB activation, but does not affect total TLR4 levels and minimally impacts TLR4 cell-surface expression

Because the magnitude of LPS responses depends on TLR4 expression (48, 49), we studied the impact of the D299G and T399I polymorphisms on TLR4 levels and signaling in TLR4-deficient HEK293 cells (50) transfected with engineered WT or mutant YFP-TLR4 species, CD14, and MD2 (to impart LPS sensitivity) (51–53). Fusion of YFP with TLR4 at the C terminus allows for improved detection of TLR4, while not affecting its localization and functions (44, 54). Because TLR4 activates MAPKs and NF-κB via MyD88- and TRIF-dependent pathways (24, 55), we measured expression of these integral parameters to examine receptor signaling. Because phosphorylation of p38 closely correlates with activation of its kinase activity (56), LPS-mediated p38 phosphorylation was used as a readout. LPS induced robust phosphorylation of p38 in 293T/CD14/MD2 transfectants expressing WT YFP-TLR4, whereas a blunted response was observed in cells transfected with the D299G YFP-TLR4 under conditions of comparable expression of YFP-TLR4, Flag-MD2, p38, and tubulin (Fig. 1A). These data indicate that deficient LPS-induced p38 phosphorylation via D299G TLR4 was not due to lower expression of receptor components, differences in total p38 levels, or unequal protein loading. Compared to WT TLR4, D299G species exhibited 59% decreased LPS-induced activation of the NF-κB–dependent pELAM-Luc reporter, T399I TLR4 showed no statistically significant differences, whereas TNF-α similarly stimulated NF-κB in cells expressing WT or mutant TLR4 (Fig. 1B).

Total YFP-TLR4 expression was determined based on YFP fluorescence of expressed TLR4 species and their immunoreactivity with anti-GFP Ab that cross-reacts with YFP (44, 54, 57).
Confocal imaging and FACS showed comparable fluorescence intensities of WT, D299G, and T399I YFP-TLR4 in 293/CD14/MD2/TLR4 stable transfectants (Fig. 2A, 2B), and immunoblot analyses revealed similar amounts of the WT versus D299G YFP-TLR4 and Flag-MD2 proteins (Fig. 2C). To determine TLR4 cell-surface expression, we stained 293/MD2/CD14 transfectants stably expressing WT or mutant YFP-TLR4 with PE-conjugated anti-TLR4 (HTA125) or isotype control Abs, followed by FACS analyses. Because the D299G polymorphism most profoundly inhibits TLR4 signaling (Fig. 1) (25, 29, 38), we compared cell-surface expression of WT versus D299G TLR4. In contrast to TLR4-deficient HEK293 cells, TLR4-expressing 293/CD14/MD2 transfectants stained with HTA125-PE showed a shift to the right in the PE fluorescence compared with the isotype control Ab staining (Supplemental Fig. 1), indicating specific detection of TLR4. HTA125 staining of 293/CD14/MD2 cells expressing WT and D299G YFP-TLR4 showed identical peaks and only marginal differences in the shoulder patterns (Fig. 2B). Thus, the D299G and T399I polymorphisms do not affect total and minimally impact cell-surface TLR4 expression.

The D299G and T399I polymorphisms do not affect LPS binding to the TLR4/CD14/MD2 complex and TLR4 interactions with MD2

Because the D299G and T399I polymorphisms are located in the TLR4 ectodomain (25) that is involved in ligand-sensing and coreceptor interactions (2, 20, 58), they could affect LPS binding to the TLR4/CD14/MD2 complex or TLR4-MD2 assembly. To test this hypothesis, 293/CD14/MD2 transfectants expressing WT or mutant YFP-TLR4 were incubated with medium or biotinylated LPS and washed, and bound LPS was detected by SA-allophycocyanin staining followed by FACS analysis. In the absence of biotinylated LPS, no differences in SA-allophycocyanin binding to the analyzed cell lines were observed, which was comparable to the autofluorescence profiles in medium-treated cells (Fig. 3A). Cells incubated with biotinylated LPS and SA-allophycocyanin showed mean fluorescence intensity (MFI) values of 1318, 1514, and 1432 for 293/CD14/MD2 transfectants expressing WT, D299G, and T399I YFP-TLR4, respectively, significantly more than the 58.7 MFI detected in pcDNA3-transfected cells (Fig. 3A, 3B). Thus, the D299G and T399I polymorphisms do not affect LPS binding to the TLR4/MD2/CD14 receptor complex.

