NMR Studies of Hexaacylated Endotoxin Bound to Wild-type and F126A Mutant MD-2 and MD-2-TLR4 Ectodomain Complexes

Host response to invasion by many Gram-negative bacteria depends upon activation of Toll-like receptor 4 (TLR4) by endotoxin presented as a monomer bound to myeloid differentiation mutant (F126A) MD-2 binary and ternary complexes with, respectively, wild-type or chains of LOS present in TLR4-agonistic and -antagonistic use of NMR to examine structural properties of the fatty acyl chain of each of the bound fatty acyl chains both in the binary MD-2 complex and in the ternary LOS-MD-2-TLR4 ectodomain complex. In both wild-type and mutant LOS-MD-2 complexes, one of the six fatty acyl chains of LOS is more susceptible to paramagnetic attenuation, suggesting protrusion of that fatty acyl chain from the hydrophobic pocket of MD-2, independent of association with TLR4. These findings indicate that re-orientation of the aromatic side chain of Phe126 is induced by binding of hexaacylated E, preceding interaction with TLR4. This re-arrangement of Phe126 may act as a “hydrophobic switch,” driving agonist-dependent contacts needed for TLR4 dimerization and activation.

Recognition of endotoxin (E), unique glycolipids present on the surface of Gram-negative bacteria (GNB), is pivotal for host responses to many GNB (1, 2). The ability to respond to minute (pm) concentrations of E depends on the ordered action of four extracellular and cell surface host proteins: lipopolysaccharide-binding protein (LBP), soluble (s) and GPI-linked membrane (m)-associated forms of CD14, secreted and TLR4-associated MD-2 and TLR4 (1, 3–5). Among the various TLRs, TLR4 plays the major role in recognition and response to E and is unique in that its activation leads to both MyD88-dependent (NFκB-mediated) and TRIF-dependent (interferon-mediated) cellular responses (6–11). The concerted action of LBP, CD14 and MD-2 dramatically alters the physical presentation of E, such that individual E monomers are extracted from GNB or purified E aggregates to form monomeric E-protein complexes that, at pm E concentrations, indirectly (E-CD14 with MD-2-TLR4) or directly (E-MD-2 with TLR4) engage TLR4 and cause receptor and cell activation (3, 12–14).

Recent biochemical and structural studies have significantly advanced understanding of the molecular and structural basis of ligand-receptor interaction, and how this interaction results in TLR4 activation (3, 12–17). MD-2 forms a heterodimer with TLR4 by agonist-independent binding to sites within the N-terminal and central leucine-rich repeat (LRR) domains of the TLR4 ectodomain (TLR4ecd) (15, 17). Comparison of the published crystal structures of MD-2 bound to TLR4 antagonists (lipid IVA and eritoran) to that of the agonist-driven heterologimer, [TLR4ecd-MD-2-LPS], has revealed that binding of TLR4-activating LPS to MD-2 induces additional agonist-dependent contacts of MD-2 and LPS with TLR4 involving sites within the C-terminal LRR domain of the ectodomain of a second TLR4 molecule (15–17). When tetraacylated antagonists, such as lipid IVA and eritoran, are bound to MD-2, each of the four tightly packed fatty acyl chains fits within the deep, hydrophobic cavity of MD-2 and is protected from the aqueous solvent (15, 16). In contrast, when hexaacylated LPS is bound to the MD-2-TLR4ecd heterodimer, the tail of one fatty acyl chain of LPS protrudes from the pocket of MD-2 of one ternary com-
plex leading to contacts between this fatty acyl chain and TLR4ectd of a second ternary complex (15, 17).

An apparently critical determinant for agonist (i.e. hexaacylated lipid A/endotoxin)-dependent activation of TLR4 is the presence of an aromatic or hydrophobic amino acid, most often phenyalanine 126, at the mouth of the hydrophobic cavity of MD-2 (18–20). F126A mutants of both human and murine MD-2 do not support endotoxin-dependent activation of TLR4 (21–23). Monomeric complexes of endotoxin-human (h)MD-2 wild-type (wt) and endotoxin-hMD-2 F126A display the same high affinity (Kd ~ 100–200 pm) for TLR4, but the mutant complex has ≤ 1% the TLR4 agonist activity of the wt complex and is a TLR4 antagonist (12, 13, 22). Phe126 in hMD-2 clearly plays an essential role in agonist-dependent contacts between two ligand-MD-2-TLR4 ternary complexes. Comparison of the crystal structures of MD-2 complexed with non-activating ligands (lipid IVA, eritoran, myristic acid) with the crystal structure of [TLR4ecd-MD-2-LPS]2, revealed a re-arrangement of the Phe126-containing loop (“Phe126 loop”) of MD-2 apparently driven by re-orientation of the Phe126 aromatic side chain from outside the pocket when bound to non-activating ligand, to inward toward the hydrophobic cavity when activating hexaacylated LPS was bound (15–17). As suggested by Park et al. (17), this local conformational re-arrangement in MD-2 involving Phe126 could be critical for LPS-triggered TLR4 activation by influencing the position of the fatty acyl chains of bound activating LPS and fatty acyl:TLR4 contacts that drive TLR4 dimerization and activation. In addition, the re-arrangement of the Phe126 loop could promote MD-2-TLR4 contacts at the dimerization interface.

Because the structure of a potent TLR4-activating endotoxin bound to MD-2 in the absence of TLR4 has not been reported, it is not known whether the inability of the fatty acyl chains of hexaacylated agonist lipid A to fit completely into the MD-2 pocket helps drive dimerization of LPS-MD-2-TLR4 or whether contact with the second TLR4 stabilizes the extrusion of the sixth fatty acyl chain and subsequent conformational changes in MD-2. It also is not clear if agonist-induced re-orientation of Phe126 in MD-2 is triggered by the binding of a TLR4-activating lipid A/endotoxin to MD-2 independent of TLR4 or if binding to TLR4 is required to stabilize this altered conformation.

