Heterotrimeric G Proteins Physically Associated with the Lipopolysaccharide Receptor CD14 Modulate both In Vivo and In Vitro Responses to Lipopolysaccharide

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Abstract

Septic shock induced by lipopolysaccharide (LPS) triggering of cytokine production from monocytes/macrophages is a major cause of morbidity and mortality. The major monocyte/macrophage LPS receptor is the glycosylphosphatidylinositol (GPI)-anchored glycoprotein CD14. Here we demonstrate that CD14 coimmunoprecipitates with G_i/G_o heterotrimeric G proteins. Furthermore, we demonstrate that heterotrimeric G proteins specifically regulate CD14-mediated, LPS-induced mitogen-activated protein kinase (MAPK) activation and cytokine production in normal human monocytes and cultured cells. We report here that a G protein binding peptide protects rats from LPS-induced mortality, suggesting a functional linkage between a GPI-anchored receptor and the intracellular signaling molecules with which it is physically associated. (*J. Clin. Invest.* 1998. 102:2019–2027.) Key words: lipopolysaccharide • G proteins • signal transduction • monocytes/macrophages • endotoxin shock

Introduction

Lipopolysaccharide binding to its major receptor (CD14) expressed on monocytes triggers a cascade of signaling events ultimately leading to cytokine production and in vivo to septic shock. Although the precise signal transduction components that are stimulated by the LPS/CD14 interaction have not been completely elucidated, it is known that stimulation of monocytes by LPS leads to the activation of p38 mitogen-activated protein kinase (MAPK),[1] production of various cytokines (i.e., TNF, IL-6), and to other signaling events (1). The LPS/CD14 interaction has been demonstrated to be enhanced by the serum factor LPS-binding protein (LBP; references 2, 3). The interaction of LPS/LBP with CD14 causes the exchange of LPS with lipids in target membranes (4–6). It has been suggested that this lipid transfer is responsible for LPS-induced signal transduction. The rate of the exchange reaction depends on the lipid composition of the target membranes, which has led to speculation that CD14 functions only to direct LPS insertion into particular membrane domains (6).

CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein and, as such, has neither transmembrane nor cytoplasmic amino acid sequences. Although in the last few years it has been demonstrated that GPI-anchored proteins expressed on many cell types are physically associated with lipid-linked signal transduction molecules (7–12), GPI-anchored proteins (including CD14) cannot interact with intracellular signal transduction molecules through amino acid sequences in the way transmembrane receptors interact with intracellular signaling molecules.

It has been hypothesized that the association between GPI-anchored proteins and signal transduction molecules is a consequence of the fact that GPI-anchored proteins and certain signal transduction molecules reside in nonionic detergent insoluble membrane fractions or microdomains (often referred to as detergent insoluble glycolipid rafts or DIGs) (7, 13–18). These DIGs self-assemble in the trans–Golgi network (TGN) and contain high concentrations of sphingolipids and cholesterol (19, 20). It is the unique lipid composition of these DIGs that imbues them and the proteins that are resident within them with detergent insolubility (21–23). Not all nonionic detergent insoluble membrane fractions are equivalent. In some cell types (e.g., endothelial cells) there are at least two different types of DIGs (18, 24): those that contain GPI-anchored proteins and those that are organized into caveolae, which are striated plasmalemmal vesicles organized and marked by the protein caveolin (16, 25–29). The situation in hematopoietic cells is greatly simplified by the fact these cells do not express caveolin and do not have morphological caveolae (11, 30, 31). The DIGs of hematopoietic cells are likely to be free-floating lipid rafts, lacking the higher level of membrane organization found in caveolae.

Lipid-linked signal transduction molecules such as src family nonreceptor tyrosine kinases and heterotrimeric G proteins are found in DIGs (15–17, 32) and coimmunoprecipitate with GPI-anchored proteins in hematopoietic cells (7, 8, 11, 12). While a physical association between GPI-anchored proteins and their associated signal-transducing proteins has been

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1. *Abbreviations used in this paper:* DIGS, detergent insoluble glycolipid rafts; GPI, glycosylphosphatidylinositol; MAPK, mitogen-activated protein kinase; NC, nitrocellulose.

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demonstrated, a functional association has not been clearly shown.

Here it is demonstrated that CD14 associates physically with various α subunits of heterotrimeric G proteins, that LPS-induced signaling including p38 MAPK activation, and that cytokine production is regulated by heterotrimeric G proteins. In addition, we demonstrate that modulation of heterotrimeric G proteins activity in vivo reduces LPS-induced lethality. These results suggest that targeting heterotrimeric G proteins with pharmacological agents may have profound effects on the clinical outcome of sepsis.

Methods

Antibodies. The following antibodies were used in these studies: FMC17 (anti-CD14, Accurate Chem. and Scientific Corp., Westbury, NY), anti–G protein (polyclonal rabbit anti-pan G protein) raised to the GTP-binding region (Gly-[X_4]-Gly-Lys) of the heterotrimeric G protein G_z (gift of Dr. Christopher Rudd, Dana-Farber Cancer Inst.), anti-G_i α1 (Calbiochem, San Diego, CA), anti-G_i α1 and anti-G_i α2 (Calbiochem), anti-G_i α3 (Calbiochem), and anti-G_o α (Calbiochem), anti-G_s α (Calbiochem), 4G10 (antiphosphotyrosine; gift of Dr. Brian Druker, Oregon Health Sciences Center, Portland, OR), MOPC (negative control; Sigma Chemical Co., St. Louis, MO), anti-fyn antiserum (gift of Dr. Christopher Rudd, Dana-Farber Cancer Inst.), anti-lck antiserum (gift of Dr. Christopher Rudd, Dana-Farber Cancer Inst.), anti-fgr polyclonal antibody (Transduction Laboratory, Lexington, KY), anti-Lyn mAb (Transduction Laboratory), anti-Hck mAb (Transduction Laboratory), anti-p38 polyclonal antibody COOH + NH_2 terminuses (Santa Cruz Biotechnology Inc., Santa Cruz, CA), antiphosphorylated p38 polyclonal antibody (New England Biolabs Inc., Beverly, MA), anti-pan Erk (Transduction Laboratory), antiphosphorylated Erk (New England Biolabs Inc.), OKT3 (anti-CD3; American Type Culture Collection, Rockville, MD), 3PT2H9 (anti-CD2; gift of Dr. Ellis Reinherz, Dana-Farber Cancer Inst.), 60bca (anti-CD14; American Type Culture Collection).

