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Treponemal Phospholipids Inhibit Innate Immune Responses Induced by Pathogen-associated Molecular Patterns*

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Host innate immune responses to microbial components, known as pathogen-associated molecular patterns (PAMPs), are regulated and modified by cellular receptors and serum proteins, including Toll-like receptors (TLRs), CD14, and LPS-binding protein (LBP). We demonstrated that a treponemal membrane lipid inhibited PAMPs-induced immune responses. The chemical structure of the lipid was elucidated as a phosphatidylglycerol (PG) derivative, which is scarce in most mammalian tissues, but relatively abundant in treponemal membrane lipids. Natural and synthetic PG counterparts as well as related natural anionic phospholipids, phosphatidylinositol, phosphatidylserine, and cardiolipin, also demonstrated an inhibitory effect. Further, we noted that PG inhibited PAMPs-induced immune responses by blocking the binding of PAMPs with LBP and CD14. In addition, PG decreased proinflammatory cytokine production in serum of LPS-injected mice and depressed abscess formation in mice infected with treponemes. These results suggest that treponemal phospholipid interfere the function of LBP/CD14 and act as a modulator of innate immune responses.

The innate immune system plays essential roles in host defense against exposure to microorganisms. Host responses to microbial components known as pathogen-associated molecular patterns (PAMPs)[1] are regulated and modified by cellular receptors and serum proteins. Toll-like receptor (TLR), which is a type I transmembrane protein, has been implicated as the major receptor for PAMPs (1). To date, ten members of the TLR family have been described and some of their ligands identified. Among them, TLR4, in combination with its adapter molecule MD-2, has been shown to be the signaling receptor for lipopolysaccharide (LPS), which is distributed on the outer membrane of Gram-negative bacteria (2, 3), while TLR2 has been found to bind peptidoglycan (PGN), a common component of bacterial cell walls, and activate host cells (3, 4). Further, TLR9 has been reported to be involved in immune response to unmethylated CpG DNA (5) and TLR3 has been identified as the receptor for double-stranded RNA (dsRNA) (6). TLRs recognize PAMPs through an extracellular leucine-rich repeat domain and activate signal transduction cascades via the cytoplasmic Toll/IL-1 receptor domain (7). The cascade involving cytoplasmic proteins, MyD88, IRAK, and TRAF6, activates NF-κB and leads to an expression of inflammatory mediator genes.

In addition to TLRs, LPS-binding protein (LBP) and CD14 have been implicated in the recognition of some PAMPs. CD14 is found on the surface of innate immune cells as a glycosylphosphatidylinositol-anchored membrane protein and in plasma in a soluble form (8). It binds to some PAMPs, such as LPS (8) and PGN (9), and enhances their recognition by TLRs (10). Further, LBP, which is an acute-phase protein found in plasma (11), increases the binding of soluble CD14 to LPS (8) and PGN (9).

Treponemes are anaerobic bacteria with a typical helical shape that have been associated with the induction of chronic human diseases, such as syphilis caused by *Treponema pallidum* (12) and periodontal diseases caused by *Treponema denticola* (13). Such chronic diseases are characterized by an inflammatory reaction induced by pathogens followed by extensive tissue loss. While treponemes lack LPS (14), their cell surface components have been shown to induce the synthesis of inflammatory mediators by host cells. The lipoproteins of treponemes have been shown to be potent virulent factors; *i.e.* the lipoproteins of *T. pallidum* (15) induce NF-κB translocation in monocytes (16) and those of *T. denticola* (17) activate macrophages (18). Recently, we described the immunostimulating activity of the outer membrane extract (OME) from several treponemes (19). In addition, glycolipids extracted from *T. denticola*, *Treponema maltophilum*, and *Treponema brennaborense* (20, 21) are reported to be potent stimulators of host cells (22, 23). Cell activation induced by these treponemal components also depends on CD14, as well as TLR2 or TLR4 (21, 23–25). Although the immunostimulating activities of treponemal components are considerably weaker than those of LPS, the former have been suggested to induce inflammatory responses in infected lesions, which are followed by damage to host tissues. Such host responses may lead to the clearance of pathogens from the infected lesion and recovery from diseases. However, in chronic infection, persistent organisms remains in the infected tissues, thus, the organisms involved likely have mechanisms for evading host defenses.

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[1] The abbreviations used are: PAMPs, pathogen-associated molecular patterns; BAL, bronchoalveolar lavage; CL, cardiolipin; dsRNA, double-stranded RNA; rh, recombinant human; LPS, lipopolysaccharide; LBP, LPS-binding protein; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MAPK, mitogen-associated protein kinase; OME, outer membrane extract; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGN, peptidoglycan; PI, phosphatidylinositol; PS, phosphatidylserine; TLR, Toll-like receptor; ELISA, enzyme-linked immunosorbent assay; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; MS, mass spectrometry.

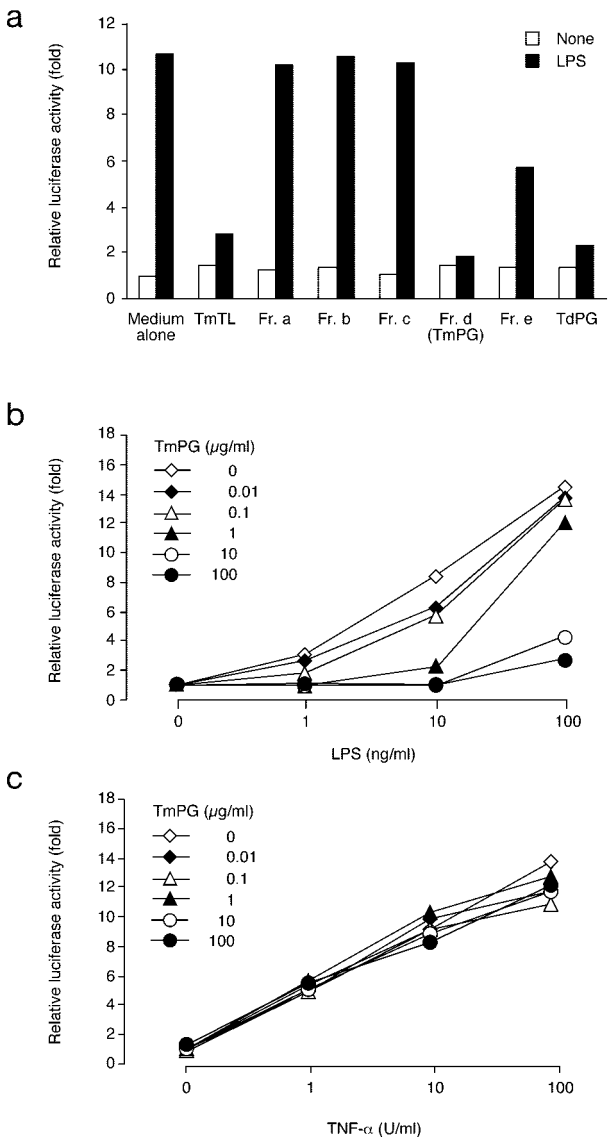

FIG. 1. **Inhibitory effects of treponemal lipids on LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells.** *a*, NF-κB activation induced by 10 ng/ml of LPS in the presence or absence of 100 μg/ml of various treponemal lipids. *b*, dose response of NF-κB activation induced by LPS in the presence or absence of TmPG at the indicated concentrations. *c*, dose response of NF-κB activation induced by TNF-α in the presence or absence of TmPG at the indicated concentrations.

