Specific High Affinity Interactions of Monomeric Endotoxin-Protein Complexes with Toll-like Receptor 4 Ectodomain*

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Potent Toll-like receptor 4 (TLR4) activation by endotoxin has been intensely studied, but the molecular requirements for endotoxin interaction with TLR4 are still incompletely defined. Ligand-receptor interactions involving endotoxin and TLR4 were characterized using monomeric endotoxin-protein complexes of high specific radioactivity. The binding of endotoxin-MD-2 to the TLR4 ectodomain (TLR4£CD) and transfer of endotoxin from CD14 to MD-2/TLR4£CD were demonstrated using HEK293T-conditioned medium containing TLR4£CD ± MD-2. These interactions are specific, of high affinity (K_D < 300 pm), and consistent with the molecular requirements for potent cell activation by endotoxin. Both reactions result in the formation of a M_r ~ 190,000 complex composed of endotoxin, MD-2, and TLR4£CD. CD14 facilitates transfer of endotoxin to MD-2 (TLR4) but is not a stable component of the endotoxin-MD-2/TLR4 complex. The ability to assign specific high affinity interactions of monomeric endotoxin-protein complexes with TLR4£CD should allow better definition of the structural requirements for endotoxin-induced TLR4 activation.

Essential arms of the innate immune system are the Toll-like receptors (TLRs).2 These receptors link recognition of unique microbial molecules to activation of host defense effector systems by rapidly triggering pro-inflammatory responses (1). Potent host responses toward many Gram-negative bacteria (GNB) are mediated by recognition and response to unique glycolipids (lipopoly- or lipooligosaccharides LOS, endotoxin) of the GNB outer membrane by TLR4.

TLR4 does not function alone but requires the accessory protein MD-2, which binds non-covalently to the N-terminal ectodomain of TLR4 (2–6). Maximally potent endotoxin-induced cell activation also requires the extracellular lipopolysaccharide-binding protein (LBP) and membrane (m) or soluble (s) extracellular CD14 (4, 7–9). The sequential action of LBP, CD14, secreted or TLR4-associated MD-2, and TLR4 confers the extraordinary sensitivity of mammalian cells to many GNB endotoxins. This ordered action implies differences in endotoxin binding specificity, with LBP having the highest affinity for endotoxin organized at lipid/water interfaces (e.g. purified endotoxin aggregates and endotoxin in the GNB outer membrane), CD14 for LBP-modified endotoxin-rich interfaces, MD-2 for monomeric endotoxin CD14 and TLR4, apparently, for endotoxin presented as a monomeric complex with MD-2 (8). Together, these proteins can convert one GNB (containing ~10^6 endotoxin molecules) to 10^6 TLR4-activating monomeric endotoxin-protein complexes (i.e. endotoxin-CD14 or endotoxin-MD-2), greatly amplifying host responsiveness to endotoxin. At pm concentrations, monomeric complexes of endotoxin-CD14 or endotoxin-MD-2 activate, respectively, mammalian cells expressing MD-2/TLR4 or TLR4 alone, triggering robust cell activation through engagement of <10^3 TLR4 molecules.

Despite the ability of endotoxin-CD14 and endotoxin-MD-2 to activate cells at pm concentrations (half-maximal cell activation is <50 pm), published estimates of TLR4-dependent cellular interactions of endotoxin have indicated an apparent K_D of 3 ± 3 nM (10–15). It is possible that brief occupation of a small subset of TLR4 is sufficient for robust cell activation. However, an important limitation of earlier studies has been the likelihood that much of the endotoxin added was not presented as the monomeric protein-endotoxin complex that is likely the preferred substrate for MD-2/TLR4 or TLR4 alone. In contrast to the remarkable progress in identification and characterization of the intracellular biochemical machinery responsible for TLR signaling, there is still virtually nothing known about the specificities of ligand-receptor interaction for any of the TLRs. In nearly all cases, TLR recognition has been inferred from the measurement of receptor activation, not binding, properties of specific compounds.

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‡ The abbreviations used are: TLR, Toll-like receptor; TLR4£CD, Toll-like receptor 4 ectodomain; AOAH, acyloxyacyl hydrolase; GNB, Gram-negative bacteria; HEK, human embryonic kidney; HSA, human serum albumin; LBP, lipopolysaccharide-binding protein; LOS, lipooligosaccharide; LOS_ag, LOS aggregate; PBS, phosphate-buffered saline; scCD14, soluble CD14; cpm, counts/min.

