

Functional analysis of recombinant bovine CD14

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Abstract – Studies in mice and humans indicate that membrane CD14 (mCD14) on the surface of monocytes, macrophages and polymorphonuclear neutrophils (PMN) mediate activation of these cells by lipopolysaccharide (LPS). Soluble CD14 (sCD14), in the circulation, binds to LPS and blocks LPS binding to mCD14. The role of bovine CD14 in cellular activation by LPS is undefined. Changes in CD18 expression on PMN and steady state levels of mRNA for tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and IL-8, sensitive markers for activation of leukocytes by LPS, were used to measure functional activity of recombinant bovine sCD14 (rbosCD14). Whole blood ($n = 3$ cows) treated with LPS alone caused CD18 expression on PMN to increase by 12% ($P < 0.02$), whereas pre-incubation of LPS with 10 or 100 $\mu\text{g}/\text{mL}$ of rbosCD14 completely inhibited increase in CD18 expression. After treating whole blood with LPS at concentrations of 1, 100 or 10^4 ng/mL for 2 h, level of mRNA for TNF- α , IL-6 and IL-8 in leukocytes and concentration of TNF- α in plasma increased. However, pre-incubation of LPS with rbosCD14 inhibited the increase in TNF- α mRNA, but not the increase in IL-6 and IL-8 mRNA. Excess amount of anti-human CD14 monoclonal antibody (MAB) also inhibited LPS-induced increase in TNF- α mRNA. Preincubation of LPS with rbosCD14, or rbosCD14 plus MAB did not affect LPS-induced increase in TNF- α in plasma. Collectively, results indicate that rbosCD14 inhibit LPS-induced increase in CD18 expression and TNF- α mRNA. However, secretion of TNF- α was not inhibited by pre-incubation of LPS with rbosCD14. The TNF- α in plasma may partially induce transcription of IL-6 and IL-8, which contribute to the CD14-independent increase in level of mRNA for IL-6 and IL 8.

CD14 / LPS / TNF- α / bovine / PMN

1. INTRODUCTION

Lipopolysaccharide (LPS) is a component of the outer membrane of all Gram-negative bacteria. The interaction of LPS

with host cells initiates production of pro-inflammatory cytokines and mediators [14, 18] necessary for host defense against infection by Gram-negative bacteria. However, overwhelming release of cytokines

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and pro-inflammatory mediators can be detrimental to the host [2]. In severe conditions such as endotoxemia or septic shock, systemic responses induced by LPS result in fever, hypertension and organ injury [4]. Due to lack of effective therapeutics, mortality associated with Gram-negative septic shock in hospitals remains high at 25–30% [8]. CD14 is a receptor that mediates activation of host cells by binding LPS [16, 26]. Two forms of CD14 exist: membrane bound CD14 (mCD14) present on the surface of monocytes, macrophages and polymorphonuclear neutrophils (PMN), and soluble CD14 (sCD14) present in serum/plasma and urine of nephrotic patients [3, 9, 17] and milk [15]. Binding of LPS to mCD14 in the presence of LPS binding protein (LBP) leads to an increase in adhesion of PMN to fibrinogen-coated surface [26], translocation of nuclear factor κ B (NF- κ B) [11] and release of tumor necrosis factor- α (TNF- α) by monocytes and macrophages [5]. Addition of sCD14 inhibits these activation effects of LPS on leukocytes in vitro, by binding LPS and preventing its interaction with mCD14 [10, 12, 16]. Inhibitory effects of human sCD14 on LPS-induced activation of leukocytes protect mice from a lethal challenge of LPS [11]. Therefore, sCD14 may function as a therapeutic tool in controlling acute inflammatory response caused by Gram-negative bacterial infections.

Bovine coliform mastitis is an inflammation of the mammary gland caused by Gram-negative bacteria, where *Escherichia coli* is the most common pathogen [19]. Around 80% of all coliform intramammary infections result in clinical mastitis, 10% of which result in acute mastitis with sudden onset of septic shock [22]. Conventional antibiotic treatment, extensive fluid supplementation and metabolic support are not effective in relieving disease symptoms [19]. Given the functional activity of human and mouse CD14, bovine CD14 is a potential candidate to control septic shock during coliform mastitis.

The objectives of the present study were to evaluate ex vivo the effect of: (1) recombinant bovine sCD14 (rbosCD14) on LPS-induced up-regulation of CD18 on PMN and (2) rbosCD14 on LPS-induced changes in mRNA for TNF- α , IL-6 and IL-8 ex vivo.

