APPLICATION OF SOLUBLE CD14 AND A TRIVALENT VACCINE TO PREVENT MASTITIS CAUSED BY *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

By Jai-Wei Lee

Department of Animal Science

McGill University, Montreal

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ABSTRACT

Escherichia coli (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are the most prevalent pathogens to induce mastitis. The pathogenesis of infections induced by *E. coli* is sophisticatedly modulated by lipopolysaccharide (LPS), LPS binding protein, membrane CD14 (mCD14), and soluble CD14 (sCD14). In the first study, administration of recombinant bovine sCD14 (rbosCD14) significantly reduced the fatality of LPS challenged mice and the severity of mouse mastitis in terms of clinical signs, bacterial load, and TNF-α production. Before investigating the potential of this strategy in dairy cows, endogenous sCD14 in milk was characterized. Based on the data of 396 quarters, the milk concentration of sCD14 was 6.67 ± 0.44 μg/ml. The stages of lactation affected the concentration of sCD14 in milk, which was higher in transitional milk (0-4 days postpartum). Milk sCD14 also increased during an intramammary LPS challenge, which paralleled with SCC increase. The protective effect of sCD14 on bovine *E. coli* mastitis was then investigated. It was shown that rbosCD14 sensitized the mammary gland to recruit leukocytes in response to LPS. To prove that the early recruitment of leukocytes plays a role in preventing intramammary *E. coli* infections, *E. coli* mastitis was induced in 9 dairy cows with or without 100 μg rbosCD14. Quarters challenged with *E. coli* plus rbosCD14 had a more rapid recruitment of neutrophils, a faster clearance of bacteria, reduced concentrations of TNF-α and IL-8 in milk, and reduced clinical symptoms than quarters injected with saline.

For *S. aureus* mastitis, a newly designed trivalent whole-cell vaccine being composed of the most dominant serotypes (T5, T8, and T336) was evaluated. The vaccine was immunized with or without either one of the two adjuvants, aluminum hydroxide

(ALUM) and Freund's incomplete adjuvant (FICA). The vaccine, with or without the presence of adjuvants, increased antigen-specific IgG_1, IgG_2, but not IgM, in serum. However, all formulations only had limited effects on lymphocyte subsets, interferon (IFN)-γ mRNA expression, and neutrophil phagocytosis in comparison with the control.

Taken together, the results indicated that increasing the concentration of sCD14 in milk might be a potential strategy to prevent or reduce severity of *E. coli* mastitis. On the other hand, both ALUM and FICA did not augment the immune responses when formulated with trivalent vaccine. A more immunostimulatory adjuvant will be required to improve the efficacy of the novel trivalent vaccine against *S. aureus* mastitis.

RÉSUMÉ

Escherichia coli (*E. coli*) et *Staphylococcus aureus* (*S. aureus*) sont les pathogènes qui induisent une mammite le plus fréquemment. La pathogénie des infections induites par *E. coli* est judicieusement modulée par le lipopolysaccharide (LPS), la protèine liant les LPS, le CD14 membranaire (mCD14) et le CD14 soluble (sCD14). Dans la première étude, l'injection de sCD14 recombinant bovin (rbosCD14) a significativement réduit la mort chez les souris injectées avec des LPS et l'intensité de la mammite chez la souris du point de vue des signes cliniques, de la charge bactérienne, et de la production de TNF-α. Avant d'étudier le potentiel de cette stratégie chez les vaches laitières, le sCD14 endogène dans le lait fut caractérisé. Selon les données de 396 quartiers, la concentration de sCD14 dans le lait était de 6.67 ± 0.44 μg/ml. Le stade de lactation influençait la concentration de sCD14 dans le lait. La concentration de sCD14 était plus élevée dans le lait transitionnel (0-4 jours postpartum). Le sCD14 du lait augmentait également lors d'une injection intramammaire de LPS, qui coïncidait avec l'augmentation du SCC. L'effet protecteur de sCD14 sur la mammite bovine causée par *E. coli* fut alors étudié. Il fut démontré que le rbosCD14 incitait la glande mammaire à recruter des leucocytes en réponse aux LPS. Pour savoir si le recrutement précose des leucocytes joue un rôle dans la prévention des infections intramammaires causées par *E. coli*, une mammite fut induite par *E. coli* chez 9 vaches laitières avec ou sans 100 μg de rbosCD14. Les quartiers infectés par *E. coli* et avec le rbosCD14 connurent un recrutement de neutrophiles plus rapide, une évacuation de bactéries plus rapide, des concentrations réduites de TNF-α et IL-8 dans le lait et des symptômes cliniques plus faibles que les quartiers injectés avec de la saline.

