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Phospholipids Inhibit Lipopolysaccharide (LPS)-Induced Cell Activation: A Role for LPS-Binding Protein

Mareile Mueller,* Klaus Brandenburg,* Russ Dedrick, ‡ Andra B. Schromm,2* and Ulrich Seydel*

The inhibition of LPS-induced cell activation by specific antagonists is a long-known phenomenon; however, the underlying mechanisms are still poorly understood. It is commonly accepted that the membrane-bound receptors mCD14 and TLR4 are involved in the activation of mononuclear cells by LPS and that activation may be enhanced by soluble LPS-binding protein (LBP). Hexaacylated Escherichia coli lipid A has the highest cytokine-inducing capacity, whereas lipid A with four fatty acids (precursor IVa, synthetic compound 406) is endotoxically inactive, but expresses antagonistic activity against active LPS. Seeking to unravel basic molecular principles underlying antagonism, we investigated phospholipids with structural similarity to compound 406 with respect to their antagonistic activity. The tetraacylated diphosphatidylglycerol (cardiolipin, CL) exhibits high structural similarity to 406, and our experiments showed that CL strongly inhibited LPS-induced TNF-α release when added to the cells before stimulation or as a CL/LPS mixture. Also negatively charged and to a lesser degree zwitterionic diacyl phospholipids inhibited LPS-induced cytokine production. Using Abs against LBP, we could show that the activation of cells by LPS was dependent on the presence of cell-associated LBP, thus making LBP a possible target for the antagonistic action of phospholipids. In experiments investigating the LBP-mediated intercalation of LPS and phospholipids into phospholipid liposomes mimicking the macrophage membrane, we could show that preincubation of soluble LBP with phospholipids leads to a significant reduction of LPS intercalation. In summary, we show that LBP is a target for the inhibitory function of phospholipids. The Journal of Immunology, 2005, 174: 1091–1096.

Bacterial LPS are major constituents of the cell wall of Gram-negative bacteria. Enterobacterial LPS consists of a sugar moiety composed of a core oligosaccharide and an O Ag with repeating sugar units, which is covalently linked to a lipid moiety termed lipid A. The release of LPS from bacterial cell walls into the host’s blood circulation causes the activation of immune cells, resulting in the production of cytokines, such as TNF-α, interleukins, NO, and tissue factors. Systemic production of these proinflammatory mediators causes the sepsis syndrome which is clinically characterized by hypotension, fever, and respiratory dysfunction and may lead to multiorgan failure and death (1, 2). Acknowledging the fact that isolated lipid A is able to cause cell activation, it is commonly referred to as the “endotoxic principle” of LPS (3).

To activate immune cells by LPS, particular physicochemical characteristics are required for its lipid A portion. The enterobacterial lipid A with the highest cytokine-inducing capacity is composed of a diglucosamine which is phosphorylated in position 1 and 4′ and carries six fatty acids in asymmetric distribution. This molecule adopts a conical shape (4, 5). Lipid A partial structures lacking one or more fatty acids, like the lipid A precursor IVa (synthetic compound 406, see Fig. 1) which carries only four fatty acids in symmetric distribution, adopts a cylindrical molecular shape, is endotoxically inactive, but may express strong antagonistic activity against LPS-induced cell activation (6, 7). Intending to define the structural prerequisites for antagonism, we asked the question whether other amphiphilic molecules that are not related to lipid A but express similar physicochemical properties as compound 406 with respect to molecular shape and net negative charge would also antagonize the cytokine-inducing capacity of endotoxically active LPS or lipid A. Earlier work of our group has shown that, for example, this is the fact for certain phospholipid (PL)-like amphiphiles with six acyl chains attached to a bisphosphorylated serine-like backbone (8, 9). In this article, we investigated the antagonistic activity of cardiolipin (CL,1 diphosphatidylglycerol), a PL which is present in the mitochondrial membrane (10) and has structural similarities to compound 406 with respect to the net negative charge [−2] and number of acyl chains [4] and a selection of negatively charged diacyl PL. The chemical structures of compound 406 and the PL used are shown in Fig. 1. The inhibition of cell activation by antagonistic structures must involve a direct interaction of the antagonist with one or more of the proteins involved in cell activation (11). An important protein mediating LPS transport to the cell surface and binding to surface structures is the soluble (s) LPS-binding protein (sLBP),

