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Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A

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Abstract

A cell surface heterodimer Toll-like receptor 4 (TLR4)/MD-2 senses lipopolysaccharide (LPS), a principal membrane component of Gram-negative bacteria. LPS binds to MD-2 and induces dimerization of TLR4/MD-2. Dimerized TLR4 activates downstream signaling. TLR4 polymorphism replacing Asp299 with Gly and Thr399 with Ile (D299G/T399I) causes LPS hyporesponsiveness, and is associated with a variety of infectious and noninfectious diseases. However, a molecular mechanism underlying the LPS hyporesponsiveness remains controversial. We here asked whether the TLR4 polymorphism influenced cell surface expression of TLR4/MD-2, ligand-dependent TLR4/MD-2 dimerization or TLR4/MD-2 responses to a weak agonist monophosphoryl lipid A (MPL). A newly established anti-TLR4 mAb detected D299G/T399I TLR4/MD-2 on Ba/F3 cells whereas a previous anti-TLR4 mAb did not, suggesting that the D299G/T399I polymorphism caused a conformational change in TLR4. Hyporesponsiveness of D299G/T399I TLR4/MD-2 was much more apparent when cells were stimulated with MPL than with lipid A. MPL-dependent TLR4/MD-2 dimerization was impaired by the D299G/T399I polymorphism. The D299G/T399I polymorphism did not alter LPS-binding to soluble TLR4/MD-2, but impaired its dimerization. These results suggest that the D299G/T399I TLR4 polymorphism impairs TLR4/MD-2 responses by altering ligand-dependent dimerization.

Keywords: human TLR4, polymorphism, MPL

Introduction

Toll-like receptors (TLRs) sense a variety of microbial products. Cell surface TLRs including TLR4/MD-2, TLR1/TLR2 and TLR6/TLR2 recognize microbial membrane lipids, whereas TLR3, TLR7, TLR8 and TLR9 reside in intracellular organelles and recognize microbial nucleic acids (1–3). TLR4/MD-2 senses lipopolysaccharide (LPS), a principal membrane component of Gram-negative bacteria. MD-2 is physically associated with TLR4 (4), directly binds to LPS (5), and induces LPS-dependent dimerization of TLR4/MD-2 (6). TLR4/MD-2 activates two distinct signaling pathways. One is mediated by the adaptor proteins TIRAP (Toll-interleukin-1 receptor domain-containing adaptor protein) and MyD88 (myeloid differentiation factor 88), which induce production of proinflammatory cytokines. The other signaling pathway is activated by a distinct set of signaling adaptors, TRAM (TRIF-related adaptor molecule)/TICAM-2 (TIR-domain-containing adaptor molecule-2) and TRIF (Toll/IL-1 receptor-containing adapter inducing IFN-β)/TICAM-1, which mediate IRF3 (interferon regulatory factor 3)-dependent type I IFN production and upregulation of costimulatory molecules.

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Two TLR4 polymorphisms, A896G and C1196T, have been identified in the human TLR4 gene, resulting in amino acid changes, D299G and T399I, respectively. These polymorphisms have been reported to cause LPS hyporesponsiveness in primary airway epithelial cells or alveolar macrophages (7). These TLR4 polymorphisms are associated with a variety of infectious and noninfectious diseases. TLR4 polymorphisms are linked with a susceptibility to infections with Gram-negative bacteria (7) or respiratory syncytial virus (8). TLR4/MD-2 has been shown to respond to endogenous ligands (9), and the responses to fatty acids predispose to obesity-induced metabolic syndrome by activating inflammatory responses in adipose tissues (10). TLR4 polymorphic forms are suggested to influence TLR4/MD-2 interaction with endogenous ligands. Along this line, TLR4 polymorphisms reduce the risk of noninfectious inflammatory diseases like atherosclerosis (11) or rheumatoid arthritis (12).

A molecular mechanism underlying LPS hyporesponsiveness of TLR4 polymorphic forms remains controversial. TLR4 polymorphisms may impair cell surface expression of TLR4 (13, 14), whereas another study failed to find impaired cell surface expression of polymorphic TLR4 (15). We here asked whether these TLR4 polymorphisms influenced cell surface expression of TLR4/MD-2 or ligand-dependent TLR4 dimerization by using a newly established anti-human TLR4 mAb and a purified soluble TLR4/MD-2 protein.

Methods

Reagents

LPS from *Escherichia coli* 055:B5, lipid A and monophosphoryl lipid A (MPL) purified from *Salmonella minnesota* (Re-595), lauric acid, palmitic acid, biotinylated anti-FLAG antibody and anti-FLAG agarose were purchased from Sigma-Aldrich. pNFκB-*hr*GFP was purchased from Stratagene. Anti-human TLR4 mAb (HTA125) and anti-mouse TLR4 mAb (Sa15-21) were previously established in our laboratory (4, 16).

Establishment of mAb to human TLR4

Another anti-human TLR4 mAb (TF901, mouse IgG1/κ) was newly established. In brief, BALB/c mice were immunized three times with Ba/F3 cells overexpressing hTLR4, hMD-2 and mCD14. Splenocytes were fused with SP2/0 myeloma cells. The mAb was purified in our laboratory.