Pour les mammites causées par *S. aureus,* un vaccin trivalent nouvellement créé et composé des sérotypes les plus dominants (T5, T8 et T336) fut testé. Le vaccin fut administré avec ou sans l'un des deux adjuvants suivant : l'hydroxide d'aluminium (ALUM) ou l'adjuvant de Freud incomplet (FICA). Le vaccin, avec ou sans la présence d'adjuvant, a augmenté dans le sérum les IgG_1, IgG_2 qui sont des antigènes-spécifiques, mais pas les IgM. Cependant, toutes les formules ont eu des effets restreints sur les sous-ensembles de lymphocytes, sur l'expression de l'ARNm de l'interféron (IFN)-γ, et sur la phagocytose par les neutrophiles comparativement au contrôle.

En somme, les résultats indiquent que l'augmentation de la concentration de sCD14 dans le lait semble une bonne stratégie pour prévenir ou réduire la sévérité des mammites causées par *E. coli.* Cependant, ALUM et FICA n'ont pu augmenter les réponses immunitaires lorsqu'utilisés dans la formule du vaccin trivalent. Un adjuvant plus immuno-stimulateur sera necéssaire pour améliorer l'efficacité du nouveau vaccin trivalent contre les mammites causées par *S. aureus*.

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CONTRIBUTIONS TO KNOWLEDGE

1. Chapter II: This is the first study to show that rbosCD14 was biologically active in mice and reduced the fatality of LPS-induced septic shock. The effect of rbosCD14 on intramammary *E. coli* infections was investigated in a mouse model as well.

2. Chapter III: For the first time, the endogenous sCD14 in bovine milk was quantified and factors affecting the level of milk sCD14 were studied. The dynamics and possible sources of sCD14 in milk were investigated during an intramammary challenge with LPS.

3. Chapter IV: The protective effect of sCD14 against bovine *E. coli* mastitis was demonstrated for the first time. Results indicated that the reduced severity and bacterial load were attributed to an early recruitment of leukocytes.

4. Chapter V: This is the first study to evaluate the novel trivalent vaccine designed for *S. aureus* mastitis. Immunization with the trivalent vaccine, increased antibody production in serum, but not persistently altered lymphocyte subpopulations, neutrophil phagocytosis, and IFN-γ production. Moreover, the two tested adjuvants did not significantly augment the immunostimulatory effects when formulated with the trivalent vaccine.

FORMAT OF THESIS

This thesis has been written in the format of manuscripts submitted to scientific journals in accordance with the **"Guidelines for Thesis Preparation, C: Manuscript-based Thesis"**, which states,

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. *a table of contents;*
2. *a brief abstract in both English and French;*
3. *an introduction which clearly states the rational and objectives of the research;*
4. *a comprehensive review of the literature (in addition to that covered in the introduction to each paper);*

5. *a final conclusion and summary;*

6. *a thorough bibliography;*

7. *Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.*

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

CONTRIBUTIONS OF AUTHORS

Four co-authored manuscripts submitted for publication are included in the thesis.

Authors of Manuscript 1 (Chapter II): J.-W. Lee, M. J. Paape, and X. Zhao.

