Vet. Res. 34 (2003) 307–316
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DOI: 10.1051/vetres:2003006

Original article

Recombinant bovine soluble CD14 reduces severity of experimental *Escherichia coli* mastitis in mice

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(Received 15 October 2002; accepted 9 December 2002)

Abstract – Endotoxin, or lipopolysaccharide (LPS), is responsible for pathogenesis of infections induced by Gram-negative bacteria, such as *E. coli*. The cellular response to LPS is modulated by interactions among LPS, LPS-binding protein (LBP) and CD14. Accumulated evidence shows that the soluble form of CD14 (sCD14) competes with membrane-bound CD14 (mCD14) for LPS and plays a pivotal role in regulating bacterial infection and septic shock caused by Gram-negative bacteria. Recombinant bovine sCD14 (rbosCD14) was produced by transfected insect sf/9 cells and its biological function was evaluated in mice. Eighty-one 8-week old BALB/cj female mice were randomly assigned to two groups, and injected intraperitoneally with either LPS (8 μg/g of body weight, $n = 41$) or LPS plus rbosCD14 (6.8 μg/g of body weight, $n = 40$). Survival rate at 24 h after injection for mice injected with either LPS or LPS plus rbosCD14 was 30 and 72%, respectively ($P < 0.01$). At 48 h survival rate was 7 and 37%, respectively ($P < 0.01$). To investigate the protective effect of rbosCD14 on experimentally induced mastitis in mice, two abdominal contralateral mammary glands of 7 lactating BALB/cj mice were injected through the teat canal with 10–20 colony-forming units (CFU) of *Escherichia coli.* One gland simultaneously received rbosCD14 (6 μg) and the other saline. At 24 h after challenge, glands that received rbosCD14 had less swelling and hemorrhaging, significantly lower bacterial counts ($P < 0.05$) and lower concentrations of TNF-α ($P < 0.05$). Results indicate that rbosCD14 is biologically functional and reduces mortality in mice from endotoxin shock and severity of intramammary infection by *E. coli*.

mastitis / CD14 / LPS / *Escherichia coli* / TNF-α

1. INTRODUCTION

Exposure to Gram-negative bacteria leads to infection, and in severe cases life-threatening "septic shock". Recognition of LPS, a cell wall component of Gram-negative bacteria, by the innate immune system is critical for initiation of inflammatory responses. The cellular receptor for LPS is CD14, a 53–55 kDa glycosylated phosphatidylinositol-anchored protein expressed on monocytes/macrophages and neutrophils [13, 31, 43]. In addition, a soluble form of CD14 (sCD14) has been found in normal

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serum, urine, and milk [3, 10, 21, 41]. Binding of LPS to mCD14 is upregulated by LPS-binding protein (LBP), an acute phase protein released by the liver during inflammation [12]. In response to LPS/LBP complexes, monocytes/macrophages release a spectrum of cytokines, including TNF-α, IL-1, IL-6 and IL-8, that initiate host defense response [26]. However, overwhelming production of TNF-α is the main causation of multiple organ failure and death as seen in "septic shock" [40].

The biological functions of sCD14 have been extensively studied. Recombinant human (rh) sCD14 was able to inhibit LPS-induced TNF-α production by monocytes in whole blood [14]. Mice intraperitoneally challenged with LPS plus rhsCD14 had decreased fatality and blood TNF-α level compared to mice that received LPS alone [15, 39]. Moreover, LBP/sCD14 complexes bind LPS and transport it to high-density lipoprotein (HDL) [44]. This process leads to detoxification of LPS in plasma. Presumably, sCD14 competes with mCD14 to interact with LPS and prevents activation of CD14-expressing immune cells. On the other hand, acquisition of LPS is required for sCD14 to activate epithelial cells as characterized by production of IL-8 [33], a potent chemoattractant for neutrophils. Neutrophils form the first line of cellular defense in combating bacterial infections.

