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Tetraacylated Lipid A and Paclitaxel-Selective Activation of TLR4/MD-2 Conferred through Hydrophobic Interactions

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LPS exerts potent immunostimulatory effects through activation of the TLR4/MD-2 receptor complex. The hexaacylated lipid A is an agonist of mouse (mTLR4) and human TLR4/MD-2, whereas the tetraacylated lipid IVa and paclitaxel activate only mTLR4/MD-2 and antagonize activation of the human receptor complex. Hydrophobic mutants of TLR4 or MD-2 were used to investigate activation of human embryonic kidney 293 cells by different TLR4 agonists. We show that each of the hydrophobic residues F438 and F461, which are located on the convex face of leucine-rich repeats 16 and 17 of the mTLR4 ectodomain, are essential for activation of with lipid IVa and paclitaxel, which, although not a structural analog of LPS, activates cells expressing mTLR4/MD-2. Both TLR4 mutants were inactive when stimulated with lipid IVa or paclitaxel, but retained significant activation when stimulated with LPS or hexaacylated lipid A. We show that the phenylalanine residue at position 126 of mouse MD-2 is indispensable only for activation with paclitaxel. Its replacement with leucine or valine completely abolished activation with paclitaxel while preserving the responsiveness to lipid IVa and lipid A. This suggests specific interaction of paclitaxel with F126 because its replacement with leucine even augmented activation by lipid A. These results provide an insight into the molecular mechanism of TLR4 activation by two structurally very different agonists. The Journal of Immunology, 2014, 192: 000–000.
species-dependent electrostatic interactions to explain the agonistic properties of lipid IVa toward mTLR4/MD-2. However, a role for hydrophobic interactions at the dimerization interface of TLR4/MD-2/lipid IVa complexes has recently been supported by the crystal structure of murine TLR4/MD-2/lipid IVa (12), as the lipid IVa has a very similar arrangement of the acyl chains as the hexaacylated lipid A. Because paclitaxel contains no charged groups, a role for electrostatic interactions in activation of mTLR4/MD-2 by paclitaxel seems unlikely (13). Thus, similar hydrophobic interactions could be important for activation of mTLR4/MD-2 by lipid IVa as well as paclitaxel.

We now show by functional analysis of wild-type (wt) and mutant mTLR4 that phenylalanine residues of the hydrophobic binding site on the TLR4ECD are essential for mTLR4/MD-2 activation by both lipid IVa and paclitaxel. An essential role of F438 implies a crucial importance of hydrophobic interactions within the dimerization interface involving solvent-exposed hydrophobic groups of lipid IVa and paclitaxel bound to murine MD-2 (mMD-2) that is supported by molecular docking studies. Comparison of the mutations of the homologous phenylalanine residues in mouse and hTLR4 on activation by hexacylated LPS suggest that differences in the number and/or strength of other interactions within and surrounding the dimerization interface could further contribute to the selective ability of mouse (versus human) TLR4/MD-2 to be activated by lipid IVa or paclitaxel.

Materials and Methods

Cell culture and reagents

The plasmid encoding the mMD-2 was a gift from Dr. C. Kirschning (Technical University of Munich, Munich, Germany). The plasmid encoding the mouse mTLR4 was purchased from InvivoGen. The human embryonic kidney (HEK) 293 cells were provided by Dr. J. Chow (Eisai Research Institute, Andover, MA). HEK293T cells were used for the analysis of complex formation among lipooligosaccharide (LOS), MD-2, and TLR4, by using gel-filtration chromatography. [3H]LOS aggregate (LOSagg) or [3H]LOS:sCD14 chromatography (20, 21).

