Transfer of Monomeric Endotoxin from MD-2 to CD14

CHARACTERIZATION AND FUNCTIONAL CONSEQUENCES

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Athmane Teghanemt, Polonca Prohinar, Theresa L. Gioannini, and Jerrold P. Weiss

From the Department of Internal Medicine and the Inflammation Program, the Department of Microbiology, and the Department of Biochemistry, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242 and the Veterans Affairs Medical Center, Iowa City, Iowa 52246

Potent Toll-like receptor 4 (TLR4)-dependent cell activation by endotoxin depends on sequential transfer of monomers of endotoxin from an aggregated form to CD14 via the lipopolysaccharide-binding protein and then to MD-2. We now show that monomeric endotoxin can be transferred in reverse from MD-2 to CD14 but not to lipopolysaccharide-binding protein. Reverse transfer requires a ~1000-fold molar excess of CD14 to endotoxin-MD-2. Transfer of endotoxin from MD-2 to extracellular soluble CD14 reduces activation of cells expressing TLR4 without MD-2. However, transfer of endotoxin from MD-2 to membrane CD14 (mCD14) makes cells expressing MD-2-TLR4 sensitive to activation by the endotoxin-MD-2 complex. An endotoxin-mutant (F126A) MD-2 complex that does not activate cells expressing TLR4 alone potently activates cells expressing mCD14, MD-2, and TLR4 by transferring endotoxin to mCD14, which then transfers endotoxin to endogenous wild-type MD-2-TLR4. These findings describe a novel pathway of endotoxin transfer that provides an additional layer of regulation of cell activation by endotoxin.

Endotoxins are unique, highly abundant surface glycolipids of Gram-negative bacteria. They have the capacity to potently induce proinflammatory responses of many multicellular hosts. With the discovery of the acute phase protein, lipopolysaccharide-binding protein (LBP), CD14, and, later, the Toll-like receptor 4 (TLR4) and MD-2, it has become clear that each of these proteins plays a key role in the ability of many mammalian hosts to mount highly sensitive responses to endotoxin (1–5). MD-2 is required for TLR4-dependent cell activation of mammalian cells by endotoxin, whereas LBP and CD14 are needed for maximal sensitivity. This sensitivity is needed to permit host cells to respond to pg/ml concentrations of endotoxin and thereby trigger defensive host responses to small numbers of Gram-negative bacteria soon after bacterial invasion, before the host is overwhelmed by bacterial growth.

We have made use of metabolically radiolabeled purified endotoxin to better define how LBP, CD14 and MD-2 together make possible potent TLR4-dependent cell activation by endotoxin (6–8). On the basis of these and many other studies, we and others have proposed that potent TLR4-dependent cell activation by endotoxin depends on sequential protein-endotoxin and protein-protein interactions between endotoxin, LBP, CD14, MD-2, and TLR4 (1–4, 9–11). Thus, LBP binds to endotoxin as presented natively in the bacterial outer membrane or as aggregates after extraction and purification (4, 12–15). In so doing, LBP catalyzes delivery and transfer of individual molecules of endotoxin to CD14 (either as a soluble extracellular protein (sCD14) or GPI-linked membrane protein (mCD14)) (4, 7, 13–16). The monomeric endotoxin-CD14 complex that is formed is the preferred substrate for MD-2, whether present in soluble extracellular form (sMD-2) or membrane-associated as a complex with TLR4 (2, 9, 11). The transfer of endotoxin from CD14 to MD-2, coupled with binding of MD-2 to TLR4, is apparently required for TLR4 activation by endotoxin (2, 11).

Consistent with this ordered pathway of protein/endotoxin and protein/protein interactions, cells expressing heterodimeric MD-2-TLR4 complexes (without mCD14) can be potently activated by monomeric endotoxin (E)CD14 but not by purified endotoxin aggregates with or without LBP or by monomeric E-MD-2 complex (2, 16). In contrast, cells expressing TLR4 without MD-2 (e.g. airway epithelial cells) can be potently activated by E-MD-2 complex but not by ECD14 (17). The inability of the monomeric E-MD-2 complex to potently activate cells expressing MD-2-TLR4 is consistent with the inability of endotoxin in E-MD-2 to be readily transferred to MD-2 (bound to TLR4) or the soluble E-MD-2 complex to readily exchange with MD-2 that is bound to TLR4 (9). Thus, under circumstances (e.g. infection and inflammation) in which increases in MD-2 expression (17–20) may lead to both increased formation of extracellular E-MD-2 and increased occupation of TLR4 by MD-2, the fate of the extracellular E-MD-2 is unclear.