Expression constructs and transfectants

Ba/F3 cells were cultured in 10% FBS/RPMI 1640 supplemented with penicillin G (100 U ml^{-1}), streptomycin sulfate (100 μg ml^{-1}), 50 μM 2-mercaptoethanol and IL-3. For the NF-κB reporter gene assay, p55IgκLuc (17) or pNFκB-*hr*GFP was electroporated into the Ba/F3 cells (BaκB-luci or BaκB cells). GFP-tagged human TLR4 or human–mouse chimeric TLR4 [human TLR4 (1–286) with mouse TLR4 (286–661) or mouse TLR4 (1–285) with human TLR4 (287–663)] were cloned into a pCAGGS vector (18). The cDNAs encoding human TLR4 (including A896G and C1196T mutants), hMD-2 and hCD14 were cloned into the retrovirus vector pMX or pMX-puro. BaκB cells expressing human TLR4, MD-2 and CD14 were established by retroviral transduction using FuGENE 6 transfection reagent (Roche). Plat-E was used as packaging cell line (19).

HEK293T cells were maintained in DMEM supplemented with 10% FBS, penicillin G (100 U ml^{-1}), streptomycin sulfate (100 μg ml^{-1}) and 50 μM 2-mercaptoethanol. The cDNAs encoding human TLR4, human MD-2 tagged with FLAG and (His)6 at the C terminus were cloned into the expression vector, pEFBOS. HEK293T transiently expressing human TLR4 and human MD-2 were established by transfection of these constructs with Lipofectamine 2000 (Invitrogen).

Immunoprecipitation and immunoblotting

Cells were harvested and washed with HBSS, and lysed in the lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonylfluoride in EtOH and 1% Triton X-100]. After 30 min of incubation on ice, lysates were coupled with mAb-conjugated beads for 2 h at 4°C. Beads were washed three times and boiled at 95°C for 5 min in the sample buffer for SDS-PAGE. Bound proteins were subjected to SDS-PAGE and western blot analysis. The primary antibody was anti-FLAG (M2), and the secondary antibody was goat anti-mouse IgG conjugated with alkaline phosphatase (American Qualex).

Cell staining and flow cytometric analysis

Cells were incubated for 15 min at 4°C with the primary antibodies diluted in staining buffer (PBS containing 2.5% FBS and 0.01% NaN_3). In the cross-blocking experiment, cells were incubated with 30 μg ml^{-1} antibody for 30 min at 4°C before primary antibody incubation. Cells were washed with staining buffer, and incubated with PE-conjugated streptavidin (Biolegend) for 15 min at 4°C. Flow cytometry analysis was performed on the FACSCalibur system (BD Biosciences).

Luciferase assay

Cells were seeded onto the 96-well plate (1×10^5 per well) and pretreated with or without anti-TLR4 antibodies (20 μg ml^{-1}) for 30 min at 4°C. After pretreatment, cells were stimulated with LPS (100 ng ml^{-1}) or lipid A (100 ng ml^{-1}) for 3.5 h and lysed in 50 μl of lysis buffer. The luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Nippon Gene). The luminescence was quantitated by a luminometer (Berthold Japan).

NF-κB-GFP reporter gene assay

Ba/F3 cells expressing human TLR4/MD-2, human CD14 and NFκB-*hr*GFP were seeded onto the 96-well plate (1×10^5 per well) and stimulated with ligands for 24 h (Fig. 4A and B). For fatty-acid stimulation, the cells were washed three times and stimulated with fatty acids in 1% FBS Ba/F3 medium for 48 h (Fig. 5A). The fatty acids were dissolved in EtOH and then conjugated with BSA. After stimulation, NF-κB activity was analyzed by GFP expression level with FACSCalibur system.

LPS-binding assay with purified TLR4/MD-2

Purified, soluble TLR4/MD-2 was added to LPS-coupled or control beads (Fig. 4C) for 1 h at room temperature. Beads

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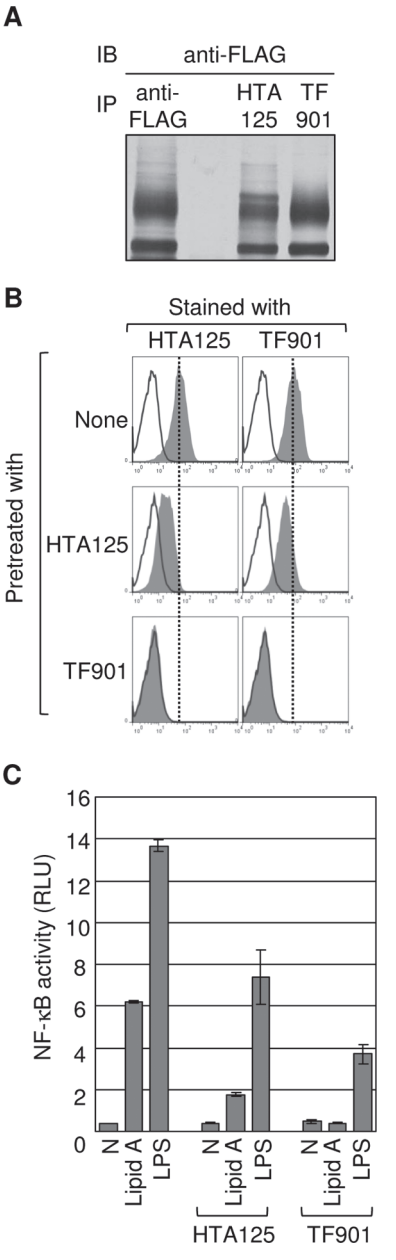

