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# The Role of Lipopolysaccharide Structure in Monocyte Activation and Cytokine Secretion

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## Abstract

**Background—**The lipopolysaccharide (LPS) molecule is composed of a hydrophobic lipid region (Lipid A), an oligosaccharide core, and an O-Antigen chain. Lipid A has been described as the molecular region responsible for inducing activation of immune cells. We hypothesize that the O-Antigen plays a critical role in the activation and responsiveness of mononuclear cell immune function.

**Methods—**Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated with LPS, LPS with attenuated O-Antigen (RF5), or Lipid A (DPL), which lacks an O-Antigen. Selected cells were pre-treated with a blocking antibody to CD14. Western blots were performed to determine activation of mitogen activated protein kinases (MAPK) p38, ERK, and JNK at selected time-points. RNA was extracted for RT-PCR quantification of TNF-α and IL-10 gene transcription. Supernatants were harvested and analyzed by ELISA for tumor necrosis factor alpha (TNF-α) and interleukin 10 (IL-10).

**Results—**LPS elicited maximal response, including phosphorylation of p38, ERK, and JNK, synthesis of TNF-α and IL-10 mRNA, and secretion of TNF-α and IL-10. Stimulation with RF5 activated the same pathways to a lesser degree. DPL led to increased phosphorylation of p38 and ERK and increased secretion of IL-10. CD14 blockade was associated with a significant decrease in cytokine secretion by LPS, and abolished cytokine secretion in cells stimulated with RF5 or DPL.

**Conclusions—**Structural variants of LPS activate monocytes differentially. The complete O-Antigen is important for maximal activation of MAPK, cytokine synthesis, and cytokine secretion. LPS with attenuated O-Antigen and Lipid A activate only certain components of these pathways. LPS with a complete O-Antigen stimulates cytokine secretion which is partially independent of CD14, but shortening or removal of the O-Antigen inhibits this secretion.

### Keywords

LPS; O-Antigen; CD14; sepsis

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## Introduction

Gram-negative sepsis is a leading cause of morbidity and mortality in the ICU and is associated with the development of multiple organ dysfunction syndrome (MODS) [1]. MODS develops as a result of dysregulated immune cell activation and function during infection and following exposure to lipopolysaccharide (LPS) from the cell wall of infecting gram-negative bacteria [2]. Immune cell activation leads to the release of pro- and anti-inflammatory cytokines as a normal component of the physiologic response to infection. In many cases this inflammatory response is transient and cytokine homeostasis is quickly restored. When it is not, poorly regulated cytokine release following LPS exposure is a key factor in progressive cellular injury and the development of organ failure.

LPS has been shown to activate monocytes through the cell surface Toll-like receptor 4 (TLR4) complex [3]. TLR4 complex activation begins when LPS binds to LPS binding protein (LBP), an acute phase protein. LBP transfers LPS to CD14, which occurs both in soluble form and anchored to the monocyte cell surface via a glycosylphosphatidylinositol (GPI) anchor. We have previously demonstrated that this interaction is essential to the activation of monocytes, and lack of LBP is associated with minimal to no response to LPS. The complex of LPS, LBP, and CD14 facilitates the transfer of LPS to the TLR4/MD-2 complex. This leads to TLR4 receptor dimerization on the lipid raft, which activates downstream adaptors via signals generated through the intracellular regions of the TLR4 receptor [4, 5]. The activated TLR4 complex stimulates complicated intracellular mitogen-activated protein kinase (MAPK) pathways through an IRAK-1 mediated pathway and leads to downstream synthesis and secretion of pro- and anti-inflammatory cytokines [6].

The *E. coli* LPS molecule is composed of three covalently bound regions: the O-Antigen, the core oligosaccharide, and Lipid A. The hydrophilic O-Antigen is composed of up to 50 repeating oligosaccharide units, with each unit composed of 2-8 covalently bound monosaccharides (Figure 1) [7]. The O-Antigen structure varies between as well as within bacterial species, and may serve to protect bacteria from phagocytosis and attack by components of the human immune system [7]. It is covalently bound to the core oligosaccharide, which is hydrophilic, composed of highly conserved monosaccharide units [7], and contains a 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) moiety. Lastly, *E. coli* Lipid A is a glucosamine-containing disaccharide with a β (1 (→) 6) linkage and two phosphate groups attached at the 1 and 4′ positions. Lipid A derives its hydrophilic nature from up to 7 attached fatty acyl chains, which vary in length as well as number. These fatty acyl chains anchor LPS into the outer leaflet of the outer cell membrane in gram-negative bacteria [8].