Cell lines and freshly isolated human blood cells. U373 cell CD14 transfectants (U373-CD14) were maintained in Earle's minimum essential medium supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin at 37°C in 5% CO_2. Freshly isolated human PBMCs and monocytes were obtained from leukopaks (discarded leukocytes from platelet donations). Cells were fractionated on Ficoll-Hypaque gradients, washed, treated with Tris-buffered NH_4Cl to eliminate red blood cells, and washed (PBMC). Monocytes were obtained by depleting the PBMCs of T and NK cells by negative selection. T and NK cells were removed by treatment with anti-CD3 (OKT3) and anti-CD2 (3PT2H9) followed by goat anti–mouse Ig–conjugated magnetic beads (Advanced Magnetics Inc., Cambridge, MA) at a 10:1 bead/cell ratio. The monocyte preparations were 80–85% monocytes, as determined by anti-CD14 staining and forward and side light scatter analysis using a FACScan® (Becton Dickinson, Elmhurst, IL). Less than 2% of contaminating cells in the monocyte preparations were T cells and no NK cells could be detected. Monocytes were maintained in Ham's F-12 10% FCS, L-glutamine, and penicillin/streptomycin at 37°C in 5% CO_2.

Mastoparan, pertussis toxin, and LPS. Mastoparan (Quality Control Biochemicals, Hopkinton, MA) treatment of human PBMCs, monocytes, and U373 transfectants were performed with various concentrations of mastoparan or MAS-17 (QCB; see figure legends) in culture medium for either 1 h (p38 analysis) or for 6–18 h (for cytokine analysis) at 37°C. Human monocytes were treated with 50–250 ng/ml pertussis toxin (Sigma Chemical Co.) for 12–18 h at 37°C in supplemented culture medium before activation with agents and subsequent measurement of cytokines and p38 MAPK analysis. After various treatments, human PBMCs, monocytes, and U373 cell transfectants were incubated with or without various concentrations of LPS (*Escherichia coli* 0111:B4; Sigma Chemical Co.) for various times (see figure legends) in culture medium at 37°C.

Immunoprecipitation, in vitro kinase assay, and reimmunoprecipitation. Cells were washed 3 times in cold PBS (or HBSS) and were lysed on ice for 30 min in lysis buffer containing 0.5% NP-40, 300 mM NaCl, 50 mM Tris, pH 7.6, 0.15 U/ml aprotinin (Sigma Chemical Co.), 10 mM iodoacetamide, 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 1 mM PMSF. Insoluble debris were removed by microcentrifugation (10,000 rpm) for 20 min and the lysates were precleared (30 min each) with 100 μl (10% wt/vol) rabbit anti-mouse–coated protein A Sepharose beads (1 mg/ml) followed by 200 μl (10% wt/vol) protein A Sepharose beads. The lysates were then incubated for 2 h at 4°C with mAbs previously bound to protein A Sepharose beads. After 2 h the beads were washed four times in lysis buffer and once in kinase buffer (25 mM Hepes, 1 mM $MnCl_2$, and 100 μM Na_3VO_4). The immunoprecipitates were then resuspended in 50 μl of kinase buffer with 20 μCi [γ-^{32}P]ATP (New England Nuclear, Boston, MA) and incubated for 15 min at room temperature. Next, the samples were washed four times in lysis buffer with 15 mM EDTA. Samples were either eluted in 0.5% SDS at 70°C for 3 min or boiled in 1% SDS for 5 min and diluted 10-fold with cold lysis buffer. The eluate was either subjected directly to SDS-PAGE analysis or was subjected to reimmunoprecipitation with various mAbs or polyclonal antisera (see figures) and 20 μl protein A Sepharose beads for 2 h at 4°C. Reimmunoprecipitated samples were washed four times in lysis buffer, resuspended in reducing Laemmli sample buffer, boiled, and subjected to electrophoresis through a 10% SDS-PAGE gel.

Immunoblotting. For detection of p38, phosphorylated p38, and phosphorylated Erk kinases in cell lysates, treated cells were lysed in boiling reducing Laemmli sample buffer, subjected to electrophoresis through a 10% SDS-PAGE gel, and then transferred to nitrocellulose (NC). After washing twice with TBS–Tween 20 (0.1%), the NC was placed in a solution of Ponceau S dye (to ensure equal loading) and left in blocking buffer (1× TBS, 0.1% TBS, 5% milk) for 1 h. After three washes with TBS–Tween 20, the NC was incubated with anti-p38 antibodies for 2–3 h in blocking buffer, or with antiphosphorylated p38 and Erk antibodies (New England Biolabs Inc.) for 18 h. Membranes were washed three times in TBS–Tween 20 and were incubated for 30 min with horseradish peroxidase–conjugated donkey-α-rabbit antibody in blocking buffer (Amersham Corp., Arlington Heights, IL). Membranes were washed an additional six times (3× TBS–Tween 20, 3× TBS), and were developed by exposure to enhanced chemiluminescence (ECL) chemicals (Amersham Corp., Arlington Heights, IL) and visualized by exposure to film.

Measurement of p38 kinase activity. p38 kinase activity was assessed by measurement of ATF_2 phosphorylation subsequent to immunoprecipitation of activated p38 MAPK using a p38 MAPK kit (New England Biolabs Inc.).