All experiments and data analysis were carried out by J.-W. Lee. Dr. Paape and Zhao assisted J.-W. Lee in the experimental design. The manuscript was written by J.-W. Lee, and revised by both co-authors.

Authors of Manuscript 2 (Chapter III): J.-W. Lee, M. J. Paape, T. H. Elsasser, and X. Zhao.

Determination of TNF-α concentrations in milk samples was performed by Dr. Elsasser. All other experiments and data analysis were performed by J.-W. Lee. Dr. Paape and Zhao assisted J.-W. Lee in the experimental design. The manuscript was written by J.-W. Lee, and revised by all co-authors.

Authors of Manuscript 3 (Chapter IV): J.-W. Lee, M. J. Paape, T. H. Elsasser, and X. Zhao.

Determination of TNF-α concentrations in milk samples was performed by Dr. Elsasser. All other experiments and data analysis were performed by J.-W. Lee. Dr. Paape and Zhao assisted J.-W. Lee in the experimental design. The manuscript was written by J.-W. Lee, and revised by all co-authors.

Authors of Manuscript 4 (Chapter V): J.-W. Lee, C. N. O'Brien, A. J. Guidry, K. A. Shafer-Weaver, and X. Zhao.

The experiment was designed by Dr. Guidry and Ms. Shafer-Weaver. Titration of antibodies was conducted by Dr. O'Brien. The phagocytosis assay was performed by J.-W. Lee and Dr. O'Brien. All the other experiments and data analysis were carried out by

J.-W. Lee, Ms. Shafer-Weaver, and Dr. Zhao. The manuscript was written by J.-W. Lee, and reviewed by all co-authors.

TABEL OF CONTENT

LIST OF ABREVIATION

ALUM..... aluminum hydroxide
ANOVA..... analysis of variance
APC..... antigen presenting cell
BAL..... bronchoalveolar lavage
BSA..... bovine serum albumin
CFU..... colony forming unit
CD..... cluster of differentiation
Clf..... clumping factor
CLT..... cytotoxic T lymphocyte
CP..... capsular polysaccharide
CR..... complement receptor
CSF..... cerebrospinal fluid
DEPC..... diethylpyrocarbonate
DIM..... day in milk
DXS..... dextran sulfate
Eap..... extracellular adherence protein
ECM..... extracellular matrix
Efb..... fibrinogen binding protein
ELISA..... enzyme-linked immunosorbent assay
EU..... endotoxin unit
FACS..... fluorescence-activated cell sorter
FCA..... Freund's complete adjuvant
FICA..... Freund's incomplete adjuvant
FITC..... fluorescence isothiocyanate
FnBP..... fibronectin binding protein
GPI..... glycosylphosphatidylinositol
HBSS..... Hank's balanced salt solution
HDL.....high-density lipoprotein
ICAM..... intercellular adhesion molecule
IFN..... interferon
IκB..... inhibitory κB
IKK..... inhibitory κB kinase
IL..... interleukin
IMI..... intramammary infection
IRAK..... interleukin-1 receptor-associated kinase
KDa..... kilo dalton
LAL..... limulus ameobocyte lysate
LBP..... lipopolysaccharide binding protein
LPS..... lipopolysaccharide
MAC..... membrane-attack complex
mCD14..... membrane CD14
MHC..... major histocompatibility complex
MSCRAMM..... microbial surface components recognizing adhesive matrix molecule