We now report NMR studies of the acyl chain properties of hexaacylated endotoxin bound to wt or mutant F126A hMD-2 and as part of a monomeric ternary complex of E:MD-2, wt or F126A-TLR4ecd. These studies were designed to address two questions: 1) Are protrusion of a single fatty acyl chain and re-orientation of the aromatic side chain of Phe126 intrinsic properties of TLR4-activating hexaacylated E:MD-2 complexes induced before contact with TLR4? 2) Does Phe126 of MD-2 affect positioning of the acyl chains of bound hexaacylated E that may drive contacts with TLR4 at the dimerization interface leading to TLR4 activation? Key to these studies was our ability to metabolically label uniformly and efficiently the acyl chains of endotoxin with alternating [13C] and [12C] atoms and generate mg quantities of purified monomeric, functional, and stable endotoxin-MD-2, TLR4ecd, and endotoxin-MD-2 wt and F126A-TLR4ecd complexes. Results of the NMR studies indicate that protrusion of one of the six fatty acyl chains of endotoxin bound to MD-2 precedes interaction with TLR4 when endotoxin is bound to either wt or F126A MD-2. Consequently, the presence of a protruding fatty acyl chain is not a distinguishing feature of TLR4-activating E:MD-2 complexes and so is not sufficient for driving TLR4 activation. However, the position and surrounding environment of the bound fatty acyl chains in MD-2 are altered when Phe126 is substituted with alanine. In the endotoxin-MD-2 wt complex, the aromatic side chain of Phe126 is re-oriented toward the hydrophobic cavity preceding interaction with TLR4. Local rearrangements of the Phe126 loop in MD-2 may promote TLR4 dimerization and activation, by affecting both contacts between the protruded fatty acyl chain and TLR4 as well as MD-2:TLR4 contacts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Metabolically labeled [13/14C]LOS endotoxin was isolated from NMB Ace1 grown in Morse medium supplemented with 2 mM 1-[13C]2,2-[12C]-acetate (Moravek Chemicals, Brea, CA) and 1 uCi/ml of 1,2-[14C]acetate as previously described (24, 25). [13/14C]LOS contained alternating [13C] and [12C]carbon atoms starting with the [13C] terminal methyl in each fatty acyl chain of LOS (Fig. 2D) and had sufficient radioactivity (15 cpm/pmol LOS) to monitor purification and facilitate quantitative analysis. To minimize effects of possible endotoxin heterogeneity, a single preparation of purified [13/14C]-LOS was used. [3H]LOS-MD-2 (25,000 cpm/pmol) was prepared as described (13, 24). Reagents used include: human serum albumin (HSA), an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA), bovine serum albumin (BSA), and other chemical reagents from Sigma, and chromatography matrices (GE Healthcare, Piscataway, NJ). Purified Gd(DPTA-BMA) was a gift from Dr. Klaus Zangger, University of Graz, Graz, Austria.

**Production of Recombinant WT and F126A hMD-2 and TLR4 Ectodomain**—As previously described (12, 13), cDNA encoding human MD-2 wt, F126A, or TLR4 ectodomain, amino acids 24–631, was inserted using SacII and XhoI restriction sites into pBAC3 (Novagen) that provides secreted protein expression sites into pBAC3 (Novagen) and 1 uCi/ml of 1,2-[14C]acetate as previously described (24, 25). [13/14C]LOS contained alternating [13C] and [12C]carbon atoms starting with the [13C] terminal methyl in each fatty acyl chain of LOS (Fig. 2D) and had sufficient radioactivity (15 cpm/pmol LOS) to monitor purification and facilitate quantitative analysis. To minimize effects of possible endotoxin heterogeneity, a single preparation of purified [13/14C]-LOS was used. [3H]LOS-MD-2 (25,000 cpm/pmol) was prepared as described (13, 24). Reagents used include: human serum albumin (HSA), an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA), bovine serum albumin (BSA), and other chemical reagents from Sigma, and chromatography matrices (GE Healthcare, Piscataway, NJ). Purified Gd(DPTA-BMA) was a gift from Dr. Klaus Zangger, University of Graz, Graz, Austria.

**Preparation of LOS:MD-2 Complexes**—[13/14C]LOS-albumin complexes were generated and purified from [13/14C]LOS-aggregates solubilized in buffer A (100 mM Tris-HCl 5 mM EDTA, pH 8.0) as previously described (26). LOS-albumin complexes (1 mg LOS, 30 ml) were diluted 10-fold with buffer A and incubated with 2 liters of conditioned insect medium containing His6-sMD-2, wt or F126A (~1 µg/ml active, monomeric sMD-2) for 3 h at 37 °C. An aliquot of the reaction mixture was analyzed on Sephacryl S200 to measure generation of [13/14C]-LOS:MD-2. The reaction mixture was dialyzed (Spectrapor1) against buffer B (20 mM phosphate, 0.5 mM NaCl, 5 mM imidazole, pH 7.6) and loaded onto Ni FF Sepharose (5 cm × 25 cm) at 4 °C.
at a flow rate of 3 ml/min (GE Healthcare Explorer FPLC). After washing the column with 20 mM imidazole in buffer B, the adsorbed material was eluted by an imidazole gradient in buffer B. Gradient fractions containing radioactive LOS were combined, concentrated to ~2 ml (Millipore Centricon-70), and applied to Sephacryl S100 (1.6 × 100 cm) equilibrated in buffer C (20 mM phosphate, 150 mM NaCl, pH 7.1). Radioactive fractions consistent with the M_r of LOS-MD-2 were combined and concentrated to ~1 ml (Millipore Centricon MWCO 10K). Purity of the samples was confirmed by Coomassie Blue stain of 10–15% gradient PhastGels (GE Healthcare). Each LOS-MD-2 complex analyzed by NMR was derived from reactions of 12–14 liters of conditioned medium containing MD-2. The final samples (1 ml) contained 240 and 260 μM of LOS-MD-2 wt and F126A, respectively. \(^{13}C\)LOS-MD-2 was prepared by the identical procedure described above.

The functional activity of purified LOS-MD-2 complexes was measured by TLR4-dependent cell activation. HEK293 cells, parent, or TLR4-containing, were seeded in a 96-well plate (1 × 10^4 cells/well) in DMEM/10% FBS and incubated overnight at 37 °C in 5% CO_2/95% humidity. The next day, cells were washed twice with PBS, pH 7.4. Increasing doses of purified LOS-MD-2 wt and F126A were added in 200 μl of DMEM/0.1% HSA. After 18 h at 37 °C, cell supernatants were collected and tested for accumulation of extracellular IL-8 by ELISA (BD Biosciences).