Preparation of the [3H]LOSaggregate and [3H]LOS:sCD14 complex

The [3H]LOS aggregate (LOSagg) and [3H]LOS:sCD14 complex was prepared as previously described (11, 21, 22). Briefly, [3H]LOS (Mg > 20 × 106) was obtained after the heat phenol extraction of [3H]LOS, followed by the ethanol precipitation of [3H]LOSagg and ultracentrifugation. Monomeric [3H]LOS:sCD14 complex (Mm ~60,000) were prepared via the treatment of [3H]LOSagg for 30 min at 37˚C with saucithiocephyrin IPS-binding protein (molar ratio 100:1 LOS:IPS-binding protein) and 1–1.5 × molar excess of sCD14, followed by gel-exclusion chromatography (Sephacryl S200, 1.6 × 70 cm column) in PBS buffer (pH 7.4) and 0.03% HSA to isolate monomeric [3H]LOS:sCD14 complex. The radiochemical purities of [3H]LOSagg and [3H]LOS:sCD14 were confirmed via Sephacryl S200 (LOSagg) or S200 ([3H]LOS:sCD14) chromatography (20, 21).

Production of secreted MD-2/TLR4ECD and its reaction with the [3H]LOS:sCD14 complex

HEK293T cells were plated in the six-well plate with 10% FBS in DMEM. On the next day, these cells were transfected with either an expression plasmid encoding MD-2 alone or cotransfected with expression plasmids encoding MD-2 and TLR4ECD by using PolyFect reagent (Qiagen). After 12–16 h, 1.5 ml serum-free medium (293; Invitrogen) plus 0.1% HSA (2 × 106) was added. The serum-free medium was spiked with [3H]LOS:sCD14 at the time of the addition of the medium to the transfected cells to permit the reaction of MD-2 ± TLR4ECD with [3H] LOS:sCD14 upon secretion. Reaction products were analyzed via Sephacryl HR S200 (1.6 × 70 cm) chromatography in PBS. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min at room temperature by using an AKTA Purifier or Explorer 100 fast protein liquid chromatography (GE Healthcare). Radioactivity in the collected fractions was analyzed via liquid scintillation spectroscopy (Beckman LS liquid scintillation counter; Beckman Coulter). Recoveries of [3H]LOS were ≈70% in all cases. All solutions used were pyrogen-free and sterile-filtered.

HEK293 cell activation assay and luciferase reporter assay

HEK293 cells were seeded into 96-well Costar plates (Corning) at 5 × 104 cells/well and incubated overnight in a humidified atmosphere (5% CO2) at 37˚C. The next day, the cells were cotransfected with pUNO-mTLR4-HA and pEFBOS-mMD-2-FLAG-His, together with NF-κB-dependent (or IFN-β-dependent) luciferase and constitutive Renilla reporter plasmids, by using JetPEI (Polyplus Transfection). The following day, the cells were stimulated with TLR4 agonists for 16 h. The cells were lysed in 1× reporter assay lysis buffer (Promega) and analyzed for reporter gene activities by using a dual-luciferase reporter assay system on a Mithras LB940 luminometer. Relative luciferase activity was calculated via normalizing each sample’s luciferase activity for constitutive Renilla activity, which was measured within the same sample. When plotting the data, the value of the control (wt TLR4/MD-2 stimulated with LPS) was normalized to 100, and other values were adjusted accordingly.

Site-directed mutagenesis

All mutations were introduced into the pEFBOS-mMD-2-FLAG-His and pUNO-mTLR4-HA plasmids by using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Several clones that resulted from the mutational procedure were sequenced to confirm the mutation. Primer sequences will be made available upon request.

Immunoblotting

We expressed mTLR4 or mTLR4 mutants in HEK293T cells, which do not express mTLR4 without transfection with expression plasmids encoding TLR4 (21, 23). HEK293T cells were therefore transiently transfected with wt or mutant TLR4 by using PolyFect (Qiagen) as a transfection reagent. After 24 h, the cells were lysed in isyfer buffer (50 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], 1 mM EGTA [pH 8], 150 mM NaCl, 1% Triton X-100, 1% Na-DOc, 10% glycerol, 1 mM Na2VO4, 50 mM NaF, and 1 mM β-glycerophosphate) containing a mixture of protease inhibitors (Roche). The samples were then mixed with a DTT-containing loading buffer and resolved on SDS-PAGE electrophoresis (Bio-Rad mini gel system; Bio-Rad) via a 10% acrylamide gel (Tris/HEPES/SDS buffer) and transferred to a nitrocellulose (NC) membrane. The NC membrane was washed with TBS (pH 7.5) containing 0.05% Tween-20 and 0.2% Triton X-100 (TBST) and then blocked with 3% BSA in TBST for 1 h at 25˚C to reduce nonspecific background. To detect hemagglutinin (HA)-labeled wt and mutant mTLR4, an anti-HA Ab (rabbit anti-HA tag; Sigma-Aldrich; 1:2000 dilution) was used. We incubated the NC membrane with the anti-HA Abs in TBST overnight. After washing with TBST, the blot was incubated with goat anti-rabbit IgG-HRP Abs (Abcam; 1:4000 dilution) for 1 h at 25˚C in TBS and washed with TBST exhaustively. Blots were developed using the Pierce SuperSignal substrate system (Pierce).