NF-κB..... nuclear factor-κB
NIK..... nuclear factor-κB-inducing kinase
NK..... nature killer
OD..... optical density
PBS..... phosphate buffered saline
PBS-T..... phosphate buffered saline – Tween 20
PKC..... protein kinase C
PMN..... polymorphonuclear neutrophil
PTK..... protein tyrosine kinase
rbosCD14..... recombinent bovine soluble CD14
rhsCD14..... recombinant human soluble CD14
RAP..... RNAIII-activaing protein
RIP..... RNAIII-inhibiting protein
RT-PCR..... reverse transcriptase – polymerase chain reaction
SCC..... somatic cell count
sCD14..... soluble CD14
TCR..... T cell receptor
TNF..... tumor necrosis factor
TLR..... toll like receptor
TRAF..... tumor necrosis factor receptor-associated factor
TSB..... trypticase soy broth
VLA..... very late activation antigen

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CHAPTER I. GENERAL INTRODUCTION AND REVIEW OF LITERATURE

GENERAL INTRODUCTION

Bovine mastitis induced by *E. coli* or *S. aureus* is responsible for tremendous economic losses. The treatment for mastitis is strongly relied on administration of antibiotics which has been an issue of public concerns. Requirement of alternative strategies to combat this disease is imperative. The pathogenesis of *E. coli* infections is mediated by the interaction among lipoplysaccharide (LPS), LPS-binding protein, and CD14, the cellular receptor of LPS. CD14 is expressed on monocytes/macrophages and neutrophils (mCD14). Shedding of mCD14 from the plasma membrane becomes a soluble form of CD14 (sCD14). Both forms of CD14 have been shown to react with LPS. Activation of mCD14-expressing leukocytes by LPS leads to production of TNF-α which is responsible for deleterious inflammatory responses at high concentrations. Administration of exogenous sCD14 has been demonstrated to reduce TNF-α production and fatality in LPS challenged mice. However, whether sCD14 has the protective effect on bacterial infections has not been well documented. In the present study, sCD14 was characterized in milk from normal and LPS-challenged glands. In addition, by using mouse as the model and then bovine, the effect of sCD14 administration on *E. coli* infections was investigated.

Numerous attempts have been made to develop vaccines against *S. aureus*. However, most of these vaccines have received poor responses and never prevented new intramammary infections (IMIs) caused by *S. aureus*. This may be attributed, at least partially, to the capsular polysaccharide (CP) produced by *S. aureus* which significantly

increases the resistance to phagocytosis. Ninety-four to one hundred percent of *S. aureus* isolated from mastitic cows are encapsulated. A recent study indicated that all the *S. aureus* isolates from the United States belong to three categories, T5, T8, and T336, based on the serotype of CP. It has been proposed that using CP as the antigen is essential to develop an effective vaccine against *S. aureus*. Thus, a trivalent (T5, T8, T336) whole cell vaccine has been designed to prevent bovine *S. aureus* mastitis. The efficacy of a vaccine can be remarkably affected by its adjuvant. Aluminum Hydroxide (ALUM) and Freund's Incomplete Adjuvant (FICA) are the most common and safe adjuvants used in veterinary vaccines. Therefore, the last objective of our study was to evaluate the trivalent vaccine, with or without either one of the adjuvants.

In summary, two different approaches were investigated: administration of sCD14 and immunization of a trivalent vaccine to reduce or prevent bovine mastitis caused by *E. coli* and *S. aureus*. This study presented information fundamental to exploring alternative strategies which may prevent or alleviate bovine mastitis by enhancing the host immunity.

REVIEW OF LITERATURE

1. Bovine Mastitis

1.1. Overview

Mastitis is classically defined as an inflammation with redness, swelling, heat, pain and loss of function of the mammary gland. Bovine mastitis is most frequently associated with bacteria and more commonly, a subclinical manifestation. Normally, mastitis develops as a result of passage of pathogenic bacteria through the teat canal, followed by

their multiplication within the milk. A total of 137 different pathogens, including *Staphylococcus*, *Streptococcus*, and Coliform bacteria, have been isolated from bovine mastitis samples (Watts, 1988). Taken together, these bacteria comprise about 90% of the isolates from subclinical cases and about 84% from clinical cases (Sandgren, 1991). Mastitis-causing pathogens can be classified into two categories, environmental and contagious. Environmental pathogens, including *Escherichia coli* (*E. coli*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), and *Streptococcus uberis* (*S. uberis*), are mainly transmitted from the environment to the cow. On the other hand, *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*) are the major contagious pathogens and are spread from one cow to another (Radostits et al., 1994).