Preparation of His\textsubscript{6}-TLR4ecd and LOS-MD-2-HisTLR4ecd Complexes—Conditioned insect cell medium (2–3 liters) containing His\textsubscript{6}-TLR4ecd was dialyzed overnight against buffer B at 4 °C and then applied to Ni FF Sepharose (5 ml) in DMEM/10% FBS and incubated overnight at 37 °C. The next day, cells were washed twice with PBS, pH 7.4. Increasing doses of purified LOS-MD-2 wt and F126A were added in 200 μl of DMEM/0.1% HSA. After 18 h at 37 °C, cell supernatants were collected and tested for accumulation of extracellular IL-8 by ELISA (BD Biosciences).

Production and Purification of \(^{13}C\)LOS-MD-2—To better define the effect of Phe\textsuperscript{126} of MD-2 on the positioning of the fatty acyl chains of bound hexacylated endotoxin, we produced and purified \(^{13}C\)endotoxin-MD-2 complexes using wt and F126A hMD-2. Metabolic labeling of the endotoxin (lipooligosaccharide, LOS) of a Neisseria meningitidis serogroup B Acet1 mutant during growth in Morse medium supplemented with 2 mM 1-[\(^{13}C\)]acetate resulted in \(^{13}C\) labeling of alternating C atoms in each fatty acyl chain, starting with the terminal methyl group of each fatty acid. Trace amounts of \(^{14}C\)acetate (one part in 200 of \(^{12}/^{13}C\)acetate) were added to the growth medium to yield sufficient radiolabeling of LOS (15 cpm/pmol) to facilitate quantitative monitoring of purification and recovery of desired products. All of the experiments described here were done with a single preparation of purified LOS to preclude sample-to-sample differences due to endotoxin heterogeneity.

Monomeric LOS-MD-2 complexes are most efficiently generated by LBP-catalyzed extraction and transfer of LOS monomers from LOS aggregates to sCD14 and subsequent transfer of monomeric LOS from sCD14 to (s)MD-2 (3, 12, 26). The amounts of \(^{13}C\)LOS-MD-2 needed for NMR analysis would have necessitated production of at least 25 mg of recombinant sCD14. Therefore, we took advantage of an alternative method we have recently developed for generation of LOS-MD-2 in high yield independent of (s)CD14 (26). This alternative method depends upon generation of monomeric endotoxin-albumin complexes during overnight incubation of E aggregates in a divalent cation-depleted environment supplemented with albumin. The resultant E-albumin complexes are efficient donors of E monomers to insect cell-derived His\textsubscript{6}-TLR4ecd containing TLR4ecd.

NMR Spectroscopy—All NMR samples were exchanged into 10 mM sodium phosphate, pH 6.5, in 100% D_2O. The LOS-MD-2 complexes (~250 μM) were stable at 4 °C for at least 6 months as judged by NMR profile and size exclusion chromatography. Aliquots of the LOS-MD-2-TLR4ecd samples were chromatographed after NMR to check for stability. NMR spectra were collected at 25 °C on a Bruker Avance II 800 MHz NMR spectrometer equipped with a sensitive TCI Cryoprobe. High resolution \(^{13}C/H\) HSQC spectra (28) of the \(^{13}C\)H\textsubscript{2} region were collected to provide maximal resolution of the individual LOS \(^{13}C\)H\textsubscript{2} groups. Relative solvent/surface exposure of individual LOS \(^{13}C\)H\textsubscript{2} groups in LOS-MD-2 complexes was examined using the PRE NMR method (29–31) by acquiring \(^1H\) T_2 relaxation rates of the LOS \(^{13}C\)H\textsubscript{2} groups of the \(^{13}C\)LOS-MD-2 complexes in the absence and presence of a neutral chelating gadolinium paramagnetic agent Gd(DPTA-BMA), 2–16 mM, as previously described (29–31). The \(^1H\) T_2 Carr-Purcell-Meiboom-Gill (CPMG) delays used in these experiments were 0.92, 14.71, and 27.58 ms. NMR spectra were processed with the NMRPipe package (32) and analyzed using NMRView software (33).
The identical procedure produced in similar yield and purity LOS/H18528 MD-2 F126A when insect cell conditioned medium containing His6-MD-2 F126A was used. In contrast to LOS/H18528 MD-2 wt, the purified LOS/H18528 MD-2 F126A complex did not activate HEK/TLR4 cells (Fig. 1 D) and acted as a TLR4 antagonist (data not shown).

NMR Analyses of WT and Mutant [13C]LOS—High resolution 13C/1H HSQC spectra acquired on a 800 MHz NMR spectrometer for the 13CH3 region of [13C]LOS complexed to wt and F126A MD-2 provided optimal resolution of the six acyl chain 13CH3 groups (labeled as M1-M6) (Fig. 2, A and B). Interestingly, M1-M4 signals are clustered together, while M5 and M6 formed a separate cluster, reflecting the distinct local environment surrounding each 13CH3 group of LOS when bound to MD-2. To ascertain that these crosspeaks were all derived from [13C]LOS and not from 13C natural abundance signals of MD-2, [12C]LOS-MD-2 wt complex was prepared. NMR spectra collected under identical conditions for this control sample showed that there were virtually no detected crosspeaks in this region (data not shown), thus confirming that the observed signals in Fig. 2, A and B were derived specifically from the fatty acyl chains of [13C]LOS bound to MD-2. Overlay of the spectra of the 13CH3 region of [13C]LOS-MD-2 wt and F126A revealed significant chemical shift perturbation of all LOS 13CH3 groups with the F126A mutation (Fig. 2C and supplemental Table S1). When the high resolution HSQC spectra of these complexes were plotted at a lower contour level, additional crosspeaks likely corresponding to minor forms of bound LOS were observed. (supplemental Fig. S1, A and B). These minor forms could be due to a reverse orientation of the lipid A backbone of the bound pseudosymmetric LOS (Fig. 2 D) as suggested by recent molecular modeling studies of LOS-MD-2 complexes (34). Overlay of these lower contour spectra revealed that the peak positions for these minor forms were also significantly different between these two complexes, i.e. they experienced significant chemical shift perturbation by the F126A mutation of MD-2 (supplemental Fig. S1 C). Phe126 is located in a loop adjacent to the entry of the LOS-binding pocket on MD-2. This loop has a large B-factor (17) and is mobile as shown by molecular dynamics simulation (34). The Phe126 side chain is completely exposed when tetraacylated LOS binds to MD-2 (an inactive complex) (15, 16). The observed significant chemical shift perturbation of the hexaacylated LOS 13CH3 groups upon F126A mutation strongly suggests that the Phe126 side chain has reoriented toward the bound LOS to interact with the fatty acids of the acyl chains of the lipid A backbone of the bound pseudosymmetric LOS (Fig. 2 D).
acyl chains and this re-orientation of the aromatic side chain in MD-2 was induced by the bound, TLR4-activating hexaacylated LOS before interaction with TLR4. The focus of additional analyses presented below is on the major form of the bound LOS.