Cytokine production. Human PBMCs, human monocytes, and U373-CD14 cells were incubated with various concentrations of LPS (*E. coli* 0111:B4; Sigma Chemical Co.) (Fig. 2), 100 ng/ml PMA, or were untreated in culture medium with or without various concentrations of mastoparan (see Figs. 2 and 3) for 18 h at 37°C in 24-well tissue culture dishes. IL-6 and TNF-α levels were determined by ELISA (Endogen Inc., Boston, MA) from supernatants harvested at 4 h (for TNF-α) and at 18 h (for IL-6).

LPS treatment of rats. Wistar rats (200 g) obtained from Charles River Laboratories (Wilmington, MA) were treated with 3 mg/kg mastoparan by intravenous injection in the tail vein, immediately followed by 15 mg/kg lead acetate and 1–5 μg/kg LPS 0111:B4 intravenously. Mortality was assessed up to 96 h after LPS treatment. Mortality frequency was compared by Fisher exact test.

Results

CD14 is physically associated with src kinases and heterotrimeric G proteins. To begin understanding the mechanism of

LPS-induced signal transduction mediated through CD14, we immunoprecipitated CD14 from freshly isolated human monocytes (Fig. 1) and assessed the association of CD14 with phosphorylated proteins using in vitro kinase assays (Fig. 1, *A* and *B*). In vitro kinase assays performed on CD14 immunoprecipitated from human monocytes indicated the presence of multiple phosphorylated species (Fig. 1 *A*). Reimmunoprecipitation of the products of these in vitro kinase assays with an antiphosphotyrosine-specific monoclonal antibody indicated that all the major phosphorylated species were tyrosine phosphorylated. Immunoprecipitation of the products of the in vitro kinase assay with heterosera and various mAbs recognizing src family tyrosine kinases indicated that in human monocytes fyn, lyn, and fgr src family kinases were all present in substantial quantities (Fig. 1 *B*) and a small amount of hck could be observed on overexposed autoradiographs. There was no evidence for lck in the immunoprecipitates from human monocytes.

In vitro kinase assays of CD14 revealed the presence of a 40-kD tyrosine phosphorylated species (Fig. 1, *A* and *B*). This protein was immunoprecipitated from the CD14 in vitro kinase assays with an anti-pan G protein antiserum that recognizes the GTP binding site of a variety of G proteins (12), indicating that this species was a G protein. Immunoprecipitation of the products of the CD14 in vitro kinase assays (Fig. 1 *B*) by antisera specific for various α subunits of heterotrimeric G proteins indicated that this 40-kD protein consisted of a combination of heterotrimeric G protein α subunits: a small amount of G_i α1 (sometimes only observable on overexposed autoradiographs), larger amounts of G_i α2, G_i α3, and G_o α. There was no immunoprecipitation of G_s α subunits from the CD14 in vitro kinase assay. Similar patterns of heterotrimeric G proteins and src family kinases were found to coimmunoprecipitate with CD14 from Chinese hamster ovary- and U373-CD14 transfectants (data not shown). These patterns of phosphoproteins are similar to those we and others have noted to associate with other GPI-anchored proteins (7–12).

Mastoparan inhibits LPS-induced cytokine production from human monocytes and U373 transfectants. The fact that CD14 immunoprecipitates contained G_i and G_o heterotrimeric G proteins suggested that G proteins might be involved in LPS-induced signaling. To reveal the functional consequences of the CD14/heterotrimeric G protein association, we determined the effect of mastoparan (a peptide which specifically stimulates G_i and G_o heterotrimeric G proteins; references 33–35) on LPS-induced cytokine production from both freshly isolated human cells (PBMCs and monocytes) and U373 cell transfectants expressing CD14 (U373-CD14).

Initially we tested the effect of mastoparan and its inactive analogue, MAS-17, on cytokine production from human PBMCs. Freshly isolated human PBMCs were treated with LPS and/or peptides (mastoparan or the MAS-17 control peptide)