Soluble CD14 plays a crucial role in the pathogenesis of Gram-negative bacteria. *E. coli* is a common mastitis pathogen in dairy cows and causes a large economic loss to the dairy industry. There is a general agreement that LPS is the key molecule involved in pathogenesis of mastitis induced by Gram-negative bacteria [5]. LPS is widely used to simulate *E. coli* infection in studying bovine mastitis [30, 36]. The concentration of sCD14 in milk increases following intramammary injection of LPS [23]. Recombinant bovine sCD14 (rbosCD14), previously cloned and expressed in our laboratory, sensitized bovine mammary glands to LPS and resulted in recruitment of neutrophils [41]. However, the role of sCD14 in the pathogenesis of *E. coli* mastitis has not been elucidated.

In the present study, mice were injected intraperitoneally with rbosCD14 and LPS to determine if rbosCD14 was biologically functional in mice. Further, a mouse mastitis model, initially introduced by Chandler in 1970 [7], was modified and used for studying experimentally induced *E. coli* mastitis. A mouse mammary gland has only one streak canal and is functionally and anatomically independent from the others, which is similar to the bovine mammary gland. Two abdominal mammary glands of each lactating mouse were challenged with either *E. coli* or with *E. coli* together with rbosCD14. The effect of rbosCD14 on *E. coli* mastitis was evaluated by comparing clinical signs, bacterial counts and TNF-α concentrations in the mammary gland between the two treatments. Results demonstrate that the technique of inducing intramammary infection in lactating mice is a feasible model for studying bovine mastitis. Moreover, rbosCD14 was able to reduce severity of experimentally induced *E. coli* mastitis.

2. MATERIALS AND METHODS

2.1. Peritoneal challenge with LPS

Eighty-one 8-week-old female BALB/cj mice (17–22 g) (Jackson Laboratory, Bar Harbor, ME, USA) were injected intraperitoneally with LPS (8 μg/g of body weight) (L-1887, prepared from *Salmonella abortus equi* and chromatographically purified by gel filtration (Sigma Chemical Co., St. Louis, MO, USA)). Mice were randomly assigned to either phosphate buffered saline (PBS) group (n = 41) or rbosCD14 (6.8 μg/g of body weight) group (n = 40). The rbosCD14 was produced in a baculovirus expression system as described [41]. Briefly, rbosCD14, with a deletion of 15 amino acids at the C-terminal end, was

generated by insect sf/9 cells infected with recombinant virus containing the gene. Both LPS and rbosCD14 were diluted in non-pyrogenic saline to desired concentrations. Survival rate was recorded every 12 h for 3 days.

2.2. Intramammary challenge with *E. coli*

Seven lactating BALB/cj mice, 5 to 10 days after parturition, were used for the bacterial challenge. Pups were removed from mothers for 4 h and allowed to suck for 30 min before experimental challenge of two abdominal mammary glands (L4 and R4). The 30 min of nursing was performed to elongate the teats that facilitated insertion of the micropipette into the teat canal. Control and treated glands received either *E. coli* or *E. coli* plus rbosCD14.

Glass Drummund Precision disposable micropipettes (5 μL, Fisher, Hampton, NH, USA) were heated by a Bunsen burner and pulled horizontally to reduce the diameter to less than 75 μm. The micropipettes were broken by a glass-cutter followed by fire polishing in a microforge. Thereafter, micropipettes were washed with 70% ethanol and air-dried to prevent contamination.