Lipid A has historically been implicated as the region of the molecule responsible for inducing an inflammatory cell response during infection [9]. However, there is data suggesting that the O-Antigen also plays a part in immunostimulation [10]. Additionally, much of the early investigation into LPS-mediated immune cell activation was conducted using murine models, which may not closely mimic the interactions between LPS and human immune cells [11]. Thus, the purpose of this study is to further elucidate the contribution of each region of the LPS molecule to stimulating cytokine synthesis and

secretion by human mononuclear cells. We hypothesize that the presence of the O-Antigen is necessary for full monocyte activation and maximal cytokine secretion in response to LPS exposure.

## Materials & Methods

### Reagents

Wild-type LPS from *E. coli* K12 was obtained from List Biological Laboratories (Campbell, CA). Lipopolysaccharide from *E. coli* F583 with a short polysaccharide chain (RF5) was obtained from Sigma-Aldrich (Saint Louis, MO). Lipopolysaccharide Lipid A from *E. coli* F583 (DPL) was obtained from Sigma-Aldrich (Saint Louis, MO) (Figure 1).

### PBMC isolation

Heparinized venous blood was collected from healthy male volunteers between the ages of 20 and 40 years in accordance with a protocol approved by the Institution Review Board for Human Subjects at the University of Washington. Volunteers were screened for recent illnesses, history of immune or bleeding disorders, and use of nonsteroidal anti-inflammatory drugs (NSAIDs) or acetaminophen within 7 days prior to inclusion in the study. Each 15ml aliquot of whole blood was diluted with 15ml of DPBS (Dulbecco's PBS without calcium or magnesium; BioWhittaker, Walkersville, MD) and underlayed with 15ml Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ). The samples were centrifuged at room temperature for 30 minutes at 1500 rpm. The buffy coat was retrieved, washed, and re-suspended in RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) plus 50μg/ml of gentamicin (BioWhittaker).

### PBMC stimulation

Cell counts were adjusted to $2\times 10^6$ cells/ml and PBMCs were plated at 1ml/well in tissue culture polystyrene plates (Corning, Corning, NY). PBMCs were allowed to adhere at 37°C for 2 hours, then were washed and re-suspended in RPMI with gentamicin plus 10% sterile, heat-inactivated adult bovine serum (ABS; Hyclone, Logan, UT) as a source of LPS binding protein (LBP). PBMCs were then stimulated with LPS (100ng/ml), DPL (100ng/ml), or RF5 (100ng/ml) for 8 hours at 37°C.

### Protein extraction and Western blot

Following 30 or 60 minutes of stimulation, total cellular protein from 5 subjects (n=5) was extracted on ice in 100μl lysis buffer (20mM Tris pH 8.0, 137mM NaCl, 2mM EDTA, 10% glycerol, 1% Triton X-100, 10μl/ml Protease Inhibitor Cocktail [Sigma-Aldrich], 10μl/ml Phosphatase Inhibitor Cocktail 2 [Sigma-Aldrich]). Protein concentration of the supernatants was measured using the BCA protein assay (Thermoscientific Pierce, Rockford, IL) and Softmax analysis.

Total cell protein (20μg/well) was separated using electrophoresis in a 10% SDS-PAGE gel and transferred onto 0.2μm pore Hybond ECL nitrocellulose membrane (Amersham Pharmaceuticals). The membranes were blocked for 1 hour at room temperature with 5% milk (1% BSA for JNK). Immunoblotting was then performed overnight at 4°C with

primary antibody to phosphorylated p38 (Cell Signaling Technology, Beverly, MA), JNK (Promega, Madison, WI), or ERK (Cell Signaling Technology). The membranes were incubated for 1 hour at room temperature with anti-rabbit IgG labeled with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) as a secondary antibody. The signal was visualized with the Super Signal West Pico detector system (Thermoscientific Pierce) and exposed on Genemate film. All gels were stripped and re-blotted for un-phosphorylated p38, JNK, or ERK (all by Santa Cruz Biotechnology). Densitometry was performed using the NIH ImageJ program. The results (Figure 2) represent densitometric data that is normalized to, and expressed as fold-change from, PBMCs stimulated with LPS for 30 minutes.