Fig. 1. Newly established anti-human TLR4 antibody TF901 shows higher affinity than previous antibody HTA125. (A) Ba/F3 cells expressing human TLR4-FLAG were immunoprecipitated with anti-FLAG, HTA125 or TF901. After 7.5% SDS-PAGE and transfer to a PVDF membrane, precipitated TLR4 was probed with anti-FLAG mAb. (B) Cross-blocking study was conducted with Ba/F3 cells expressing human TLR4 (wild type)/MD-2, human CD14 and NFκB-*hr*GFP. Ba/F3 cells were pretreated with HTA125 or TF901 at 30 μg ml^{-1}, stained with 10 μg ml^{-1} bio-HTA125 or 0.5 μg ml^{-1} bio-TF901, followed by streptavidin-PE, and analyzed by flow cytometry. Shaded histograms show the staining by the indicated antibody. Open histograms show the staining with secondary antibody alone. (C) Ba/F3 cells expressing NF-κB-luciferase, human TLR4-FLAG and human MD-2-FLAG were preincubated with HTA125 (20 μg ml^{-1}) or TF901 (20 μg ml^{-1}) at 4°C. After 30 min culture, cells were stimulated with lipid A (100 ng ml^{-1}) or LPS (100 ng ml^{-1}) as indicated at 37°C. After incubation for 3.5 h, cells were harvested and NF-κB activation was measured by the luciferase assay. The data are expressed as relative luciferase activity. Shown data are representative of more than three independent experiments.

were washed two times and boiled at 95°C for 5 min in the sample buffer [0.1 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 2-mercaptoethanol and bromophenol blue (BPB)]. Bound proteins were visualized by 12% SDS-PAGE and immunoprobing. The primary antibodies were polyclonal anti-human TLR4 (eBioscience) and polyclonal anti MD-2. The bound antibody was detected by goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad).

Native-PAGE

Drosophila S2 cells were cotransfected with the human TLR4 (wild type or D299G/T399I) and MD-2 vectors. The proteins were purified from the culture medium. Purified TLR4/MD-2 protein was mixed with ligands in buffer [10 mM Tris–HCl (pH 8.0), 0.15 M NaCl and 0.1% Triton X-100], and incubated for 2 h at 37°C. After incubation, loading buffer [30% glycerol/Tris–HCl (pH 8.0), BPB] was added to the samples. Samples were subjected to 10% native-PAGE and stained with coomassie brilliant blue (Fig. 4D). For the gel shift competition assay with LPS and fatty acids (Fig. 5B–D), purified TLR4 (wild type or D299G/T399I)/MD-2 were preincubated with or without LPS for 30 min at 37°C, followed by incubation with the indicated amounts of fatty acids (Sigma) for 2 h at 37°C, and then subjected to native-PAGE analyses. The fatty acids were dissolved in 2% Triton X-100.

Results

We previously established anti-human TLR4 mAb, HTA125 (4). We obtained another anti-TLR4 mAb, TF901. The specificity was validated by immunoprecipitation (Fig. 1A). Human TLR4 with the FLAG epitope was expressed on Ba/F3 cells and immunoprecipitated with anti-FLAG, HTA125 or TF901. TLR4 was immunoprobed by anti-FLAG. TF901 was able to precipitate TLR4 as much as HTA125 or anti-FLAG. To compare TF901 with the previously established HTA125, a cross-blocking study was conducted. Ba/F3 cells expressing human TLR4/MD-2 were pretreated and stained with HTA125 or TF901 (Fig. 1B). TF901 completely inhibited the binding of HTA125 as well as of itself, whereas HTA125 was much weaker than TF901 in cross-blocking either antibody. These results suggested that the epitopes of HTA125 and TF901 are close to each other and that TF901 has higher affinity than HTA125. The effect of TF901 on TLR4/MD-2-dependent NF-κB activation was next examined by using a Ba/F3 transfectant expressing human TLR4/MD-2 and NF-κB-luciferase. After stimulating with LPS or lipid A, the luciferase activity was measured (Fig. 1C). TF901 showed stronger inhibition than HTA125. Higher affinity to TLR4 would explain this stronger inhibition by TF901.

To compare the epitopes of HTA125 and TF901, we constructed cDNAs encoding human–mouse chimeric TLR4. These constructs were transiently expressed together with MD-2 in HEK293T cells, and transfected cells were stained with HTA125, TF901 or Sa15-21, an anti-mouse TLR4 mAb that reacts with the N-terminal half (amino acid 1–285) of mouse TLR4 (20). Both HTA125 and TF901 reacted with the N-terminal half (1–286) of human TLR4 (Fig. 2A). Considering that the TLR4 SNPs are both in the C-terminal half of the

