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Influence of CD14 on Ligand Interactions between Lipopolysaccharide and Its Receptor Complex[1]

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The interaction of LPS (endotoxin) with the CD14-TLR4 receptor complex modulates the host innate immune response. Several studies using partial structures of LPS have suggested that TLR4 determines the ligand specificity of this complex, and that CD14 indiscriminately serves to deliver the ligand to TLR4. This conclusion has been made despite observations that the response of TLR4$^{+/+}$,CD14$^{-/-}$ macrophages to LPS is very weak. To determine whether CD14 itself plays a role in specific ligand recognition, the influences of various partial structures of LPS on induction of the proinflammatory cytokine, TNF, by CD14$^{+/+}$ and CD14$^{-/-}$ macrophages were compared. These studies show that the ligand specificities of CD14$^{+/+}$ and CD14$^{-/-}$ macrophages are very different. When CD14 is present, the receptor complex shows exquisite specificity for smooth LPS, the major form expressed by Gram-negative bacteria; however, as increasing amounts of carbohydrate are removed from smooth LPS, the sensitivity of CD14$^{+/+}$ macrophages decreases as much as 500-fold. In contrast, CD14$^{-/-}$ macrophages are unable to distinguish between smooth LPS and its various partial structures. Furthermore, CD14$^{-/-}$ macrophages are 150,000-fold less sensitive than CD14$^{+/+}$ macrophages to smooth LPS. A similar ability to distinguish the differing LPS structures of various bacteria such as *Bacteroides fragilis* and *Salmonella abortus* are observed for CD14$^{+/+}$, but not CD14$^{-/-}$, macrophages. Thus, CD14$^{+/+}$, but not CD14$^{-/-}$, macrophages are highly sensitive to stimulation by natural forms of LPS and show the ability to distinguish between various LPS ligands, consistent with CD14 being a highly specific receptor. *The Journal of Immunology,* **2005, 175: 3940–3945.**

Lipopolysaccharide (endotoxin) is a major component of the outer membrane of all Gram-negative bacteria (1) and has been implicated as a major toxin that, in severe infection, can cause a cascade of events leading to the often fatal condition of endotoxin shock (2, 3). A primary activity of LPS is its ability to stimulate the production of proinflammatory cytokines (including IL-1, IL-6, and TNF-α) that, when produced in high doses, can initiate this cascade (4). Mechanisms regulating LPS-induced release of such proinflammatory cytokines have largely been studied in vitro, using LPS-responsive cells such as monocytes/macrophages (5, 6). Identification of the myeloid Ag, CD14, as a receptor for LPS (7) and demonstration that monocytes/macrophages lacking CD14 are highly unresponsive to pure smooth LPS (8–11) show a pre-eminent role for CD14 on monocytes/macrophages. Nevertheless, some responses can be detected when CD14-deficient macrophages are stimulated with LPS composed of different backbone structures (10), suggesting the existence of a CD14-independent pathway. More recent studies have shown that another molecule expressed on the surface of monocytes, TLR4, is required for LPS-induced signal transduction and that cells deficient in TLR4 do not respond to smooth, wild-type LPS even when CD14 is present (12, 13). These studies have led to the conclusion that CD14 and TLR4 are components of a receptor complex that includes several additional molecules, such as MD-2 and MyD88 (14, 15).

Structurally, LPS consists of a variable repeating oligosaccharide domain (O-Ag) and a less variable oligosaccharide core covalently bound to lipid A (16–18). Numerous studies have led to the conclusion that the majority of endotoxic activities exhibited by LPS, including pyrogenicity, lethal toxicity, tumor necrotizing activity, local Schwartzman reactivity, and B cell mitogenicity, reside in the lipid A portion of the molecule (19–22). Additional studies using analogues of lipid A that are antagonists for human cells yet agonists for mouse cells have shown that such species-specific responses are determined by TLR4, leading to the conclusion that TLR4 determines the ligand-specific recognition of LPS (23–26). However, because monocytes/macrophages are at least 100-fold less sensitive to lipid A than to smooth LPS (5, 6), the form normally expressed by most Gram-negative bacteria, these conclusions may not reflect the true role of CD14 in ligand recognition. Accordingly, we sought to determine whether CD14 itself might also play a role in specific ligand recognition for more natural forms of LPS.