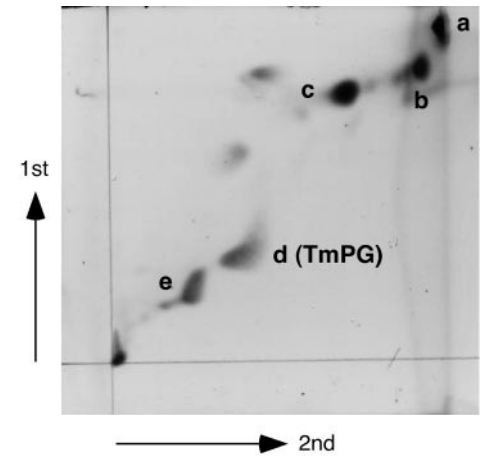

FIG. 2. **Two-dimensional TLC profile of TmTL.** The TLC profile was developed using a chloroform/methanol/ammonia solution (65:25:5) for the first dimension followed by chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5) for the second dimension. The spots were visualized using anisaldehyde-sulfuric acid reagent.

The structural features of the outer membrane of treponemes are considered to be involved with evasion from the immune system. For example, the outer membrane of *T. pallidum* is populated by a remarkably low concentration of transmembrane proteins (26, 27), which limits its antigenicity and may lead to inertness against the immune system. Treponemes are also known to be rich in lipids, as they comprise ~20% of the dry cell weight (28). Further, the antibody against cardiolipin (CL), a major lipid of *T. pallidum* (29), has been detected in the sera of patients with syphilis and used for diagnosis (30). These findings indicate that lipid components are released from organisms and play some kind of role in the host. Herein, we describe an inhibitory role for a treponemal membrane phospholipid against PAMPs-induced innate immune response.

EXPERIMENTAL PROCEDURES

Reagents—Phospholipids, phosphatidylglycerol from egg yolk (EYPG), phosphatidylcholine from egg yolk (EYPC), and bovine heart (BHPC), phosphatidylethanolamine from egg yolk (EYPE), cardiolipin from bovine heart (BHCL), phosphatidic acid from egg yolk (EYPA), phosphatidylinositol from bovine liver (BLPI), phosphatidylserine from bovine brain (BBPS), 1,2-dioleoyl-phosphatidylglycerol (DOPG), 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), 1,2-dipalmitoyl-phosphatidylglycerol (DPPG), *Escherichia coli* O55:B5 LPS, and dsRNA were purchased from Sigma-Aldrich. *Staphylococcus aureus* PGN was prepared as previously described (31), as was CpG DNA (5). OME from *Treponema medium* (TmOME) was prepared as previously described (19). Recombinant human (rh) TNF-α was obtained from Dainippon Pharmaceutical (Osaka, Japan). RhCD14 and rhLBP were purchased from R&D systems (Minneapolis, MN) and soluble-type human CD14 (sCD14) was kindly provided from Dr. Y. Kuroki, Department of Biochemistry, Sapporo Medical University School of Medicine (32). Anti-LBP polyclonal antibody was obtained from HyCult biotechnology (Uden, Netherlands) and anti-CD14 polyclonal antibody was purchased from Genzyme Techne (Minneapolis, MN).

Preparation of Treponemal Lipids—*T. medium* ATCC 700293 and *T. denticola* ATCC 35405 were grown anaerobically in trypticase-yeast extract-gelatin-volatile fatty acids-rabbit serum broth as described previously (19). Bacterial total lipids were extracted using the chloroform-methanol method of Bligh and Dyer (33), and designated TmTL for *T. medium* and TdTL for *T. denticola*. To separate the lipid components, TmTL and TdTL were each subjected to silica gel column chromatography using solvent system A, followed by preparative silica gel TLC using solvent systems B and C, as follows: *A*, chloroform/methanol/water (65:25:4); *B*, chloroform/methanol/ammonia solution (65:25:5); *C*, chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5). Spots on the TLC were visualized using anisaldehyde sulfuric acid reagent.

Analytical Procedures—Proton and ^{13}C NMR spectra were measured using a Unity Inova spectrometer (Varian) at 500 and 126 MHz, respectively, and obtained at 297 K. The chemical shifts are expressed in δ-values with chloroform (δ 7.25 and 77.0) as the internal standard for ^{1}H and ^{13}C spectra, respectively. MALDI-TOF-MS was measured using a QSTAR Pulsar i (Applied Biosystems) instrument equipped with an oMALDI ion source. Samples were combined with 2,5-dihydroxybenzoic acid as a matrix and placed on a sample plate. Spectra were obtained in positive ion mode.

Cells—Ba/F3 cells stably expressing murine TLR4/MD-2 and p55Igκ-Luc, an NF-κB-dependent luciferase reporter construct (Ba/mTLR4/mMD-2 cells), were kindly provided by Dr. K. Miyake, Division of Infectious Genetics, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, IL-3, and 50 μM of 2-mercaptoethanol (34). A mouse macrophage cell line, J774 (Dainippon Pharmaceutical), and murine fibroblast cell line, Balb-3T3 (Health Science Research Bank, Osaka, Japan), were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum, 50 μg/ml of gentamicin, and 50 ng/ml of amphotericin B.