To determine the impact of the D299G polymorphism on TLR4–MD2 interactions, transfected WT or D299G YFP-TLR4 species were immunoprecipitated with anti-GFP Ab, and the amount of TLR4-interacting Flag-MD2 was determined by immunoblotting of YFP immune complexes with anti-Flag Ab. Immunoblot analyses and densitometric quantification of WT versus D299G YFP-TLR4 proteins immunoprecipitated from cells treated with medium or LPS revealed comparable intensities of protein bands immunoreactive with anti-GFP and anti-Flag Abs (Fig. 3C, 3D, top panels, 3E). Whole-cell lysates obtained from 293T/CD14/Flag-MD2 cells complemented with WT or D299G YFP-TLR4 showed comparable total levels of Flag-MD2 proteins (Fig. 3C, second panel from bottom). Thus, similar amounts of Flag-MD2 interacted with WT or D299G YFP-TLR4 variants under conditions of similar total expression of the interacting proteins, indicating that the D299G polymorphism does not affect TLR4–MD2 interactions.

The D299G polymorphism impairs the ability of TLR4 to recruit MyD88 and activate MyD88-dependent cytokine genes

Recruitment of MyD88 to TLR4 is one of the earliest events of TLR4 signaling (2, 20). To determine the impact of the D299G TLR4 polymorphism on this process, we used coimmunoprecipitation to study LPS-inducible association of transfected AU1-MyD88 with WT or D299G YFP-TLR4 in 293/CD14/TLR4 stable cell lines expressing the respective TLR4s, CD14, and HA-MD2. LPS induced robust association of WT YFP-TLR4 with AU1-MyD88 (3.2–14.4-fold increase over medium controls), whereas the D299G YFP-TLR4 had a reduced (0.98–1.8-fold increase) capacity to recruit AU1-MyD88, leading to 32–82% inhibition (Fig. 4). Comparable total levels of HA-MD2 (Fig. 4A), YFP-TLR4 species, and AU1-MyD88 were seen (Fig. 4A, 4B), indicating that
differences in MyD88 recruitment to WT versus D299G TLR4 were not due to variations in total expression of TLR4, MD2, or MyD88.

Next, we studied the impact of the D299G and T399I polymorphisms on transcription of MyD88-dependent cytokine genes IL-8 and TNF-α (59–61). LPS increased the levels of IL-8 mRNA in 293T/CD14/MD2 cells transiently and stably transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. After recovery for 20 h, cells were incubated for 30 min at 37°C with medium or biotinylated LPS (1 µg/ml), washed, stained with SA-allophycocyanin (1 µg/ml), and analyzed by FACS to detect LPS binding (allophycocyanin fluorescence). B Quantification of FACS results (MFI) shown in (A) using the FlowJo program (Tree Star). HEK293T cells were transiently transfected with vectors encoding WT or D299G YFP-TLR4 species, along with pcDNA3-CD14/pEFBOS-Flag-MD2. After recovery, cells were treated with medium (C) or stimulated with 100 ng/ml LPS (D), and cell lysates were immunoprecipitated with anti-GFP Ab (YFP-TLR4 pulldown) and analyzed by immunoblotting with anti-Flag Ab (to determine Flag–MD2 interactions with YFP-TLR4s). Tubulin immunoblot was used to control protein loading. The data of a representative experiment (n = 3) are shown. E Densitometric quantification of the data shown in (C) and (D). The results are presented as arbitrary units representing ratios of normalized densitometric values of MD2–TLR4 complexes detected in samples transfected with pcDNA3 (background) or vectors expressing WT or D299G YFP-TLR4. Normalization of densitometric values was calculated as a/b/c/d, where a, b, c, and d correspond to the densitometric values of MD2-TLR4, total TLR4, total MD2, and tubulin, respectively.