2. MATERIALS AND METHODS

2.1. Experimental design and animals

Ten clinically normal mid-lactating Holstein cows from the Beltsville Agricultural Research Center dairy herd (Beltsville, MD, USA) served as blood donors for the isolation of leukocytes. Tail vein blood was collected using sodium heparin as an anticoagulant. The leukocytes ($n = 7$ cows) were used in studies to determine effect of rbosCD14 on expression of CD18, and for examination of changes in mRNA transcripts for TNF- α , IL-6 and IL-8 after activation by LPS ($n = 3$ cows). Use of animals for this investigation was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

2.2. Effect of rbosCD14 on CD18 expression of PMN stimulated with LPS

The rbosCD14 was produced in a baculovirus expression system as described [25]. Briefly, rbosCD14, with a deletion of 14 amino acids at the C-terminal end, was generated by insect *Sf9* cells infected with recombinant virus containing the gene. The rbosCD14 was purified from culture supernatant using Ni-NTA superflow agarose beads and FPLC system with a typical yield of 4–6 mg/L of culture supernatant. Blood (100 μ L, $n = 7$ cows) was treated with either LPS (*E. coli* 0111:B4, Sigma Chemical Co., final concentrations of 0, 1, 10, or 100 ng/mL), LPS pre-incubated with rbosCD14 (final concentrations of 10 and 100 μ g/mL), or LPS pre-incubated with

BSA (final concentrations of 10 and 100 $\mu\text{g}/\text{mL}$) at 37 °C in 5% CO_2 for 90 min. Blood was then incubated with anti-bovine CD18 monoclonal antibody (MAB, 1 $\mu\text{g}/\text{mL}$ blood) (Dr. Jean-Jacques Letesson, Facultés Universitaires Notre Dame de la Paix, Namur, Belgium) on ice for 30 min. After lysis of red blood cells with lysis buffer (2.6 g Tris/ 100 mL water +7.4 g NH_4Cl /900 mL water), the leukocytes were incubated with FITC-conjugated goat-anti-mouse IgG (H+L) at a final concentration of 1:200. (Kirkegaard & Perry Labs Inc., Gaithersburg, MD, USA) at 4 °C for 30 min. The washed leukocytes were fixed in 2% paraformaldehyde. Binding of anti-bovine CD18 MAB on PMN was analyzed by flow cytometry (Profile, Coulter Electronics, Hialeah, FL, USA).

2.3. Effect of rbovCD14 on abundance of mRNA transcripts for TNF- α , IL-6 and IL-8

Five milliliters of blood ($n = 3$ cows) were incubated with 300 μL of activation medium at 37 °C, 5% CO_2 for 2 h. Activation medium consisted of LPS + rbovCD14 (10 $\mu\text{g}/\text{mL}$), LPS + BSA (endotoxin-free, 10 $\mu\text{g}/\text{mL}$), LPS + rbovCD14 + anti-human CD14 MAB (10 $\mu\text{g}/\text{mL}$), or LPS + polymyxin B sulfate (10 $\mu\text{g}/\text{mL}$). Final concentrations of LPS for each media was 0, 1, 100, and 10000 ng/mL. Activation media were incubated at 37 °C for 1.5 h before combining with blood. After incubation, plasma was collected by centrifugation of blood at $300 \times g$ for 5 min at 4 °C, then stored at -20 °C. The concentration of TNF- α in plasma was determined by radio-immunoassay as described [13]. The RBC were lysed by incubating with 7 mL of lysis buffer on ice for 10 min. Leukocytes were pelleted at $200 \times g$ for 5 min, washed once with ice-cold PBS at $200 \times g$ for 5 min at 4 °C. The cell pellet was lysed in Tri-reagent (Sigma). Total RNA was isolated according to the manufacturers instructions. First-strand cDNA was synthesized using Superscript RT II system

(GIBCO-BRL Life Technologies, Gaithersburg, MD, USA) and oligo(dT) primer.