The organism used was *E. coli* strain P4 (serotype O32:H37), which originally had been isolated from a clinical case of bovine mastitis [4], and had been used in studies of *E. coli* mastitis in cows [24] and mice [1]. Before challenge exposure, a tube of brain-heart infusion broth (Baltimore Biological laboratories, Cockeysville, MD, USA) was inoculated with frozen *E. coli* and incubated for 18 h at 37 °C. The resulting broth culture was streaked onto a Trypticase soy blood agar plate to determine its purity. After incubation, a single colony was transferred into 10 mL of non-pyrogenic Trypticase soy broth (TSB) (Difco, Detroit, MI, USA) and incubated for 18 h at 37 °C. After incubation, bacteria were centrifuged at 2500 × *g*, 4 °C, for 10 min followed by 3 washes with non-pyrogenic PBS. The pellet was resuspended in non-pyrogenic PBS, and the suspension was diluted to a transparency of 80% at 610 nm. The concentration of bacteria approximated 10^8 CFU/mL. Serial dilutions were made in non-pyrogenic PBS to approximately 400 CFU/mL and kept on ice until the injection. The actual number of CFU injected (between 10 to 20 CFU) was confirmed by spreading 50 μL of the inoculum onto a blood agar plate and counting number of CFU after overnight incubation at 37 °C.

Intramammary injection of mice was carried out as described [29]. Briefly, a micropipette was fitted to a microdispenser held by a micromanipulator and filled with 10 to 20 CFU of *E. coli* in 50 μL of non-pyrogenic PBS (1% India ink) with or without 6 μg rbosCD14. The addition of India ink was used to confirm success of injection after dissection, and has been shown to have no effect on bacterial growth [19]. At 24 h post injection, mice were anesthetized, the abdominal surface disinfected with 70% ethyl alcohol, and positioned on the platform of the dissecting microscope. The tip of the micropipette was carefully inserted into the teat canal, and the solution was slowly injected into the mammary gland with a Hamilton syringe attached to the microdispenser. When injection of both abdominal glands was completed, mice were disinfected again and allowed to recover on a warmed (37 °C) plate. At 24 h after injection, mice were sacrificed by cervical dislocation. The skin over the abdomen was cut with scissors to expose the glands, for confirming success of injection and observing clinical symptoms.

Mammary glands were removed, weighed and homogenized in non-pyrogenic PBS (1 mL PBS/100 mg of tissue). Pour plates were prepared for determining number of CFU by mixing 40 μL of various dilutions of the sample with 13 to 15 mL pre-warmed MacConkey agar (Difco) and

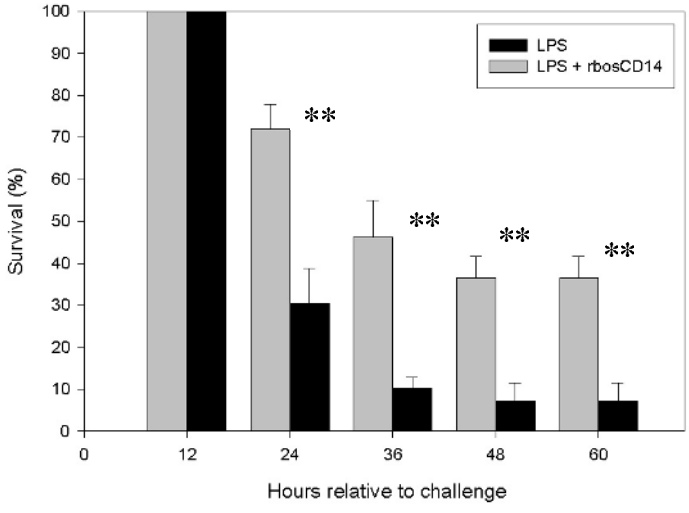

Figure 1. Effect of rbosCD14 on survival of mice injected intraperitoneally with LPS (8 μg/g of body weight, $n = 41$) or LPS plus rbosCD14 (6.8 μg/g of body weight, $n = 40$). Results are presented as the mean and standard error of the mean from three experiments. * * ($P < 0.01$).

incubated for 18 h at 37 °C. A small portion of the sample was plated on a 5% blood agar plate for observation of colony morphology. One colony was removed and Gram-stained. The remaining sample was centrifuged at 15 000 × *g*, for 30 min at 4 °C, and the supernatant collected for measuring concentration of TNF-α using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA).