FIGURE 3. LPS binding to the TLR4/CD14/MD2 complex and TLR4–MD2 interactions in HEK293/CD14/MD2 cells expressing WT, D299G, or T399I YFP-TLR4 species. (A) HEK293 cells stably expressing WT, D299G, or T399I YFP-TLR4 species were transiently transfected with pcDNA3-CD14 and pEFBOS-Flag-MD2. After recovery for 20 h, cells were incubated for 30 min at 37°C with medium or biotinylated LPS (1 µg/ml), washed, stained with SA-allophycocyanin (1 µg/ml), and analyzed by FACS to detect LPS binding (allophycocyanin fluorescence). (B) Quantification of FACS results (MFI) shown in (A) using the FlowJo program (Tree Star). HEK293T cells were transiently transfected with vectors encoding WT or D299G YFP-TLR4 species, along with pcDNA3-CD14/pEFBOS-Flag-MD2. After recovery, cells were treated with medium (C) or stimulated with 100 ng/ml LPS (D), and cell lysates were immunoprecipitated with anti-GFP Ab (YFP-TLR4 pulldown) and analyzed by immunoblotting with anti-Flag Ab (to determine Flag–MD2 interactions with YFP-TLR4s). Tubulin immunoblot was used to control protein loading. The data of a representative experiment (n = 3) are shown. (E) Densitometric quantification of the data shown in (C) and (D). The results are presented as arbitrary units representing ratios of normalized densitometric values of MD2–TLR4 complexes detected in samples transfected with pcDNA3 (background) or vectors expressing WT or D299G YFP-TLR4. Normalization of densitometric values was calculated as a/b/c/d, where a, b, c, and d correspond to the densitometric values of MD2-TLR4, total TLR4, total MD2, and tubulin, respectively.
The D299G polymorphism inhibits TLR4-mediated activation of the TRIF pathway

To discern whether the D299G and T399I polymorphisms affect the TRIF pathway, we examined LPS-induced recruitment of TRIF to TLR4 and activation of the TBK1–IRF3–IFN-β signaling axis. LPS induced recruitment of transfected Flag–TRIF to WT YFP-TLR4 within 15–30 min poststimulation, whereas no TRIF-associated TLR4 was detected in 293/CD14/MD2 cells expressing WT, D299G, or T399I TLR4 (Fig. 7C, 7D) and increased the levels of IFN-β mRNA by 2.8-fold (Fig. 7E). Cells expressing the D299G TLR4 showed significantly decreased LPS-driven activation of IFN-β–promoter reporter (Fig. 7C, 7D) and IFN-β gene expression (Fig. 7E) compared with the responses observed in WT TLR4-transfected cells. 293/CD14/MD2 cells transfected with WT or T399I TLR4 showed no statistically significant differences in LPS-mediated p125-Luc reporter activation and IFN-β gene expression (Fig. 7A–E). To confirm our data in cells with the macrophage phenotype, we assessed TLR4-inducible induction of IFN-β mRNA in iBMDM cell lines that have been reported to replicate LPS responses of primary macrophages (63). LPS treatment of TLR4-deficient iBMDMs complemented with WT YFP-TLR4 increased expression of IFN-β mRNA by ~5-fold, whereas pcDNA3-transfected cells did not show IFN-β mRNA induction (Fig. 7F). In contrast, complementation of TLR4<sup>−/−</sup> iBMDMs with D299G YFP-TLR4 led to only a 2-fold increase in IFN-β mRNA in response to LPS.
The D299G polymorphism impairs the ability of TLR4 to recruit TRIF in response to LPS stimulation. 293TLR4 stable cell lines expressing WT or D299G YFP-TLR4 species were cotransfected with pcDNA3-CD14, pEFBOS-Flag-MD2, and pEFBOS-Flag-TRIF. After recovery for 20 h, cells were treated with medium or stimulated for the indicated times with 100 ng/ml LPS, and cell lysates were prepared and immunoprecipitated with anti-GFP or anti-Flag Abs. Immune complexes were analyzed by immunoblotting with anti-GFP, anti-Flag, or isotype control IgG Abs to determine total TLR4 expression and amounts of TRIF recruited to TLR4 proteins. Data of a representative (n = 3) experiment are shown.