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In this study, we have made use of our ability to produce and isolate stable endotoxin-protein complexes of high specific radioactivity (~25,000 cpm/pmol) to more rigorously and directly characterize ligand–receptor interactions involving endotoxin and TLR4. For this purpose, we have expressed, in HEK293T cells, the predicted ectodomain of human TLR4 (amino acids 24–634, TLR4ECD) (16) with or without human MD-2 to permit direct assay of endotoxin interactions with TLR4ECD ± MD-2. We describe, for the first time, the direct binding of endotoxin-MD-2 complex to the predicted TLR4 ectodomain in the absence of any other cellular or extracellular co-factors and the direct transfer of endotoxin from CD14 to MD-2/TLR4ECD. These interactions are highly specific and of very high affinity ($K_d \approx 300$ pm) and fully consistent with the molecular requirements for potent mammalian cell activation by endotoxin. Both binding of endotoxin-MD-2 to TLR4ECD and transfer of endotoxin from endotoxin-CD14 to MD-2/TLR4ECD result in the formation of a stable complex that is composed of endotoxin, MD-2, and TLR4ECD, without CD14.

**EXPERIMENTAL PROCEDURES**

**Materials**—LBP and sCD14 were gifts from Xoma (Berkley, CA) and Amgen Corp. (Thousand Oaks, CA) respectively. Acylloxyacyl hydrolyase (AOAH) was a gift from Dr. R. Munford (University of Texas Southwestern, Dallas, TX). Soluble MD-2 containing a hexapolyhistidine tag on the C-terminal end was prepared as previously described (17). Human serum albumin (HSA) was obtained as an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA). [3H]LOS (25,000 cpm/pmol) from an acetate auxotroph of *Neisseria meningitidis* serogroup B was metabolically labeled and isolated as described previously (18). Chromatography matrices (Sephacryl HR S200 and S300, Ni²⁺ FF-Sepharose) were purchased from GE Healthcare (Piscataway, NJ). Anti-FLAG M2-agarose and streptavidin-agarose were purchased from Sigma.

**Production of Recombinant Protein—**HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells (~80% confluency in T75 flasks) were transfected with 12 µg of DNA using PolyFect reagent (Qiagen). After 12 h, plates were rinsed in PBS and 8 ml of serum-free medium (293 SFM, Invitrogen) was added. Medium containing expressed proteins was collected 24–48 h later. Medium was concentrated 10–20-fold using Millipore Centricon-10 before use. Conditioned medium containing purified TLR4ECD and MD-2/TLR4ECD proteins maintained activity to react with [3H]LOS:MD-2 or [3H]LOS:CD14 for at least 6 months when stored at 4 °C. Expression vectors containing the DNA of interest for production of FLAG-TLR4ECD amino acids 24–634, (pFLAG-CMV-TLR4) and MD-2-FLAG-His (pEF-BOS) have been previously described and characterized (16).

cDNA encoding MD-2 and sCD14 (1–156) were inserted (17) into pBAC11 (Novagen) using Xhol and NotI-sensitive restriction sites. This provides a 5’-flanking signal sequence (gp64) to promote secretion of protein and a C-terminal six-residue polyhistidine tag. Sf9 cells were used for transfection and amplification of baculovirus, whereas High Five cells in serum-free medium were used for protein production (17).

Conditioned medium containing secreted MD-2-His$_6$, was used directly for generation of LOS:MD-2. Conditioned medium containing sCD14 (1–156)–His$_6$ was dialyzed against 20 mM phosphate, 0.5 mM NaCl, adsorbed to Ni²⁺ FF-Sepharose equilibrated in the same buffer, washed, and the adsorbed protein eluted by an imidazole gradient (Explorer 100 fast protein liquid chromatography, GE Healthcare).

**Preparation of [3H]LOS:Protein Complexes—**[3H]LOSagg, [3H]LOS:CD14, and [3H]LOS:MD-2 complexes were prepared as previously described (8, 17, 19). Briefly, [3H]LOSagg (M$_r$ > 20 x 10$^6$) was obtained after phenol extraction of [3H]LOS followed by ethanol precipitation of [3H]LOSagg and ultracentrifugation. Monomeric [3H]LOS:CD14 complexes (M$_r$ ~ 60,000) were prepared by treatment of [3H]LOSagg for 30 min at 37 °C with substoichiometric LBP (molar ratio 200:1 of LOS:LBP) and equimolar sCD14 followed by gel exclusion chromatography (Sephacryl S200, 1.6 x 70-cm column) in PBS, pH 7.4, 0.03% HSA to isolate monomeric [3H]LOSagg, and [3H]LOS:MD-2 (30 min at 37 °C) with High Five insect cell medium containing MD-2-His$_6$ followed by isolation of [3H]LOS:MD-2 by S200 chromatography.