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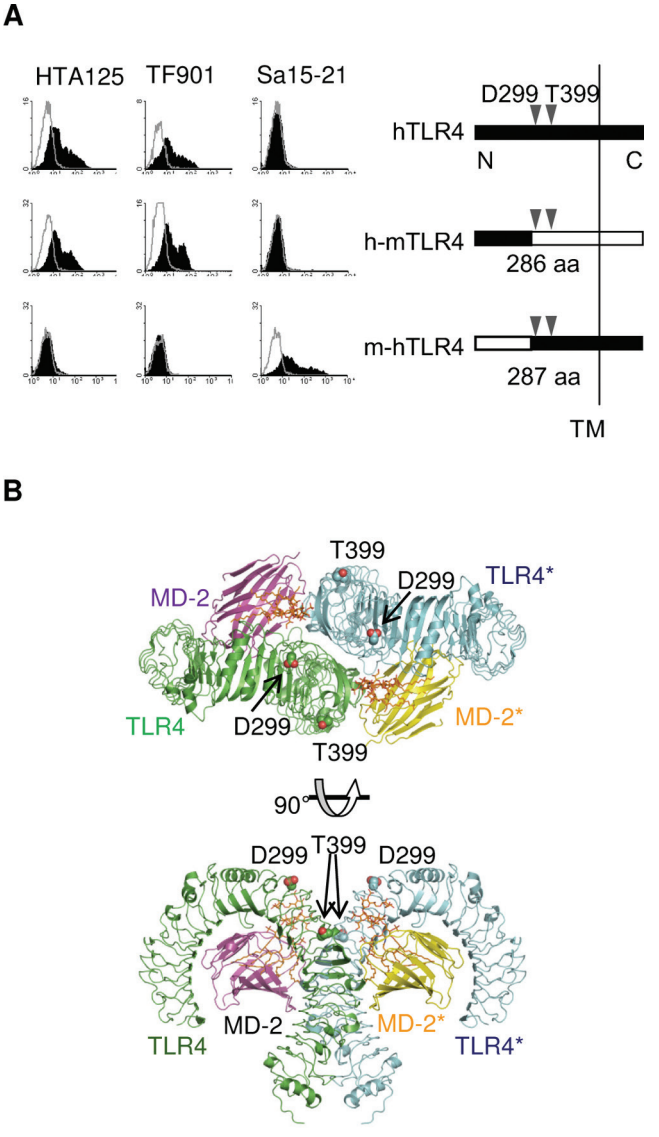

Fig. 2. The epitopes of anti-hTLR4 antibodies are not directly affected by TLR4 SNPs. (A) A human kidney cell line, HEK293T, was transfected with expression vectors encoding the indicated human–mouse chimeric TLR4 tagged with GFP in conjunction with human MD-2. Transfected cells were stained with biotinylated HTA125 (left), Sa15-21 (right) or supernatant of TF901 hybridoma (middle), followed by streptavidin-PE (left and right) or goat anti-mouse-Ig-PE. Open histograms indicate the samples stained with the second reagent only. The right scheme stands for the extracellular domain of mouse (□) and human(■) TLR4. TM, transmembrane. (B) Both D299 and T399 are located in the C-terminal half of the extracellular domain, which is not the epitope of HTA125 nor TF901. Overall ribbon representation exhibits the crystal structure of 2:2:2 human TLR4/MD-2/LPS complex (6) (PDB ID: 3FXI). The dimerization partners of the 1:1:1 human TLR4/MD-2/LPS complex are indicated by asterisks. Side chains of D299 and T399 are shown in a space-filling representation. This figure was prepared with PyMOL (28).

extracellular domain (Fig. 2A and B), TLR4 SNPs do not seem to directly alter the binding of HTA125 or TF901.

By using HTA125, TLR4 polymorphisms are reported to impair cell surface expression of human TLR4 on epithelial cells (13, 14). TF901 and HTA125 were used to detect cell surface expression of TLR4/MD-2 on Ba/F3 transfectants. TF901 detected all the wild type and polymorphic forms of TLR4/MD-2, and showed higher staining than HTA125 (Fig. 3A). Interestingly, the D299G or T399I polymorphism selectively impaired HTA125 binding, but not TF901 binding. Further, the D299G/T399I polymorphism completely abolished the binding of HTA125 but not of TF901 (Fig. 3A). These results suggested a conformation change by the D299G/T399I polymorphism. To ask whether the D299G/T399I polymorphism directly alters TLR4 conformation, soluble TLR4/MD-2 was immunoprecipitated with HTA125 or TF901, and precipitated TLR4/MD-2 was compared (Fig. 3B). HTA125 was able to precipitate D299G/T399I TLR4, albeit less than wild type TLR4 (compare lanes 7 and 8 with 9 and 10 in Fig. 3B). The effect of the D299G/T399I polymorphism on HTA125 antibody binding was more pronounced in cell surface staining than immunoprecipitation using soluble TLR4/MD-2. In addition to direct alteration of TLR4 conformation by the D299G/T399I polymorphism, an indirect effects of the D299G/T399I polymorphism on TLR4 conformation on the cell surface is possible (see the first paragraph of the Discussion). MD-2 was coprecipitated with D299G/T399I TLR4 as much as with wild type TLR4, indicating that MD-2 association was not altered by the D299G/T399I polymorphism.