### TNF-α and IL-10 PCR

PBMCs from 5 subjects (n=5) were isolated by centrifuging whole blood in CPT tubes (BD, Franklin Lakes, NJ) for 25 minutes at 1900g. The buffy coat was retrieved and centrifuged for 10 minutes at 400g. Cell counts were adjusted to $1 \times 10^6$ cells/ml and plated with 3ml/well in polystyrene tissue culture plates (Corning).

PBMCs were allowed to adhere at 37°C for 2 hours, re-suspended in RPMI with gentamicin and 10% ABS, and incubated for 30 minutes at 37°C. Cells were stimulated for four hours at 37°C with 100ng/ml of LPS, DPL, or RF5. Total RNA was harvested with the RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse transcribed to cDNA with the High Capacity cDNA RT Kit (Invitrogen). TNF-α and IL-10 expression were measured by real time PCR using Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Grand Island, NY) and Taqman probes for TNF-α, IL-10, and β-actin for normalization (all Invitrogen, primer sequences in Table 1). The comparative $C_T$ ($\Delta\Delta C_T$) method for quantifying relative gene expression was used to evaluate differences in transcription of TNF-α and IL-10 between cells treated with LPS, RF5, or DPL, using β-actin as an endogenous control [12].

The fold-changes of transcripts were calculated with DataAssist Software v3.0 (Life Technologies Corporation, Carlsbad, CA). The results (Figure 3) are normalized to, and expressed as fold-change from, PBMCs stimulated with LPS.

### TNF-α and IL-10 Secretion

Following stimulation with LPS, RF5, or DPL (see “PBMC Stimulation”), cell supernatants from 5 subjects (n=5) were harvested and TNF-α and IL-10 concentrations were measured using BD OptEIA ELISA kits (BD Biosciences, San Jose, CA). The results (Figure 4) are expressed in pg/ml.

### CD14 blockade

PBMCs from 5 subjects (n=5) were prepared as described for TNF-α and IL-10 secretion assays. Cells were treated for 30 minutes with a demonstrated blocking monoclonal mouse $IgG_1$ antibody to human CD14 [13] (Clone #134620, 10μg/ml; R&D Systems, Minneapolis, MN) and stimulated with LPS (100ng/ml), DPL (100ng/ml), or RF5 (100ng/ml) for 8 hours at 37°C. Supernatants were harvested and TNF-α and IL-10 concentrations were measured

using the BD OptEIA ELISA kits (BD Biosciences, San Jose, CA). The results (Figure 5) are expressed in pg/ml.

### Statistical Analysis

Data was analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. *P* values ≤ 0.05 were considered significant. Cytokine secretion (Figure 4) and CD14 blockade (Figure 5) data was log transformed prior to analysis and back-transformed for presentation in the manuscript.

## Results

### LPS, RF5, and DPL Stimulation Resulted in Differential MAPK Phosphorylation

As a major component of the cell wall in gram-negative bacteria, LPS induces an inflammatory response in immune cells. To investigate how the structure of the LPS molecule contributes to this inflammatory response, we stimulated PBMCs with LPS, RF5, or DPL and measured phosphorylation of ERK, p38, and JNK at 30 and 60 minutes post-stimulation. The greatest increase in phosphorylation was seen 30 minutes after stimulation with LPS (Figure 2A-C).

Shortening the O-Antigen had the greatest effect on phosphorylation of pJNK at 30 minutes. Stimulation with RF5 resulted in JNK phosphorylation that was 57% of that seen in cells stimulated with LPS. Removal of the O-Antigen completely (stimulation with DPL) led to JNK phosphorylation that was 32% of that seen in cells stimulated with LPS (Figure 2C).