Tetraacylated LOS (specific activity ~16,000 cpm/pmol) was prepared by partial deacylation of [3H]LOS with AOAH according to the method of Munford and Erwin (20), and [3H]LOS:AOAH:MD-2 was prepared and isolated as described previously (21). The extent of deacylation of [3H]LOS by AOAH was monitored by separation of released [3H]-free fatty acids from partially deacylated and remaining intact [3H]LOS by ethanol precipitation. Ethanol-soluble radioactivity representing released [3H]fatty acids was analyzed by liquid scintillation spectroscopy (20, 21); ≥ 90% [3H]LOS was deacylated (21). Radiochemical purity of [3H]LOSagg, [3H]LOS:CD14, and [3H]LOS:MD-2 was confirmed by Sephacryl S500 (LOSagg) or S200 [3H]LOS:CD14, [3H]LOS:MD-2) chromatography (17, 18).

**Reaction of Secreted TLR4ECD and MD-2/TLR4ECD with [3H]LOS:Protein Complexes—**[3H]LOSagg, [3H]LOS:CD14, or [3H]LOS:MD-2 (1 nm or as indicated) was incubated with concentrated (8–10×) conditioned medium ± proteins diluted to a final volume of 0.5 or 1 ml in PBS, pH 7.4, for 30 min at 37 °C. In most experiments, conditioned medium harvested after a 48-h culture of transfected HEK293T cells in serum-free medium was used for incubations with [3H]LOSagg, [3H]LOS:CD14, or [3H]LOS:MD-2. However, in selected experiments, the serum-free medium was spiked with [3H]LOS:CD14 (1 nm) at the time of the addition of medium to the transfected cells to permit reaction of MD-2 with [3H]LOS:CD14 upon secretion. Medium harvested without [3H]LOS:CD14 is expressed (see Figs. 1 and 2–5) as recombinant protein secreted in cm, whereas medium spiked with [3H]LOS:CD14 during cell culture are represented as HEK/recombinant protein(s) secreted + [3H]LOS:CD14 (see Fig. 4). Reaction products were analyzed by Sephacryl HR S200 or S300 (1.6 x 70 cm) chromatography in PBS, pH 7.4, ± 0.5 mM Mg$^{2+}$, 1 mM Ca$^{2+}$, 0.03% HSA. Divalent cations and HSA were used in columns with LOSagg to improve recoveries of LOSagg. Fractions (0.5 ml) were collected at a flow rate of 0.3 ml/min at room temperature using AKTA Purifier or Explorer 100 fast
Endotoxin-Protein Interactions with TLR4 Ectodomain

protein liquid chromatography (GE Healthcare). Radioactivity in collected fractions was analyzed by liquid scintillation spectroscopy (Beckman LS liquid scintillation counter). Recoveries of \(^{3}H\)LOS were \(\pm 70\%\) in all cases. All solutions used were pyrogen-free and sterile-filtered. After chromatography, selected fractions were sterile-filtered (0.22 \(\mu m\)) and kept at 4 \(^\circ C\) for 3–6 months with no detectable changes in chromatographic or functional properties. The same conditioned medium was used for all concentrations of \(^{3}H\)LOS-sCD14 or \(^{3}H\)LOS-MD-2 used for Scatchard analysis. Multiple preparations of conditioned medium contained similar amounts of TLR4ECD or MD-2/TLR4ECD, i.e. 1–2 and 3–4 pmol/ml, respectively.

**Characterization of \(^{3}H\)LOS-containing Complex**

**Determination of Apparent Size**—Sephacryl S300 used for determination of apparent \(M_r\) was calibrated with the following proteins: blue dextran (2 \(\times 10^6\), \(V_g\)), thyroglobulin (650,000), ferritin (440,000), catalase (232,000), IgG (158,000), HSA (66,000), ovalbumin (44,500), myoglobin (17,500), vitamin B\(_{12}\) (1200, \(V_{t}\)). For size determination, the complex containing \(^{3}H\)LOS was resolved in the presence of at least three protein standards. Protein standards were detected by \(A_{280}\) and the \(^{3}H\)LOS-containing complex by liquid scintillation spectroscopy. \(M_r\) was calculated using GraphPad Prism version 4.