Eight-week-old female C3H/HeN mice and BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). The animals received humane care in accordance with our institutional guidelines and the legal

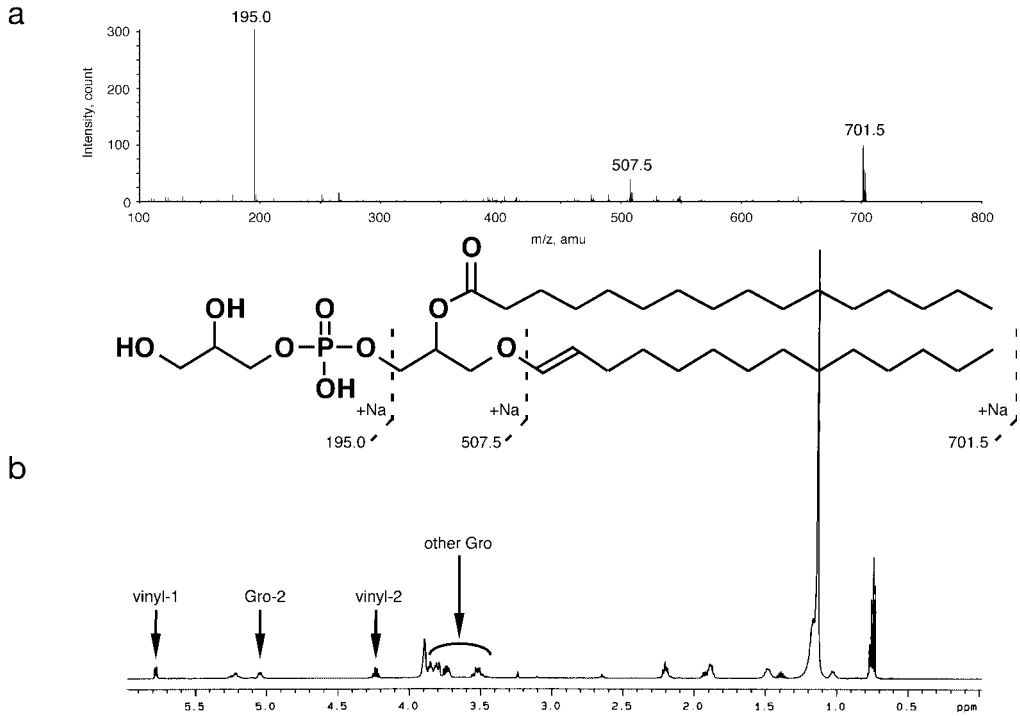

FIG. 3. **Identification of TmPG by MALDI-TOF MS/MS and ^{1}H NMR.** *a*, tandem mass spectrum of the parent ion at *m/z* 701.5 in the positive ion mode MALDI-TOF-MS of TmPG. Target structures are shown in the lower portion and the fragmentation patterns are indicated. *b*, a part of the ^{1}H NMR spectrum of TmPG. Significant signals are assigned as shown.

requirements of Japan. Alveolar macrophages were obtained from mice using a modified technique of bronchoalveolar lavage (BAL) as described previously, with a slight modification (35). Elicited peritoneal macrophages were obtained from mice 3 days after intraperitoneal inoculation of 1.0 ml of 3% sterile Brewer's thioglycolate broth (BD Biosciences). BAL cells and peritoneal exudate cells were centrifuged and suspended in RPMI 1640 supplemented with 10% fetal bovine serum, 50 μg/ml of gentamicin, and 50 ng/ml of amphotericin B at 1×10^6 cells/ml. These cells were then distributed to each well of a 96-well flat-bottomed plate (Falcon) at 2×10^5 cells/200 μl, after which they were incubated for 2 h at 37 °C in humidified air containing 5% CO_2. Each well was washed twice with phosphate-buffered saline to remove non-adherent cells, and those attached to the culture plate served as alveolar and peritoneal macrophages.

Luciferase Assays—Ba/mTLR4/mMD-2 cells were inoculated onto 96-well flat-bottomed plates at 1×10^5 cells in 100 μl of RPMI 1640 supplemented with or without 5% fetal bovine serum, and stimulated with the indicated doses of LPS together with or without the test specimens. In some experiments, Ba/mTLR4/mMD-2 cells were stimulated with test specimens in the presence or absence of 500 ng/ml of CD14 and 50 ng/ml of LBP. After 4 h at 37 °C, 100 μl of Bright-Glo™ luciferase assay reagent (Promega) was added to each well, and luminescence was quantified with a luminometer (Promega). Results are shown as relative luciferase activity, which was the ratio of stimulated activity to non-stimulated activity, in each cell line.

mRNA Expression—J774 cells were stimulated with 1 ng/ml of LPS with or without 1 μg/ml of POPG in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum for 4 h at 37 °C. Total RNA was extracted by a single-step extraction method with TRIzol reagent (Invitrogen). RT-PCR was performed with extracted total RNA using an RNA PCR kit (TaKaRa Biomedicals, Shiga, Japan), with sense and antisense oligonucleotide primers specific for TNF-α, IL-1β, IL-6, or β-actin (36, 37). For a negative control, a non-RT sample was amplified by PCR reaction. PCR products were detected by electrophoresis on a 1% agarose gel.

Western Blotting—J774 cells were stimulated with 1 ng/ml of LPS in the presence or absence of 1 μg/ml of POPG in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum for 4 h at 37 °C. Cells were collected and p38 mitogen-associated protein kinase (MAPK) phosphorylation was analyzed by Western blotting according to the manufacturer's instructions (Cell Signaling, Beverly, MA).

Cytokine Assays—Cells were stimulated with the indicated doses of the test specimens together with or without 10 μg/ml of POPG in culture medium supplemented with 1% fetal bovine serum for 24 h at 37 °C. After incubation, culture supernatants were collected and used for the cytokine assay. In some experiments, C3H/HeN mice were injected intraperitoneally with 100 μg of LPS with or without 1 mg of POPG in 1 ml of saline. Serum specimens were then obtained 1.5–3 h after injection. The test specimens were analyzed using an ELISA kit for secreted TNF-α, IL-6 (eBioscience, San Diego, CA), IL-1β (Endogen, Woburn, MA), and KC (Genzyme Techne), and results determined using a standard curve prepared for each assay.

Activation of Limulus Clotting Factor C—LPS was incubated with or without the indicated dose of POPG for 30 min at 37 °C. LPS-induced activation of *Limulus* clotting factor C was measured using an Endospecy™ Test (Seikagaku, Tokyo, Japan) according to the manufacturer's instructions.

Binding of LPS to LBP and CD14—Biotinylated LPS was prepared as previously described (38). Binding of biotinylated LPS to rhLBP that was captured by anti-LBP adsorbed onto ELISA plates was measured as previously described, with a slight modification (39). The binding was performed for 30 min at 37 °C in 0.1% bovine serum albumin/phosphate-buffered saline. Binding of biotinylated LPS to rhCD14 adsorbed onto the ELISA plates was detected as previously described (40). Binding was carried out for 30 min at 37 °C in 0.1% bovine serum albumin in phosphate-buffered saline containing 50 ng/ml rhLBP. Binding of biotinylated LPS to sCD14 that was captured by anti-CD14 adsorbed onto ELISA plates was measured as follows. Anti-CD14 (50 μl, 10 μg/ml) was placed onto an ELISA plate, incubated at 4 °C for 12 h, and then blocked with 1% bovine serum albumin/phosphate-buffered saline at 37 °C for 1 h. Biotinylated LPS (1 μg/ml) and sCD14 (5 μg/ml) were incubated at 37 °C for 13 h with or without the indicated doses of the phospholipid. After washing the plate with phosphate-buffered saline containing 0.1% Tween 20, the mixture was placed and incubated at 37 °C for 1 h to capture sCD14. Bound biotinylated LPS was detected using streptavidin-peroxidase and TMB substrate (KPL, Guildford, UK).