Molecular docking

The structures of the ligands paclitaxel, docetaxel, and lipid IVa were taken from the crystal structures or built using polar hydrogen atoms and docked into the structures of hMD-2, and mouse MD-2. The tertiary structures of MD-2 were taken from the inactive (hMD-2 with lipid IVa [2ES9] and hMD-2 with myristates [2ES6]) or active complexes (hMD2 with LPS and hTLR4 [3FXI] and mMD-2 with lipid IVa or LPS and mTLR4 [3VQ1, 3VQ2]). The end of the hydrophobic pocket of MD-2 was filled with the residing fatty acids in the same orientation as in the Protein Data Bank entry 2E56 (24). Ligands were built using ISIS Draw 2.4 (ACSLab Software) and geometry optimized with Hyperchem (Hearne Scientific Software). Docking was performed using the AutoDock Vina algorithm, which has recently been shown to have superior docking accuracy and speed for docking small ligands to proteins (25). For ligand docking to TLR4/MD-2 complex, the Autogrid grid point spacing was set to 0.375 Å, center coordinates of the grid box were 12.972, −10.395, and −1.168 (x, y, and z), and number of grid points in x, y, and z were 26, 26, and 26, respectively. MD-2 and TLR4 were treated as a rigid molecular, whereas the ligand was allowed to sample torsions around all rotatable bonds. The recommended scoring function of the PDBbind set and the default optimization parameters for the iterated local search global optimizer of Vina were used. Twenty docking modes
Results

Identification of hydrophobic residues in the mTLR4ECD required for LPS activation

Point mutations within the hTLR4ECD (i.e., F440A and F463A) confirmed the essential role of these hydrophobic residues in the LPS-induced activation of hTLR4/MD-2 (7), which was corroborated by the crystal structure of the complex (5). We replaced each of those two phenylalanine residues in the ectodomain of mTLR4 (F438 and F461), corresponding to F440 and F463, respectively, of the hTLR4 and tested the response to different TLR4/MD-2 agonists (Fig. 1), starting with LPS. In contrast to hTLR4 mutants (F440A and F463A) (7), each of the corresponding mTLR4 mutants (F438A and F461A) retained a considerable extent (20–50%) of the response of wt TLR4 to LPS (Fig. 2A, 2B). Expression levels of wt and mutant mTLR4 were comparable (Fig. 2C), indicating that differences in TLR4-dependent cell activation reflect differences in the response of wt versus mutant mTLR4 to LPS. Double mTLR4 mutation mF438A/F461A completely abolished activation with LPS (Fig. 2A, 2B). Similar findings were obtained when single and double mTLR4 mutants were coexpressed with mouse (Fig. 2A) or hMD-2 (Fig. 2B), although the decrease of activation of the F438A TLR4 mutant on LPS responsiveness of mTLR4 was somewhat greater when mTLR4 was coexpressed with mMD-2.