In this study, we have considered the possibility that CD14 could provide an alternative target for the monomeric E-MD-2 complex and thereby provide an alternative route for TLR4-dependent cell activation and, perhaps, for clearance of E-MD-2 complexes. Using endotoxin (lipooligosaccharide; LOS) from an acetate auxotroph of Neisseria meningitidis, which we have
previously described (6), we show direct transfer of endotoxin from MD-2 to CD14 that, depending on the properties of the host cell and surrounding extracellular fluid, can either attenuate or promote cell activation by endotoxin. These findings thus describe a novel pathway of endotoxin transfer that provides an additional layer of regulation of cell activation by endotoxin.

EXPERIMENTAL PROCEDURES

Materials—LBP and sCD14 were provided by Xoma Corp. (Berkeley, CA) and Amgen Corp. (Thousand Oaks, CA), respectively. Both parental human embryonic kidney HEK293 cells and stable transfectants of these cells expressing TLR4 (HEK293/TLR4) were a gift from Dr. Jessey Chow (Eisai Institute, Andover, MA); HEK293/mCD14/MD-2/TLR4 stable cell lines were purchased from InvivoGen, (San Diego, CA). Human serum albumin (HSA) was obtained as an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA). Chromatography matrices (Sephacryl HR S200 and S300, nickel FF-Sepharose) were purchased from GE Healthcare. Murine monoclonal anti-CD14 antibodies, MEM18 and MY-4, were purchased from Accurate Chemicals & Scientific Corp. (Westbury, NY), and 18E12 was a gift from Johnson & Johnson Corp. (New Brunswick, NJ).

Recombinant Proteins—Insect-derived soluble MD-2 containing a hexahistidine tag on the C-terminal end was prepared as previously described (11). Preparative amounts of the mutant MD-2F126A were generated from infections of High Five insect cells with baculovirus containing the gene for human MD-2F126A inserted into pBAC11. MD-2 cDNA was isolated, linearized, and inserted, using NcoI and XhoI-sensitive restriction sites, into the baculovirus transfection vector pBAC11 (Novagen) that provides a 6-residue polyhistidine (His$_6$)$_{tag}$ tag at the carboxyl-terminal end of MD-2 and 5’-flanking signal sequence (ggp64) to promote secretion of the expressed protein. DNA encoding MD-2F126A was sequenced in both directions to confirm fidelity of the product. The fragment of human TLR4 corresponding to the predicted ectodomain, amino acid residues 24–634, (TLR4ECD) was generated by transient transfection of HEK293T cells. HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Expression vectors containing 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 (11). Preparative amounts of the gene for human TLR4ECD, amino acids 24–634 (pFLAG-CMV-TLR4), and vectors containing DNA of interest for production of FLAG-HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (293 SFM; Invitrogen) plus 0.4% HSA was added.

Media containing expressed proteins were collected 24–48 h later and stored at 4°C until used. The reactivity of conditioned medium containing secreted TLR4ECD/MD-2 maintained reactivity with [3H]LOS·sCD14 for at least 6 months at 4°C.

Preparation of Metabolically Labeled Endotoxins—Lipooligosaccharide (14C]LOS (600 cpm/ng) or [3H]LOS (5000 cpm/ng)) was isolated after growth and metabolic labeling of an acetate auxotroph of N. meningitides serogroup B as previously described (6). An msbB derivative of N. meningitidis serogroup B, NMBA11K3cap-, was obtained from Dr. Michael Apicella (University of Iowa). The strain was grown in minimal medium supplemented with radioactive [14C]acetate or [3H]acetate, and LOS (500 or 80 cpm/ng, respectively) was isolated as previously described (22, 23). After extraction of LOS with hot phenol/water, precipitation with ethanol and resuspension by sonication in distilled water, LOS aggregates were further purified by ultracentrifugation (16, 22, 23). [14C]LOS or [3H]LOS aggregates (apparent $M_r$ ~ 20 million) and [14C]LOS·sCD14 or [3H]LOS·sCD14 ($M_r$ ~ 60,000) were prepared and purified by size exclusion chromatography as previously described (6, 16).