Murine Abscess Model—Abscess formation assay was performed as described previously (41). Briefly, BALB/c mice were injected subcutaneously with living *T. medium* cells (10^9 cells) with or without 1 mg of POPG in 0.5 ml of saline on the posterior dorsolatal surface. After challenge, lesion size (length and width) was measured daily for 9 days with a caliper gauge, and the area was expressed in square millimeters.

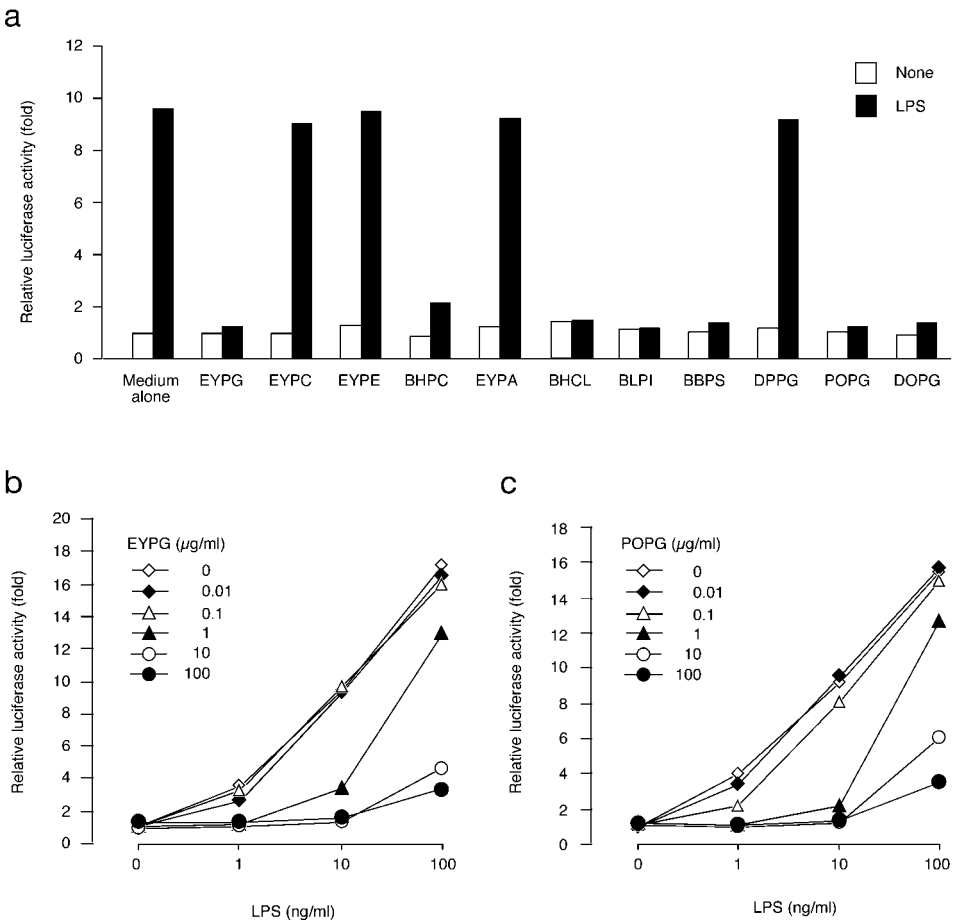

FIG. 4. **Inhibitory effect of phospholipids on LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells.** *a*, NF-κB activation induced by 10 ng/ml of LPS in the presence or absence of 100 μg/ml of various phospholipids. *b*, dose response of NF-κB activation induced by LPS in the presence or absence of EYPG at the indicated concentrations. *c*, dose response of NF-κB activation induced by LPS in the presence or absence of POPG at the indicated concentrations.

RESULTS

Treponemal Phospholipid Inhibits LPS-induced Immune Responses—T. medium is a medium-sized oral spirochete found in the subgingival plaque of patients with adult periodontitis (42) and has been reported to be associated with chronic periodontal diseases (43, 44). We investigated the immunobiological activity of its lipid components. Total membrane lipids (TmTL) were extracted from *T. medium* using the Bligh-Dyer method (33), which yielded ca. 25% from dried cells. TmTL possessed no immunostimulating activity with mammalian immune cells, whereas they were found to have an inhibitory effect on LPS-induced innate immune responses. This inhibitory effect was examined using Ba/mTLR4/mMD-2 cells (34), in which TmTL inhibited LPS-induced NF-κB activation (Fig. 1*a*). Since TmTL was shown to contain several components (Fig. 2), the lipids were subjected to column chromatography to yield five fractions, Fr. a–e. Among them, Fr. d, which was comprised of ~15% TmTL, was found to possess a strong dose-dependent inhibitory effect with an IC_{50} of 0.12 μg/ml at an LPS concentration of 10 ng/ml (Fig. 1, *a* and *b*). Since Fr. d did not decrease TNF-α-induced NF-κB activation in Ba/mTLR4/mMD-2 cells (Fig. 1*c*), cell viability was not responsible for the effect. Fr. e showed a weak inhibitory effect (IC_{50} = ~100 μg/ml), while the others had none (Fig. 1*a*).

PG Is Responsible for Inhibitory Effect—Earlier studies of treponemal membrane lipid (45–47) indicated that Fr. a–e corresponded to neutral lipid, sterols, galactosylglycerolipid, PG, and PC, respectively. In the positive ion mode MALDI-TOF mass spectra of Fr. d, ions at *m/z* 687.5, 701.5, 715.5, and 755.5 were mainly observed. These ions represented a PG type structure; however, it substituted a vinyl ether group for an acyl group: *e.g. m/z* 701.5 was a sodium ion adduct of PG consisting of 15:0 fatty acid and 14:0 vinyl ether, and *m/z* 755.5 was that of 18:1 fatty acid and 14:0 vinyl ether. The fragmentation patterns of the precursor ions at *m/z* 701.5 proved the structure (Fig. 3*a*). The position of the vinyl ether substitution was determined to be the 1-position of glycerol by NMR spectra (Fig. 3*b*). Thus, Fr. d was designated as TmPG. A similar PG fraction (TdPG, ~2.6% from dried cells) was obtained from a closely related bacterium, *T. denticola*, and it also inhibited LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells (Fig. 1*a*).