**Co-capture Analyses**—The protein composition of \(^{3}H\)LOS-containing complexes generated from incubation of \(^{3}H\)LOS-MD-2-His\(_6\) with medium containing FLAG-FLAG4ECD or of \(^{3}H\)LOS-sCD14 with medium containing MD-2-FLAG-His\(_6\) and FLAG-FLAG4ECD was determined by monitoring \(^{3}H\)LOS adsorption to resins that specifically interacted with either TLR4 or MD-2. Co-capture of \(^{3}H\)LOS associated with MD-2-FLAG-His\(_6\) (HEK293T cell-derived) or MD-2-His\(_6\) (insect cell-derived) was performed using Ni\(^{2+}\)-FF-Sepharose resin pre-equilibrated in either PBS (product containing MD-2-FLAG-His\(_6\)) or 20 \(mM\) phosphate and 0.5 \(mM\) NaCl, pH 7.4, (product containing MD-2-His\(_6\)). Resin (\(\sim 50\mu l\)) was incubated with 0.2 pmol \(^{3}H\)LOS-containing complexes for 1 h at room temperature. The resin was spun down, supernatant was removed, and the resin was washed with PBS, pH 7.4, or 20 \(mM\) phosphate, 0.5 \(mM\) NaCl, pH 7.4, before elution with 2% SDS or 0.5 \(mM\) imidazole. \(^{3}H\)LOS absorbed to the resin was evaluated by liquid scintillation spectroscopy.

Detection of FLAG-FLAG4ECD in \(^{3}H\)LOS-containing complexes was determined by adsorption to anti-FLAG-agarose (for the product of the reaction of \(^{3}H\)LOS-MD-2-His\(_6\) + (FLAG-FLAG4ECD)\(_{\text{cm}}\)) or by reaction with biotinylated HTA125 anti-TLR4 antibody followed by adsorption to streptavidin-agarose (for the product of the reaction of \(^{3}H\)LOS-sCD14 with (MD-2-FLAG-His\(_6\)/FLAG-FLAG4ECD)\(_{\text{cm}}\)). \(^{3}H\)LOS-containing complex (0.2 pmol) was incubated with 75 \(\mu l\) of anti-FLAG-agarose in 0.5 ml of buffer (PBS, pH 7.4, or 20 \(mM\) phosphate and 0.5 \(mM\) NaCl, pH 7.4) overnight at 4 \(^\circ C\) on a rotating wheel. The supernatant was collected after spinning (2000 revolutions/min, 5 min, 4 \(^\circ C\)), and the sedimented beads were washed three times with 20 \(mM\) phosphate and 0.5 \(mM\) NaCl before \(^{3}H\)LOS adsorbed to beads was determined by liquid scintillation spectroscopy. Immunocapture by anti-TLR4 anti-body of \(^{3}H\)LOS-containing complex (0.2 pmol) was performed by incubation of the complex with 2 \(\mu g\) of biotinylated HTA125 anti-TLR4 antibody (or non-biotinylated HTA125 as a negative control) in PBS, pH 7.4. After overnight incubation at 4 \(^\circ C\) with the sample, 50 \(\mu l\) of PBS-equilibrated streptavidin-coupled agarose beads were added for an additional 1 h at room temperature. Adsorbed \(^{3}H\)LOS-containing complex was determined by measuring \(^{3}H\)LOS associated with the washed agarose.

**RESULTS**

**Soluble TLR4ECD Binds \(^{3}H\)LOS-MD-2 with Picomolar Affinity**—We have previously described the generation and isolation of a monomeric \(^{3}H\)LOS-MD-2 complex that potently activates cells expressing TLR4 without MD-2 (17, 21, 22). \(^{3}H\)LOS-MD-2, but not the same \(^{3}H\)LOS (25,000 cpm/pmol) presented as LOS aggregates (LOS\(_{agg}\)) or monomeric LOS-sCD14, associates with these cells in a TLR4-dependent fashion (17). However, detection of cell-associated \(^{3}H\)LOS-MD-2 required several hours of incubation (17), suggesting that levels of surface-expressed TLR4 in these cells were too low to measure direct binding.