Materials and Methods

Reagents

Fig. 1 summarizes the structures of the LPS compounds used in this study. These include wild-type smooth LPS (*Escherichia coli* 015), the rough chemotypes Ra LPS (*E. coli* EH100) and Re LPS (*E. coli* F515), lipid A, and a form of Re LPS that lacks phosphate. The rough forms (Ra and Re) lack the repeating O-Ag that is expressed by wild-type smooth LPS and have decreasing amounts of core carbohydrate. The lipid A form resulted from removal of the Kdo carbohydrate component from the Re LPS (F515). The smooth LPS purified from *E. coli* serotype 015 and the rough

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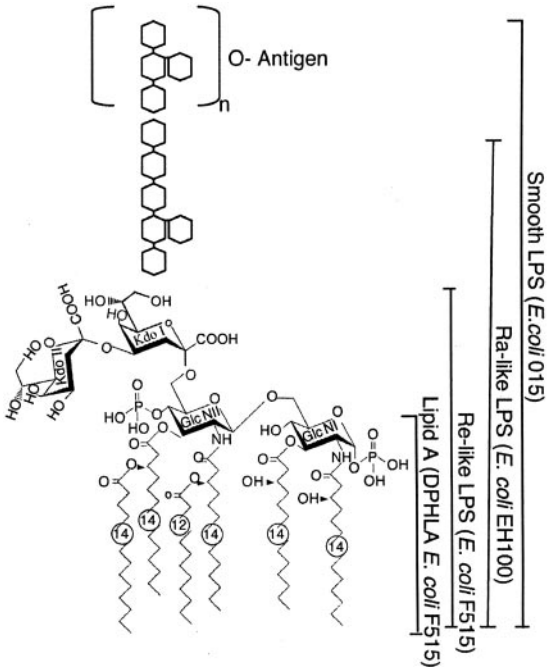

FIGURE 1. Diagram of the molecular structure of *E. coli* LPS (smooth and rough (Re) chemotypes) and lipid A.

mutant strains *E. coli* EH100 Ra LPS, *E. coli* F515 Re LPS, and dephosphorylated Re LPS (DeP-Re LPS)[3] molecules were prepared and purified as previously described (27, 28). Because of naturally occurring, incomplete bacterial biosynthesis of LPS, these preparations are heterogeneous in several respects, including the number of acyl groups in their lipid A moiety, and have, in general, a distribution of hexa (~70%), penta (~20%), and tetra (~10%) acyl groups in their lipid A moiety. The lipid A used in these studies was prepared from Re LPS (27, 28), and the hexa-acylated form was purified by preparative layer chromatography (27, 28). It was found to be homogeneous by TLC, MALDI-mass spectrometry, and nuclear magnetic resonance analysis (27, 28). The biological activity of all preparations was confirmed by functional analyses (29, 30). Stock solutions (1 mg/ml), aliquoted and stored at −80°C, were thawed, sonicated, diluted with RPMI 1640 (Invitrogen Life Technologies) containing 1% autologous serum, and vortexed each time before use.

Mice

CD14-deficient mice from the 10th backcross on BALB/c, C3H/HeN, or C3H/HeJ were produced in our laboratory (8). Control mice were obtained from The Jackson Laboratory (C3H/HeJ) or Harlan Sprague Dawley (BALB/c and C3H/HeN). All animals were housed under specific pathogen-free conditions, and all animal experiments were conducted in accordance with the National Institutes of Health and American Association for Assessment of Laboratory Animal Care guidelines, using protocols approved by the North Shore-Long Island Jewish Research Institute animal care and use committee.