2.4. Competitors for bovine TNF- α , IL-6, and IL-8

Total RNA and cDNA were derived from leukocytes in whole blood treated with LPS (10 $\mu\text{g}/\text{mL}$). A 456 bp fragment of bovine IL-6 cDNA was PCR amplified using the sense primer IL-6F1 (5'-GGG GCTGCTCCTGGTGAT-3') and the anti-sense primer IL-6R1 (5'-TTTGTGGCTGGAGTGGTTATTAGA-3'), and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). One clone, designated as BoIL-6 native # 8, was picked and confirmed to contain a 456 bp of bovine IL-6 DNA by automated sequencing. A competitor for the BoIL-6 was generated essentially as described [27]. Inverse PCR was performed on the cloned BoIL-6 sequence using the sense and anti-sense primers IL-6F2 (5'-ACTGTCGACAGAACGAGTATGAGGGAAATC-3') and IL-6R2 (5'-GATTGTCGACATTTCTGCCAGTGTCTCC-3'), respectively, where each contained a Sall site proximal to their 5'-end (underlined). The amplified PCR product was purified, digested with Sall, ligated and transformed into *E. coli* DH5 α cells. One clone, designated as BoIL-6M#3, was picked and confirmed to contain a 351 bp of bovine IL-6 DNA by restriction enzyme digestion and PCR amplification using IL-6F1 and IL-6R1. Therefore, BoIL-6M#3 was used as a competitor molecule to study transcriptional changes of bovine IL-6. Competitor molecules for bovine TNF- α , IL-8 and hypoxanthine phosphoribosyltransferase (HPRT) were constructed in a similar manner [27].

2.5. Competitive RT-PCR

The cDNAs' derived from 1 μg of total RNA were normalized to HPRT levels. The normalized cDNA was PCR amplified with a constant amount of each cytokine competitor. Competitive PCR was run by

Table I. Primer sequences used in competitive RT-PCR.

Primer name	Primer sequence
HPRT forward	5'-GGAGATGATCTCTCAACTTTAACTGG-3'
HPRT reverse	5'-CATTATAGTCAAGGGCATATCCCAC-3'
TNF- α forward	5'-CAAGAATTCAGGTCTCTTCTCAAGCCTCAAGTAAC-3'
TNF- α reverse	5'-TTTGGATCCCGGCAGGTTGATCTCAGCACTGAGG-3'
IL-6 forward	5'-GGGGCTGCTCCTGGTGAT-3'
IL-6 reverse	5'-TTTGTGGCTGGAGTGGTTATTAGA-3'
IL-8 forward	5'-GAATTCATGACTTCCAAACTGGCTGTTGC-3'
IL-8 reverse	5'-TCATGGATCTTGCTTCTCAGC TC-3'

co-amplifying cDNA and competitor in a reaction containing 67 mM Tris-HCl (pH 8.8) (Sigma), 16 mM $(\text{NH}_4)_2\text{SO}_4$ (Sigma), 0.8 μM EDTA (Sigma), 0.3% β -mercaptoethanol (Biorad, Hercules, CA, USA), 0.1 mg/mL BSA (Sigma), 1.5 mM MgCl_2 (Sigma), 200 μM dNTP (Invitrogen), 0.25 μM of each primer and 0.625 units of AmpliTaq polymerase [14]. The PCR mixture was cycled 30 times at 94 °C for 40 s, 55 °C for 45 s and 72 °C for 1 min. Primer sequences for HPRT, TNF- α , IL-6 and IL-8 are listed in Table I. The PCR products were separated on a 2% Metaphor:GTG (1.8:0.1 wt/wt) (FMC) agarose gel and stained with ethidium bromide (Sigma). Band intensities were determined by scanning using the UVP gel documentation system (UVP, Inc., Upland, CA, USA). For TNF- α and IL-6, ratio of intensity between cDNA to competitor was determined and normalized to the HPRT content. For IL-8 competitive RT-PCR, each normalized cDNA was diluted and run with 1200 fg of IL-8 competitor in a PCR. A standard curve using a fixed amount of cDNA with various amount of IL-8 competitor was constructed as described [23]. The calculated amount of IL-8 was used for statistical analysis.

2.6. Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA). Data are expressed as the mean \pm SE.

3. RESULTS

3.1. Effect of rbosCD14 on CD18 expression on PMN in whole blood stimulated with LPS

The percentage of PMN fluorescing in both experiments was 100%. However, expression of CD18 on the cell surface of PMN increased when cells were treated with LPS at a concentration of 100 ng/mL (Fig. 1A). This concentration was chosen as the optimal activation concentration in subsequent studies. Pre-incubation of LPS with rbosCD14 at 10 or 100 $\mu\text{g}/\text{mL}$ abolished the increase in CD18 expression induced by LPS alone (Fig. 1B). Pre-incubation of LPS with BSA had no adverse effects on expression of CD18 on the cell surface of PMN. In addition, rbosCD14 or BSA alone at both 10 and 100 $\mu\text{g}/\text{mL}$ did not affect expression of CD18 on PMN.