2.3. Statistical analysis

Analysis of survival rate of mice after LPS challenge was made using GENMOD of SAS [34]. Comparisons of bacterial counts and TNF-α concentrations after intramammary infection with *E. coli* was made using PROC MIXED [34].

3. RESULTS

3.1. The effect of rbosCD14 in preventing endotoxin shock

The concentration of LPS (8 μg/g of body weight) used in the present study induced severe endotoxin shock in mice. The response to LPS was rapid. Most of the mice became lethargic 30 min after challenge and developed "rough coats" within 2 to 3 h. All deaths occurred between 12 to 48 h postinjection. At the end of the study period, fatality was reduced in rbosCD14 treated mice ($P < 0.01$). Survival rates were 7.0 ± 4.5% and 36.5 ± 8.8% for LPS and LPS plus rbosCD14 injected mice, respectively (Fig. 1). Furthermore, administration of rbosCD14 delayed outbreak of endotoxin shock induced deaths. Seventy percent of the mice in the control group died between 12 and 24 h after challenge, whereas only 30% of rbosCD14-treated mice died during the same period ($P < 0.01$).

3.2. Intramammary challenge of mice with *E. coli*

No deaths were observed after intramammary challenge exposure. However, all mice developed slight "rough coats" and became inactive 24 h after challenge. There was more visible swelling of

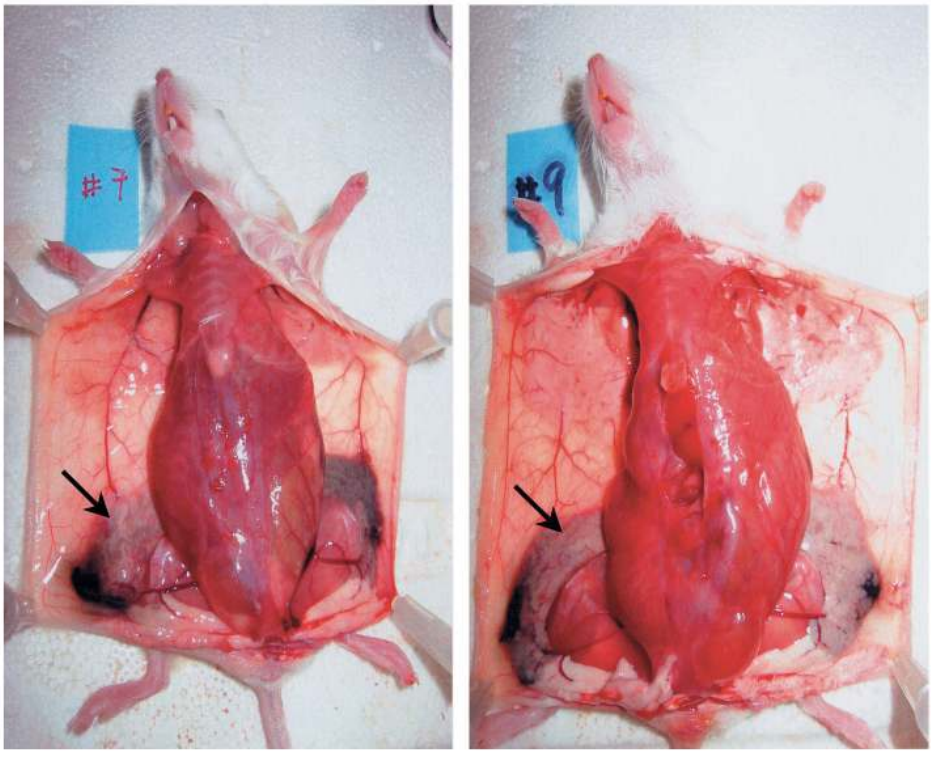

Figure 2. Appearance of abdominal mammary glands 24 h after experimental challenge with *Escherichia coli* (10 to 20 CFU). The right abdominal glands (arrow) exhibited more severe clinical signs that included bloody exudation and dilated vessels compared to glands that received rbosCD14 (6 μg). Pictures were randomly selected from pictures of seven challenged mice.