An impact of TLR4 polymorphisms on LPS responses was next addressed. Ba/F3 cells used in Fig. 3A expressed NF-κB-GFP, which was able to detect NF-κB activation by GFP induction. We confirmed that the amounts of cell surface TLR4, MD-2 and CD14 were similar to each other (Fig. 3A and data not shown). These cells were stimulated with lipid A and GFP induction was analyzed. D299G/T399I TLR4/MD-2 showed slightly lower responses than wild type, D299G or T399I TLR4/MD-2(Fig. 4A). The impaired response of D299G/T399I TLR4/MD-2 was more apparent when TLR4/MD-2 was stimulated with MPL. As MPL is a modified lipid A whose agonistic activity is much weaker than lipid A (21), GFP induction by MPL was much lower than that by lipid A (Fig. 4A and 4B). MPL stimulation revealed that D299G or T399I TLR4/MD-2 showed weaker GFP induction than wild type TLR4/MD-2 did. The D299G/T399I polymorphism further impaired MPL-dependent GFP induction (Fig. 4B).

To gain insight into a mechanism underlying hyporesponsiveness due to the D299G/T399I polymorphism, wild type and D299G/T399I TLR4/MD-2 were further compared. LPS binds to MD-2 (5), whereas TLR4 is directly involved in the following ligand-dependent TLR4/MD-2 dimerization (6, 22). It is, however, possible that the D299G/T399I TLR4 polymorphism indirectly influences MD-2 interaction with its ligand LPS. To address this possibility, LPS binding to TLR4/MD-2 was studied. LPS was coupled to agarose beads and mixed with soluble TLR4/MD-2. Bound TLR4/MD-2 was detected by SDS-PAGE and immunoprobing (Fig. 4C). Both wild type and D299G/T399I TLR4/MD-2 showed much higher binding to LPS-coupled beads than to control beads (Fig. 4C, compare lanes 3 and 6 for wild type TLR4/MD-2 and lanes 4 and 8 for D299G/T399I TLR4/MD-2). We could not find any difference between wild type and D299G/T399I TLR4/MD-2 in LPS binding.

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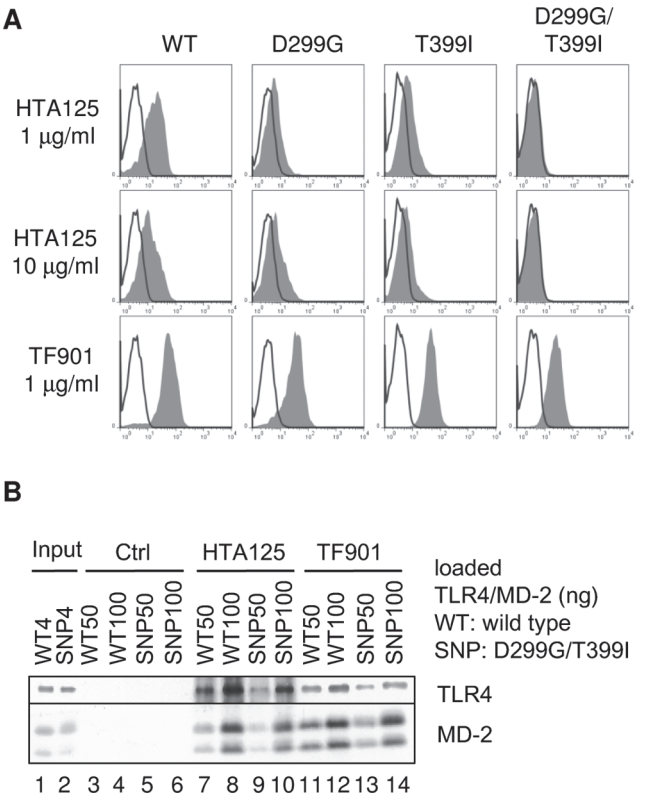

Fig. 3. TF901, but not HTA125, detects cell surface TLR4/MD-2 with the D299G/T399I polymorphism. (A) Ba/F3 cells expressing human TLR4, MD-2, CD14 and NFκB-*hr*GFP were stained with biotinylated anti-hTLR4 antibodies and analyzed by flow cytometry. Shaded histograms show the staining by indicated antibody. Open histograms show the staining with secondary antibody alone. (B) Purified TLR4 (wild type or D299G/T399I)/MD-2 proteins were added to mAb–beads for 60 min at room temperature. Samples were eluted from the beads with sample buffer and subjected to SDS-PAGE analyses. Anti-CD14-coupled beads were used as a control (Ctrl). In lanes 1 and 2, soluble TLR4/MD-2 used for the pull down assay was directly loaded as a control for precipitated TLR4 or MD-2. Shown data are representative of more than three independent experiments.

We next examined the effect of TLR4 polymorphism on ligand-dependent dimerization by using native-PAGE. Soluble TLR4/MD-2 protein was incubated with lipid A or MPL, and subjected to native-PAGE to detect ligand-bound TLR4/MD-2 dimers. As reported previously (23), lipid A induced TLR4/MD-2 dimerization (Fig. 4D). Almost all of the wild type TLR4/MD-2 dimers after the addition of 0.05 μg lipid A. Although almost all of the D299G/T399I TLR4/MD-2 also showed dimerization at 0.05 μg lipid A, wild type TLR4/MD-2 showed a higher ratio of dimer to monomer at 0.0125 μg lipid A than D299G/T399I TLR4/MD-2 did (Fig. 4D). MPL also induced TLR4/MD-2 dimerization, but TLR4/MD-2 monomer remained even though 0.2 μg MPL was added. MPL-dependent dimerization of wild type TLR4/MD-2 was seen in the 0.0125 μg MPL lane, whereas that of D299G/T399I TLR4/MD-2 was apparent only in the 0.2 μg MPL lane (Fig. 4D). The D299G/T399I polymorphism impaired ligand-dependent dimerization, which was most apparent when TLR4/MD-2 was stimulated with a weak agonist, MPL.