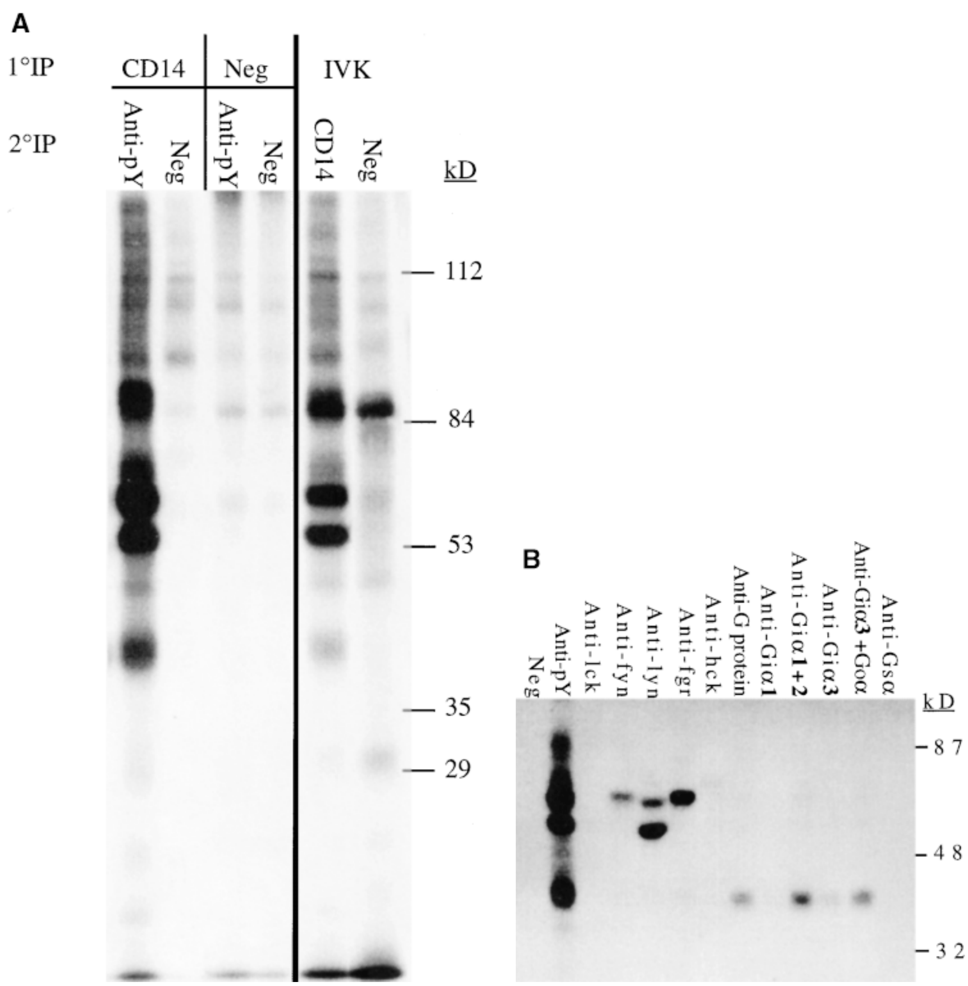
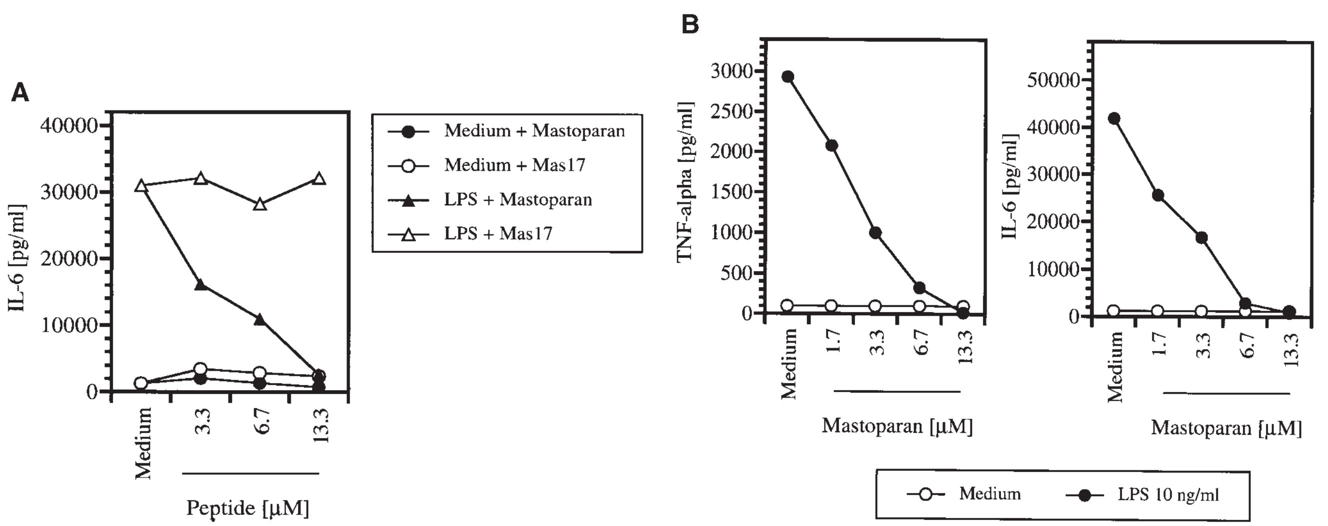


Figure 1. CD14 is associated with src kinases and heterotrimeric G proteins. (*A*) CD14 expressed on human monocytes is associated with tyrosine phosphorylated proteins. Human monocytes were lysed in a buffer containing 0.5% NP-40 and the lysate was subjected to anti-CD14 or negative control antibody immunoprecipitation. In vitro kinase assays were performed on the immunoprecipitates, and the labeled products were either eluted in 0.5% SDS at 70°C and reimmunoprecipitated with negative control or antiphosphotyrosine mAb, or were directly subjected to electrophoresis through a 10% SDS-PAGE gel. (*B*) CD14 is associated with various src kinases and heterotrimeric G proteins. In vitro kinase assays were performed as described above and the labeled eluate was immunoprecipitated with antiphosphotyrosine, anti-src kinase family member, anti-heterotrimeric G protein α subunit mAbs, or rabbit heterosera, and subjected to electrophoresis through a 10% SDS-PAGE gel.

and IL-6 levels were measured in the tissue culture supernatants of the cells (Fig. 2 *A*). PBMCs produced IL-6 in response to LPS, whereas neither mastoparan or MAS-17 stimulated IL-6 production from the PBMCs. Mastoparan was a potent inhibitor of LPS-induced IL-6 production from the LPS-stimulated PBMCs, whereas MAS-17 had no effect on cytokine production. The effect of mastoparan on cytokine production was dose dependent, and at a concentration of 13.3 μM, mastoparan totally ablated LPS-induced IL-6 production from the PBMCs.

To determine if the effect of mastoparan on LPS-induced cytokine production was due to a direct effect on monocytes, we produced a highly enriched monocyte population and tested the effect of LPS and mastoparan on cytokine production from these cells. Freshly isolated human monocytes were treated with mastoparan and LPS, after which IL-6 and TNF cytokine levels were measured in the tissue culture supernatants of the cells (Fig. 2 *B*). Untreated monocytes did not produce detectable levels of cytokines, verifying that the isolation procedure had not activated these cells. LPS caused a dose-dependent stimulation of cytokines from isolated monocytes (data not shown), whereas mastoparan induced neither IL-6 nor TNF production from these cells. When mastoparan was used in conjunction with LPS, cytokine production was diminished. Concentrations of mastoparan as low as 1.7 μM caused dramatic reductions in both IL-6 and TNF production from LPS-stimulated monocytes. Mastoparan at 13.3 μM concentration totally ablated LPS-induced cytokine production from these cells. Mastoparan had little or no effect on cytokine production by PMA-stimulated cells (Fig. 2 *C*), indicating the specificity of mastoparan action and lack of mastoparan toxicity. Mastoparan had no effect on cell viability as measured by trypan blue uptake even after 36 h of continuous mastoparan incubation.