While the data did not reach statistical significance, phosphorylation of ERK at 30 minutes decreased with shortening of the O-Antigen to 72% of that seen in cells stimulated with LPS (Figure 2A). There was considerable variation in phosphorylation when the O-Antigen was removed completely, but significant phosphorylation was retained overall (Figure 2A).

Lastly, p38 was most heavily phosphorylated in cells stimulated for 30 minutes with LPS. There was a trend toward decreasing p38 phosphorylation with shortening of the O-Antigen; phosphorylation in cells stimulated with RF5 for 30 minutes was 90% of that seen in cells stimulated with LPS. With removal of the O-Antigen, phosphorylation of p38 was partially preserved (74% of LPS-stimulation levels, Figure 2B) after 30 minutes of stimulation.

### Stimulation with Shortened O-Antigen Resulted in Decreased IL-10 and TNF-α mRNA Transcription Compared to Wild-Type LPS

In order to investigate whether changes in cytokine secretion in response to LPS are the result of altered cytokine synthesis or altered secretion of pre-formed cytokine, we measured the change in TNF-α and IL-10 mRNA transcription after stimulation with each structural variant of LPS. LPS stimulation maximally increased transcription of TNF-α and IL-10 mRNA (Figure 3). PBMCs stimulated with RF5 also demonstrated increased TNF-α mRNA transcription, but only to 28% of the levels seen in cells stimulated with LPS (Figure 3A). Similarly, IL-10 mRNA transcription was 31% of that seen in cells stimulated with LPS (Figure 3B). Complete removal of the O-Antigen (DPL stimulation) did not result in up-regulated mRNA synthesis for either cytokine (Figure 3).

### LPS, RF5, and DPL Stimulation Resulted in Differential Secretion of IL-10 and TNF-α

PBMC stimulation with wild-type LPS led to increased secretion of TNF-α and IL-10 (Figure 4). Similarly, PBMCs stimulated with RF5 secreted increased concentrations of both cytokines, but there was a trend toward lower TNF-α and IL-10 secretion following RF5 stimulation when compared to cells stimulated with wild-type LPS (Figure 4). Cells stimulated with DPL did not secrete significant amounts of TNF-α (Figure 4A), but there was a trend in these cells toward increased secretion of IL-10 (Figure 4B). As previously discussed, DPL treatment did not lead to increased IL-10 mRNA synthesis. Thus, IL-10 secreted by cells stimulated with DPL does not appear to be the result of *de novo* synthesis.

### Wild-type LPS Stimulated CD14-Independent TNF-α and IL-10 Secretion

We next set out to investigate whether alteration of the O-Antigen affects interactions between CD14 and the LPS molecule. TNF-α and IL-10 secretion was measured in PBMCs pretreated with blocking antibody to CD14 and stimulated with 100ng/ml LPS, RF5, or DPL. CD14 blockade resulted in significant inhibition of TNF-α and IL-10 secretion from cells stimulated with 100ng/ml wild-type LPS, as has been previously demonstrated [14], but modest amounts of cytokine secretion did occur. With attenuation or removal of the O-Antigen, secretion of both cytokines in the presence of CD14 antibody decreased to control levels (Figure 5). DPL, even in 1000ng/ml concentrations, was unable to stimulate secretion of TNF-α in the presence of CD14 blockade (data not shown).

## Discussion

Previous work has suggested that the Lipid A region of the LPS molecule is responsible for inducing the inflammatory response [9]. However, there is also evidence that while Lipid A alone stimulates cytokine release, it is unable to do so to the same degree as the complete LPS molecule [15]. Additionally, much of the early research into LPS function was performed using murine or other animal models, which have been shown to be less representative of the human inflammatory state than was previously thought [11]. Thus, the purpose of our study was to determine whether the presence of the intact LPS molecule is necessary to fully activate intracellular signaling in human PBMCs which results in secretion of pro- and anti-inflammatory cytokines.