Stimulation of thioglycolate-elicited murine peritoneal macrophages

Eight-week-old mice were injected i.p. with 3 ml of 3% (w/v) Brewer thioglycolate broth (Difco). Four days later, cells were harvested by peritoneal lavage with 10 ml of RPMI 1640 (Invitrogen Life Technologies), containing 2 mM L-glutamine and supplemented with 100 U of penicillin and 100 μg of streptomycin/ml. The cells were washed twice in RPMI 1640, resuspended in the above medium supplemented with 1% autologous serum, and then added to the wells (5×10^5 macrophages/well) of a 24-well tissue culture plate (Nunc). The cells were incubated for 3 h at 37°C in a humidified 5% CO_2 incubator to allow the macrophages to adhere. The wells were then washed twice with 1 ml of medium before treatment with the various stimuli as previously described (10). Briefly, 10-fold serial dilutions of the different LPS and lipid A samples were added to adherent macrophages (0.5 ml/well). After a 3-h incubation, cell-free supernatants were assayed for murine TNF-α by ELISA (Genzyme) according to the manufacturer's instructions. The lower detection limit of TNF-α was 10 pg/ml.

Statistical analysis

To compare the relative abilities of the different forms of LPS to stimulate $CD14^{+/+}$ and $CD14^{-/-}$ macrophages, the amount of each form of LPS required to induce the release of 1.5 ng/ml TNF-α was determined from each dose-response curve. Each form of LPS was tested three times, and the mean and SD for the amount of LPS required to induce 1.5 ng/ml TNFα were determined. A two-tailed unpaired *t* test was used to compare the significance of differences in the means for different forms of LPS using PRISM software (GraphPad).

Results

To determine whether CD14 plays a role in ligand recognition by the CD14/TLR4 complex, we tested whether the expression of CD14 alters the production of TNF when normal ($CD14^{+/+}$) or CD14-deficient ($CD14^{-/-}$) macrophages are stimulated with different forms of LPS. These forms included smooth, wild-type *E. coli* 015 LPS and two mutant chemotypes, Ra LPS and Re LPS, that lack the O-Ag (Fig. 1). In addition, responses to dephosphorylated-Re LPS and lipid A (Fig. 1) were measured. The latter two forms of LPS were used in these studies because others have used similar forms to assess the role of TLR4 in the recognition of LPS. To assess their ability to trigger responses, each form of LPS was used to stimulate macrophages at the same time at concentrations ranging from 10^{-4} to 5×10^4 nM, and the molar concentrations of the ligands that elicited 1.5 ng/ml TNF were compared. This value of TNF-α was selected because this amount is significantly ($p = 0.001$) above background and lies on the linear part of the curve. Furthermore, we did not select higher values because some of the partial forms of LPS were unable to induce appreciably >1.5 ng/ml TNF. In all cases, studies were performed in the presence of serum rather than under serum-free conditions because we wished to simulate physiological conditions as much as possible.

Recognition of smooth, Ra, and Re LPS by $CD14^{+/+}$ and $CD14^{-/-}$ macrophages

Smooth LPS and the two chemotypes, Ra LPS and Re LPS, have decreasing amounts of carbohydrate (Fig. 1). As shown in Fig. 2, these forms of LPS stimulate $CD14^{+/+}$ macrophages in a manner distinctly dependent on the amount of carbohydrate present in the LPS, with decreasing potency corresponding to the reduction in carbohydrate. The concentrations of smooth LPS, Ra LPS, and Re LPS required to induce a constant amount of TNF-α (1.5 ng/ml) by