Structural Requirement of Phospholipid for Inhibitory Effect—To investigate the structure-activity relationship, we examined the inhibitory effects of various phospholipids on LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells (Fig. 4*a*). EYPG, a PG that contains diacylglycerol instead of 1-vinyl-2-acylglycerol in TmPG, showed an inhibitory effect. The effect of EYPG was dose-dependent and the IC_{50} was 0.47 μg/ml (Fig. 4*b*), which was comparable to that of TmPG. EYPC and EYPE, which are also the diacyl type but have a different head group, each had no inhibitory effect at the highest concentration tested (100 μg/ml). BHPC, which includes 1-vinyl-2-acylglycerol similar to TmPG, showed a weak inhibitory effect (IC_{50} = ~50 μg/ml). In the negatively charged phospholipids, EYPA possessed no inhibitory effect. In contrast, BHCL, BLPI, and BBPS showed inhibitory effects (IC_{50} = 0.03, 0.25, 0.35 μg/ml, respectively) similar to PG. To determine the effect of fatty

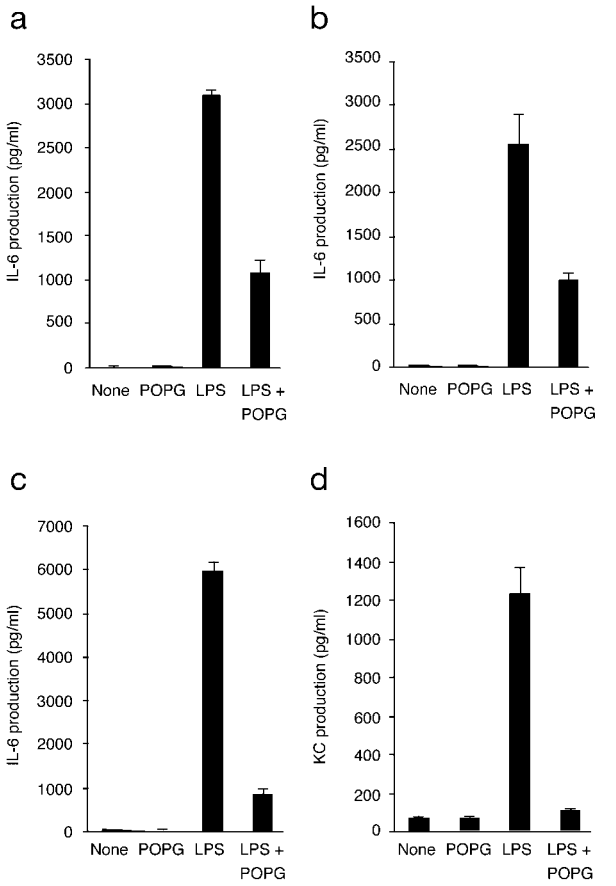

FIG. 5. **Inhibitory effect of POPG on LPS-induced cytokine production.** *a*, J774 cells; *b*, peritoneal macrophages; *c*, alveolar macrophages; *d*, Balb-3T3 cells. LPS (10 ng/ml) and POPG (10 μg/ml) were used.

acids moiety, the inhibitory effects of synthetic specimens were also examined (Fig. 4, *a* and *c*). POPG and DOPG, which contain unsaturated fatty acids, inhibited LPS-induced NF-κB activation (IC_{50} = 0.31, 0.36 μg/ml, respectively), whereas DPPG, which includes only saturated fatty acids, showed no effect.

PG Inhibits PAMPs-induced Cell Stimulation—To elucidate the details of the effect of PG on the innate immune system, we performed experiments with POPG as a synthetic model compound of PG. POPG inhibited LPS-induced IL-6 production by J774 cells (Fig. 5*a*), and was also effective with peritoneal macrophages, alveolar macrophages, and Balb-3T3 cells (Fig. 5, *b–d*). POPG also diminished the LPS-induced mRNA expression of proinflammatory cytokines, TNF-α, IL-1β, and IL-6, in J774 (Fig. 6*a*). Since cytokine production and mRNA expression are associated with the activation of NF-κB, the results with J774 cells and Ba/mTLR4/mMD-2 cells coincided. POPG blocked the LPS-induced phosphorylation of p38 MAPK in J774 cells (Fig. 6*b*), which is upstream of the signaling cascade of NF-κB activation. The NF-κB cascade is activated by various PAMPs through TLRs. To determine the upstream of the signaling cascades, ligand specificity was investigated in regards to the inhibitory effect of cytokine production by J774 cells (Figs. 5 and 6*c*). IL-6 production mediated by PGN as well as by LPS was inhibited by POPG, and the production of TmOME, a mixture of treponemal surface components, was also inhibited, whereas IL-6 production by dsRNA and CpG DNA was not. LPS- and PGN-induced cell activation are known to be mediated by CD14 alone or in combination with LBP and TLRs (8, 9). These observations indicate the possibility that POPG modulates the function of the PAMPs-LBP-CD14-TLR system.