We next determined whether mastoparan would affect LPS-induced cytokine production from an LPS responsive, CD14-transfected cell line. LPS treatment of U373-CD14 transfectants induced an LPS dose-dependent production of IL-6 (Fig. 3). At low concentrations of LPS (10–100 ng/ml), LPS-induced IL-6 responses were completely inhibited by treatment of the U373-CD14 cells with an anti-CD14 mAb. IL-6 production induced by 10 ng/ml LPS was also ablated by treatment of the cells with mastoparan, whereas at 100 ng/ml of LPS, mastoparan reduced IL-6 levels by approximately one



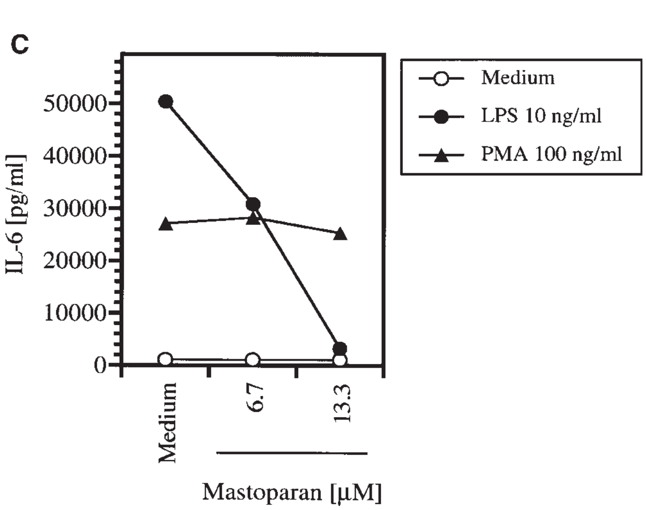


Figure 2. Mastoparan inhibits cytokine production from LPS-stimulated PBMCs and monocytes. (*A*) Mastoparan but not its inactive analogue (MAS-17) inhibits LPS-induced cytokine production. Freshly isolated human PBMCs were either left unstimulated or were stimulated with 1 ng/ml LPS in supplemented RPMI with various concentrations of mastoparan or MAS-17 for 4–18 h at 37°C. Supernatants were harvested and IL-6 levels were determined by ELISA. (*B*) Mastoparan dose-dependent inhibition of IL-6 and TNF-α production in human monocytes. Freshly isolated human monocytes were incubated with or without 10 ng/ml LPS in supplemented RPMI with or without various concentrations of mastoparan for 4–18 h at 37°C. Supernatants were harvested and IL-6 and TNF-α levels were determined by ELISA. (*C*) Mastoparan inhibits LPS-, but not PMA-, induced cytokine production from human monocytes. Freshly isolated human monocytes were either left unstimulated, were stimulated with 10 ng/ml LPS, or with 100 ng/ml PMA in supplemented RPMI with or without various concentrations of mastoparan for 4–18 h at 37°C. Supernatants were harvested and IL-6 levels were determined by ELISA. Cytokine levels were determined in duplicate and all SD are < 10% of indicated value. Experiments are representative of six different assays with very similar results.

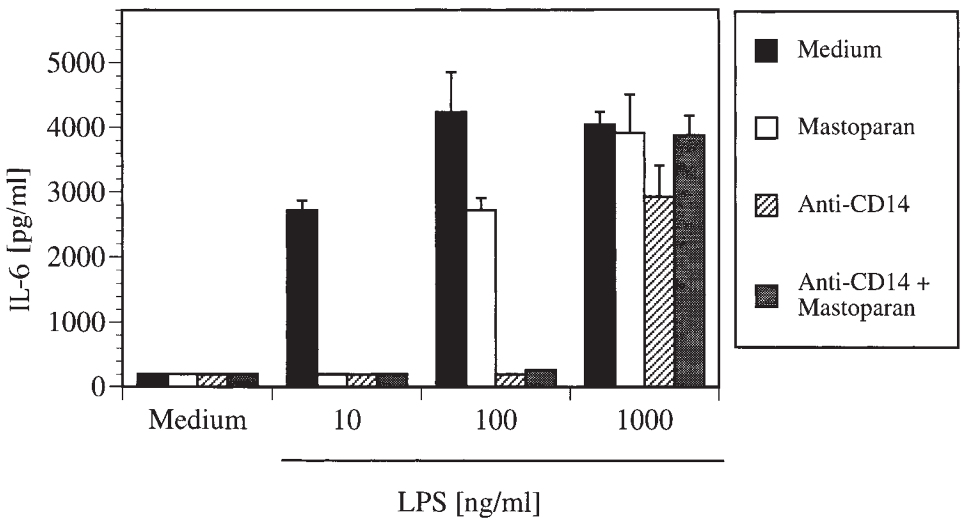


Figure 3. Mastoparan only inhibits CD14-dependent LPS-induced signal transduction. U373-CD14 cells, cultured in 24-well tissue culture plates, were treated with 13.3 μM mastoparan and/or 10 μg/ml 60 bca anti-CD14 mAb. Cells were incubated at 37°C for 1 h before the addition of various concentrations of LPS. Supernatants were harvested 18 h later for determination of IL-6 secretion by ELISA.

third. At high concentrations of LPS (1 μg/ml), the IL-6 responses of these cells were not inhibited by treatment with the anti-CD14 mAb. Thus, at a high concentration of LPS, U373 cells exhibit CD14-independent LPS-induced cytokine responses. At LPS concentrations of 1 μg/ml, mastoparan was ineffective at reducing cytokine responses from these cells, i.e., mastoparan inhibition of cytokine secretion was overcome at high ligand concentrations.