Our study demonstrates the importance of the O-Antigen and core oligosaccharide in stimulating cytokine secretion. Intact LPS elicited maximal cellular response, including phosphorylation of three MAP kinases, increased transcription of TNF-α and IL-10 mRNA, and increased secretion of both cytokines, while shortening the O-Antigen resulted in lower level activation of all these pathways. These findings suggest that the complete LPS molecule stimulates cytokine secretion primarily through up-regulation of intracellular phosphorylation pathways that result in *de novo* synthesis of TNF-α and IL-10. This is consistent with prior literature suggesting that TNF-α and IL-10 secretion by cells stimulated with LPS results from MAP kinase activation and up-regulation of cytokine transcription factors [16, 17]. More importantly, they suggest a role for the O-Antigen in activating PBMCs to produce pro- and anti-inflammatory cytokines. There is a wealth of data detailing how alteration of Lipid A structure affects stimulation of the immune response

[18], but relatively little investigating the involvement of the O-Antigen. However, our findings are supported by Burd et al, who found that blocking antibodies to the *E. coli* O-Antigen led to a decrease in TNF-α secretion by RAW 264.7 cells.

We also found that *E. coli* Lipid A was unable to activate the full complement of pathways activated by intact LPS, which is consistent with the findings of Gaekwad et al that the KDO residue in the core oligosaccharide increases the potency of the cytokine response to LPS, potentially due to hydrogen bonding between the KDO residue and the TLR4 receptor [19]. The preferential p38 phosphorylation and IL-10 secretion is similarly supported by evidence that p38 phosphorylation regulates IL-10 production [17], but it is surprising that we did not observe increased transcription of IL-10 mRNA in response to Lipid A. This finding suggests that Lipid A stimulates release of IL-10 that is not newly synthesized in response to PBMC stimulation. Previous work has extensively described two pathways which are activated by LPS: the MyD88 pathway, which activates release of inflammatory cytokines (TNF-α, IL-6) via cell-surface TLR4/CD14 activation, and the TRIF-dependent pathway, which stimulates release of IFN-type cytokines (including IL-10) via internalization of TLR4 into endosomes [20]. Our data suggests that the Lipid A component of LPS may function by preferentially activating the TRIF-dependent pathway, although there is likely some degree of intracellular crosstalk between the two paths. Alternatively, the potential exists for an additional as yet unrecognized pathway for stimulation of pre-formed IL-10 within the cell.

Lastly, we confirmed that TNF-α and IL-10 secretion largely necessitates functional CD14. However, wild-type LPS elicited low-level secretion of both cytokines in the presence of CD14 blockade, while RF5 and DPL (even in higher concentrations) did not. This finding suggests that the complete O-Antigen may partly stimulate cytokine secretion via a pathway independent of the CD14 molecule, and attenuation of the O-Antigen abolishes this pathway.

Although this study provides insight into the effect of the O-Antigen, it has several limitations. First, this study provided an *in vitro* evaluation of a complex interaction between LPS, LBP, CD14, and the TLR4 receptor. Although each interaction is vital to appropriate activation, only the interaction of LPS and CD14 were investigated. Furthermore, we did not investigate the impact of altering LPS structure on the function of downstream effector proteins known to play a role in the response to LPS [21, 22]. Second, this study only examined partial components of the O-Antigen on a number of LPS molecules. These slight changes in the O-Antigen may be associated with alterations in the Lipid A tail, which could be responsible for the activation patterns demonstrated in this study. Further investigations will be required to determine these potential roles. Lastly, in this study PBMCs were isolated from healthy male volunteers. Previous research has demonstrated gender differences in the humoral cell response to stress, including cytokine secretion [23]; we did not address these gender differences in our study.

In conclusion, the complete O-Antigen was necessary for full monocyte activation, including MAPK phosphorylation, increased synthesis of TNF-α and IL-10 mRNA, and increased secretion of both cytokines, which occurred in a largely CD14-dependent manner. Partial loss of the O-Antigen resulted in decreased activation of each of these cellular pathways in

comparison to cells stimulated with wild-type LPS. Lipid A treatment led to selective phosphorylation of p38 and ERK without activation of inflammatory cytokine transcription. Despite the lack of mRNA transcription, isolated Lipid A resulted in secretion of pre-formed IL-10. Lastly, the wild-type LPS molecule stimulated cytokine secretion independent of the CD14 receptor; CD14-independent cytokine secretion was not, however, seen in cells stimulated with LPS variants containing a shortened O-Antigen or lacking the O-Antigen entirely.