Preparation of [3H]LOS·Protein Complexes—[3H]LOS·sCD14 and [3H]LOS·MD-2 complexes were prepared as previously described (2, 11, 16). Monomeric [3H]LOS·sCD14 complexes ($M_r$ ~ 60,000) were prepared by treatment of [3H]LOS aggregates for 30 min at 37°C with substoichiometric LBP (molar ratio 200:1, LOS/LBP) and a 1–1.5-fold molar excess of sCD14 to LOS followed by gel exclusion chromatography (Sephacryl S200; 1.6 × 30 cm) in phosphate-buffered saline, pH 7.4, plus 0.03% HSA to isolate monomeric [3H]LOS·sCD14 complex. [3H]LOS·MD-2·His$_6$ ($M_r$ ~ 25,000) was generated by treatment of [3H]LOS·sCD14 (30 min at 37°C) with High Five insect cell medium containing either wild-type or F126A MD-2·His$_6$ followed by isolation of [3H]LOS·MD-2 by S200 chromatography (1.6 × 70 cm). To minimize contamination of LOS·MD-2 with any small remaining amount of LOS·sCD14, the peak and downslope fractions of LOS·MD-2 were repurified by Sephacryl S200 using a long column (1.6 × 70 cm) to further increase resolution of LOS·MD-2 from LOS·sCD14.

Samples were applied in 1-ml fractions and collected (flow rate, 0.3–0.5 ml/min) at room temperature using AKTA or AKTA Purifier FPLC (GE Healthcare). Aliquots of the collected fractions were analyzed by liquid scintillation spectroscopy and radioactivity was detected using a Beckman LS liquid scintillation counter. Radioactivity was confirmed by Sephacryl S500 chromatography, and that of [3H]LOS·sCD14 and of [3H]LOS·MD-2 was confirmed by Sephacryl S200 chromatography (6, 11).

After chromatography, selected fractions to be used in bioassays were pooled and sterile-filtered (0.22-µm pore size) with >90% recovery. Fractions were stored under sterile conditions at 4°C until needed. Sephacryl S200 columns were calibrated with Bio-Rad gel filtration standards that included thyroglobulin, globulin, ovalbumin, myoglobin, vitamin B$_{12}$, and human serum albumin.

Cell Activation Assays—Human umbilical vein endothelial cells were cultured on collagen-coated plasticware (Costar, Cambridge, MA) at 37°C, 5% CO$_2$, and 95% relative humidity in
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**RESULTS**

**Transfer of Endotoxin from MD-2 to CD14**—To test the hypothesis that endotoxin could be transferred from a monomeric E-MD-2 complex to CD14, we first examined the effect of increasing concentrations of sCD14 on the ability of purified LOS-MD-2 to activate HEK293/TLR4 cells. In contrast to E-MD-2, monomeric sCD14 does not activate these cells (2, 11). Thus, transfer of endotoxin from E-MD-2 to sCD14 should be accompanied by reduced cell activation.

As shown in Fig. 1A, sCD14 produced a dose-dependent inhibition of cell activation by LOS-MD-2 in HEK/TLR4 cells. Nearly complete inhibition by added sCD14 required a 1000–3000-fold molar excess of sCD14 to E-MD-2. There was virtually quantitative conversion of [3H]LOS-MD-2 to CD14-dependent activation by E-MD-2 of Cells Expressing mCD14, MD-2, and TLR4—Although transfer of endotoxin from E-MD-2 to sCD14 inhibited TLR4-dependent activation of cells expressing TLR4 without MD-2 or mCD14, it seemed possible that the same intermolecular transfer of endotoxin could promote activation of cells that expressed mCD14 as well as MD-2 and TLR4. To test this hypothesis, we compared the ability of LOS aggregates plus substoichiometric concentrations of LBP, monomeric LOS+sCD14, and LOS-MD-2 complexes to activate HEK293/mCD14/MD-2/TLR4 cells. Remarkably, LOS-MD-2 was nearly as potent as LOS+sCD14 and at least as potent as LOS aggregates plus LBP in inducing activation of these cells (Fig. 2A).