Saturated fatty acid is known to stimulate TLR4/MD-2, and this interaction has been implicated in obesity (10). As the TLR4 polymorphism has also been involved in obesity and atherosclerosis (11, 24), we studied the effect of fatty acids such as laurate, myristate and palmitate on TLR4/MD-2-dependent NF-κB activation and TLR4/MD-2 dimerization. Ba/F3 cells expressing TLR4/MD-2, CD14 and NF-κB-GFP were stimulated with fatty acids, and GFP induction was examined. Although myristate at 100 μM slightly induced GFP induction, comparable GFP induction was also seen in the control cells that did not express TLR4/MD-2 (Fig. 5A). TLR4/MD-2-dependent GFP induction by these fatty acids was not detected. D299G/T399I TLR4/MD-2 also failed to induce GFP in the presence of the fatty acids (Fig. 5A).

The effect of fatty acids on TLR4/MD-2 dimerization was next analyzed with native-PAGE analyses (Fig. 5B–D). As reported previously (25), palmitate interacted with TLR4/MD-2 and thereby made TLR4/MD-2 shift faster in native-PAGE (Fig. 5B, compare lane 1 with lane 2 or 3). Despite interaction with palmitate, TLR4/MD-2 did not form a dimer (Fig. 5B, lanes 2 and 3). Instead, palmitate inhibited LPS-induced TLR4/MD-2 dimerization (Fig. 5B, lanes 5–7), suggesting that palmitate competes against LPS for binding to the hydrophobic pocket of MD-2. Laurate and myristate were similar to palmitate in that they inhibited LPS-induced TLR4/MD-2 dimerization (Fig. 5B and C). Interestingly, myristate seemed to be more potent than palmitate or laurate in inhibiting LPS-induced TLR4/MD-2 dimerization (Fig. 5D). The D299G/T399I polymorphism did not alter fatty acid-dependent inhibition of LPS-induced TLR4/MD-2 dimerization (Fig. 5B and C, lanes 14 and 15).

Discussion

The present study addressed the effect of the human TLR4 polymorphisms on the cell surface expression and the function of TLR4/MD-2. The D299G/T399I polymorphism abolished the reactivity of HTA125 to the surface TLR4/MD-2 expressed on Ba/F3 cells. Although the HTA125 epitope is close to the TF901 epitope, only the HTA125 epitope was influenced by the D299G/T399I polymorphism. The loss of HTA125 reactivity could be due to a conformation change in TLR4, but not to the lack of cell surface expression of TLR4/MD-2, as TF901 antibody was able to detect cell surface expression of TLR4/MD-2 with the D299G/T399I polymorphism. Interestingly, the effect of the D299G/T399I polymorphism on HTA125-binding was more pronounced in cell surface TLR4/MD-2 than in soluble TLR4/MD-2. In addition to a possibility that the D299G/T399I polymorphism directly alters TLR4 conformation, an indirect effect needs to be considered. It is possible that the D299G/T399I-dependent TLR4 conformation change is exaggerated on the cell surface by another cell surface molecule. This possibility will be addressed in future studies.

The effect of D299G/T399I TLR4 polymorphism was much more apparent when it was stimulated with MPL rather than with lipid A (Fig. 4A and B). MPL is known to be a weak TRIF-biased ligand when it is compared with lipid A (21, 26). The present study suggested that MPL was weaker than lipid A in an ability to induce ligand-dependent TLR4/MD-2 dimerization (Fig. 4D), and that MPL-induced dimer formation was negatively regulated by the D299G/T399I TLR4