3.2. Effect of rbosCD14 on abundance of mRNA transcripts for TNF- α , IL-6 and IL-8 in leukocytes in whole blood stimulated with LPS

Whole blood treated with LPS at concentrations of 1, 100 or 10⁴ ng/mL resulted in an increase in levels of mRNA for TNF- α , IL-6, and IL-8 in leukocytes ($P < 0.05$); however, the transcription of

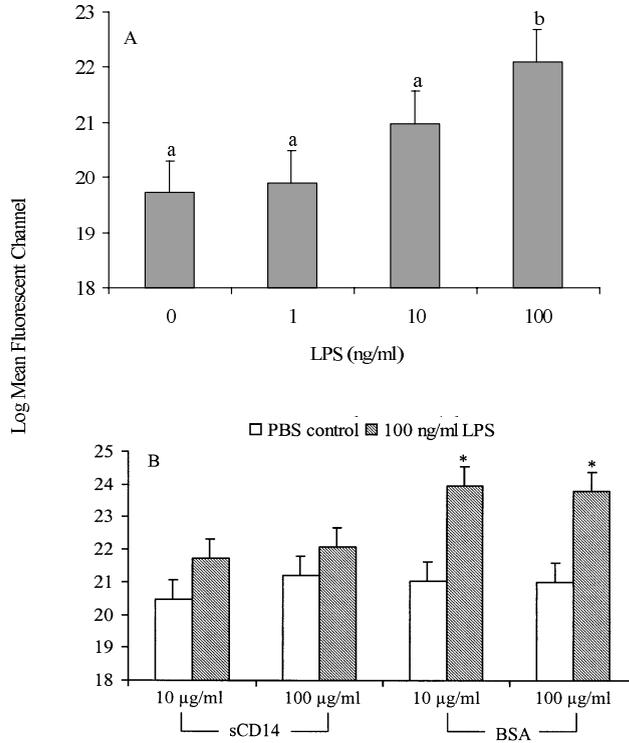


Figure 1. Effect of rbovCD14 on expression of CD18 on the cell surface of PMN in whole blood stimulated with LPS (100 ng/mL). (A) Whole blood (100 µL) was incubated with LPS at 37 °C in 5% CO₂ for 90 min. (B) BSA or rbovCD14 was incubated with PBS (□) or LPS (▣) at 37 °C in 5% CO₂ for 60 min, and incubated with whole blood at 37 °C in 5% CO₂ for 90 min. The binding of anti-bovine CD18 MAB to PMN was determined by flow cytometry. The log mean fluorescence channel was used as a measure of MAB binding. The data from seven cows are expressed as mean and standard error of the mean. ^{a,b} Means with different superscript differ (*P* < 0.05). * Denotes that means within the same concentration of rbovCD14 or BSA differ (*P* < 0.05).

mRNA for each cytokine was not significantly different across all three LPS concentrations (data not shown). Pre-incubation of LPS with rbovCD14 (14.1 µg/mL) or the immune complex of rbovCD14 (14.1 µg/mL) and anti-human CD14 mAb (20 µg/mL) resulted in lower levels of TNF-α (Fig. 2A), but did not affect mRNA levels for IL-6 and IL-8 (Figs. 2B and 2C). Pre-incubation of LPS with BSA (14.1 µg/mL) did not affect the LPS-induced changes in mRNA levels for any of the three cytokines analyzed (Figs. 2A, B and C).