Comparison of the crystal structures of hexaacylated LPS-MD-2-TLR4ecd and tetraacylated lipid A-MD-2-TLR4ecd complexes revealed that in the complex containing activating hexaacylated LPS, one of the six fatty acyl chains partially protrudes from the hydrophobic cavity of MD-2, permitting fatty acyl:TLR4 contacts that are apparently important in TLR4 dimerization and activation (15–17). Our ability to identify by high-resolution 13C/1H HSQC the crosspeaks in the methyl region of LOSwt and F126A complexes permitted us to probe by paramagnetic relaxation enhancement (PRE), whether a single, more surface exposed fatty acyl chain is: 1) a distinguishing characteristic of TLR4-activating endotoxin (LOS)-MD-2 complexes manifest before interaction with and dimerization of TLR4 and/or 2) dependent on Phe126 of MD-2. The relative surface exposure of the individual 13CH3 groups was determined using PRE by measuring the 1H T2 relaxation rates of the LOS 13CH3 groups in the presence and absence of a neutral chelated paramagnetic gadolinium compound Gd(DPTA-BMA) (Fig. 3A) (29–31). Perturbation by Gd(DPTA-BMA) reflects the relative solvent/surface accessibility of the individual fatty acyl chains (i.e. CH3 groups) in LOSwt and F126A complexes as the less sequestered groups will be more readily affected by the paramagnetic reagent (30, 35). In both 13C/1H LOSwt and F126A complexes, five of the 13C/1H LOS methyl crosspeaks (M2-M6) showed similar PRE rates (Fig. 3B) indicating that the methyl groups have similar distances from their respective atomic coordinates to the protein surface of MD-2. However, one (M1) methyl signal in each complex had more significant PRE effects.
subsequent NMR analysis. TLR4ecd was expressed in insect cells to produce and purify ternary complexes with soluble TLR4ecd for LOS bound hexaacylated endotoxin in the ternary complex, the same. To determine the relative solvent/surface exposure of the individual $^{13}$CH$_3$ signals from each of the six lipid A fatty acyl chains, $^1$H$^2$ relaxation data of the LOS $^{13}$CH$_3$ were acquired for the individual $^{13}$C-LOS-MD-2 wt and F126A complexes in the absence and presence of 4 mM Gd(DTPA-BMA). The PRE rates, $\gamma_p$, were calculated by subtracting the diamagnetic relaxation rates $R_{2,\text{dia}}$ (obtained in the absence of the paramagnetic agent) from the paramagnetic relaxation rates $R_{2,\text{para}}$ (obtained in the presence of the paramagnetic agent) and by using data collected with $^1$H$^2$ Carr-Purcell-Meiboom-Gill (CMPG) delays of 0.92 and 14.71 ms as previously described (29). Peak names correlate with the crosspeak labels shown in Fig. 2. Sample concentrations were 240 and 260 $\mu$M for $^{13}$C-LOS-MD-2 wt and $^{13}$CLOS-MD-2 F126A, respectively. A concentrated stock solution of Gd(DTPA-BMA) was used to minimize the change in $^{13}$C-LOS-MD-2 concentration upon addition of the reagent.

(Fig. 3B) suggesting that the $^{13}$CH$_3$ group of this fatty acyl chain was less sequestered and more solvent/surface accessible, i.e. protruding out of the hydrophobic pocket of both wt and F126A MD-2, independent of LOS-MD-2 association with TLR4. These findings suggest that protrusion of one of the six fatty acyl chains is a feature of monomeric complexes of hexaacylated lipid A/endotoxin (LOS) with MD-2 that precedes interactions of this complex with TLR4. The apparent similarity of this property in LOS-MD-2 wt and F126A complexes suggests that protrusion of a single fatty acyl chain of LOS bound to MD-2 is not dependent on Phe$^{126}$ and so not sufficient for TLR4 activation.

Monomeric Ternary Complexes of $^{13}$C-LOS-MD-2-TLR4ecd—According to current theory, TLR4 activation by hexaacylated LOS bound to MD-2 is not dependent on Phe$^{126}$ and so not sufficient for TLR4 activation.

Prior to reaction with $^{13}$C-LOS-MD-2, $^{13}$C$/^1$H HSQC, and HSQC NMR spectra were collected on the concentrated sample of TLR4ecd to ensure that any crosspeaks detected due to the $^{13}$C natural abundance of the purified TLR4ecd did not overlap with $^{13}$C-LOS signals. As shown in supplemental Fig. S2, the crosspeaks derived from both the methyl (CH$_3$) and methylene (CH$_2$) groups of $^{13}$C-LOS were clearly resolved from signals due to $^{13}$C natural abundance of the purified TLR4ecd.