To circumvent these limitations and permit more direct assay of the molecular requirements for endotoxin–TLR4 interactions, we transiently expressed, in HEK293T cells, an N-terminal fragment of recombinant human TLR4 (residues 24–634) corresponding to the predicted ectodomain of TLR4 (TLR4ECD) and containing an N-terminal FLAG tag. Harvested control and TLR4ECD-containing culture media were incubated with 1 \(nM\) of purified \(^{3}H\)LOS aggregates (LOS\(_{agg}\)), monomeric \(^{3}H\)LOS-sCD14, or \(^{3}H\)LOS-MD-2-His\(_6\). Interaction of the various forms of \(^{3}H\)LOS with TLR4ECD was monitored by gel filtration analysis to assay for TLR4ECD-dependent changes in the physical state of \(^{3}H\)LOS. Under these experimental conditions, only \(^{3}H\)LOS-MD-2 reacted with TLR4ECD (Fig. 1). Incubation of \(^{3}H\)LOS-MD-2 with conditioned medium containing TLR4ECD (but not control medium) yielded a novel \(^{3}H\)LOS-containing product (Fig. 1A, encircled peak) whose formation depended upon TLR4ECD and the presentation of \(^{3}H\)LOS as \(^{3}H\)LOS-MD-2 (Fig. 1, compare A to B and C). Neither \(^{3}H\)LOS\(_{agg}\) nor \(^{3}H\)LOS-sCD4 showed interaction with TLR4ECD (Fig. 1, B and C), even when added at 200× greater LOS concentrations (data not shown). These findings strongly suggest a specific and direct interaction of \(^{3}H\)LOS-MD-2 with TLR4ECD.

Rechromatography on Sephacryl S300 of the newly formed \(^{3}H\)LOS-containing product recovered after incubation of \(^{3}H\)LOS-MD-2-His\(_6\) with medium containing FLAG-FLAG4ECD (Fig. 1A, encircled fractions) yielded a single symmetrical peak that, by comparison to elution of protein standards, gave a predicted \(M_r\) \(\sim 190,000\) (Fig. 1D). \(^{3}H\)LOS in the product could be captured by anti-FLAG (Fig. 1F) antibodies as well as by Ni\(^{2+}\)-chelating resin (Fig. 1E), indicating the presence of MD-2-His\(_6\) as well as \(^{3}H\)LOS and FLAG-FLAG4ECD in the \(M_r\) \(\sim 190,000\) complex.

The high specific radioactivity of \(^{3}H\)LOS-MD-2 permitted quantitative assay of the formation of the \(M_r\) \(\sim 190,000\) complex at \(pM\) concentrations of \(^{3}H\)LOS-MD-2 and under
conditions where the concentration of TLR4EC, was limiting. Formation of the $M_r \sim 190,000$ complex was dependent on $[^3H]LOS$-MD-2 concentration and saturated at $\sim 1$ nM $[^3H]LOS$-MD-2 with half-maximal formation at $\sim 200–300$ pM $[^3H]LOS$-MD-2 (Fig. 1H). Equilibrium conditions were met after 30 min at 37 °C as equal amounts of product were formed at 30 and 120 min. Scatchard analysis (Fig. 1H) indicated an apparent $K_D$ of $\sim 300$ pM for the reaction of $[^3H]LOS$-MD-2 with TLR4EC. The interaction of $[^3H]LOS$-MD-2 (25,000 cpm/pmol) with TLR4EC could be competed with weakly labeled $[^14C]$LOS-MD-2 (625 cpm/pmol) but not $[^14C]$LOS$_{agg}$ or LOSsCD14 (data not shown), further demonstrating the specificity of the interaction of LOS-MD-2 with TLR4EC.

Transfer of $[^3H]LOS$ from $[^3H]LOS$sCD14 to MD-2/TLR4EC

The inability of $[^3H]LOS$sCD14 to react with TLR4EC (Fig. 1B) is consistent with the requirements of co-expression of MD-2 and TLR4 for sensitive cellular responses to monomeric endotoxin-sCD14 complex (22). To demonstrate more directly the requirement of MD-2 for interaction of $[^3H]LOS$sCD14 with complexes containing TLR4EC, we co-transfected HEK293T cells with expression plasmids for FLAG-TLR4EC and MD-2-FLAG-His$_{6}$. In contrast to incubations with control medium or medium containing TLR4EC alone, incubation of $[^3H]LOS$sCD14 with harvested culture medium containing both secreted MD-2 and TLR4EC yielded an $[^3H]LOS$-containing product that eluted at $M_r >[^3H]LOS$sCD14 (Fig. 2A). In contrast to the efficient reaction of $[^3H]LOS$sCD14 with MD-2 and TLR4EC, there was little or no reaction of $[^3H]LOS$-MD-2 (Fig. 2B) or $[^3H]LOS_{agg}$ (Fig. 2C) under otherwise the same experimental conditions. The product formed by incubation of $[^3H]LOS$sCD14 with the culture medium containing TLR4EC and MD-2 (Fig. 2A) was the same as that observed when the culture medium containing TLR4EC alone was incubated with $[^3H]LOS$-MD-2, as judged by gel filtration chromatography (Fig. 2D) and co-capture analyses (Fig. 2, E and F). With limiting amounts of culture medium containing MD-2 and TLR4EC, formation of the $M_r \sim 190,000$ complex increased with increasing concentrations of $[^3H]LOS$sCD14 (Fig. 2G) and was saturable with an apparent $K_D$ of $\sim 130$ pM (Fig. 2H).