mammary glands for mice challenged with *E. coli* compared to glands challenged with *E. coli* plus rbosCD14. After dissection, control mammary glands showed more severe clinical signs, such as bloody exudation and dilated vessels (Fig. 2). However, average weight of control glands was similar to rbosCD14 treated glands (data not shown). Bacteriological diagnosis indicated presence of *E. coli* in all of the challenged glands. The resulting colonies on blood agar plates were confirmed to be *E. coli* by colony morphology and Gram staining. The rbosCD14 reduced number of CFU by 65%. Number of CFU for the control and rbosCD14 treated glands averaged $14.1 \pm 5.8 \times 10^7$ and $4.96 \pm 2.6 \times 10^7$ ($P < 0.05$) (Fig. 3). Uninjected inguinal mammary glands remained sterile, indicating that no crossover contamination from infected glands occurred in vivo or during dissection of the glands.

There was no detectable TNF-α in non-challenged inguinal mammary glands. Concentrations of TNF-α averaged 262 ± 43.5 and 164.7 ± 23.6 pg/mL in glands challenged with *E. coli* and *E. coli* plus rbosCD14, respectively ($P < 0.05$) (Fig. 4).

4. DISCUSSION

Mastitis is a major infectious disease of dairy cows and accounts for an annual

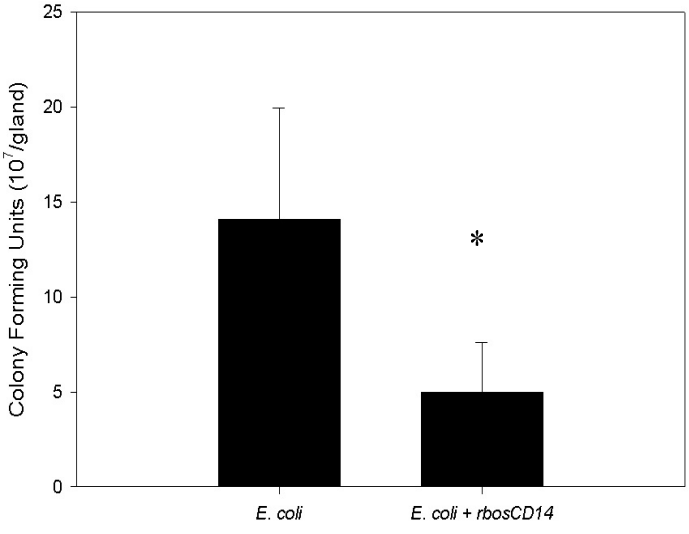

Figure 3. Effect of rbosCD14 on growth of *Escherichia coli* 24 h after experimental infection of two abdominal mammary glands with either *E. coli* (10 to 20 CFU) or *E. coli* plus rbosCD14 (6 µg). Results are presented as the mean and standard error of the mean of 7 animals. * ($P < 0.05$).