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3 Abbreviations used in this paper: CL, cardiolipin; FRET, fluorescence resonance energy transfer; LBP, LPS-binding protein; mLBP, membrane-bound LBP; sLBP, soluble LBP; MØ, macrophage; MNC, mononuclear cell; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE; PAPC, 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine; PC, phosphatidylycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; RhPE, N-(rhodamine B sulfonyl)-PE; SM, sphingomyelin.
a 55-kDa acute-phase serum protein produced by hepatocytes (12). Because of its positive charges, sLBP binds not only to LPS, but also to a variety of negatively charged lipids like phosphatidylinositol (PI), phosphatidylserine (PS), or CL (Ref. 13 and our unpublished data). It accelerates LPS binding to the cell membrane, thus also enhancing cell activation (14). Targeted deletion of the gene for LBP in mice has underlined the role of LBP in cell activation by LPS (15) as well as its importance in combating infections with Gram-negative bacteria (16). Recently, we were able to show that besides transporting LPS or PL into PL membranes, LBP itself intercalates into cell membranes and assumes a transmembrane configuration (17). This membrane-bound form of LBP (mLBP) plays an important role in cell activation, as described for CD14, possibly by binding LPS aggregates and intercalating these into the cell membrane (18).

Another important cell surface structure involved in cell activation is the membrane-bound protein mCD14, a 55-kDa protein anchored to the membrane by a glycosylphosphatidylinositol anchor. Because of its lack of a transmembrane domain, mCD14 cannot transfer a signal across the cell membrane. Instead, probably the most important function described for mCD14 is the binding and transfer of LPS or lipid A to other proteins, facilitating the most important function described for mCD14 is the bind-

**Materials and Methods**

This article seeks to define the basic molecular structures that present data showing that not only CL, but also the PL phosphatidyglycerol (PG) and PI exhibit strong antagonistic activity against LPS and provide evidence suggesting that mLBP is a target for this antagonistic activity.

**Lipids and other reagents**

Deep rough mutant LPS from *Escherichia coli* strain F515 was extracted by the phenol/chloroform/petroleum ether method (25), purified, lyophilized, and transferred into the triethylamine salt form. All LPS preparations are routinely tested by mass spectrometry for purity to exclude contaminations. LPS was suspended in buffer (100 mM KCl and 5 mM HEPES, pH 7.0) by thorough vortexing. The suspension was temperature-cycled at least twice between 4 and 56°C, each cycle followed by intense vortexing for a few minutes, and then stored at 4°C for at least 12 h before measurement. LPS suspensions were aliquoted and stored at −20°C.

CL from bovine heart, phosphatidylcholine (PC) and PG from egg, PI from bovine liver, sphingomyelin (SM) and phosphatidylserine (PS) from bovine brain, and PE from *E. coli* were purchased from Avanti Polar Lipids. All PL, were used without further purification. Recombinant human TNF-α was purchased from PepTech. The fluorescent dyes N-(7-nitro-2,1,3-benoxadiazol-4-yI)-PE (NBD-PE) and N-(rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes. Recombinant human LBP (456-aa holoprotein rLBP50) in 10 mM HEPES (pH 7.5) was prepared as described in detail elsewhere (26). The monoclonal mouse anti-mouse LBP Ab biG33 cross-reacting with human LBP was obtained from Biometic.

**Stimulation of human mononuclear cells (MNC) and macrophages (Mb) by LPS**

In experiments aiming at the determination of the cytokine-inducing capacity of LPS, human MNC or Mb were stimulated with LPS, and TNF-α production by the cells was determined in the supernatant. Blood for human MNC preparation was obtained from healthy volunteers. MNC were separated from whole blood using Ficoll-Hypaque as previously described (18). To obtain Mb, MNC were separated into monocytes and lymphocytes by elutriation as described elsewhere (27). The monocytes were cultivated for 7 days in RPMI 1640 containing 4% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 ng/ml M-CSF to allow them to differentiate into Mb.

For stimulation experiments, cells were suspended in RPMI 1640 (containing 4% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 105 cells/ml for MNC and 106 cells/ml for Mb and transferred to 96-well culture plates at 200 μl/well. LPS or LPS/PL mixtures were serially diluted in serum-free RPMI 1640 and added to the cultures at 20 μl/well. The cultures were incubated for 4 h at 37°C and 5% CO2 for induction of TNF-α or 24 h for induction of IL-8. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 g and stored at −20°C until determination of cytokine content. To investigate the dependence of antagonistic effects on serum proteins and mLBP, in some experiments cells were washed several times in serum-free medium to completely remove soluble proteins and resuspended in serum-free medium for stimulation. To investigate the antagonistic effect of PL on LPS-induced TNF-α production, PL were either added to the cells 15 min before stimulation or preincubated with LPS for 15 min at 37°C and the mixture added to the cells. In the case of addition of LBP Ab or recombinant human LBP Ab and rLBP were added concomitantly 10 min before stimulation or preincubated with LPS for 15 min at 37°C and the mixture added to the cells. In the case of addition of PL, PL were added 15 min before stimulation with LPS to the cells. All stimulation experiments were performed in triplicates.