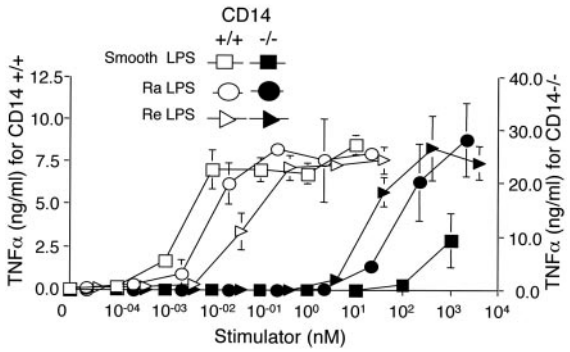

FIGURE 2. Responses of $CD14^{+/+}$ and $CD14^{-/-}$ macrophages to LPS. Murine macrophages were stimulated with smooth LPS or with the Re or Ra chemotype, and the production of TNF-α was measured. The results are a mean of duplicate determinations ± SD. Similar results were obtained in three independent experiments.

[3] Abbreviation used in this paper: DeP-Re LPS, dephosphorylated LPS.

Table I. *Concentration (nanomolar) ligand required to induce TNF-α (1.5 ng/ml) from CD14$^{+/+}$ or CD14$^{-/-}$ macrophages*[a]

Ligand	Concentration of Ligand (nM)		Relative Responses of Macrophages (CD14$^{+/+}$ to CD14$^{-/-}$)
	CD14$^{+/+}$	CD14$^{-/-}$	
Sm LPS	0.001 ± 0.0007 (2190[b])	150.00 ± 40 (1[c])	150,000
Ra LPS	0.004 ± 0.001 (548)	8.10 ± 3.28 (19)	2,025
Re LPS	0.0196 ± 0.002 (112)	3.27 ± 0.818 (46)	167
Lipid A	0.471 ± 0.247 (5)	18.71 ± 4.71 (8)	40
DeP-Re LPS	2.190 ± 0.876 (1)	3.50 ± 0.76 (43)	2

[a] Values are the mean of three different experiments.
[b] Relative strength compared with DeP-Re LPS.
[c] Relative strength compared with Sm LPS.

CD14$^{+/+}$ macrophages are significantly different for all three forms ($p = 0.001$): 0.001, 0.004, and 0.020 nM, respectively (Fig. 2 and Table I).

In contrast to what was observed in the presence of CD14, where decreasing levels of carbohydrate diminished the response, decreases in carbohydrate enhanced the response of CD14$^{-/-}$ macrophages (Fig. 2). The concentrations of Re and Ra LPS required to induce 1.5 ng/ml TNF-α by CD14 $^{-/-}$ cells were significantly different ($p = 0.05$), 3.27 and 8.10 nM, respectively, whereas a concentration of 150 nM smooth LPS was needed to induce an equivalent amount of TNF-α (Fig. 2 and Table I). Thus, removal of carbohydrate enhances the potency of LPS on CD14-negative macrophages by 20-fold (Ra LPS) and 40-fold (Re LPS) compared with smooth LPS, whereas removal of carbohydrate diminishes the response 4- and 20-fold, respectively, when CD14 is present. Furthermore, it is important to note that the sensitivity of macrophages to smooth LPS, the major form normally encountered by living cells, was at least 150,000-fold greater for CD14$^{+/+}$ cells than for CD14$^{-/-}$ cells (Fig. 2). Similarly, although not to the same extent, the sensitivity of CD14$^{+/+}$ cells was substantially greater than that of CD14$^{-/-}$ cells to Re and Ra LPS (160- and 2000-fold, respectively).

Recognition of lipid A and dephosphorylated Re LPS by CD14$^{+/+}$ and CD14$^{-/-}$ macrophages

Removal of the Kdo group from Re LPS resulted in a lipid A moiety that consisted of only two phosphorylated glucosamine residues and its long chain fatty acids (Fig. 1). Stimulation of CD14$^{+/+}$ macrophages required significantly ($p = 0.001$) more (24-fold) lipid A (0.471 nM) than Re LPS (0.02 nM) to produce an equivalent amount of TNF (1.5 ng/ml). Similarly, CD14$^{-/-}$ macrophages required more lipid A than Re LPS ($p = 0.001$) to produce an equivalent response, although this increase was only 6-fold (Table I). However, it should be noted that CD14$^{+/+}$ macrophages were 470-fold more sensitive to smooth LPS than to lipid A ($p = 0.001$), whereas CD14$^{-/-}$ macrophages were actually 27-fold less sensitive ($p = 0.001$) to smooth LPS (150 nM) than to lipid A (18.71 nM; Fig. 3 and Table I).