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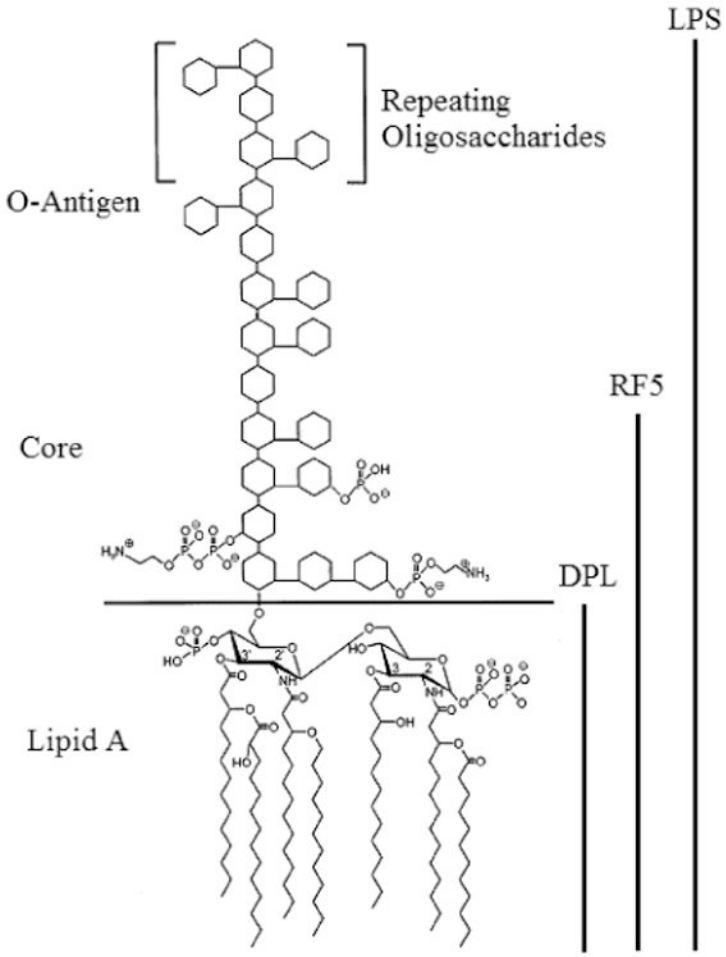


**Fig 1.**
Structure of lipopolysaccharide (LPS), RF5, and Lipid A (DPL).

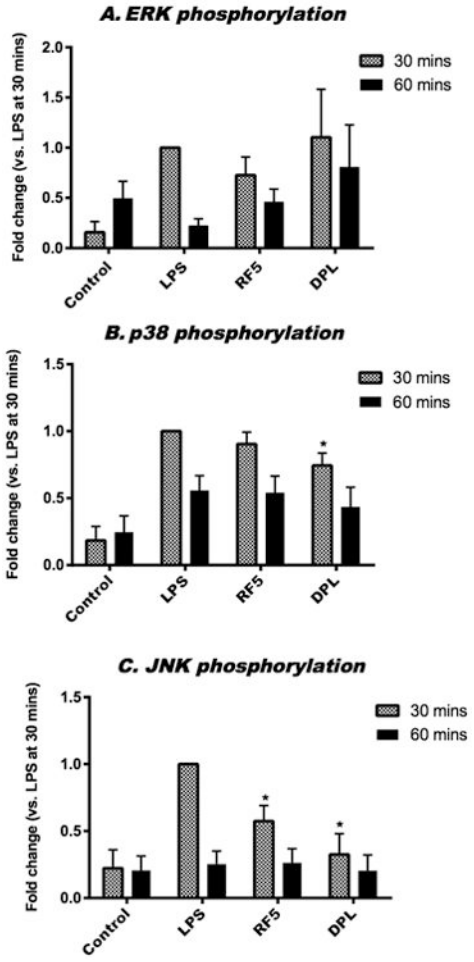


**Fig 2.**

ERK, p38, and JNK phosphorylation. PBMCs were stimulated with LPS, DPL, or RF5 for 30 or 60 minutes and cell extracts were analyzed by Western blotting as described in the Materials and Methods Section. Graphs represent densitometric data as mean ± SEM, expressed as fold-change from LPS at 30 mins. (A) ERK phosphorylation (n=5); (B) p38 phosphorylation (n=5); (C) JNK phosphorylation (n=5). * $p \leq 0.05$, vs. LPS at 30 mins. LPS, complete lipopolysaccharide molecule; RF5, LPS with attenuated O-Antigen; DPL, Lipid A.