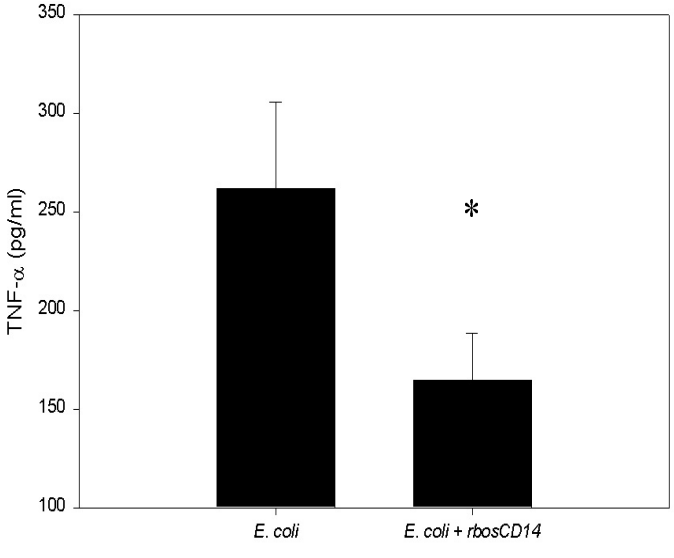

Figure 4. Concentration of TNF-α in mouse mammary glands homogenized in sterile PBS (1 mL PBS/100 mg of tissue) 24 h after experimental infection of two abdominal mammary glands with either *E. coli* (10 to 20 CFU) or *E. coli* plus rbosCD14 (6 µg). Results are presented as the mean and standard error of the mean of 7 animals. * ($P < 0.05$).

$1.8 billion economic loss to the dairy industry in the United States [28]. Coliform bacteria are ubiquitous to the environment of dairy cows, making control of intramammary infection by these organisms very difficult. Infection caused by Gram-negative bacteria may result in septic shock and is associated with a high mortality rate in severe cases [11]. It is estimated that 80 to 90% of all coliform cases of mastitis become clinical, and that 10% of these cases result in death [38]. We were therefore interested in knowing if our rbosCD14 could be used to reduce endotoxin shock and mastitis caused by *E. coli*. Because of the cost involved in using dairy cows for mastitis research, a mouse model was developed [7]. Advantages of using a mouse model are reduced management and high cost associated with dairy cattle, minimized quantity of reagents needed, in our case rbosCD14, and similarity between cows and mice in anatomical structure of the mammary gland, such as a single teat duct. Mouse mastitis had been induced by either direct injection of bacteria through the skin and into the gland, or by exposure of teats that had their teat-ends removed to a suspension of bacteria [2, 7]. In order to avoid damage to mammary tissue and teats, we chose to use an ultra-fine (< 75 μm in diameter) micropipette, fitted to a micromanipulator. This allowed for easy penetration into the teat duct without noticeable damage to the gland or teat. The success of introducing bacteria into the gland was determined by dispersion of India ink throughout the gland and recovery of bacteria. Only successfully challenged mice were used in this study.

Since the discovery that CD14 was a cellular receptor for LPS, numerous studies have been carried out on the role of CD14 in septic shock. Recombinant human sCD14 (rhsCD14) has been shown to reduce mortality in mice challenged with LPS, attributed to reduced production of circulating TNF-α [15, 39]. Before adopting the mouse as a mastitis model for our CD14 research, our first objective was to determine if rbosCD14, which is 61–73% homologous to mouse, rabbit and human sCD14 [17], was biologically functional in mice. Similar to results obtained with rhCD14, our results also showed that rbosCD14 simultaneously injected with LPS increased survival rate of LPS-challenged mice. In studies with rhsCD14 higher survival rates (68–100%) were observed [15, 39] compared to our study (37% at 48 h post injection). It is possible that the BALB/cj mice used in our study were more sensitive to LPS challenge than the C57BL/6j strain used in the study with rhsCD14 [39]. Also, differences due to age of the mice, source of LPS [45] and concentration of sCD14 probably contributed to differences among the studies in mortality rates.

A second objective of the present study was to determine if rbosCD14 reduced severity of *E. coli* mastitis in mice. Unlike intraperitoneal injection with LPS, intramammary *E. coli* challenge does not usually cause deaths in mice. This is probably due to the fact that a large portion of LPS is detoxified locally in the mammary gland and does not get into the circulation [8]. However, we were able to demonstrate that simultaneous administration of rbosCD14 (6 μg) with an inoculum of *E. coli* reduced inflammatory symptoms of the mammary gland, and reduced both the concentration of TNF-α and number of CFU in the mammary gland by 37.2 and 64%. Our results are in contrast to those from an earlier study where presence of excess mouse sCD14 increased growth of *Streptococcus pneumoniae* and concentration of TNF-α in cerebrospinal fluid (CSF) of challenged mice [6]. Reasons for increased bacterial growth and elevated concentration of TNF-α in that study were not determined. However, the authors speculated that direct or indirect modulation of sCD14 on bacterial growth and interference of excess sCD14 in the ability of phagocytic cells to recognize