By removing the phosphates from Re LPS to yield DeP-Re LPS, the ability to induce a response from CD14$^{+/+}$ macrophages was reduced even further by 5-fold ($p = 0.001$; Fig. 4 and Table I). In contrast, the response of CD14$^{-/-}$ macrophages to DeP-Re LPS was actually enhanced 5-fold relative to lipid A ($p = 0.001$). As a consequence, the responses of CD14$^{-/-}$ macrophages to Re LPS and dephosphorylated Re LPS (DeP-Re LPS) were comparable, whereas the responses of CD14$^{+/+}$ macrophages to these two forms of LPS differed by >100-fold ($p = 0.001$).

Recognition by CD14$^{+/+}$ and CD14$^{-/-}$ macrophages of LPS from different Gram-negative bacterial strains

To further assess the role of CD14 in ligand recognition, the responses of CD14$^{+/+}$ and CD14$^{-/-}$ macrophages to other naturally occurring wild-type LPSs from various Gram-negative bacteria were compared. These included smooth LPS from the *Salmonella* strains *Salmonella abortus* and *Salmonella minnesota* and the natural forms of LPS from *Neisseria meningitidis* and *Rhodopseudomonas sphaeroides* that normally lack the O-Ag (31–34). In addition, LPS from *Bacteroides fragilis*, which normally lacks a typical O-Ag and one of the phosphate groups (35, 36), was used.

Smooth LPS from *S. abortus* and *S. minnesota* were found to be 4-fold more potent than the Ra-like LPS from *N. meningitidis* ($p = 0.02$) in their ability to stimulate CD14$^{+/+}$ macrophages (Table II). In contrast, they were only 1/10th as potent as the Ra-like LPS from *N. meningitidis* in stimulating CD14$^{-/-}$ macrophages ($p =$

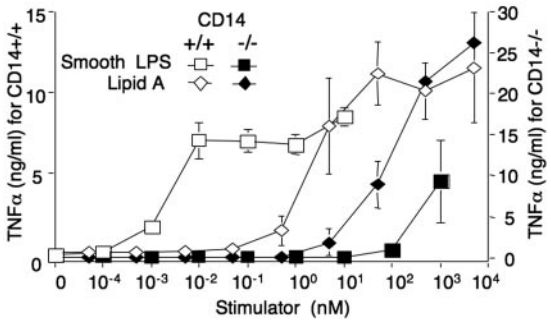

FIGURE 3. Responses of CD14$^{+/+}$ and CD14$^{-/-}$ macrophages to lipid A. Murine macrophages were stimulated with smooth LPS or lipid A, and the production of TNF-α was measured. The results are a mean of duplicate determinations ± SD. Similar results were obtained in three independent experiments.

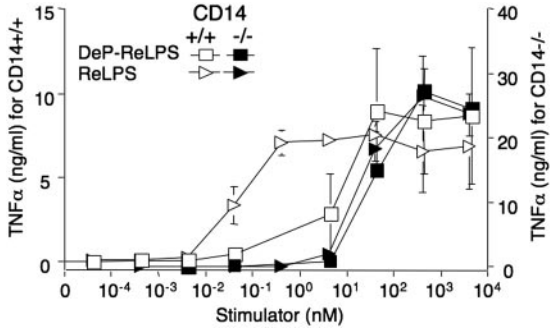

FIGURE 4. Responses of CD14$^{+/+}$ and CD14$^{-/-}$ macrophages to dephosphorylated Re LPS. Murine macrophages were stimulated with Re LPS or DeP-Re LPS, and the production of TNF-α was measured. The results are a mean of duplicate determinations ± SD. Similar results were obtained in three independent experiments.