Because mCD14 is very highly expressed in these stable cell transformants, we repeated the same experiments with primary cells (i.e. PBMCs containing human monocytes) expressing mCD14/MD-2/TLR4. Fig. 2B shows that LOS-MD-2 also induced dose-dependent secretion of IL-8 from PBMC (monocytes), although the potency of LOS-MD-2 toward these cells was 3–10-fold less than that of LOS+sCD14. Activation of monocytes by LOS-MD-2 as well as by LOS aggregates plus LBP and by LOS+sCD14 at 10 psL LOS was nearly completely inhibited by an antibody to CD14 (MY-4) that blocks the endotoxin-binding site of CD14 (Fig. 2C). This is consistent with a role for endotoxin transfer from MD-2 to CD14 in activation of monocytes by monomeric LOS-MD-2. Similar observations were made with cultured endothelial cells that express MD-2 and TLR4 and low levels of mCD14 (data not shown) (6, 16, 25).

These cells were potently activated by LOS+sCD14 but much less by LOS-MD-2 (Fig. 2D). Cell activation by LOS-MD-2 was further reduced by pretreatment of the endothelial cells with anti-CD14 antibody (data not shown), strongly suggesting that transfer of LOS from LOS-MD-2 to mCD14 was necessary in these cells as well for maximal activation by LOS-MD-2.

**Contrasting Properties of LOS-MD-2 Complexes Containing either Mutant LOS or Mutant MD-2**—We have recently characterized mutant LOS-MD-2 complexes containing either pentacylated LOS or MD-2F126A that have markedly reduced TLR4 agonist properties when tested with cells expressing only TLR4 (e.g. HEK293/TLR4 cells) (Fig. 3A) (23, 37). These mutant LOS-MD-2 complexes contained the same intermolecular transfer of endotoxin as described above and achieved full activation of TLR4-expressing cells (Fig. 3B). These results reveal that the structure of the LOS-MD-2 complexes is an important determinant for TLR4 agonist activity.
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FIGURE 2. CD14-dependent activation by LOS-MD-2 of cells containing mCD14/MD-2/TLR4. Activation of HEK/CD14/MD-2/TLR4 cells (A), PBMC (B and C), and human umbilical vein endothelial cells (HUVEC) (D) was measured as LOS-induced extracellular accumulation of IL-8 in cell supernatants after a 20-h incubation by enzyme-linked immunosorbent assay. (See “Experimental Procedures.”) C, PBMC were preincubated at 4 °C for 30 min in Dulbecco’s modified Eagle’s medium, 0.1% HSA with or without 10 μg/ml anti-CD14 monoclonal antibody MY-4. Results shown represent the mean ± S.E. of triplicate samples from three separate experiments. Error bars that are not visible reflect S.E. that are less than the size of the data point symbols. LOSagg, LOS aggregates.

FIGURE 3. Comparison of the ability of wild-type and variant LOS-MD-2 complexes to induce cell activation. Activation of HEK/TLR4 cells (A) or PBMC (B) by increasing concentrations of LOSWT-MD-2WT (○), LOSagg-MD-2WT (●), or LOSWT-MD-2F126A (□) was measured as described under “Experimental Procedures.” C, PBMC were preincubated at 4 °C for 30 min in Dulbecco’s modified Eagle’s medium, 0.1% HSA with or without 10 μg/ml anti-CD14 monoclonal antibody MEM18 prior to the addition of 60 pM WT or variant LOS-MD-2 as indicated. After overnight incubation, extracellular IL-8 was measured by enzyme-linked immunosorbent assay. Results shown are mean ± S.E. of triplicates from three separate experiments.
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\(2^{F126A}\), and \(\text{LOS}^{\text{mbB}, \text{MD}-2^{\text{WT}}}\) was nearly completely inhibited by neutralizing anti-CD14 antibody.