The concentration of TNF-α in the cell supernatant was determined in a sandwich ELISA as described elsewhere (28). Microtiter plates (Greiner) were coated overnight at room temperature with a monoclonal mouse anti-human TNF-α Ab (Intex) followed by three washings with water. One hundred-microliter samples of culture supernatants were dispensed into the wells and incubated with HRP-conjugated rabbit anti-human TNF-α Ab (Intex) for 16–24 h at 4°C. After washing, the color reaction was started by addition of tetramethylbenzidine/H2O2 and stopped by the addition of 1 M sulfuric acid. Serial dilutions of human rTNF-α (Intex) provided a standard curve. Plates were read at 450 nm with an ELISA photometer. Quantification of TNF-α was determined with detection ranges of 0–500 pg/ml. TNF-α content was determined for each condition and the data given are average values ± SD. The concentration of IL-8 was determined by the
Fluorescence resonance energy transfer (FRET) spectroscopy

The fluorescence resonance energy transfer (FRET) technique was used as a probe dilution assay (29, 30) with the fluorescent dyes NBD-PE and Rh-PE to obtain information on the intercalation of LBP and LPS into liposomes PL<sub>348</sub> made from a lipid mixture resembling the lipid composition of human M<sub>0</sub> [PC][PE][SM][PS] = 38:1:27:3:19:4:15:2 molar ratios (31). For the FRET experiments, PL<sub>348</sub>-liposomes were double labeled with NBD-PE and Rh-PE in chloroform (lipid):[NBD-PE][Rh-PE] of 100:1:1 molar ratios. The solvent was evaporated under a stream of nitrogen, the lipids resuspended in a bathing solution containing 100 mM KCl and 5 mM HEPES at pH 7.0, mixed thoroughly, and sonicated with a Branson sonicator for 1 min (1-ml solution). Subsequently, the preparation was cooled for 30 min at 4°C, heated for 30 min at 56°C, and recooled to 4°C. Preparations were stored at 4°C overnight before measurement. A preparation of 900 μl of the double-labeled lipid liposomes (0.1 mM) at 37°C was excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE, and the acceptor Rh-PE (593 nm) were measured simultaneously on the fluorescence spectrometer SPEX F1T11 (SPEX Instruments). LBP, PL aggregates, and LPS aggregates were added after 50, 100, and 150 s, respectively. Since FRET spectroscopy is used here as a probe dilution assay, intercalation of unlabeled molecules such as LBP, PL, and LPS causes an increase of the distance between donor and acceptor and, thus, leads to a reduced energy transfer. This again causes an increase of the donor and decrease of the acceptor intensities. For a qualitative analysis of experiments, the quotient of the intensities of the donor dye and the acceptor dye are plotted against time (denoted in the following as the FRET signal). The data shown are representative for at least three independent experiments.

Results

Preincubation of MNC with CL reduces the TNF-α production after LPS stimulation

As a measure of the biological activity of LPS, the TNF-α production by human MNC or M<sub>0</sub> was determined. To investigate the influence of CL on the LPS-induced activation of MNC, the cells were stimulated with 1 ng/ml LPS in the presence of CL in various molar ratios and under various incubation conditions. CL was either preincubated with MNC for 15 min before addition of LPS or was preincubated along with LPS for 15 min at 37°C before addition of the mixture (CL + LPS) to the cells. Under serum-containing conditions (Fig. 2a), stimulation of the cells with LPS alone resulted in a TNF-α production of 1266 ± 82 pg/ml. Preincubation of the cells with CL before addition of LPS led to a considerable reduction of TNF-α production. Under the latter conditions, a strong inhibitory effect of CL was observed at a molar ratio of [CL]:[LPS] >10:1. Higher molar ratios of CL led to a complete inhibition of LPS-induced TNF-α production. In addition, the stimulation by a preincubated mixture of CL and LPS enhanced the inhibitory effect ~100-fold.