Table II. *Concentration (nanomolar) of different wild-type LPSs required to induce TNF-α (1.5 ng/ml) from CD14$^{+/+}$ or CD14$^{-/-}$ macrophages*

Ligand	Concentration (nM)		Relative Responses of Macrophages (CD14$^{+/+}$ to CD14$^{-/-}$)
	CD14$^{+/+}$	CD14$^{-/-}$	
S. abortus	0.002 ± 0.0007	56.58 ± 14.3	28,290
S. minnesota	0.002 ± 0.001	23.43 ± 6.7	11,715
N. meningitidis	0.008 ± 0.002	7.49 ± 3.3	936
R. sphaeroides	5.000 ± 0.18	707.00 ± 121	141
B. fragilis	14.600 ± 4.3	462.80 ± 82.6	32

0.001). Even more striking were the differences when LPS from *R. sphaeroides* and *B. fragilis* were used; although *S. abortus* LPS was 2500- and 7300-fold, more potent than *R. sphaeroides* and *B. fragilis* LPS, respectively, in stimulating CD14$^{+/+}$ macrophages ($p = 0.001$), *S. abortus* LPS was only 8- and 12-fold more potent than *R. sphaeroides* and *B. fragilis* LPS, respectively, in stimulating CD14$^{-/-}$ macrophages ($p = 0.001$). In all cases, less LPS was required to stimulate CD14$^{+/+}$ macrophages than CD14$^{-/-}$ macrophages; however, this difference ranged from 30,000-fold for *S. abortus* LPS to only 32-fold for *B. fragilis* LPS.

Role of TLR4 in CD14-independent LPS responses

To confirm that the responses of CD14$^{-/-}$ macrophages to the various forms of LPS studied require TLR4, macrophages expressing CD14, but not TLR4, and macrophages deficient in both TLR4 and CD14 were stimulated with smooth LPS, Re LPS, and lipid A. As shown in Fig. 5, none of these forms of LPS was able to stimulate TNF production by *TLR4*$^{-/-}$ macrophages or by *CD14*$^{-/-}$,*TLR4*$^{-/-}$ macrophages even at high doses of LPS.

Discussion

CD14 and TLR4 are part of a receptor complex for LPS, a major toxin of Gram-negative bacteria. Both CD14 and TLR4 are required for strong responses to LPS, as demonstrated by inhibition with Abs (7, 37) and by the lack of responsiveness of mice deficient in CD14 (8–11) or TLR4 (13, 38, 39). Several studies using partial structures of smooth LPS such as lipid A in combination with transfected cells overexpressing TLR4 from different species have suggested that TLR4 is responsible for the ligand specificity (23–26) of the receptor complex and that CD14 serves merely to concentrate the ligand and present it to TLR4. However, because of the complex and artificial nature of these experiments and because partial structures of LPS that are several orders of magnitude less potent than smooth LPS were used in these studies, this interpretation is open to question. Consequently, we decided to use conditions that were more physiologically relevant to determine the relative roles of CD14 and TLR4 in ligand recognition. Accordingly, macrophages from normal and CD14-deficient mice were examined for their ability to respond to smooth LPS and a variety of LPS partial structures. These partial structures are similar to those used by others to assess the role of TLR4 in ligand recognition.