Discussion
The D299G and, to a lesser extent, T399I polymorphisms in the TLR4 ectodomain have been linked to sepsis, Gram-negative infections, RSV bronchiolitis, Mediterranean spotted fever, and chlamydial Hsp60, whereas the T399I polymorphism has either a significantly reduced or no inhibitory impact on TLR4 functions (25–31, 35, 36, 38). The molecular mechanisms responsible for the observed signaling deficiencies are unclear, and controversial data have been reported regarding the impact of these polymorphisms on TLR4 expression (38, 64).

Association of the ectodomain of TLR4 with chaperones gp96 and CNPY3 regulate TLR4 trafficking and expression (65–67), critical parameters defining the magnitude of LPS responses (48). Because the D299G and T399I polymorphisms are located in the ectodomain of TLR4 (25), it is possible that they could affect TLR4 cellular localization and/or expression levels by impacting TLR4 assembly with chaperones. However, the data provided in this report do not support this hypothesis and show comparable total expression of transfected WT versus polymorphic D299G and T399I YFP-TLR4 variants, as demonstrated by confocal microscopy, FACS, and immunoblot analyses, and using both transient and stable transfectants. Furthermore, FACS analysis revealed only minimal differences in cell-surface expression of WT and mutant TLR4 species, and confocal imaging showed their comparable localization on the plasma membrane and in intracellular compartments, reported to be the Golgi apparatus and endoplasmic reticulum (44, 54). Our results support similar data reported with transfected untagged TLR4 (38) and indicate that the D229G and T399I polymorphisms do not significantly alter TLR4 expression, suggesting that signaling deficiencies of the mutant TLR4 species are not due to changes in their expression levels.

MD2 is the principal coreceptor that confers LPS responsiveness by binding LPS and presenting it to TLR4 (51). Mice with a targeted deletion of MD2 exhibit a phenotype identical to TLR4 knockout mice, with both knockouts lacking LPS responsiveness (61, 68). Although the D299G and T399I polymorphisms are located outside of the F440-, F463-, and L444–MD2 interaction interface of TLR4 (58), it could be theorized that they distantly impose conformational changes, affecting TLR4–MD2 interactions, as previously suggested (38). To address this possibility, we studied whether the D299G polymorphism, associated with the strongest inhibition of TLR4 signaling (25, 27, 29, 38), affects the ability of transfected YFP-TLR4 to interact with Flag-MD2 under basal state and upon LPS stimulation. Comunnoprecipitation experiments revealed no measurable differences in TLR4–MD2 interactions regardless of whether WT or D299G TLR4s were present, indicating that compromised signaling elicited by D299G TLR4 cannot be accounted for by its deficient association with MD2.

Five acyl chains of lipid A bind to a hydrophobic pocket within MD2, imposing conformational changes and reorientation of the critical F126 residue in MD2 that presents the remaining acyl chain to the neighboring TLR4–MD2 complex, promoting TLR4 homodimerization (58). Hydrophobic interactions of lipid A with the TLR4 residues F440, F463, and L444 and interactions of the two phosphate groups of lipid A with positively charged K388, K264, K341, and K362 residues within TLR4 are important for promoting receptor homodimerization (58). Because the D299G polymorphism compromises the ability of TLR4 to elicit LPS-induced recruitment of TRIF to TLR4, phosphorylation of TBK1 and IRF3, and activation of IRF3-driven reporter and IFN-β mRNA.

**FIGURE 5.** The impact of the D299G and T399I TLR4 polymorphisms on LPS-induced expression of MyD88-dependent cytokine genes. HEK293 transient (A) and stable (B) transfectants expressing WT, D299G, or T399I YFP-TLR4 were cotransfected with pcDNA3-CD14-1pEHFOS-Flag-MD2. (C) C57BL/6 (TLR4 WT) and TLR4 D299G TLR4–MD2 complex, promoting TLR4 homodimerization (58). Because the D299G polymorphism compromises the ability of TLR4 to elicit LPS-induced recruitment of TRIF to TLR4, phosphorylation of TBK1 and IRF3, and activation of IRF3-driven reporter and IFN-β mRNA.