Serum proteins like sLBP and sCD14 play important roles in cell activation by LPS. To investigate the involvement of serum proteins in the antagonistic action of PL, similar experiments were performed under serum-free conditions (Fig. 2b). Stimulation with 1 ng/ml LPS alone led to a TNF-α release of 377 ± 29 pg/ml. It should be noted that MNC release significantly more TNF-α under serum-containing conditions than washed MNC under serum-free conditions. A notable reduction of LPS-induced TNF-α production could be observed at molar ratios of [CL]:[LPS] = 1:1, regardless whether CL was preincubated with the cells or with the LPS. The inhibitory activity of CL under serum-free conditions is enhanced, probably due to the absence of CL-binding proteins which are present in human serum.

To exclude cytotoxic effects that have been described for high concentrations of CL, the viability of cells after PL treatment was estimated by the trypan blue exclusion test, and no change in viability was observed even at the highest CL concentrations (data not shown).

Addition of sLBP can abolish the effect of an LBP Ab

To investigate whether the inhibition of TNF-α production by CL is dependent on serum proteins such as sLBP, M<sub>0</sub> were washed three times in serum-free medium to remove all soluble proteins. Stimulation of these washed cells with LPS alone led to a TNF-α production of 1200 ± 65 pg/ml (Fig. 3, bar a). Subsequent addition of sLBP (100 ng/ml) led to an enhanced LPS-induced TNF-α production by a factor of 3.5 (Fig. 3, bar b). The LPS-induced TNF-α production could be completely blocked, when an LBP Ab (10 μg/ml) was added to the washed cells 30 min before stimulation in the absence of sLBP, indicating that mLBP is essential for cell activation by LPS under serum-free conditions (Fig. 3, bar c). The inhibition of cell activation by the LBP Ab could be abolished by addition of sLBP (100 ng/ml). The subsequent stimulation with LPS resulted in a TNF-α production of 3214 ± 608 pg/ml (Fig. 3, bar d). To elucidate whether LBP is a target for the inhibitory function of CL, sLBP (100 ng/ml) was preincubated with CL at a molar ratio 1:1 for 15 min at 37°C. Addition of this mixture to the cells led to a dramatic decrease of the TNF-α induction which...
could not be enhanced by subsequent addition of LPS (Fig. 3, bar e). This result indicates a direct interaction between CL and LBP.

**CL decreases the LBP-mediated intercalation of LPS in PL liposomes when preincubated with LBP**

An important attribute of LBP is its capacity to bind lipids and deliver them to acceptor systems such as PL membranes. We used FRET spectroscopy to investigate the effect of PL on LBP-mediated transport and intercalation of LPS into PL liposomes. Liposomes were prepared from a PL mixture resembling the lipid composition of the cytoplasmic Mφ membrane and labeled with the donor and acceptor dyes. The addition of LPS (10 μM) to the liposomes (10 μM) at t = 50 s did not change the FRET signal; however, the subsequent addition of LBP (100 nM) at t = 100 s led to an increase (Fig. 4, trace a), indicating an LBP-mediated intercalation of LPS into the liposomes. From trace b (Fig. 4) it can be taken that LBP does not intercalate into PLMφ liposomes to a noticeable degree. In trace b (Fig. 4), subsequent addition of LBP, CL (10 μM), and LPS to PLMφ liposomes resulted in an increase of the FRET signal, suggesting an LBP-mediated intercalation of CL and LPS into PLMφ liposomes. Obviously the interaction of CL with LBP led to a reduction of the LBP-mediated intercalation of LPS into the liposomes. In trace c (Fig. 4), again LPS was added to the liposomes, which did not lead to an increase of the signal. In contrast to trace a (Fig. 4) not LBP alone was added but rather LBP preincubated with CL (30 min, 37°C). Under these conditions, only a minor change of the signal was observed, indicating that LBP preincubated with the PL is only marginally able to interact with LPS and to promote its intercalation.

It should be noted that the intercalation of LBP itself into PL liposomes depends on their net negative surface charge, i.e., most effectively into liposomes made from negatively charged PL (30). Since the changes in the FRET signal upon addition of LBP to PLMφ are poorly visible in our experimental system, we did analogous experiments using liposomes made from the negatively charged PS. In this case, addition of LBP to PS liposomes caused a clear change of the FRET signal, and the effects measured for the addition of CL under various conditions were comparable (data not shown).