When the relative strengths of smooth LPS and partial structures of LPS are compared for their ability to induce TNF, the ligand specificities of CD14$^{+/+}$ and CD14$^{-/-}$ macrophages are found to be very different (Table I and Fig. 6). When CD14 is present, the receptor complex is highly specific for smooth LPS, the form expressed by the majority of pathogenic Gram-negative bacteria. As increasing amounts of carbohydrate are removed from smooth LPS in a stepwise manner, the sensitivity of CD14$^{+/+}$ macrophages decreases dramatically to the point where nearly 500 times as much lipid A as smooth LPS is required to produce an equivalent response. In contrast, CD14$^{-/-}$ macrophages show very little variation in sensitivity to the various partial forms of LPS and, indeed, are the least sensitive to smooth LPS. Furthermore, the optimal response of CD14$^{-/-}$ macrophages is at best 3000-fold less than the optimal response of CD14$^{+/+}$ macrophages to smooth LPS (Table I).

These differences in ligand specificity between CD14$^{+/+}$ and CD14$^{-/-}$ macrophages can also be observed when natural LPS from different Gram-negative bacteria are used. For example, LPS from *S. abortus* and *B. fragilis* differ by 7300-fold in their ability to induce an equivalent response from CD14$^{+/+}$ macrophages, whereas there is only an 8-fold difference in their ability to induce a response in CD14$^{-/-}$ macrophages (Table II). Because these two forms of LPS differ in both their carbohydrate and lipid moieties, it is not clear which of these components is responsible for the differences in ligand specificity observed with CD14$^{+/+}$ macrophages (32–36). For LPS from some bacteria (*R. sphaeroides* and *B. fragilis*), CD14$^{+/+}$ macrophages are not only highly insensitive

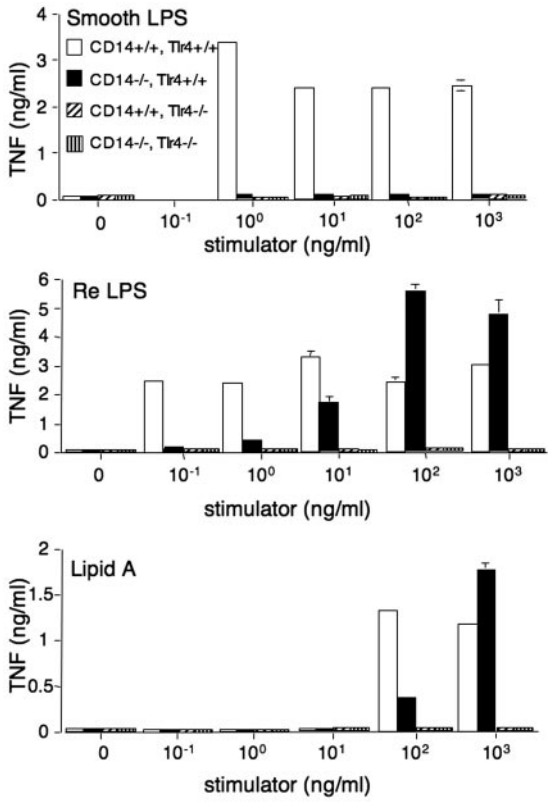

FIGURE 5. TNF-α response of macrophages from normal mice or mice deficient in CD14, *Tlr4*, or both *Tlr4* and CD14 to smooth LPS, Re LPS, or lipid A. The results are representative of three independent experiments.