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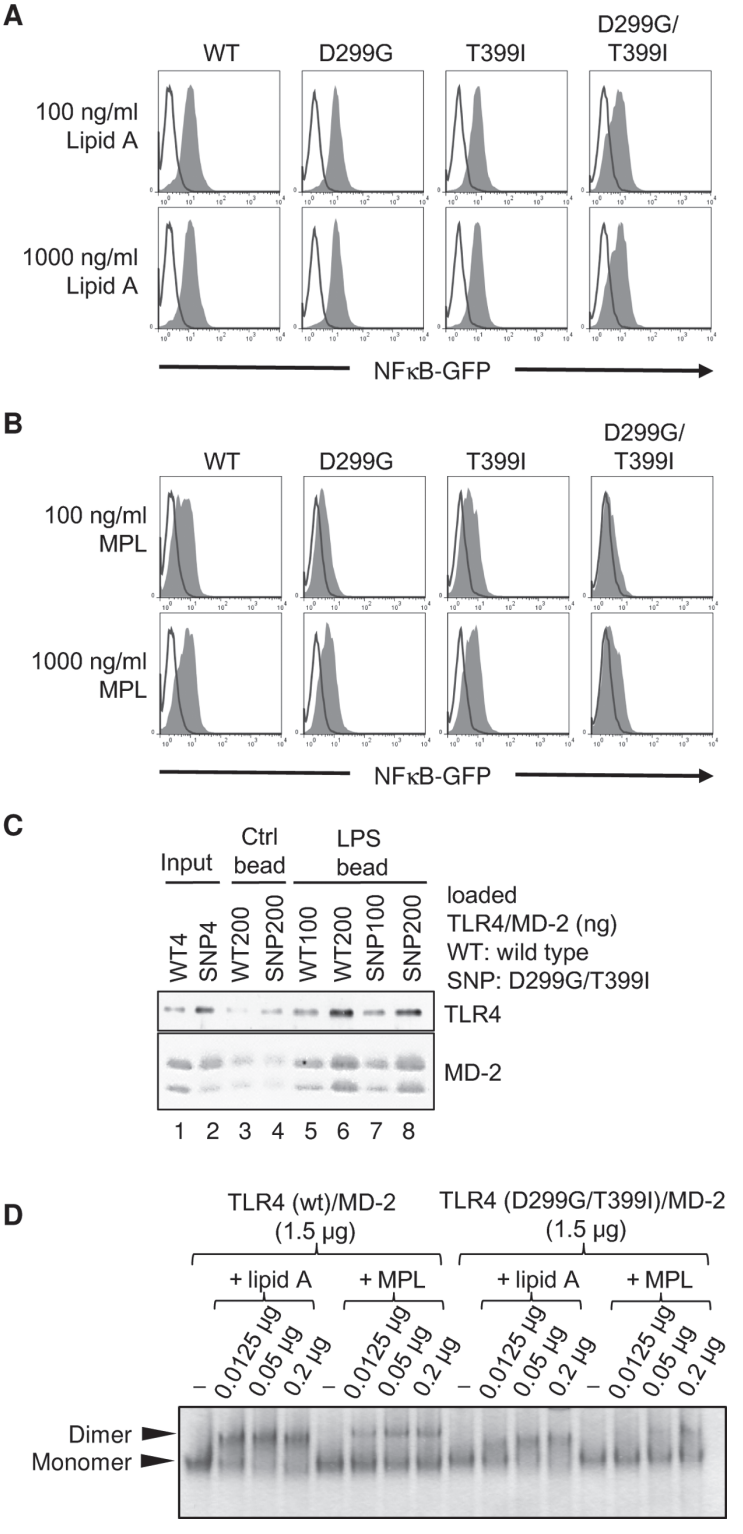

Fig. 4. Hyporesponsiveness of D299G/T399I TLR4/MD-2 in responses to a weak agonist MPL. (A and B) Ba/F3 cells expressing human TLR4/MD-2, human CD14 and NFκB-*hr*GFP were stimulated with lipid A (A) or MPL (B) for 24 h. Cells were harvested and GFP expression levels were analyzed by flow cytometry. Shaded and open histograms show the stimulated cells and unstimulated cells, respectively. (C) Purified, soluble TLR4 (wild type or D299G/T399I)/MD-2 proteins were added to LPS-bound beads for 60 min at room temperature. Samples were eluted from the beads with sample buffer and subjected to 12% SDS-PAGE analyses. Plain beads were used as controls. (D) Purified TLR4 (wild type or D299G/T399I)/MD-2 proteins were incubated with lipid A or MPL and subjected to 10.0% native-PAGE and stained with coomassie brilliant blue. Monomer indicates unbound TLR4/MD-2 complex or 1:1:1 TLR4/MD-2/ligand complex and dimer indicates 2:2:2 TLR4/MD-2/ligand complex. Shown data are representative of more than three independent experiments.

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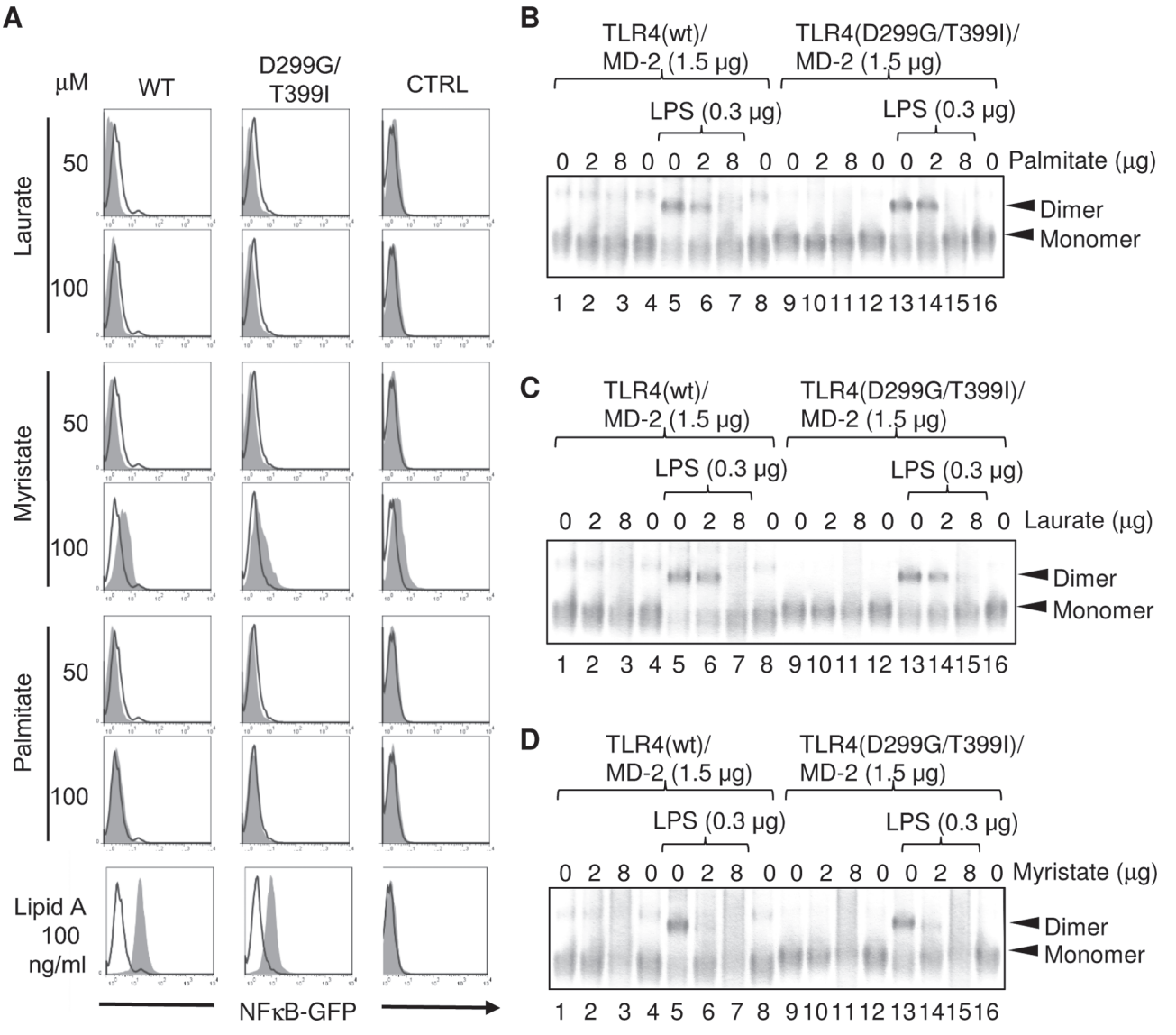

