Gi proteins regulate lipopolysaccharide and Staphylococcus Aureus induced cytokine production but not (1→3)-Beta-D-glucan induced cytokine suppression

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1. ABSTRACT

Previous studies have demonstrated that bacterial lipopolysaccharide (LPS) and heat killed Staphylococcus aureus (SA) activation of inflammatory cells depended in part upon activation of heterotrimeric Gi proteins. It has also been shown that (1→3) beta-D-glucan can suppress inflammatory cell activation by microbial products although the cellular mechanism of the glucan effect remains to be clearly defined. We hypothesized that Gi proteins function as a common convergent signaling pathway for both LPS and SA leading to monocyte mediator production. Additionally, we hypothesized that soluble glucan suppresses LPS and SA induced cytokine production via Gi protein coupled signaling. Human THP-1 promonocytic cells were pretreated with pertussis toxin (PTx, 100ng/ml or 1 microgram/ml) 6 hours prior to stimulation with LPS (10 microgram/ml) and SA (10 microgram/ml) and/or soluble glucan (10 microgram/ml). Both LPS and SA significantly (p<0.05) induced cytokine production IL-6 >TNF alpha >IL-1 beta >GM-CSF >IL-10 >IFN gamma. The induction of these cytokines was significantly (p<0.05) suppressed by PTx. Glucan treatment alone had no effect on cytokine production but suppressed (P<0.05) LPS and SA induced cytokines. PTx further augmented (p<0.05) the inhibitory effect of glucan on the LPS and SA cytokine expression. The data support the hypothesis that Gi proteins function as a common signaling protein for both LPS and SA induction of pro-and anti-inflammatory cytokines and that soluble glucan effectively suppresses cytokine production to the microbial stimuli. In contrast, the effect of soluble glucan on inhibiting cellular activation by LPS and SA is Gi protein independent.

2. INTRODUCTION

Septic shock is most commonly induced by gram-negative and gram-positive bacteria (1,2). Activation of inflammatory cells by bacteria or their cell wall components induces pro-inflammatory mediators, which may play a central role in septic shock. The membrane glycolipid LPS appears to be the main mediator of immune responses to gram-negative bacteria while several gram-positive bacterial components including peptidoglycans, lipoteichoic acid and lipoproteins on the cell surface mediate activation (3-5).

Macrophage pro-inflammatory cytokine gene induction by LPS, and Staphylococcus aureus (SA) occurs through activation of different cell receptors. The first identified LPS receptor CD14, expressed on the surface of macrophage and monocytes, binds LPS with high affinity (6-8). Toll-like receptor 4 (TLR4) also plays a critical role in the LPS mediated immune response (9,10). The gram-positive bacteria SA induced signaling pathways are mainly mediated primarily through Toll-like receptor 2 (TLR2) (3,11).

The extent to which gram-negative and gram-positive bacteria show common post-receptor signaling is an area of active investigation. LPS signals through the TLR4/CD14/MD2 cell receptor complex while SA signals through the TLR2/CD14 pathway (12). Although, they interact with different receptors, LPS and SA affect common downstream signaling proteins in the TLR signaling cascade, such as MyD88, IRAK1, and TRAF6 leading to downstream pro-inflammatory gene expression.
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Although the fact that both agonists activate similar signaling proteins and cytokines (13,14), there are differences in the signaling pathways. For instance, blockade of the p38 pathway with antibodies prevents LPS-induced TNF-alpha production, but has no effect on SA-induced TNF-alpha production (14). LPS signaling involves MD2 while SA does not (15). TLR4 ligands unlike TLR2 ligands can also signal through a MyD88 independent pathway leading to inflammatory gene expression (16,17).

Hereotrimetric Gi proteins are a novel class of post-receptor binding proteins involved in LPS signaling. Gi proteins co-immunoprecipitate with the CD14 receptor (18). Studies utilizing PTx that specifically inhibits receptor-Gi coupling by catalyzing ADP-ribosylation have demonstrated inhibition of LPS-induced transcription of TNF-alpha, IL-1-beta mRNA, activation of mitogen-activated protein (MAP) kinase, p38, JNK, ERK1/2 and production of prostaglandin E2 (PGE2), thromboxane B2 (TxB2), and NO in different cell types (19-22). Gi proteins appear to modulate signaling of both gram-negative and gram-positive microbial stimuli. It has also been observed that SA induced cytokine expression was inhibited by mastoparan, which functions as a G i protein antagonist (23). Also our previous studies have demonstrated that PTx inhibited LPS and SA induced TNF-alpha production in murine J774.1A cells and human promonocytic THP-1 cells (24). However, the effect of inhibition of Gi proteins on synthesis of other cytokines has not been investigated.