and phagocytose bacteria contributed to elevated concentration of TNF-α and increased bacterial growth. In our study, addition of rbosCD14 into milk inoculated with *E. coli* did not alter the rate of growth (data not shown). Therefore, other cellular responses induced by rbosCD14 may have contributed to reduction in bacterial counts. Both mCD14 and sCD14 bind to bacteria [9, 18], and together with LBP increase phagocytosis of serum-opsonized *E. coli* [35]. However, LBP-mediated phagocytosis of *E. coli* was only slightly decreased after removal of mCD14 from the surface of rat alveolar macrophages [20]. Further, binding and uptake of *E. coli* was not decreased by macrophages isolated from CD14-deficient mice [27]. It would appear that binding of mCD14 to bacteria plays a minimal role in promoting phagocytosis.

Our finding of reduced mammary inflammation and reduced number of CFU in mice injected with rbosCD14 is partially supported by reports that CD14-deficient mice intraperitoneally challenged with *E. coli* [16] or *Bacteroides fragilis* [42] reduced dispersion of bacteria throughout the peritoneum. The authors proposed that monocytes in CD14-deficient mice were not activated by bacteria due to lack of mCD14, and this in turn reduced production of inflammatory cytokines, such as TNF-α and IL-1, which accelerate growth of bacteria [25, 32]. The authors postulated that reagents capable of blocking or neutralizing mCD14 on monocytes might also inhibit growth of Gram-negative bacteria. Similarly, our results demonstrated that intramammary administration of rbosCD14 reduced TNF-α production by phagocytes in milk by possibly competing with mCD14 for LPS.

The reduction in number of CFU of *E. coli* in mammary glands injected with rbosCD14 was more likely the result of early infiltration of neutrophils. It is known that sCD14/LPS complexes activate epithelial cells by binding to Toll-like receptor 4 on epithelial cells, causing them to secrete IL-8, a potent chemoattractant of bovine neutrophils [22, 33, 41]. Activation of epithelial cells by sCD14/LPS complexes and recruitment of leukocytes was verified in a recent study in our laboratory [41]. In that study, a concentration of LPS was used that did not increase milk somatic cells when injected into mammary glands of lactating cows. When the same concentration of LPS was injected together with rbosCD14, identical to the rbosCD14 used in the present study, concentration of milk somatic cells increased to 500 000 cells/mL of milk. Intramammary injection of the same concentration of rbosCD14 did not induce an increase in milk somatic cells. Early recruitment of neutrophils is crucial to clearance of bacteria from the mammary gland and the outcome of intramammary infection [37]. In a previous study, the leukocyte count in CSF of *S. pneumoniae* challenged mice did not increase after administration of rhsCD14 [6], and clearance of bacteria may have been attributed to up-regulation of bactericidal functions of neutrophils. Further investigation will be required to verify whether rbosCD14 can enhance phagocytic and bactericidal function of neutrophils in milk.

In conclusion, rbosCD14 protected mice from endotoxin shock. Intramammary administration of rbosCD14 reduced deleterious inflammatory responses, TNF-α production, and bacterial growth in a mouse mastitis model. Results indicate that rbosCD14 could be a potential therapeutic agent to minimize the impact of bovine mastitis caused by Gram-negative bacteria.

ACKNOWLEDGMENTS

This study was partially supported by a grant from Natural Science and Engineering Research Council of Canada to X. Zhao. Authors express sincere appreciation to Dr. Robert J. Wall (USDA, Beltsville, MD, USA) for his generous assistance.

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