Fig. 5. The D299G/T399I polymorphism does not influence inhibition by saturated fatty acids of LPS binding to TLR4/MD-2. (A) Ba/F3 cells expressing human TLR4/MD-2, human CD14 and NFκB-*hr*GFP were washed and stimulated with fatty acids in 1% FBS Ba/F3 medium for 48 h. Cells were harvested and GFP expression levels were analyzed by flow cytometry. Shaded histograms show the stimulated cells and open histograms show the unstimulated cells. As a negative control (CTRL), Ba/F3 cells expressing only NFκB-*hr*GFP were used. (B–D) Purified TLR4 (wild type or D299G/T399I)/MD-2 proteins were preincubated with or without LPS for 30 min at 37°C, followed by the incubation with the indicated amounts of fatty acids for 2 h at 37°C, and then subjected to native-PAGE analyses. Proteins were stained with coomassie brilliant blue.

polymorphism. According to our results, this negative effect of the D299G/T399I polymorphism seems to be not strong enough to affect TLR4/MD-2 dimerization by a potent ligand, lipid A.

LPS directly binds to MD-2 and induces TLR4/MD-2 dimerization (5, 22, 23). Ligand-dependent TLR4/MD-2 dimerization is controlled by interaction between TLR4 and MD-2. MD-2 interacts with TLR4 at two distinct sites. The first site is used for constitutively interacting with TLR4, whereas the second is activated only when MD-2 binds to a ligand, leading to ligand-dependent dimerization of TLR4/MD-2 (23). Neither D299 nor T399 is, however, close to these MD-2 binding sites, negating a possibility that D299G and T399I polymorphisms directly influence MD-2 interaction with TLR4. MD-2 coprecipitation with soluble TLR4 was not altered by the D299G/T399I polymorphism. The constitutive interaction between MD-2 and TLR4 is not altered by the D299G/T399I polymorphism. On the other hand, the D299G/T399I polymorphism was suggested to influence ligand-dependent TLR4/MD-2 dimerization. The D299G/T399I polymorphism might indirectly affect the second, ligand-dependent MD-2-TLR4 interaction. Structural information on D299G/T399I TLR4/MD-2 could reveal a mechanism behind hyporesponsiveness due to the D299G/T399I polymorphism.

Recent studies suggest that weak endogenous ligands activate TLR4/MD-2 and resultant inflammation predisposes

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to noninfectious chronic diseases including obesity and atherosclerosis (24). In native-PAGE, saturated fatty acids such as laurate (C12:0), myristate (C14:0) and palmitate (C16:0) inhibited LPS-induced TLR4/MD-2 dimerization (Fig. 5B–D), demonstrating that these fatty acids share the binding site on MD-2 with LPS. Despite binding to MD-2, these fatty acids failed to induce ligand-dependent TLR4/MD-2 dimerization. Laurate (C12:0) has been shown to induce TLR4/MD-2 dimerization when assessed using the cell-based assay system (27), but not when assessed using native-PAGE as shown in the present study, suggesting that native-PAGE with soluble TLR4/MD-2 protein may not have been sensitive enough to detect weak TLR4/MD-2 dimerization. Alternatively, an additional molecule is required for saturated fatty acids to induce TLR4/MD-2 dimerization. NF-κB-dependent GFP induction by saturated fatty acids was not detected. TLR4/MD-2-dependent NF-κB activation by saturated fatty acids would be weak and may require development of a novel assay system. Further study is required to look for the effect of TLR4 SNPs on TLR4/MD-2 dimerization induced by endogenous TLR4/MD-2 ligands.

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