Glucans are natural product biological response modifiers composed of (1->3)-beta-D-linked polymers of glucose units (25). In animal studies glucans have been shown to be effective in attenuating experimental peritonitis (26-29). Clinical studies suggest that administration of glucans to trauma and surgical patients will stimulate conversion from anergy, decrease the incidence of septic complications, and improve survival (30-33). Soluble glucan mitigates the severity of in vivo endotoxemia (34) and SA infection (35) in rodents. In vivo treatment with soluble glucans has been shown to suppress ev vivo LPS, staphylococcal enterotoxin B and toxic shock syndrome toxin induction of TNF-alpha, IFN-gamma, IL-2 and IL-6 in murine macrophages or lymphocytes (36,37). However, the molecular mechanisms whereby glucan mediates its cellular effects remains to be clearly defined. Recently, soluble glucan has been shown to activate cells through binding to dectin-1, scavenger and TLR2 receptor (38-41). Scavenger receptors function through Gi protein coupled signaling pathways (42) and inhibition of Gi proteins has been shown to inhibit SA (a TLR2 ligand) activation (24). This suggests that the cellular effects of soluble glucan may be mediated, in part, by Gi protein coupled signaling.

In the present study, we examined the effect of inhibition of Gi protein function on LPS and SA activation of multiple pro-inflammatory cytokines (IL-6, TNF-alpha, IL-1-beta, and IFN-gamma), the anti-inflammatory cytokine IL-10 and GM-CSF in human promonocytic THP-1 cells. Additionally, we examined the effect of soluble glucan on LPS and SA induced mediator production and the possible involvement of Gi proteins.

3. MATERIAL AND METHODS

3.1. Cell culture
THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Cellgro Mediatech Inc., Herndon, VA), supplemented with 10% heat inactivated fetal calf serum (FCS) (Sigma, St. Louis, MO), 50U/ml penicillin, 50microgram/ml streptomycin (Cellgro Mediatech Inc., VA) in 150cm2 tissue culture flasks and maintained at 37°C in 5% CO2, 95% incubator air. The confluent THP-1 cells within 10 passages were used for experiments.

3.2. Cell stimulation
THP-1 cells at a density of 1x10^6 cells/ml were pretreated with or without PTx (List Biological Laboratories Inc., Campbell, CA) or vehicle at a concentration of 100ng/ml or 1 microgram/ml for 6 h. The cells were washed and treated with or without soluble (1->3) beta-D-glucan together with LPS (10 microgram/ml) from Salmonella enteritidis (Sigma, St. Louis, MO), or heat killed SA (10 microgram/ml, heat killed SA were prepared as described previously 43) for 24 h.

Viability was quantitated by Trypan Blue exclusion. LPS, SA, PTx, and soluble (1->3) beta-D-glucan at the concentrations of drugs do not affect cell viability (>95% viability).

3.3. Luminescence Analysis
TNF-alpha, IFN-gamma, IL-1–beta, IL-6, IL-10, and GM-CSF were measured using an enzyme-linked immunosorbant assay (ELISA) with luminex ELISA kits (Biosource, Camarillo, CA).

3.4. Statistical analysis
Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by analysis of variance (ANOVA) with Fisher’s probable least-squares difference test using Statview software (SAS Institute Inc., Cary, NC). p< 0.05 was considered significant.

4. RESULTS
4.1. Effects of PTx on LPS or SA induced cytokine production
To test the hypothesis that Gi proteins are involved in both gram-negative and gram-positive bacteria induced pro-inflammatory cytokine release, human promonocytic THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) followed by stimulation with LPS, or SA. PTx has no affect on cytokine production. LPS and SA significantly induced IL-6, TNF-alpha, IL-1-beta, GM-CSF, IL-10, and IFN-gamma production. PTx inhibited LPS induced IL-6 production by 80±4% and 87±1% (n=3, p<0.05, Figure 1A), TNF-alpha production by 93±1% and 95±2% (n=3, p<0.05, Figure 2A), IL-1-beta
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Figure 1. Effects of PTx and glucan pretreatment on LPS and SA induced IL-6 production in THP-1 cells. THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10µg/ml) for 24 h. LPS and SA induced IL-6 production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p< 0.05 compared to basal group. †, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