A molar excess (1.2×) of concentrated TLR4ecd was incubated with $^{13}$C$/^{14}$C-LOS-MD-2 (wt and F126A) for 30 min at 37 °C. Complete conversion of $^{13}$C-LOS-MD-2 to $^{13}$C$/^{14}$C-LOS-MD-2-TLR4ecd was verified by Sephacryl S300 size exclusion chromatography (Fig. 4C). Comparison of $^{13}$C$/^1$H HSQC spectra of the binary $^{13}$C-LOS-MD-2 and ternary $^{13}$C-LOS-MD-2-TLR4ecd complexes containing wt or F126A MD-2 (Fig. 5, A and B) revealed a significant chemical shift perturbation upon TLR4ecd binding for all $^{13}$CH$_3$ groups of LOS bound to MD-2. More severe and selective line broadening was observed for LOS M1-M4 $^{13}$CH$_3$ groups, which includes the $^{13}$CH$_3$ group M1 that protrudes out of the LOS-binding pocket of MD-2. Overlay of the $^{13}$C$/^1$H high resolution HSQC spectra of the two ternary complexes (Fig. 5C) showed chemical shift perturbation of the LOS $^{13}$CH$_3$ groups by the F126A mutation of MD-2 in the LOS-MD-2 F126A-TLR4ecd ternary complex, consistent with the essential role of Phe$^{126}$ in the TLR4-activating properties of the LOS-MD-2-TLR4 complex.

Comparison of $^{13}$C$/^1$H HSQC NMR spectra of the binary $^{13}$C-LOS-MD-2 versus the ternary $^{13}$C-LOS-MD-2 wt-TLR4ecd complexes showed a remarkable broadening of the
FIGURE 4. Isolation of TLR4ecd, evaluation of reactivity with LOS MD-2, and preparation of LOS MD-2-TLR4ecd complexes. A, monomeric His6TLR4ecd was isolated on Sephacryl S200 (1.6 cm × 100 cm) at 4 °C at a flow rate of 0.5 ml/min in 20 mM phosphate, 150 mM NaCl, 0.005% BSA, pH 7.1 after application of the concentrated imidazole eluate fractions that contained His6TLR4ecd recovered from the Ni FF Sepharose column. Fractions (2 ml) from the Sephacryl S200 were collected and tested for immunoreactivity with an anti-His antibody; a blot of fractions containing His6TLR4ecd is shown. B, the reactivity of the purified TLR4ecd (5 μl of the S200 peak) was evaluated by reaction with [3H]LOS MD-2 (1.1 nM) in PBS, pH 7.4. Samples were incubated in 0.5 ml for 15 min at 37 °C and reaction products were resolved on S300 Sephacryl (1.6 cm × 70 cm) in PBS, 0.1% HSA, pH 7.4. Radioactivity in eluate fractions was determined by liquid scintillation spectroscopy. The experiment shown is a representative chromatogram; elution peaks of IgG (158,000), BSA (65,000), and LOS MD-2 (25,000) are indicated. C, molar excess (1.2) of purified TLR4ecd was incubated with [13C]LOS MD-2 wt or F126A for 30 min at 37 °C; an aliquot of the reaction mixture was analyzed by size exclusion on Sephacryl S300 equilibrated in 20 mM phosphate, 150 mM NaCl, pH 7.1. The amount of radioactivity in each fraction was evaluated by liquid scintillation spectroscopy. The data shown here are representative of multiple similar experiments (n = 4).

FIGURE 5. Comparison of the 13C/1H HSQC and HMQC NMR spectra of [13C]LOS in LOS MD-2 wt and F126A binary complexes with their respective LOS MD-2-TLR4ecd ternary complexes. High resolution HSQC spectra of the LOS 13CH3 region were overlaid for (A) [13C]LOS MD-2 wt binary complex in black and [13C]LOS MD-2 wt-TLR4ecd ternary complex in red, (B) [13C]LOS MD-2 F126A binary complex in orange, and [13C]LOS MD-2 F126A-TLR4ecd ternary complex in blue, (C) [13C]LOS MD-2 wt-TLR4ecd ternary complex in red, and [13C]LOS MD-2 F126A-TLR4ecd ternary complex in blue. The LOS 13CH3 crosspeaks corresponding to the major form of bound LOS are labeled as M1-M6 as shown in panels A and B. D, HMQC spectral overlay of [13C]LOS MD-2 wt binary complex in red, [13C]LOS MD-2 wt-TLR4ecd ternary complex in black, and [13C]LOS MD-2 F126A-TLR4ecd ternary complex in green. The sample concentrations were 90, 110, and 85 μM for [13C]LOS MD-2 wt, [13C]LOS MD-2 wt-TLR4ecd, and [13C]LOS MD-2 F126A-TLR4ecd complexes, respectively. NMR crosspeaks corresponding to the LOS methyl (13CH3) and methylene (13CH2) regions are circled in the figure. The LOS methylene (13CH2) crosspeaks were significantly reduced in intensity, particularly in the [13C]LOS MD-2 wt-TLR4ecd ternary complex. An enlarged version of the methylene region of (D) at a lower contour level (to the spectral floor noise level) and with a different color scheme is represented in supplemental Fig. S3.
[\textsuperscript{13}C]Endotoxin NMR in MD-2 Complexes ± TLR4 Ectodomain

signals derived from the \textsuperscript{13}CH\textsubscript{2} (methylene) groups of LOS fatty acyl chains, especially in the ternary complex containing wt MD-2 (Fig. 5D and supplemental Fig. S3). These changes were not due to dissociation of [\textsuperscript{13}C]LOS from the ternary complex or multimerization of these complexes. After NMR analyses, the complexes were re-examined by size exclusion chromatography. The post-NMR and pre-NMR elution profiles were identical for all complexes. These subtle differences in line broadening of the LOS-derived \textsuperscript{13}CH\textsubscript{2} signals in the wt versus F126A MD-2 containing ternary complexes could reflect differential ligand dynamics in these complexes due to the mutation of Phe\textsubscript{126}.