**Negatively charged PL inhibit LPS activation and intercalation**

In addition to CL, we included the negatively charged diacyl phospholipid PG (1 net negative charge) and PI (1 net negative charge) in our experimental scheme (Fig. 1) to gain more information on the inhibitory potential of PL. Comparable to CL, also the phospholipids PG and PI led to an inhibition of LPS-induced TNF-α production by Mφ (Fig. 5a). The dose response shows that the inhibitory effects of PG and PI were less pronounced than that of CL, and for all tested inhibitors the potency of inhibition did not depend on the presence of serum (Fig. 5). To show the specificity of inhibition, we also stimulated Mφ with TNF-α in the presence or absence of CL and measured production of IL-8 as a readout for cell activation. In contrast to stimulation with LPS, CL did not inhibit cell activation by TNF-α. The capacity of the negatively charged diacyl PL to inhibit LBP-mediated transport of LPS was comparable to that of CL, as shown in Fig. 6 for PG. LPS was intercalated into the liposomes by LBP (Fig. 6, trace a), whereas preincubation of LBP with PG led to a dramatic decrease in the FRET signal (Fig. 6, trace b).

**Discussion**

To increase our understanding of the molecular basis of antagonistic activity against LPS, we examined the effects of CL and other PL on the LPS-induced TNF-α production by human MNC and Mφ in vitro, the intercalation of LPS into PL liposomes, and the role of LBP in these experiments. In the presence of CL, LPS-induced TNF-α production was decreased in a dose-dependent manner (Fig. 2). This was similarly true for the negatively charged phospholipids PG and PI (Fig. 5). The inhibitory effect of negatively charged PL on the LPS-induced TNF-α production was observed under serum-free as well as under serum-containing conditions. Therefore, it seems likely that the target of CL and other PL, and most likely of LPS, is a membrane-bound or even a transmembrane rather than a serum protein. However, the interaction of CL with serum proteins obviously modulates its inhibitory potency. Thus, the inhibitory effect of CL when applied before LPS is reduced under serum-containing conditions, reflecting the presence of CL-binding proteins in human serum. In contrast, preincubated (CL + LPS) complexes exhibit the same potency of inhibition in the presence as well as in the absence of serum (Fig. 2), indicating that complex formation of CL and LPS interferes with

**FIGURE 3.** TNF-α production from Mφ (washed three times in serum-free buffer) after stimulation with LPS (0.5 ng/ml) in the absence and presence of the monoclonal LBP Ab bg33 (10 μg/ml), CL (25 ng/ml), and LBP (100 ng/ml). CL was preincubated for 15 min at 37°C (LBP + CL).

**FIGURE 4.** Time kinetics of changes in the FRET signal from double-labeled PLMφ liposomes (10⁻⁵ M) upon subsequent addition of LBP (10⁻⁵ M), CL (10⁻⁵ M), LPS (10⁻⁵ M), and LPS and CL preincubated (15 min, 37°C) in varying order. Time points of addition of the agents are marked as steps 1, 2, and 3.
binding of CL to proteins in serum. The observed inhibitory effect of PL appears to be specific for cell stimulation by LPS. Neither of the PL exhibited antagonistic activity on cell stimulation by TNF-α (Fig. 5 and data not shown).

LBP is a 55-kDa protein and has been shown to play an important role in LPS-induced cell stimulation (32). Our previous studies have shown that LBP is not only a serum protein but also interacts into cell membranes (17) and assumes a transmembrane configuration. Thus, LBP is present as a cell surface protein even under serum-free conditions and is in this configuration involved in cell signaling (18). We showed that endotoxin aggregates rather than monomers are the biologically active unit (33). For energetic reasons, these aggregates cannot intercalate by themselves into the immune cell membrane. The energy barrier may, however, be overcome by an assumed function of mLBP as a fusion protein, thus binding LPS aggregates and intercalating them into the membrane.

To explore the possibility that the inhibitory effect of CL and other negatively charged PL on LPS-induced TNF-α production results from the inhibition of LPS binding to LBP on the cell surface, we blocked under serum-free conditions membrane-bound LBP with a monoclonal anti-LBP Ab (Fig. 3). By adding free LBP, we could abolish the inhibitory effect of the Ab, and LPS stimulation again led to TNF-α production. Under otherwise identical conditions, preincubation of sLBP with CL, however, led to complete inhibition of LPS-induced TNF-α production. Thus, it seems very likely that binding of CL or other PL to sLBP, possibly leading to complex formation, is a reason for the inhibition of LPS-induced TNF-α production by MNC. We, therefore, focused on the elucidation of the mechanism of the inhibitory action of PL on LBP.