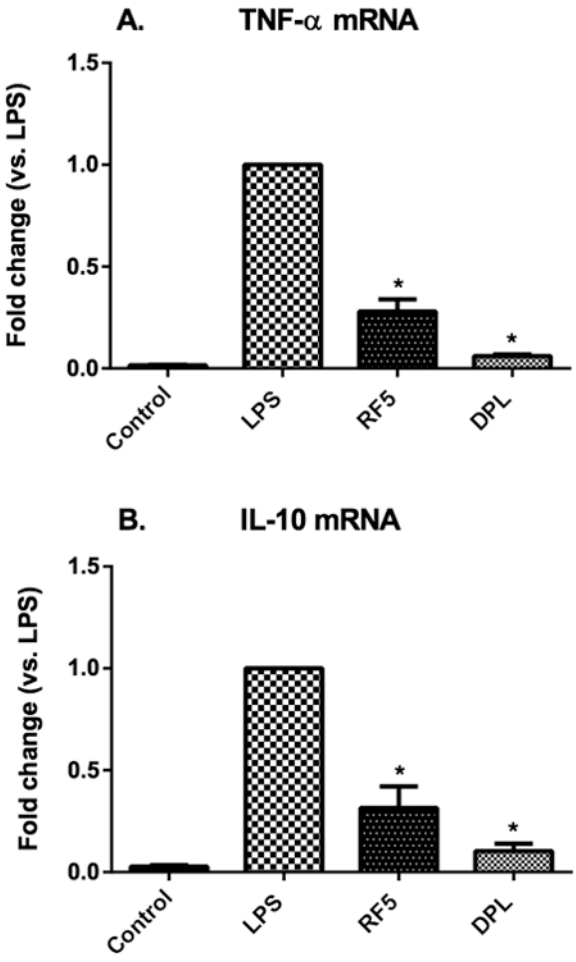


**Fig. 3.**

TNF-α and IL-10 mRNA synthesis. PBMCs were stimulated for 4 hours with LPS, DPL, or RF5. Total RNA was assayed for TNF- α and IL-10 mRNA levels. Data are expressed as fold-change from PBMCs stimulated with LPS and are represented as mean ± SEM. (A) TNF- α mRNA synthesis (n=5); (B) IL-10 mRNA synthesis (n=5). * $p \leq 0.05$, vs. LPS. LPS, complete lipopolysaccharide molecule; RF5, LPS with attenuated O-Antigen; DPL, Lipid A.