sCD14-dependent Transfer of \([^{3}\text{H}]\text{LOS}\) from \([^{3}\text{H}]\text{LOS}-\text{MD-2}\) to MD-2-TLR4\(\text{ECD}\)—The findings described above strongly suggest that endotoxin from E-\text{MD-2} can be delivered to MD-2-TLR4 in a CD14-dependent manner. To demonstrate this more directly, we made use of a novel cell-free assay (9) in which transfer of endotoxin from E-\text{MD-2} to a soluble complex of secreted MD-2 and the predicted ectodomain of TLR4 (MD-2-TLR4\(\text{ECD}\), forming a complex containing \([^{3}\text{H}]\text{LOS}, \text{MD-2},\) and TLR4\(\text{ECD}\) with \(M_r \sim 190,000\) (i.e. \((^{3}\text{H}]\text{LOS-MD-2}\text{-TLR4ECD})^2\), can be monitored at picomolar concentrations of \([^{3}\text{H}]\text{LOS}\). Incubation of \([^{3}\text{H}]\text{LOS-}\text{MD-2}\) (\(M_r \sim 25,000\)) with culture medium containing secreted MD-2-TLR4\(\text{ECD}\) yielded only a very small amount of the \(M_r \sim 190,000\) complex (Fig. 4). The addition of sCD14 to the incubation mixture markedly increased the yield of this \(M_r \sim 190,000\) complex with a corresponding reduction in \([^{3}\text{H}]\text{LOS-}\text{MD-2}\) (Fig. 4). These findings demonstrate directly the ability of CD14 to facilitate delivery of endotoxin from E-\text{MD-2} to MD-2-TLR4, presumably via formation of an E-\text{CD14} complex.

DISCUSSION

We have demonstrated, for the first time, intermolecular transfer of endotoxin from MD-2 to CD14. Many intermolecular transfer reactions involving endotoxin have been demonstrated before, most notably from LBP to CD14 and from CD14 to MD-2, reactions that are key steps in potent TLR4-dependent cell activation by endotoxin. In addition, delivery of endotoxin via E\text{CD14} to plasma lipoproteins and via LBP-coated endotoxin aggregates to scavenger receptor(s) and lipoproteins has been demonstrated (26–30). Whether endotoxin itself or the intact endotoxin-protein complex/aggregate is transferred in those reactions has not been established. The various reactions of endotoxin involving LBP and CD14 have underscored the complexity of host/endotoxin interactions and demonstrated that a single endotoxin-protein complex/aggregate may have multiple host acceptors and, as a result, be linked to different functional outcomes (e.g. MD-2-TLR4-dependent cell activation versus scavenger receptor or lipoprotein-mediated endotoxin clearance).

The reverse transfer of endotoxin from MD-2 to CD14 documented in this study reveals that E-\text{MD-2} has at least two host acceptors, TLR4 and CD14 (Fig. 5). Depending on the abundance and reactivity of acceptors of E-\text{CD14} (e.g. MD-2-TLR4, LBP, and lipoproteins), the transfer of endotoxin may, as we have shown, lead either to inhibition (Fig. 1) or promotion (Figs. 2 and 3) of TLR4-dependent cell activation by endotoxin (Fig. 5). The latter effect may be more likely in tissues or under inflammatory conditions where levels of secreted MD-2 (resulting from synthesis of MD-2 in molar excess to that of TLR4) relative to sCD14, LBP, and lipoproteins or other endo-
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toxin scavengers may favor formation of E-MD-2. Our findings indicate that both cells expressing TLR4 without MD-2 (such as airway epithelial cells) and cells containing mCD14 and MD-2-TLR4 (e.g. macrophages) could be targets of E-MD-2, the former by direct interaction of E-MD-2 with TLR4 and the latter by transfer of endotoxin from extracellular E-MD-2 to membrane CD14 and then MD-2-TLR4 (Fig. 5). The efficiency of transfer of endotoxin from extracellular E-MD-2 to mCD14 is likely to depend on the abundance of mCD14 (Fig. 2). These properties suggest that E-MD-2 could provide, pharmacologically, a particularly advantageous vehicle for delivery and transfer of endotoxin agonists and antagonists to desired tissue sites of action, especially if its relatively small mass and favorable aqueous solubility permit penetration into tissue more rapidly than transfer of endotoxin from E-MD-2 to CD14 in the circulatory system.