p38 MAPK phosphorylation is regulated by heterotrimeric G proteins. LPS stimulation of cells through CD14 leads to the induction of a MAPK signaling pathway involving the p38 MAPK, which has been shown to be specifically induced by LPS (36–38; see Fig. 4, *A* and *B*). Because p38 MAPK is involved in LPS-induced signaling, we next evaluated the effect of mastoparan on MAPK activation. For full activation, p38 MAPK requires phosphorylation on both threonine and tyrosine residues (38). Detection of dual-phosphorylated p38 MAPK by mAbs specific for the dual-phosphorylated form of p38 was used as a measure of p38 activation. Consistent with the effect of mastoparan on LPS-induced cytokine production, mastoparan reduced the LPS-induced phosphorylation of p38 MAPK in both monocytes and PBMCs (Fig. 4 *A*). Mastoparan also inhibited LPS-induced p38 MAPK activation as measured by the ability of p38 to phosphorylate ATF_2 (Fig. 4 *B*). It is interesting to note that a 50% decrease in the quantity of dual-phosphorylated p38 in these cells (Fig. 4 *A*) is accompanied by a > 90% decrease in the catalytic (kinase) activity of p38 (Fig. 4 *B*) and in cytokine secretion (Fig. 2) when the cells are treated with mastoparan. This suggests that there may be a threshold level of p38 dual phosphorylation that is necessary for activation of gene transcription and cytokine secretion.

Since it had been previously demonstrated that LPS induces phosphorylation of Erk 1 and 2 MAPK in transformed macrophage cell lines (39, 40), we examined the LPS-induced activation of Erk 1 and 2 and the effect of mastoparan on the activation of these kinases in normal human monocytes (Fig. 5). Only minimal phosphorylation of Erk 1 or 2 was seen in LPS-stimulated monocytes. The lack of substantial LPS-induced Erk kinase phosphorylation in freshly isolated monocytes is consistent with results obtained with other nontransformed human cells (41). In contrast to LPS, PMA induced substantial phosphorylation of Erk kinases. Interestingly, although mastoparan completely inhibited the Erk kinase activation induced by LPS, it had minimal effects on Erk kinase activation induced by PMA. This is consistent with the cytokine data, i.e., the effect of mastoparan was specific for the LPS signal transduction pathway and did not globally alter the ability to activate MAPKs.

Mastoparan protects rats from LPS-induced lethality. The ability of mastoparan to inhibit cytokine production and to block the activation of p38 MAPK suggested that mastoparan might have efficacy in reducing LPS-induced pathology in vivo. To determine the effect of mastoparan in vivo, we assessed the effect of mastoparan on LPS-induced lethal endotoxic shock in rats. Mastoparan significantly ($P = 0.003$) protected rats from LPS-induced mortality (Table I). These experiments demonstrate the importance of G protein–mediated events in endotoxic shock and suggest that targeting heterotrimeric G proteins with pharmacological agents may have therapeutic potential.

Effects of pertussis toxin on LPS-induced signaling. The association of GPI-anchored proteins (including CD14) with intracellular signaling molecules is likely not mediated by protein–protein interactions but is probably a consequence of the colocalization of GPI-anchored proteins and dual-acylated signaling molecules (src kinases and heterotrimeric G proteins) to detergent insoluble membrane microdomains containing

Table I. Mastoparan Treatment Protects Rats from Lethal Endotoxin Shock

Group	Mortality	Mortality percent total
Mastoparan	0/5	0%
LPS	21/27	78%
LPS + mastoparan	10/28	36%

Rats were treated with 3 mg/kg mastoparan by intravenous injection in the tail vein, immediately followed by 15 mg/kg lead acetate and 1–5 μg/kg LPS intravenously. Mortality was assessed up to 96 h following LPS treatment. The ability of mastoparan to protect rats from LPS-induced mortality was statistically significant, $P = 0.003$ using the Fisher exact test.

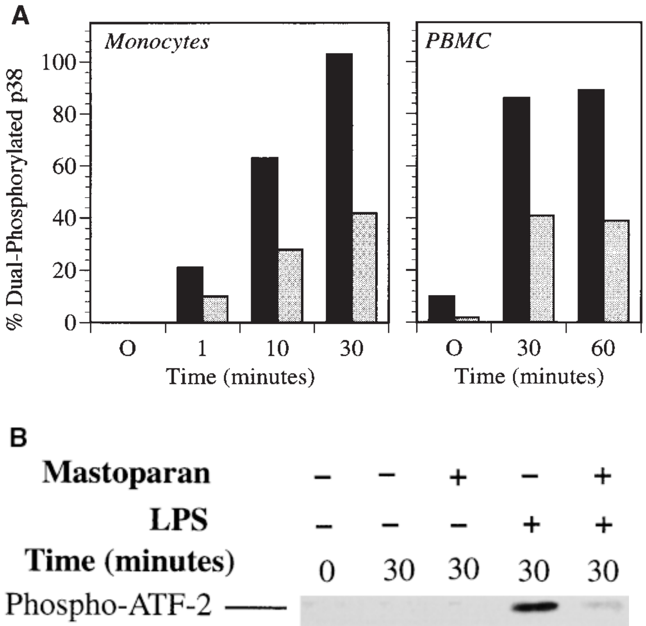


Figure 4. Mastoparan inhibits the phosphorylation of p38 MAPK in human monocytes and PBMCs. (*A*) Human monocytes and PBMCs were treated with 13.3 μM mastoparan for 1 h at 37°C and were then stimulated with 10 ng/ml LPS for the indicated period of time at 37°C. Cells were then lysed in boiling reducing Laemmli sample buffer and subjected to electrophoresis through a 10% SDS-PAGE gel. Membranes were washed and blocked as indicated in Methods, were immunoblotted with antiphosphorylated p38, were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.7; 50°C for 30 min), and were reimmunoblotted with anti-p38. Data are presented as percentage of dual-phosphorylated p38 (dual phosphorylated/total p38). *A* is representative of five separate experiments with similar results. (*B*) Mastoparan reduces LPS-induced p38 MAPK activity. Human PBMCs were treated as described for *A* and lysed in lysis buffer. p38 MAPK was immunoprecipitated from the lysates and MAPK activity was measured by the phosphorylation of ATF_2 visualized by immunoblotting with antiphospho-ATF_2 mAb subsequent to 10% SDS-PAGE and transfer to NC.