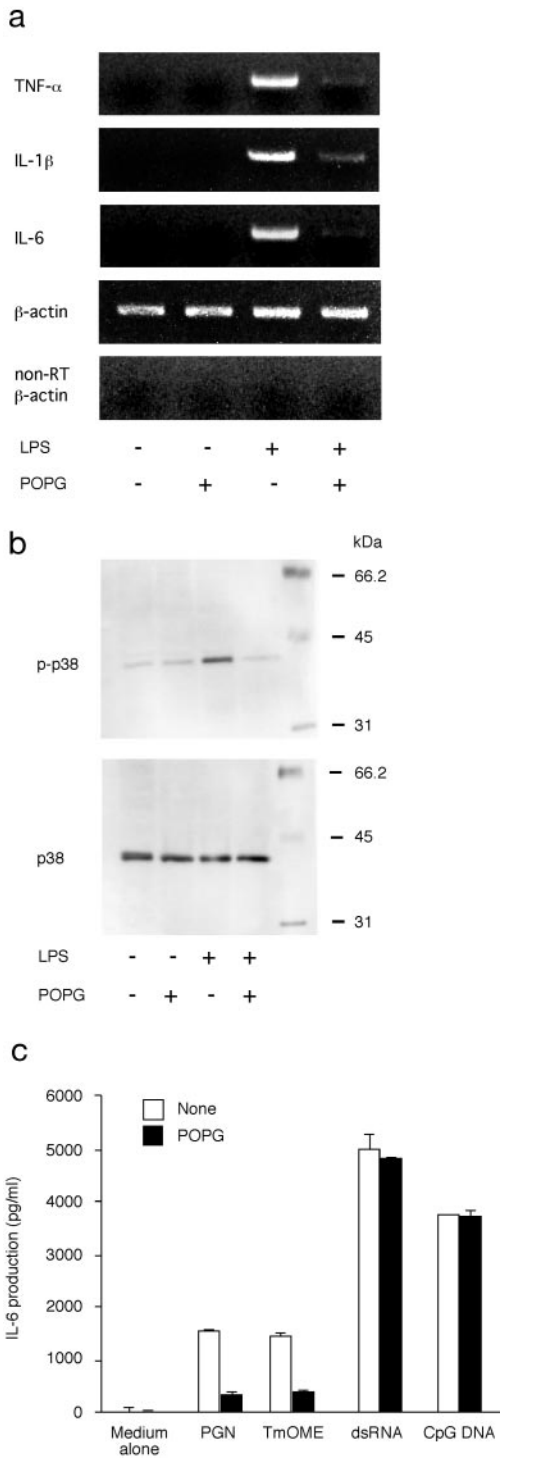

FIG. 6. **Inhibitory effect of POPG on PAMPs-induced inflammatory reactions of J774 cells.** *a*, expression of mRNA for inflammatory cytokines induced by 1 ng/ml of LPS in the presence or absence of 1 μg/ml of POPG. *b*, phosphorylation of p38 MAPK induced by 1 ng/ml of LPS in the presence or absence of 1 μg/ml of POPG. *c*, IL-6 production induced by PGN (10 μg/ml), TmOME (10 μg/ml), dsRNA (10 μg/ml), and CpG DNA (1 μM) in the presence or absence of 10 μg/ml of POPG.

PG Inhibits LBP and CD14 Functions—We attempted to determine whether any component interacts with POPG. Polymyxin B is known to bind to LPS and inhibit its endotoxic activity (48), and such binding can be detected by the inhibition of *Limulus* clotting factor C activation (49). Thus, we examined whether POPG binds to LPS. POPG displayed no inhibition

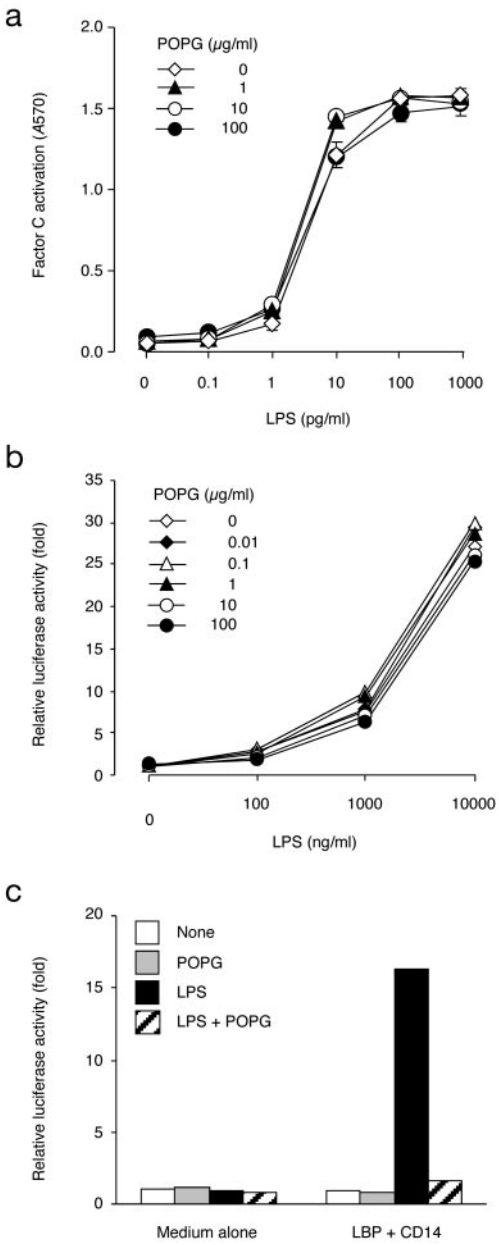

FIG. 7. **Inhibitory effect of POPG on LPS-induced activation.** *a*, *Limulus* clotting factor C activation by the indicated concentrations of LPS and POPG. *b*, NF-κB activation in Ba/mTLR4/mMD-2 cells induced by the indicated concentrations of LPS with or without POPG in the absence of serum. *c*, NF-κB activation in Ba/mTLR4/mMD-2 cells induced by 10 ng/ml of LPS with or without 10 μg/ml of POPG in the presence of LBP (50 ng/ml) and CD14 (500 ng/ml).

against LPS-induced factor C activation (Fig. 7*a*), indicating that there was no direct binding of POPG to LPS. Furthermore, POPG did not significantly affect LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells in a serum depleted condition (Fig. 7*b*), suggesting no specific interaction of POPG with TLR4-MD-2. Since CD14 and LBP have been shown to bind to various phospholipids (50, 51), the effects of POPG to CD14 and LBP were investigated. LPS-induced NF-κB activation in Ba/mTLR4/mMD-2 cells in the presence of rhLBP and rhCD14 was obviously diminished by POPG (Fig. 7*c*). POPG also inhibited the binding of biotinylated LPS to LBP (Fig. 8*a*) in a concentration dependent manner (Fig. 8*b*). Further, POPG interfered with the binding of biotinylated LPS to immobilized CD14 in the presence of LBP (Fig. 8*c*) and blocked the binding of sCD14 to LPS, whereas EYPC did not (Fig. 8*d*). These observations indicate that POPG inhibits the functions of LBP and CD14.

Inhibitory Effect of PG in Vivo—We further estimated whether PG inhibits PAMPs-induced immune response *in vivo*. After intraperitoneal injection of LPS in C3H/HeN mice, POPG decreased TNF-α, IL-1β, and IL-6 production in the serum specimens (Fig. 9*a*), showing the inhibitory effect of PG *in vivo*. Treponeme is known to induce inflammation and form lesion-containing abscess at the skin of BALB/c mice injected with the organisms (41). To evaluate the effect of PG on the treponeme-infected abscess formation, BALB/c mice were injected subcutaneously with living *T. medium* with or without POPG. The lesion of the group with POPG was decreased when compared with that of the group without POPG (Fig. 9*b*), suggesting that PG depressed abscess formation by the treponemal infection.

DISCUSSION

In the present study, we demonstrated that PG derivatives inhibited LPS-induced immune responses. LPS is an amphipathic molecule with relatively large hydrophilic and small hydrophobic regions, which forms a supramolecular aggregate (micelles) in an aqueous environment. Micellar LPS binds poorly to immune cells and fails to provoke a response, except in situations with high (μg/ml) concentrations, whereas monomeric LPS is capable of stimulating cells at low concentrations (52). The addition of LBP and CD14 has been shown to dramatically accelerate the binding of LPS to cells and each evoke cellular responses at pg/ml concentrations (53, 54). The effect of LBP and CD14 can be illustrated as follows: LBP binds to LPS with a high affinity and facilitates the process of LPS monomerization and presentation to other binding sites (55, 56), and then CD14 receives monomeric LPS from LBP (57, 58) and may transfer to TLR4-MD-2. POPG completely inhibited LPS activity up to a concentration of 10 ng/ml, though the inhibition was insufficient at 100 ng/ml (Fig. 4*c*). At high concentrations, cell activation is caused by micellar LPS as well as LBP-CD14 mediated LPS monomers. POPG could not significantly reduce micellar LPS induced activation (Fig. 7*b*), which indicates that PG inhibits LPS monomerization by blocking the function of LBP-CD14.