TLR4ECD phenylalanine mutants impair formation of TLR4/MD-2/LPS complex

Crystal structure analysis indicated that LPS-induced TLR4 activation depends on the formation of dimers of TLR4/MD-2/LPS complexes in which F440 and F463 in hTLR4 and F438 and F461 in mTLR4 contribute to hydrophobic contacts between bound LPS and MD-2 in one ternary complex with TLR4 of the second complex (5). We have previously shown that formation of dimers of ternary complexes containing TLR4ECD (Mr ~190,000) can be monitored by gel-sieving chromatography of conditioned culture medium containing TLR4ECD and MD-2 secreted from transiently transfected HEK293 cells and [3H]LOS:sCD14 as the endotoxin donor (7). We used this assay to determine if the reduced ability of the F438A ± F461A mutants of mTLR4 to be activated by LPS is...
due to differences in the formation of a dimeric ternary complex. We therefore compared the ability of wt versus single (F438A) or double (F438A F461A) mTLR4ECD mutants to form dimeric ternary complexes with MD-2 and [3H]LOS. Fig. 3 shows that when mTLR4ECD was coexpressed with mMD-2 (Fig. 3A) or hMD-2 (Fig. 3B), formation of the $M_r \sim 190,000$ complex was greatest with wt mTLR4ECD. The amount of the complex was reduced by 50–70% with F438A mTLR4ECD and virtually absent with the double mutant of mTLR4ECD (F438A F461A). These findings closely correspond to the effect of these mTLR4 mutations on TLR4/MD-2 responsiveness to LPS (Fig. 2) and, hence, support the view that these phenylalanine residues in mTLR4 are important for formation of TLR4-activating dimers of TLR4/MD-2/LPS.

**Replacement of hydrophobic residues F438 and F461 of mTLR4 abolish lipid IVa and paclitaxel, but not LPS activation**

Recent studies (18, 19) suggested a prominent role of charge-based interactions between bound lipid IVa and mTLR4/MD-2 in the agonist effects of lipid IVa. As the role of hydrophobic interactions in lipid IVa-induced activation of mTLR4/MD-2 has not been addressed previously, we compared activation of wt and the mF438A and mF461A mTLR4 mutants by lipid IVa. Either of these mTLR4 single-residue mutations resulted in a complete loss of activation of mTLR4 by tetraacylated lipid IVa, in contrast to only partial reduction in activation by hexaacylated lipid A (Fig. 4A) or LPS (Fig. 2). The addition of lipid IVa to the activating lipid A resulted in inhibition of TLR4 activation (Fig. 4A). This is consistent with retained binding of lipid IVa and lipid A, whereas induction of receptor dimerization and activation only occurred with the hexaacylated lipid A. These findings clearly demonstrate the essential role of hydrophobic interactions mediated by both F438 and F461 in activation of wt mTLR4/MD-2 by lipid IVa.

Selective activation of mTLR4/MD-2 complex with antitumor agent paclitaxel is even more puzzling, as paclitaxel does not possess any charged functional groups. Therefore, hydrophobic and van der Waals interactions must determine the mTLR4/MD-2 activation by paclitaxel. This is supported by our finding that activation by paclitaxel was completely abolished in each of the mTLR4 point mutants (Fig. 4B), similar to the activation by lipid IVa. Therefore, hydrophobic interactions involving both F438 and F461 side chains of mTLR4 with MD-2/paclitaxel complex are also required for the activation by paclitaxel.