**FIGURE 6.** The D299G polymorphism impairs the ability of TLR4 to recruit TRIF in response to LPS stimulation. 293TLR4 stable cell lines expressing WT or D299G YFP-TLR4 species were cotransfected with pcDNA3-CD14, pEFBOS-HA-MD2, and pEFBOS-Flag-TRIF. After recovery for 20 h, cells were treated with medium or stimulated for the indicated times with 100 ng/ml LPS, and cell lysates were prepared and immunoprecipitated with anti-GFP or anti-Flag Abs. Immune complexes were analyzed by immunoblotting with anti-GFP, anti-Flag, or isotype control IgG Abs to determine total TLR4 expression and amounts of TRIF recruited to TLR4 proteins. Data of a representative (n = 3) experiment are shown.
and T399I polymorphisms are located relatively close to the R264, K341, and K362 residues, they could influence LPS binding to the receptor complex TLR4/MD2/CD14. To address this possibility, we examined binding of biotinylated LPS to 293/CD14/MD2 transfectants expressing WT or mutant TLR4 variants, as revealed by FACs analysis of SA-allophycocyanin bound to cell-associated biotinylated LPS. Similar LPS binding was detected in 293/CD14/MD2 cells expressing WT, D299G, or T399I YFP-TLR4 species under conditions of comparable TLR4 expression, confirming a recent publication demonstrating comparable affinities of interactions of radioactively labeled LOS with WT, D299G, or T399I YFP-TLR4 species. (F) HEK293T cells were transiently transfected with PCV-IRF3-Luc, and cell lysates were analyzed for firefly and Renilla luciferase activities.

FIGURE 7. LPS-mediated phosphorylation of TBK1 and IRF3, activation of p125-Luc, and induction of IFN-β mRNA in cells expressing WT or D299G YFP-TLR4. (A) HEK293T cells were transiently transfected with plasmids encoding WT or D299G YFP-TLR4, along with pCDNA3-CD14 and pEFBOS-Flag-MD2. After recovery, cells were treated with 100 ng/ml LPS for 30 min, and lysates were analyzed by immunoblotting with anti-GFP (YFP-TLR4), anti-Flag (Flag-MD2), anti-p-TBK1, anti-total TBK1, and anti-tubulin Abs. (B) HEK293 cells stably expressing YFP-TLR4 WT or D299G were cotransfected with pCDNA3-CD14 and pEFBOS-HA-MD2. After recovery, cells were treated with 100 ng/ml LPS for 15 min, and cell lysates were prepared and analyzed by immunoblotting with Abs against GFP (YFP-TLR4), p-IRF3, total IRF3, and anti-HA-HRP Ab (HA-MD2). (C) HEK293T cells were transiently transfected with WT, D299G, or T399I YFP-TLR4 species, along with pCDNA3-CD14 and pEFBOS-Flag-MD2. In addition, cells were cotransfected with p125-Luc and pTK-Renilla-Luc. (D) HEK293 cells stably expressing WT or D299G YFP-TLR4 species were cotransfected with pCDNA3-CD14 and pEFBOS-Flag-MD2. After recovery, cells were stimulated for 5 h with LPS or TNF-α (100 ng/ml each). Cell lysates were analyzed for firefly and Renilla luciferase activities. (E) HEK293T cells were transiently transfected with WT, D299G, or T399I YFP-TLR4 species, along with pCDNA3-CD14 and pEFBOS-Flag-MD2. Consistent with a key role for Myddosome assembly for initiation of downstream signaling (58), deficient MyD88 recruitment to D299G TLR4 was associated with impaired LPS-mediated induction of IL-8 mRNA in the HEK293 transfection system and the failure of this mutant to impart LPS-mediated activation of TNF-α gene expression upon nucleoefection-based complementation of TLR4−/− BMDMs. Our results provide a mechanistic explanation for deficiencies of the D299G TLR4 variant in eliciting MyD88-dependent responses to LPS (this paper and 25, 38, 64), F protein of RSV, chlamydial HSP60 (29), and other TLR4-dependent ligands. Furthermore, to the best of our knowledge, this paper is the first report demonstrating deficient activation of the TRIF signaling pathway via the D299G TLR4. In line with the proposed impact of the D299G polymorphism on TLR4 dimerization that is likely to compromise assembly of docking sites within the TIR domains critical for adapter recruitment, we found impaired LPS-inducible recruitment of TRIF to D299G TLR4. Deficient LPS-inducible TRIF recruitment to D299G TLR4 translated into decreased phosphorylation of TBK1 in 293T/CD14/MD2 cells transfected with D299G TLR4, compared with a robust response observed in cells complemented with WT TLR4. Consistent with the requirement for TBK1 kinase activity for IRF3 phosphorylation and activation (70), we showed compromised TLR4-elicited phosphorylation of
IRF3 and IRF3-dependent activation of IFN-β promoter-driven luciferase reporter and induction of IFN-β mRNA. Because HEK293 transfectants are limited in TLR4-mediated responses (40, 44, 50, 62), we confirmed our data on the impact of the D299G TLR4 species on LPS-driven activation of the TRIF pathway in mouse iBMDMs that behave similarly to primary macrophages (63). Transfection-based complementation of TLR4+/− iBMDMs with human WT TLR4 imported LPS-inducible IFN-β mRNA expression, whereas transfection of the D299G TLR4 species led to very little, if any, activation of this TRIF-dependent response. In contrast, cells expressing the T399I TLR4 variants exhibited no statistically significant deficiencies in LPS-induced MyD88- or TRIF-dependent responses, in line with the reported absence or relatively mild inhibitory effects of this polymorphism on TLR4 functions (29, 38, 64). Because TIRAP/Mal and TRAM are bridging adapters required for recruitment of signaling adapters MyD88 and TRIF to TLR4 (22–24, 69), deficient MyD88 and TRIF recruitment to D299G TLR4 could be due to compromised interactions of TIRAP/Mal and TRAM with the mutant receptor. This interference could result from conformational changes imposed by the mutation that would compromise MD2-assisted LPS presentation within TLR4 complexes (even though total LPS binding and TLR4–MD2 interactions are preserved), affecting TLR4 dimerization and assembly of docking platforms for adapter recruitment. Further studies are needed to address these questions.