production by 65±5% and 73±2% (n=3, p<0.05, Figure 3A), GM-CSF production by 43±4% and 51±2% (n=3, p<0.05, Figure 4A), IL-10 production by 90±3% and 91±2% (n=3, p<0.05, Figure 5A), and IFN-gamma production by 9±1% and 10±2% (n=3, p<0.05, Figure 6A). PTx inhibited SA induced IL-6 production by 79±6% and 70±9% (n=3, p<0.05, Figure 1B), TNF-alpha production by 80±6% and 59±14% (n=3, p<0.05, Figure 2B), IL-1-beta production by 40±3% and 22±15% (n=3, p<0.05, Figure 3B), GM-CSF production by 29±1% and 23±6% (n=3, p<0.05, Figure 4B), IL-10 production by 78±3% and 69±7% (n=3, p<0.05, Figure 5B), and IFN-gamma production by 30±5% and 23±5% (n=3, p<0.05, Figure 6B).

4.2. Effects of (1→3) beta-D-glucan on LPS or SA induced cytokine production

To investigate the effect of glucan on LPS and SA induced cytokine production, human promonocytic THP-1 cells were stimulation with LPS or SA together with
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**Figure 2.** Effects of PTx and glucan pretreatment on LPS and SA induced TNF-alpha production in THP-1 cells. THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10 microgram/ml) for 24 h. LPS and SA induced TNF-alpha production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p < 0.05 compared to basal group. #, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

Glucan had no effect on cytokine production. However, glucan inhibited LPS- induced IL-6 production by 65±3% (n=3, p<0.05, Figure 1A), TNF-alpha production by 81±4% (n=3, p<0.05, Figure 2A), IL-1-beta production by 67±3% (n=3, p<0.05, Figure 3A), GM-CSF production by 32±2% (n=3, p<0.05, Figure 4A), IL-10 production by 76±2% (n=3, p<0.05, Figure 5A), and IFN-gamma production by 10±2% (n=3, p<0.05, Figure 6A). Glucan inhibited SA-induced IL-6 production by 37±9% (n=3, p<0.05, Figure 1B), TNF-alpha production by 42±10% (n=3, p<0.05, Figure 2B), IL-1-beta production by 32±4% (n=3, p<0.05, Figure 3B), GM-CSF production by 19±2% (n=3, p<0.05, Figure 4B), IL-10 production by 48±3% (n=3, p<0.05,
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Figure 3. Effects of PTx and glucan pretreatment on LPS and SA induced IL-1-beta production in THP-1 cells. THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10 microgram/ml) for 24 h. LPS and SA induced IL-1-beta production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p<0.05 compared to basal group. #, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

Figure 5B), and IFN-gamma production by 16±0% (n=3, p<0.05, Figure 6B).

4.3. Effects of PTx on glucan induced suppression of cytokine production

To further investigate the involvement of Gi proteins in glucan induced suppression of cytokine production, human promonocytic THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) followed by stimulation with LPS or SA with glucan (10 microgram/ml). PTx further decreased glucan induced suppression of LPS induced IL-6 production by 68±4% and 80±4% (Figure 1A), TNF-alpha production by 82±1% and
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Figure 4. Effects of PTx and glucan pretreatment on LPS and SA induced GM-CSF production in THP-1 cells. THP-1 cells were pretreated with PTx (100 ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10 microgram/ml) for 24 h. LPS and SA induced GM-CSF production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p<0.05 compared to basal group. #, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

91±2% (Figure 2A), IL-1 beta production by 44±5% and 55±7% (Figure 3A), GM-CSF production by 32±2% and 48±6% (n=3, p<0.05, Figure 4A), IL-10 production by 77±3% and 84±3% (Figure 5A), and IFN-gamma production by 8±4% and 12±2% (n=3, p<0.05, Figure 6A). PTx further decreased glucan induced suppression of SA induced IL-6 production by 84±3% (n=3, p<0.05, Figure 1B), TNF-alpha production by 84±5% (n=3, p<0.05, Figure 2B), IL-1-beta production by 32±4% (n=3, p<0.05, Figure 3B), GM-CSF production by 17±3% (Figure 4B), IL-10 production by 72±5% (n=3, p<0.05, Figure 5B), and IFN-gamma production by 28±4% (n=3, p<0.05, Figure 6B).