3.3. Effect of rbovCD14 on concentration of TNF-α in plasma from blood treated with LPS

Incubation of blood with LPS at 1, 10² or 10⁴ ng/mL resulted in an increase (*P* < 0.05) in plasma TNF-α by 2.8, 2.2 and 4.3 fold, respectively (Fig. 3). Plasma derived from blood treated with LPS-rbovCD14 complexes, LPS + rbovCD14 + anti-human CD14 MAB, or LPS (1 and 100 ng/mL) + BSA had similar concentrations of TNF-α when compared to plasma from blood

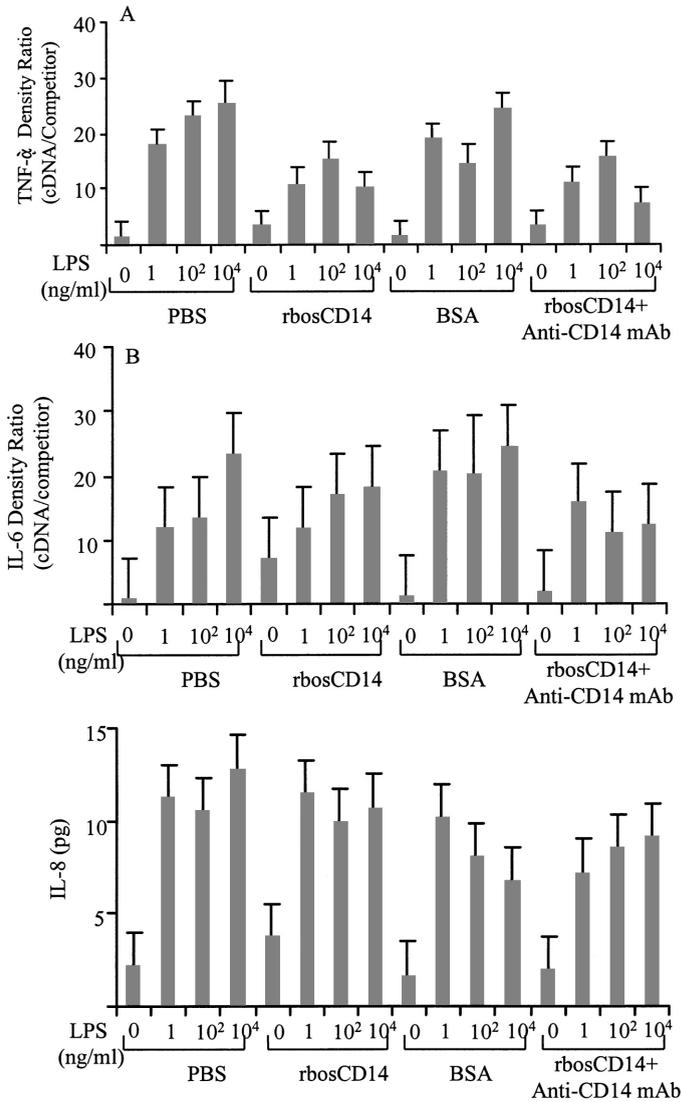


Figure 2. Effect of rbovCD14 on transcription of TNF- α , IL-6 and IL8 in leukocytes treated with LPS ex vivo. LPS was pre-incubated with PBS, rbovCD14, BSA or rbovCD14 + anti-human CD14 MAB at 37 °C, 5% CO₂ for 1.5 h, and then incubated with 5 mL of blood for 2 h. (A) Density ratio for TNF- α between target cDNA and competitor. (B) Density ratio for IL-6 between target cDNA and competitor. (C) Density ratio for IL-8 between target cDNA and competitor. Each sample was PCR amplified in triplicate. (B) The data from three cows are expressed as mean and standard error of the mean.

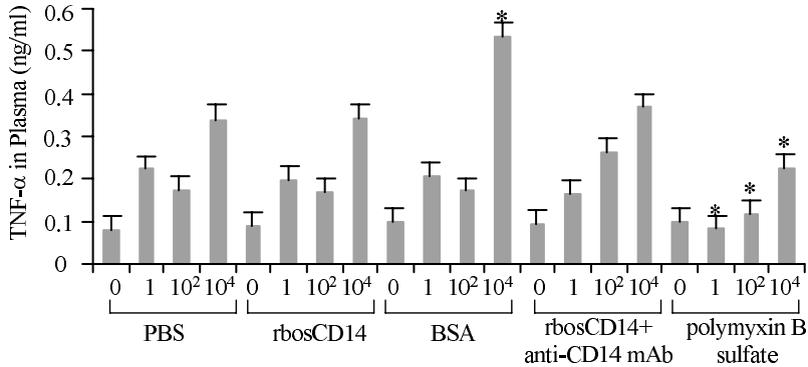


Figure 3. Effect of rbosCD14 on concentration of TNF- α in plasma from blood treated with LPS. LPS was pre-incubated with PBS, rbosCD14, BSA, rbosCD14 + anti-CD14 mAb or polymyxin B sulfate at 37 °C, 5% CO₂ for 1 h, and then incubated with 5 mL of blood for 2 h. After incubation, blood was centrifuged at 1000 \times g for 5 min at 4 °C. Plasma was collected and stored at -20 °C until analysis. The data from three cows are expressed as mean and standard error of the mean. * Significantly different from same concentration of LPS in PBS ($P < 0.05$).