**TLR4ECD mutations F438A and F461A impair activation of MPLA and pentaacylated msbB**

The requirement for both F438 and F461 residues in mTLR4 for activation of mTLR4/MD-2 by weak agonists (i.e., lipid IVa and paclitaxel) but not by stronger agonists (i.e., hexaacylated lipid A and LPS) suggested that this could be true for other lipid A–related weak TLR4 agonists as well. MPLA is a synthetic endotoxin with six acyl chains (Fig. 1). It is used as a vaccine adjuvant and has been proposed to preferentially stimulate the Toll/IL-1R domain-containing adapter inducing IFN-β signaling pathway of TLR4 (27). In TLR4/MD-2–transfected HEK293 cells, we observed activation of NF-κB– and IFN-β–responsive promoters, indicating activation of both branches of the TLR4 signaling pathway in HEK293 cells. Cell activation of both F438 and F461 mTLR4 mutants by MPLA was reduced to a slightly larger degree in comparison with the wt (Fig. 5A, 5B), although the difference in activation with LPS was not statistically significant for the IFN-β pathway. Based on the crystal structure of the TLR4/MD-2/LPS dimer, the phosphate group that is absent in MPLA does not interact with side chains of F438 and F461 (5). We also investigated the effect of F438 and F461 TLR4 mutations on activation by pentaacylated LPS msbB, which is a weaker TLR4 agonist than...
hexaacylated LPS. Cell activation with pentaacylated msbB was abolished in each of the two single-point mutants of the TLR4ECD (Fig. 5C). Thus, in contrast to activation of mTLR4/MD-2 by hexaacylated LPS and MPLA, activation by pentaacylated LPS requires both F438 and F461, similar to activation by lipid IVa. Residue F126 of mouse MD-2 is indispensable for activation by paclitaxel, but not for activation by lipid IVa. Hydrophobic interactions of the TLR4/MD-2 receptor complex with LPS are exerted primarily through MD-2. F126 is the residue that exhibits the largest conformational shift upon binding of agonist or antagonist (5, 9). It lays at the edge of the lipid A binding pocket in close proximity to the secondary binding site of the TLR4ECD. The substitution of F126 of hMD-2 with alanine does not affect either high-affinity binding of LPS to MD-2 or agonist-independent binding of MD-2 to TLR4 (14) but nearly completely prevents TLR4 dimerization and activation of both human and mouse complex (28). F126 in MD-2 is highly conserved among vertebrates, with the exception of rabbit MD-2, which contains a leucine residue at this position. Because rabbits are highly sensitive to endotoxin, we hypothesized that a F126L mutation of mMD-2 should not reduce the responsiveness of mTLR4/MD-2 to lipid A or LPS. Indeed, comparison of activation of cells expressing wt mTLR4 and wt or F126L mMD-2 by LPS as well as by lipid IVa demonstrated even greater activation of mTLR4/F126L mMD-2 than of wt mTLR4/MD-2 (Fig. 6A). Moreover, the F126L mutation in mMD-2 had no significant effect on the activation of mTLR4/F126L with MPLA or msbB (Fig. 6B). In marked contrast, expression of the F126L mutant of mMD-2 nearly completely suppressed activation of mTLR4/MD-2 by paclitaxel (Fig. 6C). Moreover, addition of a mixture of activating lipid A and nonactivating paclitaxel to cells expressing mTLR4/F126L mMD-2 yielded an intermediate level of activation. This is consistent with binding of paclitaxel to F126L MD-2 without triggering TLR4/MD-2 dimerization and thus causing inhibition (Fig. 7).

Discussion

In this report, we wanted to investigate the role of hydrophobic residues at key positions of TLR4ECD and MD-2 that interact with acyl groups of TLR4 ligand to improve the understanding of the molecular basis of the selective mTLR4/MD-2 agonist properties. Some bacteria synthesize hypoacylated LPS either constitutively or under defined conditions, such as the switch to the temperature of the warm-blooded host, which allows them to restrain activation of the innate immune response [e.g., Yersinia pestis (29)]. Organisms that recognize tetraacylated lipid A are therefore less vulnerable to this type of bacterial evasion of the innate immune response. The crystal structures of the TLR4/MD2/LPS complex...
FIGURE 5. NF-κB– and IFN-β–mediated response of mF438A and mF461A mutants on the TLR4 ECD stimulated with MPLA and pentaacylated LPS (msbB). MPLA, which lacks one of two phosphate groups, activates mF438A and mF461A through NF-κB (A) and IFN-β (B). (C) Pentaacylated msbB does not activate mF438A and mF461A. In all cases, activation was measured via dual luciferase assay of HEK293 cells transiently transfected with expression plasmids encoding wt or mutant TLR4, wt MD-2, and luciferase reporter plasmids. Each experiment shown is representative of at least three independent experiments. *p ≥ 0.05 (not significant), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-tailed t test; compared with the unstimulated control). RLA, Relative luciferase activity.