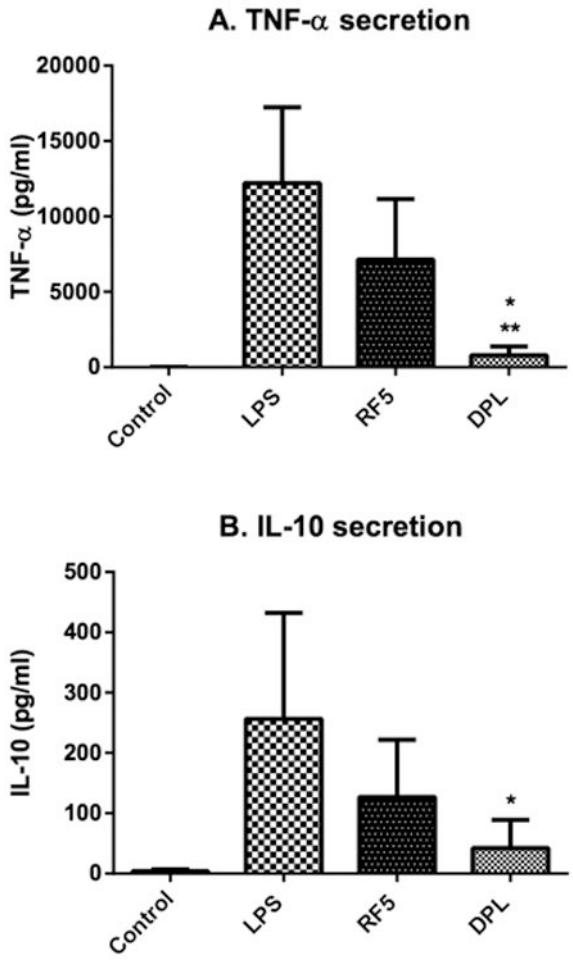


**Fig. 4.**

TNF- α and IL-10 secretion. Selected PBMCs were stimulated with LPS, DPL, or RF5 for 8 hrs. Cell supernatants were collected and TNF- α and IL-10 levels were assayed by ELISA. Data are represented as mean ± SEM. (A) TNF-α secretion (n=5); (B) IL-10 secretion (n=5). * $p \leq 0.05$, vs. LPS. ** $p \leq 0.05$, vs. RF5. LPS, complete lipopolysaccharide molecule; RF5, LPS with attenuated O-Antigen; DPL, Lipid A.

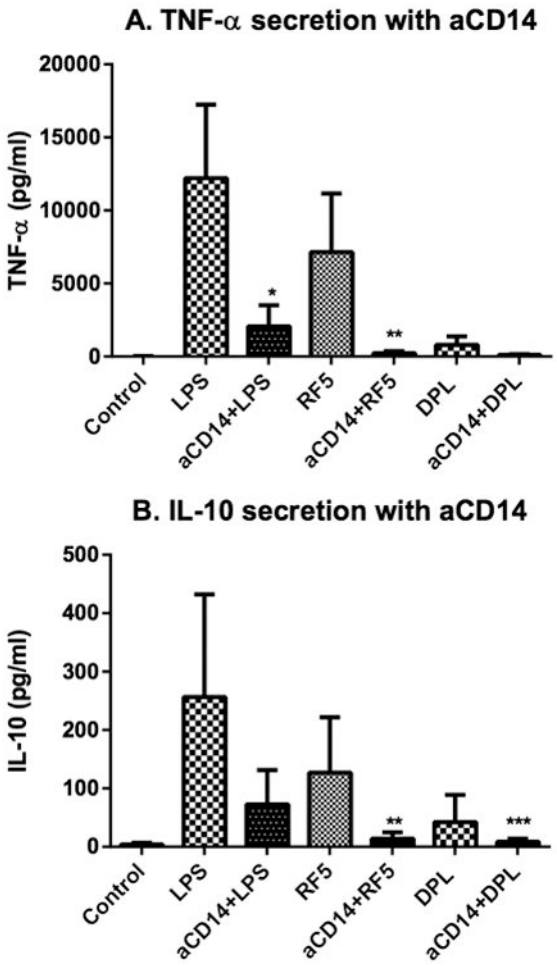


**Fig. 5.**

TNF- α and IL-10 secretion with and without blocking antibody to CD14 (aCD14). Selected PBMCs were pre-treated for 30 min with aCD14, followed by 8 hr stimulation (except controls) with LPS, DPL, or RF5. Cell supernatants were collected and TNF- α and IL-10 levels were assayed by ELISA. Data are presented in pg/ml and represented as mean ± SEM. (A) TNF-α secretion (n=5); (B) IL-10 secretion (n=5). * p≤ 0.05, vs. LPS. **p ≤ 0.05, vs. RF5. ***p ≤ 0.05, vs. DPL.LPS, complete lipopolysaccharide molecule; RF5, LPS with attenuated O-Antigen; DPL, Lipid A.

**Table 1**

Taqman primer sequences for real-time PCR of TNF-α, IL-10, and β-actin.

| Primer | Sequence (5′-3′) |
|---|---|
| TNF-α | CCATGTTGTAGCAAACCCTCAAGCT |
| IL-10 | AATAAGCTCCAAGAGAAAGGCATCT |
| β-actin | CCTTTGCCGATCCGCCGCCCGTCCA |