Production of apparently the same $M_r \sim 190,000$ complex from incubation of $[^3H]LOS$sCD14 with culture medium containing TLR4EC and MD-2 that resulted from the reaction of TLR4EC-containing medium with $[^3H]LOS$-MD-2 strongly suggested that $[^3H]LOS$sCD14 transferred $[^3H]LOS$ to a complex containing MD-2 and TLR4EC, without CD14 becoming part of this newly formed complex. To test that hypothesis, we took advantage of the fact that the ability of CD14 to bind endotoxin and transfer endotoxin to MD-2 requires only the N-terminal 152 amino acids of CD14 (full-length CD14 is 356 residues) (23–26). $[^3H]LOS$sCD14 (full-length) and $[^3H]LOS$sCD14-(1–156) were readily resolved by gel-sieving chromatography (Fig. 3). However, incubation of conditioned medium containing MD-2/TLR4EC with either $[^3H]LOS$sCD14 or $[^3H]LOS$sCD14-(1–156) yielded the same $M_r \sim 190,000$ complex (Fig. 3). That the product formed by reaction of 1) $[^3H]LOS$-MD-2 + TLR4EC, 2) $[^3H]LOS$sCD14 + MD-2/TLR4EC, and 3) $[^3H]LOS$sCD14-(1–156) + SMD-2/TLR4EC is apparently the same size strongly suggests that CD14 is neither a component of nor necessary for the formation of the $M_r \sim 190,000$ complex.
we compared the products generated when \(^{3}H\)LOS-sCD14 was incubated with culture medium harvested after transfection (as in Fig. 2A) or was spiked into the culture medium 12 h after transfection so that, immediately upon secretion, MD-2 could potentially react with \(^{3}H\)LOS-sCD14. \(^{3}H\)LOS-MD-2 was generated in significant amounts only when \(^{3}H\)LOS-sCD14 was present during secretion of MD-2. This was seen whether or not TLR4,ECD was also expressed (compare Fig. 4A with Figs. 2A and 3; Fig. 4, compare B with D). In contrast, formation of the \(M_{r} \sim 190,000\) complex occurred efficiently when \(^{3}H\)LOS-sCD14 was added either during or after culturing. These findings suggest strongly that, in contrast to free MD-2, the endotoxin binding activity (i.e. reactivity with LOS-sCD14) of MD-2 is stable in serum-free medium at 37 °C when co-expressed/associated with TLR4,ECD.

Hexaacylated and Tetraacylated LOS-MD-2 Complexes Show Similar Reactivity with TLR4,ECD—We and others (21, 27) have previously shown that differences in TLR4 agonist activity of underacylated versus hexaacylated endotoxin species reflect differences in structural and functional properties of monomeric endotoxin-MD-2 complexes. The ability of underacylated endotoxin-MD-2 complexes to inhibit cell activation by hexaacylated endotoxin-MD-2 suggested that, even though underacylated endotoxin-MD-2 complexes do not efficiently trigger receptor activation, these complexes bind to the TLR4,ECD with an affinity comparable to that of hexaacylated endotoxin-MD-2. To test this hypothesis more directly, aggregates of hexaacylated LOS were treated with the decaying enzyme acyloxyacyl hydrolase (AOAH) and used to generate monomeric complexes with MD-2 (\(^{3}H\)LOSAOAH-MD-2) containing mainly (~90%) tetraacylated LOS, as has been described previously (21). Incubation of a low concentration...
of the $M_r \approx 190,000$ complex (Fig. 5), directly demonstrating the similar reactivity of hexa- and tetraacylated LOS/MD-2 complexes with TLR4$_{ECD}$.