We clearly found inhibition of the binding of LPS to LBP by POPG (Fig. 8, *a* and *b*). The LPS-binding region of LBP has been identified within the N-terminal half of the molecule and shown to contain several positive charged amino acids (56), while polar lipid-binding pockets have been found in the central β-sheet region in LBP (59). Thus, negatively charged phospholipids are considered to bind to LBP at LPS-binding sites by electrostatic and hydrophobic interactions, and compete with LPS. Since LBP is required for the first step of LPS monomerization in physiological conditions, dysfunction of LBP may keep LPS in a micellar form, which would mean that the blocking of LBP function is responsible for the inhibitory effect. However, the LPS-binding site of CD14 is still controversial (60). PI and PS, as well as PC and PE, have also been shown to bind to CD14 (51), which is catalyzed by LBP (50). Among these phospholipids, we found that negatively charged BLPI and BBPS, as well as PG, inhibited LPS-induced cell activation, however, the neutral phospholipids, EYPC and EYPE, did not (Fig. 4*a*). These results suggest that the binding sites of negatively charged phospholipids are different from those of neutral ones, and only the former compete with that of LPS. We also demonstrated that POPG inhibited the binding of CD14 to LPS, whereas EYPC did not (Fig. 8, *c* and *d*), supporting the above results. Further, we found that POPG blocks PGN- and TmOME-induced cell activation (Fig. 6*c*). PGN is reported to bind to CD14 without the support of LBP (9), and treponemal lipoproteins were shown to stimulate cells via a CD14-dependent/LBP-independent pathway. Therefore, blocking the CD14 function contributes to the inhibitory effect.

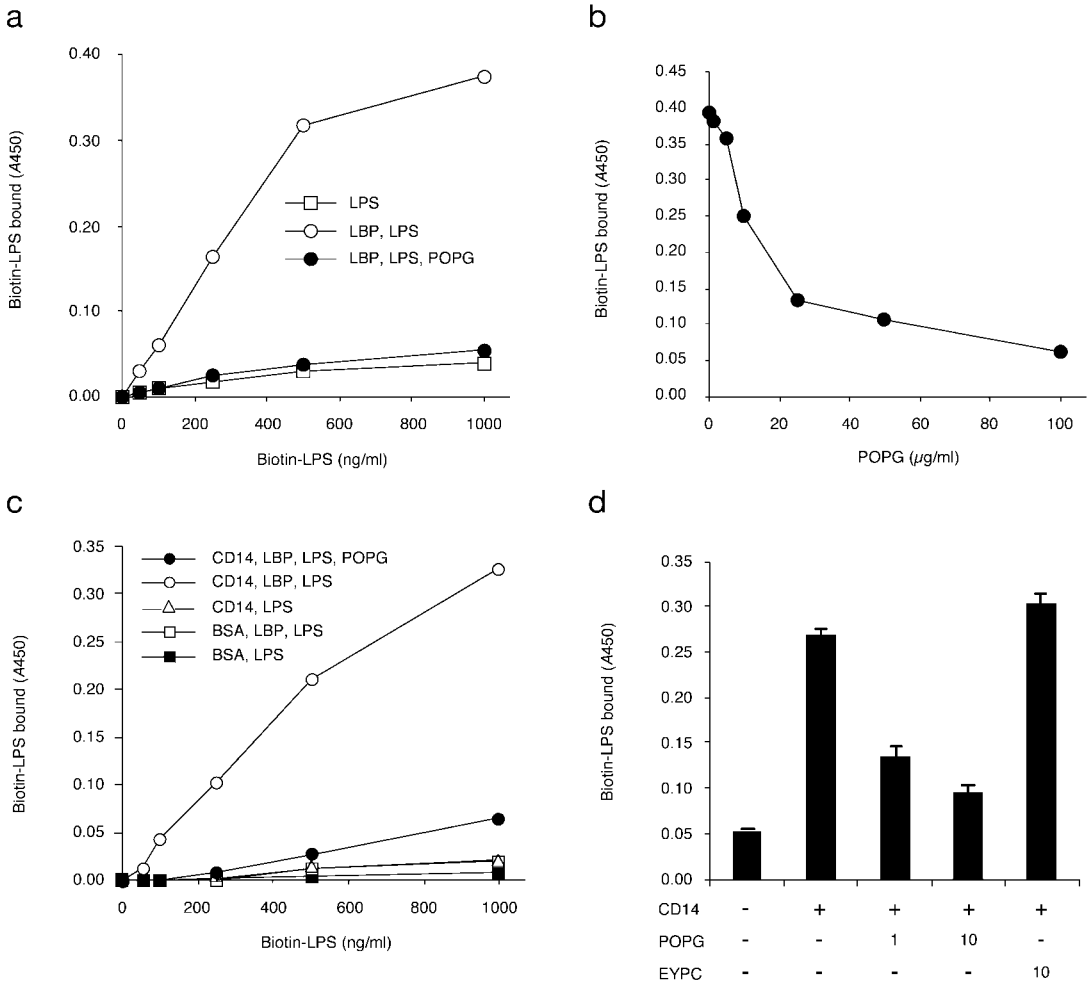

FIG. 8. **Inhibitory effects of POPG on interactions of LPS with LBP and CD14.** *a*, binding of biotinylated LPS to immobilized LBP in the presence (*filled circles*) or absence (*open circles*) of 100 μg/ml of POPG. *b*, dose response of the inhibition of LPS-LBP binding by POPG. Biotinylated LPS (1 μg/ml) was used. *c*, binding of biotinylated LPS to immobilized rhCD14 with (*filled circles*) or without (*open circles*) 10 μg/ml of POPG in the presence of LBP (1 μg/ml). *d*, binding of biotinylated LPS (1 μg/ml) to immobilized sCD14 with or without 1 or 10 μg/ml of POPG, or 10 μg/ml of EYPC.

The inhibitory effect of PG on LPS-induced activity showed a constituent dependence on acyl chain composition; *i.e.* DPPG showed no inhibition, while POPG and DOPG had inhibitory effects (Fig. 4*a*). These findings were considered to result from the membrane fluidity of the phospholipid micelles, which correlates to a gel to liquid-crystalline transition temperature (T_c). Transfer of a phospholipid with a low T_c to LBP or CD14 from the micelles is expected to occur more readily than that with a high T_c. Indeed, DPPG has a T_c of 41 °C, which is higher than the present experimental condition, whereas POPG and DOPG are estimated to have a T_c below 0 °C (61). Thus, the fluidity of phospholipid micelles is likely to affect the inhibitory effect in a manner similar to that described previously (62).