Mastitis is one of the most costly diseases of dairy cows. Approximately 38% of the cows in the United States have intramammary infections averaging 1.5 quarters (Nickerson, 1993) and the losses from them exceed 2 billion dollars per year (Eberhart et al., 1987). When converted to a per cow basis, this economic loss is consistent with the one estimated in Canada (Gill et al., 1990). Major losses from mastitis have been due to the following reasons: a) realized (clinical) and unrealized (subclinical) reduced milk production; b) milk discarded because of antibiotic content or abnormal composition; c) veterinary service; d) medications and labor; and e) culling and mortality. It has been estimated that 70-80% of the losses from mastitis is associated with reduced milk production due to subclinical mastitis (Eberhart et al., 1987).

1.2. Controlling strategies of bovine mastitis

A number of preventive strategies have been applied to control bovine mastitis in the past decades, including milking hygiene, reduced exposure to environmental pathogens, regular monitoring of udder health status, and dry cow therapy. Although the incidence of mastitis has been reduced by preventive strategies, curing of mastitis is still mainly relied on the administration of antibiotics. However, drawbacks of using antibiotics, antibiotic residues and generating resistant strains, have caused public concerns. Researchers have been trying to find new methods to prevent or cure mastitis. For example, application of an internal teat sealer during the dry period have been shown to be as effective as antibiotic treatments in terms of preventing new intramammary infections (IMI) (Huxley et al., 2002).

In addition, enhancing the immune system by vaccination to combat invading pathogens has become an interesting approach. A commercially available *E. coli* J5 vaccine has been shown to reduce the incidence and severity of clinical coliform mastitis (Hogan et al., 1992). However, in other studies, J-5, as well as a vaccine against *E. coli* ferric citrate receptor (FecA), only had a minimal effect on the clinical severity of experimental *E. coli* mastitis (Smith et al., 1999; Takemura et al., 2002). A number of vaccines, containing various virulent factors of *S. aureus* as the antigen, have been developed and evaluated. Due to the complexity of the pathogenesis of *S. aureus*, none of them has demonstrated a reliable efficacy in preventing *S. aureus* mastitis. A better understanding of the interaction between the pathogen and the host immune system is required to develop an effective vaccine.

1.3. The natural defense of bovine mammary glands

In response to invading pathogens, the natural immune system of the mammary gland elicits a spectrum of immune responses and plays a role in determining the outcome of infections. This natural defense of the bovine mammary gland can be categorized into three subgroups: anatomical, soluble, and cellular defenses (Sordillo et al., 1997).

1.3.1. Anatomical defenses

In general, pathogens gain entrance to the mammary gland through the opening of teats. The sphincter muscle located on the teat end is crucial to maintain tight closure between milking and prevents invasion of bacteria. Moreover, the teat canal is lined with a layer of keratin, a waxy material derived from ductal epithelial cells, which is able to "trap" pathogens and prevents them from moving further to the milk cistern. Cationic proteins and a number of esterified and nonesterified fatty acids, including myristic acid, palmitoleic acid, and linoleic acid, are located within the layer of keratin and act as antimicrobial agents (Treece et al., 1966). Removal of keratin has been shown to increase the susceptibility of bacterial penetration and colonization (Capuco et al., 1992).