**DISCUSSION**

The data presented demonstrate, for the first time, specific high affinity (pM) interactions of endotoxin with the ectodomain of TLR4. These interactions required presentation of endotoxin (meningococcal LOS) as a monomeric complex with MD-2 (LOS/MD-2) when TLR4$_{ECD}$ was present alone (Fig. 1) or as a monomeric complex with CD14 (LOS/sCD14) when TLR4$_{ECD}$ was co-expressed with MD-2 (Fig. 2). The characteristics of these interactions are fully consistent with the molecular requirements for TLR4-dependent cell activation by pM concentrations of meningococcal and many other endotoxin species (8, 14, 17, 21, 28, 29). How certain deep rough species of endotoxin can potently (nM) activate cells expressing TLR4/MD-2 without CD14 remains to be determined (30). The reactivity of LOS/sCD14 with culture medium containing TLR4$_{ECD}$ and MD-2 (but not TLR4$_{ECD}$ alone) (Fig. 1B) is consistent with an essential role of MD-2 in ligand (endotoxin/CD14) recognition by the MD-2/TLR4 receptor complex. Reaction of LOS/sCD14 with free MD-2 was not detected in culture medium harvested after 48 h of incubation, indicating that the reactant with LOS/sCD14 is preassociated MD-2/TLR4$_{ECD}$ and that, as previously observed, free MD-2 is unstable. The reactivity of LOS/MD-2 with TLR4$_{ECD}$ when TLR4$_{ECD}$ was expressed alone (but not when TLR4$_{ECD}$ was co-expressed with MD-2) also implies that, in the latter situation, TLR4$_{ECD}$ is preoccupied with co-expressed MD-2. The low reactivity of LOS/MD-2 with preformed MD-2/TLR4$_{ECD}$ also indicates that LOS is not readily transferred from LOS/MD-2 to MD-2/TLR4 and that MD-2 associated with TLR4$_{ECD}$ is not readily exchanged with LOS/MD-2.

Previous studies have emphasized the importance of MD-2 in the maturation and surface expression of TLR4 (14, 29, 31, 32). Our findings have revealed a role for TLR4$_{ECD}$ in stabilizing MD-2, perhaps by stabilizing an otherwise labile monomeric form of MD-2 that may be needed for reactivity with endotoxin/CD14 as well as binding to TLR4 (13, 16). The apparent stability of MD-2/TLR4$_{ECD}$ also indicates that LOS is not readily transferred from LOS/MD-2 to MD-2/TLR4 and that MD-2 associated with TLR4$_{ECD}$ is not readily exchanged with LOS/MD-2. Although our stud-

3 R. Widstrom, Taghanemt, A., Prohinar, P., Gioannini, T. L., and Weiss, J. P., unpublished observations.

**FIGURE 4.** Increased stability of endotoxin binding activity of secreted MD-2 by co-expression with TLR4$_{ECD}$ in HEK293 cells. HEK293T cells were transfected with expression vector (pEF-BOS/pFLAG-CMV) encoding MD-2-FLAG-His/FLAG-TLR4$_{ECD}$ (A), MD-2-FLAG-His$_6$ (B), or vectors alone (C); after 12 h, medium was replaced with 1 nM [H]$^3$LOS/sCD14 in serum-free medium, 0.1% HSA. Conditioned medium was collected after an additional 24 h, and 0.5 ml applied directly to a Sephacryl S200 column. D, conditioned medium from HEK293T cells transfected with pEF-BOC encoding MD-2-FLAG-His was harvested at 48 h, concentrated 10$^3$, and an aliquot incubated 30 min at 37 °C with 1 nM [H]$^3$LOS/sCD14 as indicated. Reactants and products were resolved by chromatography on Sephacryl S200. Profiles shown are representative of ≥3 experiments; overall recoveries were >80%.

**FIGURE 5.** Comparison of the reactivity of hexaacylated [H]$^3$LOS/MD-2 and tetraacylated [H]$^3$LOS$_{AOAH}$/MD-2 complexes with TLR4$_{ECD}$. Concentrated conditioned medium from HEK293T cells containing TLR4$_{ECD}$ was incubated 30 min at 37°C with 1 nM [H]$^3$ LOS/wild-type MD-2 or tetraacylated LOS$_{AOAH}$/MD-2. Products were resolved by Sephacryl S200 chromatography. Profiles shown are representative of two or more profiles for each experimental condition.

(0.6 nM) of [H]$^3$LOS$_{AOAH}$/MD-2 with medium containing secreted TLR4$_{ECD}$ followed by gel filtration of the products revealed a pattern similar to that seen with the hexaacylated [H]$^3$LOS/MD-2 complex, i.e. formation of the $M_r \approx 190,000$ complex (Fig. 5), directly demonstrating the similar reactivity of hexa- and tetraacylated LOS/MD-2 complexes with TLR4$_{ECD}$.  