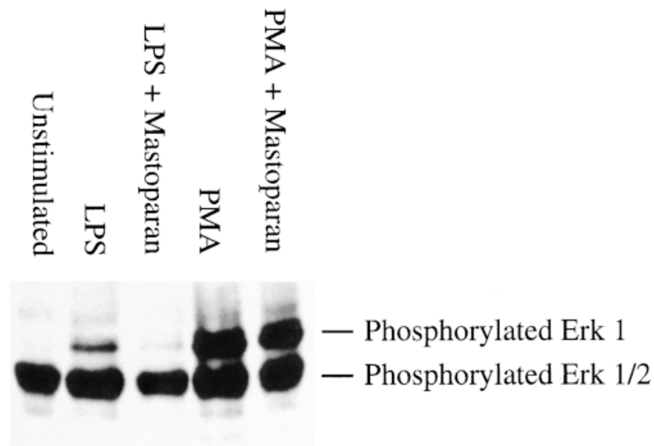


Figure 5. Mastoparan specifically inhibits LPS-induced Erk kinase activation. Monocytes were treated with mastoparan for 1 h at 37°C and then treated with 10 ng/ml LPS or 100 ng/ml PMA. (The samples shown in the figure represent the maximal activation of the Erk kinases for LPS and PMA, respectively, as determined by kinetic analysis.) Lysates from treated cells were immunoblotted with antiphosphorylated Erk 1 and 2 mAb. (This antibody reacts with both phosphorylated Erk 1 [44 and 42 kD] and phosphorylated Erk 2 [42 kD].)

high concentrations of cholesterol and sphingolipids (7–12). Pertussis toxin catalyzes the ADP ribosylation of heterotrimeric G protein α subunits in the region where the α subunits interact with serpentine (seven spanner) receptors. Pertussis toxin does not effect the ability of G proteins to bind or hydrolyze GTP, nor does it alter the effector functions of G proteins (42). Not surprisingly, pertussis toxin did not inhibit cytokine production, MAPK activation (data not shown), nor NFκB protein translocation (Solomon, K.R., E.A. Kurt-Jones, and R.W. Finberg, unpublished observations) from LPS-stimulated monocytes, since CD14 is not a serpentine receptor.

Discussion

Although the mechanism by which LPS induces cytokine production is not known and the role of CD14 in LPS-induced signal transduction is also not well understood, it is clear that the interaction of LPS and CD14 on the surface of monocyte/macrophages initiates a cascade of signaling events that have profound clinical effects. In this report we demonstrate that CD14 is physically associated with G_i/G_o heterotrimeric G proteins and that these same G proteins regulate LPS signaling in vitro and in vivo.

CD14 physically associates with heterotrimeric G proteins. We have previously demonstrated a physical association between GPI-anchored proteins and heterotrimeric G proteins in lymphocytes (12). In this report we demonstrated that CD14, the major LPS receptor, is associated with src kinases and G_i/G_o heterotrimeric G proteins. The overall pattern of tyrosine phosphorylated proteins found to coimmunoprecipitate with CD14 is very similar to that found with other GPI-anchored proteins regardless of cell type (7–12). We have previously shown that the heterotrimeric G proteins associated with CD14 are also associated with other GPI-anchored proteins in human and murine T cells (12). Thus, the association of src kinases, tyrosine phosphorylated proteins, and heterotrimeric G proteins with GPI-anchored molecules including CD14, is a general phenomenon. There was no evidence for G_s heterotrimeric G proteins in the CD14 immunoprecipitates. This observation is consistent with our previous report in which G_s heterotrimeric G proteins were not found to associate with CD48, CD59, nor Thy-1 (12). The reason for the lack of G_s in our immunoprecipitates may be that unlike G_i and G_o heterotrimeric G proteins, G_s cannot be dual acylated (43, 44). Dual acylation of intracellular proteins has been shown to be an important modification for the preservation of GPI-anchored protein/cytoplasmic protein associations in nonionic detergent lysates (9, 10). There was no evidence for β or γ subunits of heterotrimeric G proteins in the CD14 immunoprecipitates. This may be due the documented dissociation of these subunits from the α subunit during detergent lysis (17), or simply be an indication that these subunits are not phosphorylated in the in vitro kinase reaction.

GPI-anchored proteins are resistant to nonionic detergent solubilization. In fact, only a fraction of the total membrane pool of these molecules is solubilized by NP-40 (or Triton

X-100) detergent extraction (45). In our laboratory, nonionic detergent solubility for different GPI-anchored proteins ranges from 5 to 50% under conditions that completely solubilize transmembrane proteins. GPI-anchored proteins are, however, completely solubilized by octylglucoside (45, 46). In nonionic detergent lysates, GPI-anchored proteins and their associated dual-acylated signaling molecules are found in microdomains that are buoyant on sucrose gradients (47, data not shown). These domains can be disrupted by octylglucoside detergent extraction (45, 46) or by reduction of the cholesterol content of the membranes (47), indicating the critical role of lipids in maintaining microdomain structure. Immunoprecipitates of individual GPI-anchored proteins, with their associated src kinases and G proteins, do not contain multiple GPI-anchored proteins (12). We hypothesize that the reason both GPI-anchored proteins and dual-acylated signaling molecules are found in these complexes is related to their inclusion in lipid microdomains. The reason that the complexes do not pellet at 3,000 *g* and are buoyant is likely because of both their small size and their high lipid content.