In comparison with EsCD14, E-MD-2 is much more stable and soluble in aqueous environments. These properties suggest tighter binding of endotoxin to MD-2 than to CD14, with sequestration of the fatty acids of endotoxin within a hydrophobic cavity that is deep in MD-2 (31–33) but wide and shallow in CD14 (34). In support of this view, E-MD-2 is relatively resistant to the endotoxin deacetylase, acyloxyacyl hydrolase, whereas EsCD14 is a favorable substrate for this enzyme (13). Based on these biochemical and physical chemical properties, we have previously predicted that transfer of endotoxin occurs much less readily, if at all, from MD-2, in contrast to the facile transfer of endotoxin from CD14 (2, 11, 13). The findings presented in this study support this view. Although the transfer of endotoxin from CD14 to MD-2 occurs readily at molar ratios of MD-2 to EsCD14 of <10 (2, 11, 23), the reverse transfer requires molar ratios of CD14 to E-MD-2 of ≥300 (Fig. 1). Moreover, endotoxin exchange occurs readily between CD14 molecules (e.g. sCD14 and mCD14) but to only a very limited extent, if at all, between E-MD-2 and MD-2-TLR4 (Fig. 4). The facile exchange of endotoxin between CD14 molecules (i.e. sCD14 and mCD14) may explain the remarkably potent activity of LOS:sCD14 toward PBMCs (Fig. 2, B and C). Half-maximal cell activation is produced in that setting by 10 pm LOS:sCD14, well below the apparent $K_a$ (~130 pm) of LOS transfer from LOS:sCD14 to MD-2-TLR4 (9). The very limited reaction of LOS-MD-2 with conditioned medium containing MD-2 and TLR4ECD may reflect 1) limited transfer of endotoxin from E-MD-2 to MD-2-TLR4, 2) limited exchange of E-MD-2 with MD-2 associated with TLR4, or 3) limited amounts of free TLR4ECD in culture medium containing MD-2 and TLR4ECD. In any case, the addition of sCD14 in molar excess substantially increases the net transfer of endotoxin from E-MD-2 to MD-2-TLR4, fully consistent with the role of mCD14 in facilitating activation by E-MD-2 of cells containing MD-2-TLR4. The accumulation of (E-MD-2-TLR4ECD)$_2$, but little or no EsCD14, in these incubation mixtures (Fig. 4) despite a 100-fold molar excess of sCD14 underscores how favorable the transfer of endotoxin from CD14 to MD-2 is in comparison with the reverse reaction. This characteristic is consistent with and presumably necessary for the remarkable potency of cell activation by endotoxin, including in cells that may have a substantial molar excess of mCD14 to MD-2-TLR4. The efficiency of transfer of endotoxin from CD14 to MD-2-TLR4 is probably further increased when mCD14 and MD-2-TLR4 are present within the same membrane. This may explain why, at very low LOS:sCD14 concentrations, cell activation is dependent on mCD14 (i.e. blocked by anti-CD14 antibodies). At higher (>200 pm) concentrations of LOS:sCD14, cell activation is not blocked by anti-CD14 antibodies that block transfer of endotoxin between sCD14 and mCD14 (e.g. MY-4 and MEM18), probably reflecting at these higher concentrations direct transfer of LOS from extracellular LOS:sCD14 to membrane MD-2-TLR4.

In contrast to the ability of endotoxin to be transferred from MD-2 to CD14, we observed no reaction of E-MD-2 with LBP even at a 3000-fold molar excess of LBP. This suggests that the molecular requirements for transfer of endotoxin from MD-2 mirror the requirements for delivery of endotoxin to MD-2. Endotoxin is readily transferred to MD-2 from E-Carboxi but not from LBP-coated aggregates of purified E or from complexes of EsCD14 that have been induced to aggregate by reaction with LBP (11, 30). It seems likely that transfer of endotoxin between endotoxin-binding proteins is preceded by docking between the endotoxin–protein complex or aggregate and the recipient endotoxin-binding protein and thus may be driven by protein–protein as well as protein–endotoxin interactions. Therefore, the apparently specific exchange of endotoxin between CD14 and MD-2 may depend on (weak) interactions between CD14 and MD-2 that are not present between LBP and MD-2. The ability of added LBP to dampen cell activation by endotoxin and shift endotoxin away from association with MD-2 (TLR4) (26, 30, 35, 36) probably depends on the ability of mCD14 to act as an intermediate in transfer, perhaps by virtue of its ability to interact with both LBP and MD-2. Whether or not transfer of endotoxin from MD-2 to TLR4 follows engagement of E-MD-2 with TLR4 (or of endotoxin with MD-2-TLR4) and is an important step in endotoxin-induced TLR4 activation remains an open question.

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