FRET spectroscopy was used to monitor the role of LBP in the transport and membrane integration of LPS and of the possible effects of PL on these roles. Our FRET data show that preincubation of LBP with CL or the negatively charged phospholipid PG causes a dramatic decrease in the LBP-mediated intercalation of LPS into PLMΔα liposomes (Figs. 4 and 6). LBP binds to negatively charged PL and in this complexed form it is not able to mediate intercalation of LPS into PLMΔα liposomes, suggesting that LPS and negatively charged molecules compete for a common binding site of LBP (34). It should be mentioned that the zwitterionic phospholipid PC did not cause a reduction of the LBP-induced intercalation of LPS into liposomes, indicating that there is only a minor interaction between PC and LBP (data not shown).

Wang et al. (35) showed an inhibition of the LPS-induced cell activation by the negatively charged PL. These authors, however, referred this inhibition to binding of PI to mCD14. This effect was LBP dependent and could be abolished by a monoclonal LBP Ab. Since both molecules, LBP and CD14, bind LPS, it seems likely that by binding of LPS they mediate an interaction of LPS with signaling proteins. It may thus well be that cytokine production is reduced by PL by competitive inhibition of binding of LPS to CD14 or LBP. For a similar function of LBP and CD14 is the fact that in our stimulation experiments, the inhibition of LPS-induced cell activation by LBP Abs could be abolished by the addition of sCD14 (data not shown). Kutuzova et al. (11) demonstrated that diphasoryl lipid A of Rhodobacter sphaeroides binds both LBP and CD14, thus inhibiting LPS-induced cytokine production by inhibiting binding of LPS to the cell surface. Bochkov et al. (36) have demonstrated that oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) blocked both LBP and CD14, thus inhibiting LPS-induced cytokine production by up-regulation of inflammatory adhesion molecules, such as ICAM-1 or E-selectin, on human endothelial cells. These authors also showed that oxidized PAPC inhibited binding of LPS to immobilized LBP as well as to immobilized CD14, suggesting that this diminished binding is the reason for the antagonistic action of oxidized PAPC. In this context, another recent article may be of interest. Hashimoto et al. (37) have shown that PG and other negatively charged PL inhibited LPS-induced cell activation, whereas

![FIGURE 5](http://www.jimmunol.org/1095/f5.png)

**FIGURE 5.** Cytokine production by human Mø after stimulation with 1 ng/ml LPS or 100 ng/ml TNF-α in the absence and presence of CL, PI, and PG at the indicated concentrations under serum-containing (a) and serum-free conditions (b). Cells were either directly stimulated with LPS or preincubated for 15 min at 37°C with the PL and then stimulated with LPS. N.d., Not detectable.

![FIGURE 6](http://www.jimmunol.org/1095/f6.png)

**FIGURE 6.** Time kinetics of changes in the FRET signal from double-labeled PLMΔα liposomes (10⁻⁵ M) upon subsequent addition of LBP (10⁻⁵ M), PG (10⁻⁵ M), LPS (10⁻⁵ M), and LPS and PG preincubated (15 min, 37°C) in varying order. Time points of addition of the agents are marked as steps 1, 2, and 3.
PL with no net charge did not. They also showed that PG inhibited binding of LPS to LBP as well as to CD14. On the basis of their findings, the authors proposed that by reducing this binding, LBP-mediated monomerization of LPS is reduced and, therefore, LPS-induced cell activation is inhibited. This model seems rather unlikely, because of our above-mentioned findings of aggregates being the active endotoxin unit (33). Our data, showing that not only preincubation of the cells with PL but also preincubation of LPS with PL and stimulation of the cells with the mixture caused a decrease of the TNF-α release (Fig. 2), may be explained by a change of the LPS aggregate structure provoked by the PL. This would lead to interference with the presentation of LPS to the LPS-binding proteins, resulting in a reduction of the potency to stimulate immune cells. This hypothesis is supported by earlier studies which showed that incorporation of LPS into liposomes reduced its biological activity (38).

In conclusion, we have shown that CL and other negatively charged PL exhibit an inhibitory effect on LPS-induced TNF-α production by human MNC and MD, most likely by a blockade of the binding of LPS aggregates to LBP. This is accompanied by a reduction of the LBP-mediated transport and intercalation of LPS into membranes.

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