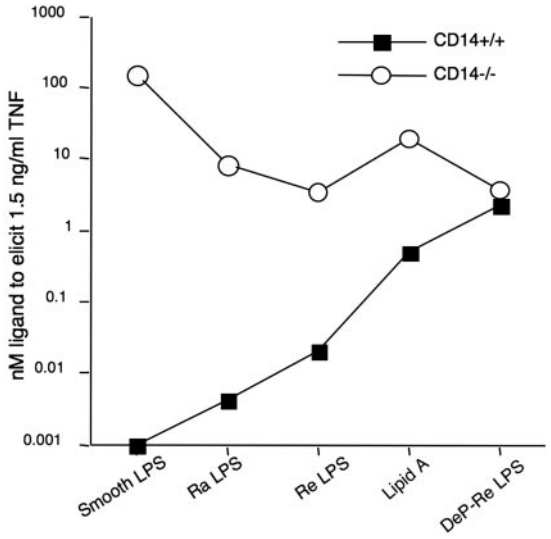

FIGURE 6. Comparison of the sensitivities of $CD14^{+/+}$ and $CD14^{-/-}$ murine macrophages to various forms of LPS. The concentration of ligand (nanomolar) required for the induction of 1.5 ng/ml TNF-α, as calculated in Table I, is shown on the *y*-axis. The different structures of LPS studied are listed on the *x*-axis in the order of decreasing sugar moieties or phosphate. Note that $CD14^{+/+}$ and $CD14^{-/-}$ macrophages differ in their sensitivity to smooth LPS by five orders of magnitude, whereas their sensitivities to DeP-Re LPS are nearly the same.

to LPS stimulation, but the response is essentially CD14 independent. Interestingly, the LPS and/or lipid A from these organisms are known to act as LPS antagonists for human cells (40, 41). Presumably, these forms can bind to TLR4 with sufficient affinity to block an LPS response, but are unable to bind to CD14 with sufficient affinity to induce a response.

In all cases tested, the residual response of $CD14^{-/-}$ macrophages was found to be TLR4 dependent (Fig. 5). However, this response, in general, required enormous amounts of LPS compared with the amount required to stimulate $CD14^{+/+}$ macrophages, especially when smooth LPS was used. This is due to the exquisite specificity and sensitivity of macrophages for smooth LPS when CD14 is present; as smooth LPS becomes sequentially truncated, the sensitivity of $CD14^{+/+}$ macrophages to these increasingly truncated forms becomes sequentially diminished. In contrast, in the absence of CD14, this specificity for smooth LPS is lost, and the resulting $CD14^{-/-}$ macrophages appear to be almost indiscriminate in their recognition of the various partial forms of LPS. Indeed, excluding smooth LPS, there is only a 6-fold difference in the sensitivities of $CD14^{-/-}$ macrophages to the various partial form of LPS, and even when smooth LPS is included in the comparison, there is only a 45-fold difference (Table I and Figs. 3 and 6). In contrast, $CD14^{+/+}$ macrophages differ by >500-fold in their sensitivity to these partial forms of LPS and by 2190-fold when smooth LPS is included in the comparison (Table I and Figs. 3 and 4). Thus, as increasingly truncated forms of LPS are used for stimulation, the responsiveness of $CD14^{+/+}$ macrophages not only decreases greatly, but also becomes increasingly CD14 independent. Importantly, it is partial structures of LPS similar to these that have been used to perform studies purporting to demonstrate that TLR4 and not CD14 is responsible for the ligand specificity of the LPS-receptor complex (23–26). Thus, when these partial forms of LPS are used, the role of CD14 in ligand recognition becomes greatly diminished. Under these conditions, and when TLR4 is overexpressed relative to CD14, as may be the case when cells are transfected with TLR4, differences in the ability of TLR4 molecules from different species to bind partial structures of LPS may completely overshadow the highly discriminating ligand specificity of CD14.

Although it may be assumed that this discriminatory ability of CD14 is based on its ability to recognize different degrees of glycosylation on LPS, whereas TLR4 lacks this ability, it should be noted that alternative explanations for our observations may be proposed. For example, it may be that the differences we observed are due to different degrees of aggregation and/or differing physical states of the micellar aggregates that are formed by the various forms of LPS used in these studies (42–44). Studies by Mueller et al. (45) suggest that aggregates, rather than monomers, are the active units of endotoxin, and that the physical states of these aggregates are a function of their hydrophobicity. In contrast, several other studies have suggested that LPS monomers, rather than LPS aggregates, are important for function (46–48). Although our studies cannot distinguish between these two very different mechanisms, they nevertheless point out the important role CD14 plays in the specific recognition of LPS.

Disclosures

The authors have no financial conflict of interest.

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