Regulation of LPS-induced p38 activation and cytokine production by heterotrimeric G proteins. Mastoparan treatment of PBMCs and monocytes suppressed LPS-induced phosphorylation of p38 MAPK. Recently it has been demonstrated that overexpression of the G_o α heterotrimeric G protein subunit inhibits p38 activation induced by adrenergic receptor stimulation (48). This suggests that mastoparan inhibits p38 activation by its stimulation of G_o α heterotrimeric G protein subunits.

The activation of p38 MAPK has been shown to be required for LPS-induced cytokine production (36–38, 49), and recently, one member of the myocyte-enhancer factor 2 (MEF2) group of transcription factors was identified as a nuclear target of the p38 MAPK (50). Thus, the profound effect of mastoparan on LPS induction of cytokine production may be related to its effects on the phosphorylation of p38 MAPK. Recent studies have revealed that heterotrimeric G protein β/γ subunits can activate MAPK, including p38 (48, 51, 52). It seems likely that G_i/G_o heterotrimeric G proteins are involved in both positive and negative regulatory LPS signaling pathways through different heterotrimeric G protein subunits (53). Mastoparan may target G proteins in a negative regulatory pathway of LPS activation of cytokine synthesis. It is interesting to note that LPS has been shown to induce changes in the intracellular G protein distribution within macrophages. Studies of Makhlouf et al. have shown that peritoneal macrophages from LPS-treated rats have lower levels of membrane-associated G proteins, particularly G_i $\alpha 3$, than cells from normal, untreated rats (54).

The effect of mastoparan on cytokine production was specific to LPS induction of cytokines, insofar as PMA-induced cytokine production was not inhibited by mastoparan. Unlike LPS, PMA induced substantial phosphorylation of Erk1 and Erk2 MAPK. Consistent with the specificity of the mastoparan effect on cytokine production, the effect of mastoparan on Erk1 and Erk2 phosphorylation, while discernible, was much less than the effect of mastoparan on p38 phosphorylation. This observation is consistent with those of previous investigators who have shown that constitutively active G_i $\alpha 2$ does not inhibit the phosphorylation of Erks (55).

Mastoparan protects rats from lethal endotoxic shock. Potentially the most important aspect of the work presented here is in the ability of mastoparan to protect rats from lethal endotoxic shock. Blood infection by gram-negative bacteria remains a serious health risk. Treatment of sepsis patients with antibiotics often does not prevent septic shock from occurring due to the large amounts of circulating LPS in the blood steam. Moreover, antibiotic treatment can lead to transient increases in LPS levels due to the LPS released from the membrane of killed bacteria. Novel methods of reducing the pathological responses to LPS are required. Mastoparan reduced LPS-induced lethality in vivo by $\sim 50\%$. Mastoparan itself was tolerated by rats at doses higher than that which protected the rats from lethal LPS challenge. The ability of mastoparan to protect rats from lethal endotoxin shock is remarkable, particularly since intravenous mastoparan has recently been shown to induce microvascular dilation, an effect that would be expected to aggravate rather than ameliorate shock (56). Further, although one would expect a peptide to be rapidly cleared, mastoparan is known to insert into the lipid bilayer of cells, where it likely exerts its effects on intracellular G protein signaling (33). The rat model of lethal endotoxin shock is complex; however, it allows screening of potentially useful therapies on large numbers of individual animals. Future studies using a rabbit model of endotoxin shock will allow us to study the effect of mastoparan on the physiologic and cytokine responses to endotoxin (57). Nevertheless, our studies in rats indicate that targeting heterotrimeric G proteins with pharmacological agents may be an effective method of reducing the morbidity and mortality associated with septic shock.

LPS, CD14, and G proteins. The current literature on LPS signaling suggests two possibilities to explain LPS-induced signal transduction. The first is that a transmembrane receptor bridges the GPI-anchored CD14 and intracellular signal transducing molecules (58). This coreceptor is postulated to accept LPS after CD14 binding and transfer. A second, related hypothesis is that CD14 transfers LPS directly to a cytoplasmic messenger, thereby activating a signal transduction cascade (59). The results presented here, while consistent with either hypothesis, suggest that heterotrimeric G proteins are involved in LPS-induced signal transduction. Interestingly, a recent publication (60) may shed light on the mechanism of G protein involvement in LPS signal transduction. In the study, Gudi et al. revealed that heterotrimeric G proteins can be activated in the absence of specific receptors by perturbation of artificial lipid bilayers via shear stress. The level of G protein activation is modulated by altering the lipid content of the bilayers, implying that local lipid environments may impact G protein signaling induced by membrane perturbation. LPS is known to be deposited in membranes after CD14 binding (4–6). The deposition of LPS may be the membrane perturbance analogous to shear stress that induces G protein activation.

Our data support the concept that colocalization of GPI-anchored proteins and signal transduction molecules to lipid microdomains may have a profound influence on signal transduction via GPI-anchored receptors. CD14, like other GPI-anchored proteins, resides in nonionic detergent insoluble microdomains (DIGs) that contain various signaling molecules. These microdomains have been found in many tissues and appear to evolutionarily conserved (61), suggesting their significance. The sorting of GPI-anchored proteins to these DIGs is a process controlled during movement of the nascent proteins through the trans-Golgi (62) and has important consequences for the surface expression and physical/biochemical properties of the GPI-anchored proteins. Thus, it is likely that the inclusion of GPI-anchored proteins in DIGs is relevant to their function. The localization of signaling molecules to DIGs is likely to also have functional consequences, i.e., proximity to effector targets. A functional relationship between GPI-anchored proteins and lipid-modified signal transduction molecules is a reasonable expectation.

The results presented here establish a role for heterotrimeric G proteins in LPS signal transduction and clarify the direct role of CD14 in LPS-mediated signaling. The results obtained also suggest important, new therapeutic approaches (the use of G protein agonists and antagonists) to prevent LPS-induced septic shock.

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