Recently, Bochkov *et al.* (63) demonstrated that 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine oxidation products (OxPAPC) bind to LBP and CD14, and inhibit LPS-induced activity, with 1-palmitoyl-2-oxovaleroyl-phosphatidylcholine (POVPC) responsible for the effect. We indicated that BHPC, which has a neutral head group but includes a 1-vinyl ether group, showed a weak inhibitory effect (Fig. 4*a*). POVPC and BHPC are not negatively charged, however, they contain aldehyde or its equivalent. Although details of the binding mechanisms of POVPC are still unknown, those of POVPC and BHPC may be different from those of negatively charged phospholipids. Our finding suggests that the vinyl ether group in TmPG contributes to the inhibition. Further, Fr. e obtained from TmTL was characterized as PC substituted with the vinyl ether group (data not shown), thus, the vinyl group is responsible for the weak inhibitory effect of Fr. e (Fig. 1*a*).

OxPAPC is considered to be absent from healthy humans and produced only at sites of bacterial inflammation, owing to its high concentrations of neutrophile-derived reactive oxygen species (63), and it is proposed to serve as a negative feedback mechanism to down-regulate acute inflammation that accompanies bacterial infection. Similarly, PG is a phospholipid that is scarce in most mammalian tissues, however, is relatively abundant in bacterial membranes, including those of treponemes (29, 45–47). CL is also present in the membrane of several bacteria, such as *T. pallidum* (29). Besides treponemes, the widely studied organism *E. coli* has up to 20% PG and 5% CL in its membrane (64). In our experiments, POPG blocked the activities of immunostimulating component of *E. coli*, LPS (Fig. 5), as well as that of treponemes, TmOME (Fig. 6*c*). POPG also reduced LPS-induced proinflammatory cytokine production and abscess formation by treponemal infection (Fig. 9). Therefore, this phospholipid-induced inhibitory mechanism may serve to modulate innate immune responses. Further, since the immunostimulating activity of treponemal components is considerably weaker than that of *E. coli* LPS, the mechanism may have a significant contribution for the chronic treponemal infection.

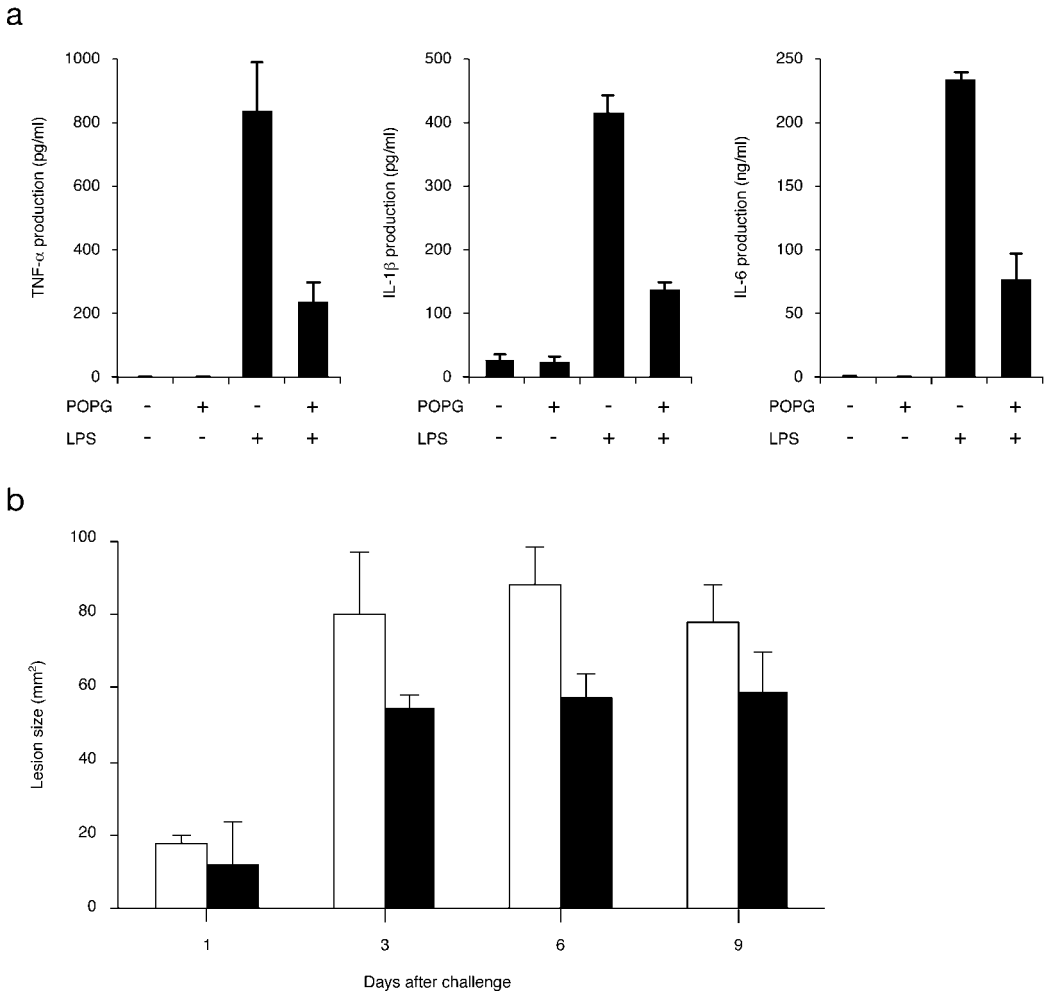

FIG. 9. **Inhibitory effects of POPG *in vivo*.** *a*, proinflammatory cytokine production in the serum of C3H/HeN mice injected intraperitoneally with 100 μg of LPS with or without 1 mg of POPG. *b*, abscess formation of BALB/c mice injected subcutaneously with 10^9 of *T. medium* cells with (*closed bars*) or without (*open bars*) 1 mg of POPG.

As an exception, PG is the dominant phospholipid next to PC in the pulmonary surfactant (65), and PI and PS are also present. The lungs contain the first line of defense against inhaled pathogens and many components are involved in pulmonary innate immunity (66). During acute lung injury, LBP levels rise substantially and enhance the activation of alveolar macrophages to release injurious mediators, whereas normal BAL fluid contains only a low level of LBP (67). We demonstrated that POPG blocks LPS-induced cytokine release by alveolar macrophages in serum (Fig. 5*c*). Thus, in a normal lung, PG and its related phospholipids in the surfactant may suppress the effect of LBP and prevent an excessive immune reaction.

In conclusion, we propose that anionic phospholipids, such as PG and CL, are possible virulent factors of treponemes to inhibit PAMPs-induced immune responses by blocking the function of LBP/CD14. These findings may provide a basis for understanding the mechanism for chronic treponemal infection.

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