treated with LPS alone. Pre-incubation with the highest concentration of LPS (10⁴ ng/mL) and BSA caused a 5.4 fold significant increase in the concentration of TNF- α in plasma when compared to LPS (10⁴ ng/mL) alone ($P < 0.05$). Pre-incubation of LPS with polymyxin B sulfate inhibited the LPS-induced increase of plasma TNF- α level ($P < 0.05$). Incubation of blood with rbosCD14, BSA, rbosCD14 + anti-human CD14 MAB or polymyxin B sulfate had no affect on TNF- α levels.

4. DISCUSSION

We chose CD18 expression on the cell surface of PMN as an LPS-sensitive parameter to evaluate effects of rbosCD14 on response of leukocytes to LPS. A whole blood assay was used to more closely mimic septic shock in vivo. Results indicate that rbosCD14 generated by the baculovirus/insect cell expression system was functionally active and capable of inhibiting the activation of PMN in whole blood stimulated by LPS.

Changes in mRNA levels for inflammatory cytokines in leukocytes treated with

LPS were also used to evaluate the functional activity of rbosCD14. Results indicate that the LPS-induced increase in TNF- α mRNA levels in leukocytes was CD14-dependent. These results were verified when addition of anti-human CD14 MAB (20 μ g/mL) prior to adding LPS caused the TNF- α mRNA levels to remain unchanged relative to controls.

Our data also show that leukocytes treated with either LPS-rbosCD14 or LPS-rbosCD14 + anti-CD14 MAB have similar levels of mRNA for IL-6 and IL-8 when compared to leukocytes treated with LPS alone. This suggests that the LPS-induced increase in mRNA levels of IL-8 and IL-6 were CD14-independent. This CD14-independency may be due to two properties of these cytokines. First, the cytokines interact with each other, where TNF- α is able to induce the production of IL-6, IL-8 and IL-1 [6]. It was reported that TNF- α is secreted slightly before IL-1 β and IL-8 in milk whey in experimentally induced *E. coli* mastitis [20, 21]. Second, cytokines function at minute concentrations. Biological activity of TNF- α can be observed at 0.01 ng/mL [20]. Tumor necrosis factor- α in plasma from blood treated with LPS

(0.17 to 0.34 ng/mL) was able to induce maximal transcription of IL-6 and IL-8. Therefore, increase in level of IL-6 and IL-8 mRNA in leukocytes treated with LPS is at least partially induced by TNF- α .

In this study, variation among cows was observed in CD18 expression on PMN after exposure to LPS; therefore, we could not evaluate effects of rboCD14 on CD18 expression of PMN from all seven cows. Cow to cow variation as well as day to day variation within cow has been reported [1], where the sensitivity of animals to LPS is genetically determined. In mice, strains that were either hypersensitive or hyposensitive to LPS have been extensively studied [24]. The hyposensitive strain has a point mutation in the *tlr4* gene, which encodes the Toll like receptor (TLR) 4 that mediates activation effect of LPS on host cells. In addition, sensitivity to LPS is also affected by experimental conditions. It is known that mice treated with hepatotoxic agents, growing malignant tumors, or infected with Gram-negative or Gram-positive bacteria will have increased sensitivity to LPS [7]. Endotoxin tolerance (hyposensitive) can also be induced by prior exposure to LPS [7]. Thus, without further data, we cannot conclude whether either genetic components or environmental conditions are the primary contributing factor to the variation observed in this study.

In conclusion, functionally active rboCD14 was generated in a baculovirus/insect cell expression system. The inhibitory effect of rboCD14 on activation of PMN and the increase in level of TNF- α mRNA by LPS may be beneficial to animals suffering acute endotoxin shock. Further characterization of rboCD14 will facilitate understanding of bovine CD14-mediated host response to LPS. The results suggest that rboCD14 may have potential as a therapeutic for relief of toxic shock resultant from inflammatory diseases caused by Gram-negative bacterial infections.

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