**DISCUSSION**

Lipid A acyl chains of endotoxin bound to MD-2 were analyzed by NMR using unique reagents comprised of TLR4-activating or -inhibiting endotoxin-MD-2 complexes containing \textsuperscript{13}C-labeled endotoxin bound either to wt or F126A MD-2, respectively. [\textsuperscript{13}C]Endotoxin, in which each of the lipid A fatty acyl chains of all LOS molecules contained a terminal \textsuperscript{13}CH\textsubscript{3} group followed by alternating \textsuperscript{12}C and \textsuperscript{13}C atoms, was obtained by metabolic labeling of endotoxin (LOS) with [1,\textsuperscript{12}C], [2,\textsuperscript{13}C]acetate. The bacterial mutant (NMB Ace1) utilized labeled acetate added to the growth medium as the source of essentially all acetyl precursors needed for fatty acid synthesis (24, 25). The stability of sMD-2 in insect cell medium and its reactivity with monomeric endotoxin-albumin complexes permitted the generation of monomeric endotoxin-MD-2 complexes in high yield (26). The remarkable stability of these monomeric endotoxin-MD-2 complexes allowed the purification and subsequent concentration of the complexes as needed for NMR analyses. NMR revealed discrete differences in the \textsuperscript{13}C/\textsuperscript{1}H high resolution HSQC spectra of the wt and F126A mutant binary LOS-MD-2 and ternary LOS-MD-2-TLR4ecd complexes. These spectra were monitored to assess the microenvironment of the fatty acyl chains upon engagement of LOS with wt or F126A MD-2 and of these binary complexes with TLR4ecd. These unique reagents and analyses have permitted us to address the following two questions raised by the observations of Park et al. (17): 1) Are protrusion of a single fatty acyl chain of hexaacylated endotoxin and re-orientation of the aromatic side chain of Phe\textsubscript{126} intrinsic properties of TLR4-activating hexaacylated endotoxin-MD-2 complexes, apparent before contact of this complex with TLR4? 2) Does Phe\textsubscript{126} affect the positioning of the acyl chains of bound endotoxin and potentially promote fatty acyl:TLR4 contacts apparently needed for TLR4 activation at the dimerization interface? \textsuperscript{13}C/\textsuperscript{1}H high resolution HSQC spectra of the \textsuperscript{13}CH\textsubscript{3} groups of LOS-MD-2 wt and LOS-MD-2 F126A showed six prominent and distinct signals in each complex (Fig. 2). These six cross-peaks correspond to signals deriving from the six terminal \textsuperscript{13}CH\textsubscript{3} groups of the bound hexaacylated LOS and reflect the distinct microenvironment of each fatty acyl chain terminus when LOS is bound to either wt or F126A MD-2. For both wt and mutant complexes, five of the six \textsuperscript{13}C/\textsuperscript{1}H methyl crosspeaks showed closely similar paramagnetic relaxation enhancement (PRE) (Fig. 3), a measure of solvent/surface accessibility. One methyl group (M1) in both complexes was more susceptible to quenching (i.e. a larger PRE rate) by Gd(DPTA-BMA), indicating that this fatty acyl chain is less sequestered and presumably protruding out of the hydrophobic pocket of both wt and F126A MD-2 complexes. These findings suggest that protrusion of one of six fatty acyl chains of hexaacylated endotoxin bound to MD-2 is a structural hallmark of hexaacylated endotoxin (lipid A)-MD-2 binary complexes, independent of and preceding interactions with TLR4. Both the geometry of fatty acyl chain substitution and the length of the fatty acyl chains differ between the TLR4-activating *Escherichia coli* LOS used in the crystal structure studies of Park et al. (17) and the TLR4-activating meningococcal LOS used in these studies (Fig. 2D).

**As indicated,** \textsuperscript{13}C/\textsuperscript{1}H high resolution HSQC spectra of LOS bound to F126A MD-2 also showed one methyl group of six that was more susceptible to PRE by Gd(DPTA-BMA), i.e. less sequestered by MD-2. Since LOS-MD-2 F126A acts as a TLR4 antagonist rather than an agonist, this finding strongly suggests that exposure of a fatty acyl chain outside of the MD-2 pocket is not sufficient for TLR4 activation. Overlay of the \textsuperscript{13}C/\textsuperscript{1}H HSQC spectra of wt and F126A complexes demonstrated differences in each \textsuperscript{13}CH\textsubscript{3} group signal between the two binary complexes consistent with a distinct microenvironment of each fatty acyl chain terminus when LOS was bound to wt versus F126A MD-2. Although some of the chemical shift perturbation induced by the F126A mutation in MD-2 could be due to effects of interactions of the aromatic ring of Phe126 with nearby fatty acyl chains, the crystal structure of Park et al. (17) suggested hydrophobic contacts between the re-oriented Phe\textsubscript{126} side chain and only two of the fatty acyl chains of bound LPS. Therefore, our findings suggest a more global effect of Phe\textsubscript{126} on the surrounding microenvironment of each of the fatty acyl chains of bound LOS, i.e. an effect on the positioning of the bound LOS as a whole.

While Phe\textsubscript{126} is apparently not necessary for protrusion of a fatty acyl chain of TLR4-activating endotoxin from MD-2, Phe\textsubscript{126} may be necessary to guide/anchor positioning of a fatty acyl chain(s) in a way that optimizes fatty acyl:TLR4 contacts at the dimerization interface and/or MD-2:TLR4 contacts. The significant chemical shift perturbation observed for the LOS methyl groups upon F126A mutation (Fig. 2C and supplemental Table S1) seems most compatible with re-orientation of the Phe\textsubscript{126} side chain toward the fatty acyl chains of bound LOS within the LOS-MD-2 wt complex, suggesting that this local conformational change in MD-2 is TLR4-independent and may represent a necessary signature of TLR4-activating endotoxin-MD-2 complexes. The presence of conserved glycines on either end of the “Phe\textsubscript{126} loop” is consistent with the apparent importance of the conformational mobility of this region and the ability of MD-2 to bind and confer TLR4 agonist (or antagonist) properties on an array of endotoxin species with
[\textsuperscript{13}C]Endotoxin NMR in MD-2 Complexes ± TLR4 Ectodomain

varying numbers, distribution, and lengths of fatty acyl chains. The flexibility of this region is reflected in the crystal structure of Park et al. (17) where the least resolved structure (i.e. region of the greatest mobility) was observed in the Phe\textsuperscript{126} loop between the βG and βH strands. The NMR data presented here support the conclusions of DeMarco and Woods (34) that utilized molecular dynamics simulations to predict that hexacylated LOS or LPS bound to MD-2 in a binary complex would have one acyl chain partially accessible to solvent lying near the protein surface and that the acyl chains within the MD-2 pocket would maintain mobility.