5. DISCUSSION

Lipopolysaccharide and SA induce synthesis of a
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**Figure 5.** Effects of PTx and glucan pretreatment on LPS and SA induced IL-10 production in THP-1 cells. THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10 microgram/ml) for 24 h. LPS and SA induced IL-10 production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p< 0.05 compared to basal group. #, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

A variety of cytokines by human THP-1 cells. These microbial stimuli induce the expression of cytokines in rank order potency IL-6>TNF-alpha>IL-1-beta>GM-CSF>IL-10>IFN-gamma. Interestingly, in all cases LPS was a more potent stimulus compared to SA suggesting a predominant TLR4 driven response of the THP-1 monocytes. PTx significantly inhibited both LPS and SA induced cytokine production. These data confirm our previous findings that Gi proteins regulate TLR4 and TLR2 ligand induction of TNF-alpha in monocytes and macrophages (24), and importantly extend these studies demonstrating that Gi proteins also regulate other pro-inflammatory cytokines, the anti-inflammatory cytokine IL-10, and the growth factor GM-CSF. Soluble glucan also suppressed mediator production induced by both LPS and SA. Inhibition of Gi protein function with PTx further
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**Figure 6.** Effects of PTx and glucan pretreatment on LPS and SA induced IFN-gamma production in THP-1 cells. THP-1 cells were pretreated with PTx (100ng/ml or 1 microgram/ml) for 6 h followed by stimulation with LPS (10 microgram/ml) (A), or heat killed SA (10 microgram/ml) (B) with or without (1→3) beta-D-glucan (10 microgram/ml) for 24 h. LPS and SA induced IFN-gamma production was analyzed by luminex analysis. Data represent the mean ± SE from three independent experiments. *, p< 0.05 compared to basal group. #, p < 0.05 compared with LPS or SA stimulated group. †, p<0.05 compared with glucans pretreated group.

augmented the glucan-induced suppression of LPS and SA induced cytokine production.

The findings that PTx blocked both LPS and SA induced multiple pro-inflammatory cytokines, a growth factor, and the anti-inflammatory cytokine IL-10 suggest that Gi proteins function as proximal signal transducers for both TLR4 and TLR2 ligands. In a recent study, Lentschat et al. (44) also concluded that Gi proteins function as proximally in the signaling cascade since Gi protein inhibition with mastoparan inhibited both MyD88 dependent and independent signaling in murine macrophages. In the latter study Gi protein inhibition did not affect activation with the soluble TLR2 ligand Pam3Cys. These findings are not in agreement with findings that PTx or mastoparan blocked the TLR2 ligand SA induced activation (23, 24). The results may suggest differences in the mechanism whereby PTx and mastoparan
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inhibit Gi protein function or to species differences ie. human monocytic cells vs murine macrophage. Another possibility is that particulate phagocytizable TLR2 ligands, ie. heat-killed SA, may activate Gi protein coupled signaling pathway not activated by soluble TLR2 ligands, ie. Pam3Cys.

Our studies demonstrate that soluble glucan alone had no effect on the induction of the cytokines. These observations are consistent with other studies that showed that soluble glucan had no effect on in vitro cytokine production by human monocytes (45) or in cultured murine BMC2.3 cells (46). Soluble glucan effectively ameliorated all cytokines induced by LPS and SA. The glucan inhibition of cellular activation by TLR2 and TLR4 ligands may be a mechanism whereby glucan improves survival in polymicrobial sepsis (47). Glucan has only a modest, but significant, effect on inhibiting IFN-gamma. Previous studies have demonstrated that soluble glucan induced IFN-gamma in mice (48). It has been suggested that the ability to induce IFN-gamma or, as in the present study, to induce a relatively modest inhibition relative to other cytokines may be beneficial in immuno-suppressed syndromes exhibiting a predominant Th2 response. Soluble glucans have been shown to bind to dectin-1 recognition domains, which in turn interact with TLR2 to transduce signaling via the TLR2/MyD88/NF-kappa-B which in turn interact with TLR2 to transduce signaling via the NF-kappa-B signaling pathway not activated by PI3 kinase (44). Thus it is feasible that glucan induced suppression of cytokine production occurs through Gi protein independent pathways leading to PI3 kinase activation.

These data support the concept that convergent Gi signaling occurs between TLR4 and TLR2 in human monocytic lineage cells treated with LPS or SA. However, since the suppression of microbial stimuli with soluble glucan occurs independent of Gi proteins, these observations raise the interesting possibility that conjoint therapeutic approaches targeted at inhibiting Gi protein function coupled with soluble glucan may provide superior beneficial effects in treating polymicrobial sepsis.

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