Activation of p38 MAPK and the transcription factor NF-κB is governed by the MyD88-dependent (early responses) and TRIF-dependent (delayed activation) pathways. The impact of the D299G and T399I polymorphisms on LPS-driven p38 and NF-κB activation was studied based on phosphorylation of p38 and induction of the NF-κB–dependent pELAM-Luc reporter. To the best of our knowledge, this is the first demonstration of compromised LPS-mediated activation of p38 MAPK as a consequence of the presence of the D299G mutation in TLR4. In keeping with previous results (29, 38), we show that complementation of 293/CD14/MD2 transfectants with D299G TLR4 reduced LPS-mediated activation of the NF-κB reporter. This reduction was specific for TLR4, as TNF-α elicited similar activation of NF-κB regardless of whether WT or mutant TLR4 species were expressed. Because the D299G polymorphism affects TLR4 signaling at the level of MyD88 and TRIF recruitment, it is likely that deficient recruitment and activation of proximal adapter/kinase modules is responsible for deficiencies in downstream signaling events. Consistent with defective activation of p38 and NF-κB, and in line with their importance in the transcriptional control of cytokine expression (2, 20), we observed impaired production of proinflammatory cytokines TNF-α and IL-8.

In summary, our data show comparable total expression of transfected WT, D299G, and T399I TLR4 species and minimal effects of the polymorphisms on TLR4 cell-surface expression and LPS binding to the TLR4/MD2/CD14 complex. This article demonstrates for the first time, to our knowledge, that deficient recruitment of signaling adapters MyD88 and TRIF to TLR4 is a mechanistic basis for D299G-mediated impairment of TLR4-elicited, LPS-induced activation of MyD88-dependent IL-8 and TNF-α genes, deficient TRIF-dependent phosphorylation of TBK1 and IRF3, transactivation of IRF3, and expression of IFN-β mRNA. TLR4-driven activation of NF-κB and p38 dependent on both pathways was also impacted by the D299G polymorphism. It will be important to extend our results to cells from human patients expressing endogenous TLR4 SNPs and to conduct studies in knockin mice expressing the humanized version of TLR4 proteins to define the impact of these mutations on susceptibility to infectious and inflammatory diseases.

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References