(5, 9) and targeted mutagenesis studies (8, 23) identified the receptor residues mediating the LPS-induced dimerization of the receptor complex but we still do not understand well why the receptor activation by lipid IVa and paclitaxel coincide. Although most of the hydrophobic residues within the secondary interaction site of TLR4 contributing to LPS-induced receptor activation are

FIGURE 6. Amino acid residue F126 of mouse MD-2 is essential for the paclitaxel, but not for the s-LPS and lipid IVa–triggered activation of TLR4. The solvent-exposed residue F126 in mMD-2 was replaced either with leucine or valine, and activation was measured via dual luciferase assay of HEK293 cells transiently transfected with plasmids encoding mouse wt TLR4, mouse wt or mutant MD-2, and luciferase reporter plasmids. Activation of wt mMD-2 and mutants mF126L and mF126V with s-LPS and lipid IVa (A), MPLA and pentaacylated LPS (msbB) (B), and paclitaxel (C). Each experiment is representative of at least three independent experiments. *p ≥ 0.05 (not significant), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-tailed t test; compared with the unstimulated control [directly above the bar] or to the s-LPS–stimulated cells [above the bracket]). RLA, Relative luciferase activity.
conserved and may thus seem less likely candidates to help elucidate the differences in activation between different TLR4 agonists, we focused our experiments on ligand-interacting hydrophobic residues of TLR4 and MD-2. We found that replacement of either one of the F438 or F461 of the mTLR4ECD with alanine reduced, but did not eliminate, mTLR4/MD-2 activation by LPS. LPS activation was abolished only when both residues were replaced (Fig. 2A). This is in contrast to the effects of analogous mutations in hTLR4, where replacement of a single phenylalanine residue at positions F440 or F463 was sufficient to preclude activation by LPS (7). This finding suggests that interactions within the mTLR4/MD-2/LPS activation complex are stronger; therefore, deletion of a contribution of a single phenylalanine residue is not sufficient to prevent the receptor dimerization. This is consistent with evidence from the crystal structures of TLR4/MD-2/LPS complexes that indicate higher numbers of interaction sites within the dimerization interface of mouse in comparison with hTLR4/MD-2-LPS complexes (5, 12). In the recently determined crystal structure of the mouse receptor complex TLR4/MD-2 with lipid IVa and LPS, both ligands adopted very similar conformations, with partial solvent exposure of the R2 chain of the lipid IVa or lipid A, allowing its interaction with the secondary binding site of the TLR4ECD (12). F461 interacts in both complexes with the hydrophobic loop of MD-2, comprising residues at positions 82, 85, and 87, which have to remain hydrophobic to support activation as we have shown before (7). Those interactions are invariant also in the human complex. The difference between lipid IVa and lipid A in hMD-2 occurs because the three terminal methylene groups of the R2 chain are exposed to the solvent and can form the hydrophobic interaction with TLR4 F438 in case of lipid A, whereas those groups are buried inside the MD-2 binding pocket and do not contribute to direct interaction with TLR4 in case of lipid IVa. Hexaacylated lipid A–mediated interaction with TLR4 is therefore stronger than interaction mediated by tetraacylated lipid IVa. In hMD-2, the interaction with hexaacylated lipid A is similar to the mMD-2, whereas lipid IVa is buried deeper inside the hMD-2 binding pocket and does not expose the R2 chain to the solvent.

In the crystal structures of mTLR4/MD-2 complexes, lipid IVa and hexaacylated Ra-LPS bind in the same orientation as Ra-LPS in the complex with hTLR4/MD-2, whereas lipid IVa bound to hMD-2 is flipped by ∼180°. Lipid IVa acts as an agonist in combination with wt mTLR4 and as an antagonist in combination with mTLR4 mutants F438A or F461A, suggesting that the orientation of the lipid IVa bound to the MD-2 is the same in both cases. The reason for antagonism of lipid IVa in case of F438A or F461A mutants is that both of the phenylalanine residues are...