3 R. Widstrom, Taghanemt, A., Prohinar, P., Gioannini, T. L., and Weiss, J. P., unpublished observations.
Endotoxin-Protein Interactions with TLR4 Ectodomain

...ies do not directly reveal to what protein endotoxin is bound in the $M_t \sim 190,000$ complex containing MD-2 and TLR4$_{ECD}$, the ability of endotoxin-CD14 to react avidly with MD-2 (but not to TLR4$_{ECD}$) suggests strongly that the primary reaction of endotoxin-CD14 is with MD-2 ($\pm$ TLR4).

Many investigators have speculated that the reaction of endotoxin-CD14 with MD-2/TLR4 results in engagement of CD14 as an essential component of an activated oligomeric MD-2/TLR4-containing receptor complex (1, 33–36). However, the ability of purified endotoxin-MD-2 complex to induce robust (MyD88-dependent) signaling in cells expressing TLR4 without MD-2 (or mCD14) (22) has suggested to us that CD14 is not an obligatory component of a TLR4 receptor complex activated by endotoxin. Our findings that reaction of LOSsCD14 with MD-2/TLR4$_{ECD}$ yields a product that contains LOS, MD-2, and TLR4$_{ECD}$ (but not CD14 (Fig. 3)) plus the formation of an apparently identical product by reaction of LOS-MD-2 with TLR4$_{ECD}$ (compare Figs. 1 and 2) are consistent with the view that the primary role of CD14 is transfer of endotoxin to MD-2 ($\pm$ TLR4). However, the requirement of mCD14 for MyD88-independent signaling after endotoxin-induced TLR4 activation suggests a more complex role of CD14 in endotoxin-driven TLR4 activation (1, 30, 37). Our experiments in solution with CD14 and TLR4$_{ECD}$ do not preclude the possibility of more stable endotoxin-induced interactions of mCD14 with activated receptor complexes containing full-length transmembrane TLR4.

Both reaction of monomeric LOSsCD14 with MD-2/TLR4$_{ECD}$ and of monomeric LOS-MD-2 with TLR4$_{ECD}$ produced a higher order complex of apparent $M_t$ of $\sim 190,000$ containing LOS, MD-2, and TLR4$_{ECD}$ (Figs. 1 and 2). This nearly matches the predicted $M_t$ of a dimer of a 1:1:1 complex of LOS-MD-2/TLR4$_{ECD}$ ($M_t$ of LOS $\sim 5,000$, of MD-2 $\sim 20,000$, of TLR4$_{ECD}$ $\sim 75,000$). Confirmation of this predicted stoichiometry will require preparative purification of $M_t \sim 190,000$ complex and chemical and immunochemical analyses. It is not yet clear whether this product reflects endotoxin-induced TLR4 dimerization or reaction of monomeric protein/endotoxin complexes with pre-existing TLR4$_{ECD}$ (MD-2) dimers in the harvested culture medium. In either case, the co-capture results shown in Fig. 1 are intriguing. The relatively efficient capture of the $M_t \sim 190,000$ complex by an activated Ni$^{2+}$-chelating resin, despite inefficient capture of the LOS-MD-2-His$_6$ (or LOS-MD-2-FLAG-His$_6$ data not shown) complex suggests that the His tag is shielded in the LOS-MD-2 complex from interaction with Ni$^{2+}$ resin but becomes more accessible upon interaction with TLR4$_{ECD}$ and formation of the $M_t \sim 190,000$ complex. This suggests a conformational change of MD-2 or of LOS bound to MD-2 upon binding of LOS-MD-2 to TLR4$_{ECD}$.

In summary, we have demonstrated for the first time $\mu$m interactions of endotoxin with TLR4$_{ECD}$. We have succeeded in demonstrating such high affinity interactions because of our use of purified endotoxin-protein complexes that are the preferred substrate(s) for (MD-2) TLR4$_{ECD}$, and that are in radiolabeled form of very high specific radioactivity, making it possible to detect and quantify intermolecular interactions at $\mu$m endotoxin concentrations. The reagents and assays described in this study should now make it possible to define the structural requirements of endotoxin and MD-2 interactions with TLR4$_{ECD}$ in a way that has not been possible before. Our findings are consistent with earlier speculations that the monomeric endotoxin-MD-2 complex, not endotoxin itself, is the ligand for TLR4 (13, 17). As such, the molecular basis of ligand recognition and receptor activation by mammalian TLR4 may resemble more closely that of Dro sophila Toll (38) than has been appreciated before. Whether or not, in comparison to other mammalian TLRs, TLR4 is idiosyncratic or instructive of the molecular basis of ligand recognition by other mammalian TLRs (39) remains to be determined.

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Endotoxin-Protein Interactions with TLR4 Ectodomain

JANUARY 12, 2007•VOLUME 282•NUMBER 2
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