In contrast to TLR3 which requires homodimerization to accommodate binding of dsRNA ligand (36), our findings (Fig. 4B) demonstrate that high affinity (pm) binding of E-MD-2 with TLR4 occurs between monomeric E-MD-2 and monomeric TLR4. Whether monomeric E-MD-2 is presented to TLR4 or an E monomer is presented to pre-assembled MD-2-TLR4, the initial product is a monomer of the ternary complex E-MD-2-TLR4. The E-MD-2-TLR4 product is the most proximal intermediate in endotoxin-induced TLR4 dimerization (i.e. formation of [E-MD-2-TLR4\textsubscript{2}]), when it is composed of TLR4-activating hexaacylated E, wt MD-2, and TLR4 or is the final product of TLR4 antagonists when containing either underacylated E and/or mutant (e.g. F126A) MD-2 (15–17). For reasons not yet clear (see below), reaction of either LOS-MD-2 wt or LOS-MD-2 F126A with the insect cell-derived recombinant human TLR4ecd yielded exclusively a monomeric ternary complex whether at pm-nM concentrations or >100 μM used for NMR analyses. This is in contrast to the dimeric ternary complexes previously reported when either hexaacylated E. coli LPS or meningococcal LOS was complexed to human MD-2-TLR4ecd (13, 17). Since the two hexaacylated endotoxin species have closely similar MD-2-TLR4-activating properties (12) and the LOS used in earlier studies (13) was the same as that used in this study, we believe the absence of dimerization of the LOS-MD-2-TLR4ecd ternary complex observed herein reflects structural differences between the recombinant TLR4ecd used in this study versus the earlier studies. Structural differences between the insect cell-derived His\textsubscript{6}-TLR4ecd (aa 24–631) produced in this study and the recombinant human TLR4ecd produced in earlier studies (aa 24–631 generated from a transfected HEK293 cell line (13) and aa 27–631 from insect cells (17)) that yielded dimeric ternary complexes include differences in epitope tags and the composition and extent of glycosylation correlating with an insect versus eukaryotic cell-derived protein. Whatever the pertinent variable(s), our findings underscore that the minimum structural requirements in TLR4 for agonist-induced TLR4 dimerization are not yet clear (37). For the purposes of this study, the exclusive formation of a monomeric ternary complex was fortuitous. It allowed us to demonstrate that pm interactions between E-MD-2 and TLR4ecd are achieved by agonist-independent interactions that occur entirely within a single ternary complex and to compare by NMR the lipid A fatty acyl chains of TLR4-activating and TLR4-inhibiting ternary complexes containing, respectively, wt and F126A MD-2.

Comparison of the [\textsuperscript{13}C]/H HSQC and HMQC spectra of LOS in the ternary complexes containing wt versus F126A MD-2 (Fig. 5) showed Phe\textsuperscript{126}-dependent differences that could be relevant to the different functional properties of these complexes. In both complexes, the engagement of TLR4 resulted in an apparent re-orientation of the fatty acyl chains since the acyl methyl groups overall underwent significant chemical shift perturbations both in position and linewidth of the signal. As indicated by the selective line broadening of the LOS methyl crosspeaks (Fig. 5, A and B) and loss of LOS methylene signals from the acyl chains, particularly in the wt MD-2-LOS-TLR4ecd ternary complex (Fig. 5D), the effect of TLR4 association was more pronounced overall on the wt MD-2 complex than in the F126A mutant complex. Although some of these changes could reflect altered tumbling properties of the ternary (M\textsubscript{r} ~ 100,000) versus the binary (M\textsubscript{r} ~ 25,000) complexes, the fact that these changes were more pronounced in the ternary complex containing wt MD-2 and included changes in the chemical shift of the major crosspeaks of the [\textsuperscript{13}CH\textsubscript{3}] groups suggests a further re-arrangement of the LOS fatty acyl chains within the TLR4-activating ternary complex that could be functionally important.

In summary, our findings demonstrate that Phe\textsuperscript{126} of MD-2 affects the position and surrounding environment of the bound fatty acyl chains of TLR4-activating hexaacylated endotoxin. These effects of Phe\textsuperscript{126} are manifest before interactions with TLR4 and are likely driven by hydrophobic interactions between the fatty acyl chains of the bound endotoxin and the aromatic side chain of Phe\textsuperscript{126}. Phe\textsuperscript{126} acts as a “hydrophobic switch” driving agonist-dependent recognition of TLR4-activating E-MD-2 by promoting formation of an agonist-dependent composite binding surface made up of optimally extruded endotoxin fatty acyl chain and an altered MD-2 surface conferred by re-arrangement of the Phe\textsuperscript{126} loop.

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REFERENCES
1. Beutler, B., and Rietschel, E. T. (2003) Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. 3, 169–176
2. Miller, S. I., Ernst, R. K., and Bader, M. W. (2005) LPS, TLR4, and infectious disease diversity. Nat. Rev. Microbio.l 3, 36 – 46
3. Gioannini, T. L., Teghanemt, A., Zhang, D., Levis, E. N., and Weiss, J. P. (2005) Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. J. Endotoxin Res. 11, 117–123
4. Miyake, K. (2003) Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. Int. Immunopharma.co.l 3, 119–128
5. Ulevitch, R. J., and Tobias, P. S. (1999) Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr. Opin. Immunol. 11, 19–22
6. Akira, S. (2009) Pathogen recognition by innate immunity and its signaling. Proc. Jpn. Acad. Ser. B. Phys. Bio.l Sci. 85, 145–156
7. Fitzgerald, K. A., and Chen, Z. J. (2006) Sorting out Toll signals. Cell 125, 834–836
8. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat.t Immunol. 11, 373–384
9. Ostuni, R., Zanoni, I., and Granucci, F. (2010) Deciphering the complexity of Toll-like receptor signaling. Cell Mol. Life Sci.e 67, 4109–4134
10. Yamamoto, M., and Akira, S. (2009) Lipid A receptor TLR4-mediated signaling pathways. Adv. Exp. Med. Biol. 667, 59–68
[13C]Endotoxin NMR in MD-2 Complexes ± TLR4 Ectodomain