FIGURE 7. Molecular model of mTLR4/MD-2 activation with the hTLR4/MD-2 antagonist paclitaxel: the cross-linking of two TLR4/MD-2/paclitaxel complexes depends on hydrophobic protein–lipid interactions. (A and B) wt mMD-2 and mF126L mutant with bound paclitaxel. The crucial amino acids, F126 in wt and L126 in the mutant mMD-2, are shown in yellow. The docking of paclitaxel on either wt mMD-2 or the mF126L mutant results in a significantly different position of paclitaxel (green versus orange). (C) A ribbon representation of the mTLR4 and MD-2 heterocomplex (in tan and gray, respectively) with bound taxol. In both cases, the remainder of the MD-2 hydrophobic cavity was filled with myristate acyl chains (not depicted). The phenylalanines 438 and 461 of mTLR4 are shown in purple and pink, respectively. Figures were prepared with the University of California San Francisco Chimera package.
required to stabilize the dimeric complex with lipid IVa. Weaker lipid IVa–mediated complex is supported by the absence of di-
merization of mTLR4ECD/MD-2/lipid IVa in the solution, whereas
dimers are formed in the crystal and at the cell membrane (12).

MPLA is a less potent activator of TLR4/MD-2 than lipid A (30),
due to the absence of a single phosphate group. Mutants mF438A
and mF461A had a similar effect on the responsiveness to MPLA
as observed for lipid A (Fig. 5A, 5B), as expected because the
phosphate group does not interact directly with either F438 or
F461. In contrast, replacement of a single phenylalanine residue
also abolished activation with pentacycated LPS (Fig. 5C).
Therefore, removal of a hydrophobic residue on TLR4 had stronger
effects on lipid A variants lacking either one or two acyl chains.

Differences in the electrostatic potential of MD-2, hydropho-
bicity, and binding pocket size have been proposed to be re-
 sponsible for the species-specific activation of TLR4 by paclitaxel
(31). The apparently similar functional TLR4 agonist properties
of lipid IVa and paclitaxel are striking given the completely different
structure of these two compounds (Fig. 1). Because paclitaxel is
very hydrophobic and does not possess any charged groups,
nonpolar interactions must play a dominant role in its activation
of mTLR4/MD-2 complex. We found that both phenylalanine resi-
dues at positions 438 and 461 on TLR4ECD are essential for ac-
tivation of TLR4 by paclitaxel (Fig. 4B); however, those residues
are present also in hTLR4. The explanation for this species-
specific difference is that intrinsically tighter interactions of the
mTLR4/MD-2 complex provide stronger contribution to the di-
merization where the exposed hydrophobic surface of paclitaxel
bound to MD-2 is sufficient to trigger receptor dimerization. We
proposed previously that activation of mTLR4 with paclitaxel
primarily involves hydrophobic interactions between the MD-2–
bound paclitaxel and hydrophobic residues in the TLR4ECD (13).
This is now supported by the unequivocal experimental results on
the effects of mMD2 F216 mutation. Despite preserved activation
by lipid IVa, replacement of F216 MD-2 with leucine or valine
was inactive for stimulation by paclitaxel (Fig. 6A, 6C). The differen-
ces in comparison with activation by lipid IVa indicate that the
side chain of the phenylalanine at position 126 is indispensable
only for the activation with paclitaxel as an agonist. The antago-
nistic effect of paclitaxel is clearly observed in the experiment
with F216L mutant and LPS/paclitaxel costimulation (Fig. 6C).
These data are consistent with paclitaxel retaining binding to the
mutated MD-2 and therefore inhibiting activation by LPS. Re-
placement of the F216 of mMD2 with leucine residue in fact even
potentiates activation by LPS, yet failed to be activated by pacl-
itaxel. This suggests that MD-2 has not evolved to maximize the
responsiveness to the LPS. F216 residue exhibits a significant
structural shift in crystal structures of MD-2 with bound agonists.
Although its side chain points toward the solvent in the structure
of MD-2, the F126 residue is involved in defining the specificity of the mouse complex for lipid
IVa. More positive charge of hMD-2 at the rim of the binding
pocket allows that the lipid IVa is pulled deeper into the pocket
of hMD-2, whereas in hexacylated lipid A, the additional acyl chains
retain it at the surface of the MD-2, available for the interaction
with TLR4ECD. We propose that lipid IVa activates the mTLR4/
MD-2 complex due to the intrinsically stronger interactions in
comparison with human orthologs. This stronger interaction is
also sufficient to support receptor dimerization by paclitaxel.

Acknowledgments
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Dr. Theresa Gioannini, who passed away just after the paper was accepted
for publication.

Disclosures
The authors have no financial conflicts of interest.

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