11. Meng, J., Gong, M., Bjorkbacka, H., and Golenbock, D. T. (2011) Genome-wide expression profiling and mutagenesis studies reveal that lipopolysaccharide responsiveness appears to be absolutely dependent on TLR4 and MD-2 expression and is dependent upon intermolecular ionic interactions. J. Immunol. 187, 3683–3693

12. Gioannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., and Weiss, J. P. (2004) Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc. Natl. Acad. Sci. U.S.A. 101, 4186–4190

13. Prohinar, P., Re, F., Widstrom, R., Zhang, D., Teghanemt, A., Weiss, J. P., and Gioannini, T. L. (2007) Specific high affinity interactions of monomeric endotoxin-protein complexes with Toll-like receptor 4 ectodomain. J. Biol. Chem. 282, 1010–1017

14. Teghanemt, A., Zhang, D., Levis, E. N., Weiss, J. P., and Gioannini, T. L. (2005) Molecular basis of reduced potency of underacylated endotoxins. J. Immunol. 175, 4669–4676

15. Kim, H. M., Park, B. S., Kim, J. L., Kim, S. E., Lee, J., Oh, S. C., Enkhhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell 130, 906–917

16. Ohno, T., Fukase, K., Miyake, K., and Satow, Y. (2007) Crystal structures of human MD-2 and its complex with antiendotoxin lipid IVa. Science 316, 1632–1634

17. Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458, 1191–1195

18. Resman, N., Oblak, A., Pristovsek, P., Gioannini, T. L., Weiss, J. P., and Jerala, R. (2009) Essential roles of hydrophobic residues in both MD-2 and toll-like receptor 4 in activation by endotoxin. J. Biol. Chem. 284, 15052–15060

19. Gruber, A., Mancek, M., Wagner, H., Kirschning, C. J., and Jerala, R. (2004) Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. J. Biol. Chem. 279, 28475–28482

20. Walsh, C., Gangloff, M., Monie, T., Smyth, T., Wei, B., McKinley, T. J., Maskell, D., Gay, N., and Bryant, C. (2008) Elucidation of the MD-2/TLR4 interface required for signaling by lipid IVa. J. Immunol. 181, 1245–1254

21. Kawasaki, K., Nogawa, H., and Nishijima, M. (2003) Identification of mouse MD-2 residues important for forming the cell surface TLR4-MD-2 complex recognized by anti-TLR4-MD-2 antibodies, and for conferring LPS and taxol responsiveness on mouse TLR4 by alanine-scanning mutagenesis. J. Immunol. 170, 413–420

22. Teghanemt, A., Re, F., Prohinar, P., Widstrom, R., Gioannini, T. L., and Weiss, J. P. (2008) Novel roles in human MD-2 of phenylalanines 121 and 126 and tyrosine 131 in activation of Toll-like receptor 4 by endotoxin. J. Biol. Chem. 283, 1257–1266

23. Kobayashi, M., Saitoh, S., Tanimura, N., Takahashi, K., Kawasaki, K., Nishijima, M., Fujimoto, Y., Fukase, K., Akashi-Takamura, S., and Miyake, K. (2006) Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering. J. Immunol. 176, 6211–6218

24. Giardina, P. C., Gioannini, T., Buscher, B. A., Zaleski, A., Zheng, D. S., Stoll, L., Teghanemt, A., Apicella, M. A., and Weiss, J. (2001) Construction of acetate auxotrophs of Neisseria meningitidis to study host-meningococcal endotoxin interactions. J. Biol. Chem. 276, 5883–5891

25. Post, D. M., Zhang, D., Weiss, J. P., and Gibson, B. W. (2006) Stable isotope metabolic labeling of Neisseria meningitidis lipooligosaccharide. J. Endotoxin Res. 12, 93–98

26. Esparza, G. A., Teghanemt, A., Zhang, D., Gioannini, T. L., and Weiss, J. P. (2012) Endotoxin:albumin complexes transfer endotoxin monomers to MD-2 resulting in activation of TLR4. Innate Immun., in press

27. Teghanemt, A., Widstrom, R. L., Gioannini, T. L., and Weiss, J. P. (2008) Isolation of monomeric and dimeric secreted MD-2. Endotoxin.sCD14 and Toll-like receptor 4 ectodomain selectively react with the monomeric form of secreted MD-2. J. Biol. Chem. 283, 21881–21889

28. Lewis. K. E., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. J. Am. Chem. Soc. 114, 10663–10665

29. Iwahara, J., Tang, C., and Marius Clore, G. (2007) Practical aspects of (1)H transverse paramagnetic relaxation enhancement measurements on macromolecules. J. Magn. Reson. 184, 185–195

30. Pintacuda, G., and Otting, G. (2002) Identification of protein surfaces by NMR measurements with a paramagnetic Gd(III) chelate. J. Am. Chem. Soc. 124, 372–373

31. Respondek, M., Madl, T., Göbl, C., Golser, R., and Zanger, K. (2007) Mapping the orientation of helices in micelle-bound peptides by paramagnetic relaxation waves. J. Am. Chem. Soc. 129, 5228–5234

32. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293

33. Johnson, B. A., and Blevins, R. A. (1994) NMR View: A computer program for the visualization and analysis of NMR data. J. Magn. Reson. 106, 8259–8262

34. DeMarco, M. L., and Woods, R. J. (2011) From agonist to antagonist: structure and dynamics of innate immune glycoprotein MD-2 upon recognition of variably acylated bacterial endotoxins. Mol. Immunol. 49, 124–133

35. Madl, T., Bernel, W., and Zanger, K. (2009) Use of relaxation enhancements in a paramagnetic environment for the structure determination of proteins using NMR spectroscopy. Angew. Chem. Int. Ed. Engl. 48, 8259–8262

36. Wang, Y., Liu, L., Davies, D. R., and Segal, D. M. (2010) Dimerization of Toll-like receptor 3 (TLR3) is required for ligand binding. J. Biol. Chem. 285, 36836–36841

37. Panter, G., and Jerala, R. (2011) The ectodomain of the Toll-like receptor 4 prevents constitutive receptor activation. J. Biol. Chem. 286, 23334–23344