1.3.2. Soluble defenses

The major components of soluble defenses in the bovine mammary gland are nonspecific bacteriostatic factors, such as lactoferrin and complements, cytokines, and immunoglobulins (IgG_1, IgG_2, IgA and IgM). They form a complicated network which functions sophisticatedly in concert with cellular defenses. Lactoferrin is an iron-binding glycoprotein which exerts both bacteriostatic and bacteriacidal activities (Sànchez et al.,

1992). Activation of the complement system can be triggered by microorganisms (the alternate pathway) and antibodies (the classical pathway). Components of the complement system are able to form a membrane-attack complex (MAC) and kill bacteria by punching holes on the bacterial membrane. Other complement fragments, known as C3a and C5a, are chemoattractants to neutrophils (Shuster et al., 1997). Immunoglobulins are synthesized by antigen-activated B-lymphocytes and work in different manners to protect the mammary gland. IgG_1, IgG_2, and IgM bind to invading bacteria and enhance the efficacy of phagocytosis. On the other hand, IgA contributes to agglutination, which prevents bacteria colonization and toxin neutralization (Musoke et al., 1987).

The cytokine network is responsible for the initiation of inflammatory responses. "Cytokine" is one term for a group of low molecular weight (<80 kDa) proteins, which are extremely sensitive, acting at very low concentrations, to their high affinity and specific receptors on the cell surface. They are produced by and released from a wide variety of cells in response to bacterial stimuli. For example, bacterial lipopolysaccharide (LPS) was able to provoke the production IL-8 from bovine mammary epithelial cells (Boudjellab et al., 1998). A number of cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8, have been shown to increase in milk during mastitis (Shuster et al., 1997). Administration of recombinant cytokines has been shown to enhancing the recruitment and bactericidal functions of phagocytes (Daley et al., 1993; Lee and Zhao, 2000).

1.3.3. Cellular defenses

Phagocytes in the mammary gland play a pivotal role in eliminating invading pathogens, especially during the early stage of infections (Nickerson, 1993). Residential leukocytes are composed of lymphocytes, macrophages, neutrophils, and nature killer (NK) cells. Lymphocytes, including T and B cells, are responsible for generating required cytokines and antigen-specific antibodies to combat infections. It usually takes at least several days to complete this specific immune process. Therefore, the elimination of bacteria is mainly relied on the non-specific immunity, bactericidal functions of macrophages and neutrophils, which is significantly enhanced by the presence of cytokines and antibodies. However, the killing efficacy of residential macrophages and neutrophils is substantially compromised by indiscriminately ingesting milk components, such as fat and casein micelle. Under this circumstance, recruitment of blood leukocytes, mainly neutrophils, is impractical to the clearance of bacteria. The promptness of leukocyte migration from blood stream to milk is critically related to the severity and duration of mastitis (Grommers et al., 1989; Hill, 1981). The importance of milk NK cells in killing bacteria is not well understood. Nevertheless, human NK cells have been shown to kill both Gram-negative and Gram-positive bacteria by an extracellular mechanism (Garcia-Penarrubia et al., 1989). Leukocytes in milk, together with a small amount of mammary epithelial cells, consist of milk somatic cell count (SCC) (Paape et al., 1963).

1.4. Somatic cell count

Under normal conditions, SCC contains macrophages (35-79 %), neutrophils (3-26 %), lymphocytes (10-24 %), and epithelial cells (2-15 %) (Lee et al., 1993; Miller et al.,

1993). The profile of SCC can be affected by the stage of lactation. The percentage of neutrophils is higher during early and late lactation, while the percentage of lymphocytes decreases (Sheldrake et al., 1983). The subpopulation of mononuclear leukocytes, including macrophages, B lymphocytes, and T lymphocytes, is also altered in late lactation (Park et al., 1992). During mastitis, the percentage of neutrophils can increase up to > 95 % (Paape et al., 1979; Kehrli & Shuster, 1994). Generally speaking, the total SCC is less than 200,000 cells/ml of milk in a healthy lactating cow (Miller et al., 1999), and it goes up to millions cells/ml within just a few hours after infection (Paape et al., 1991). Therefore, milk SCC has been used as an indicator of the existence of mastitis and the quality of milk. In the United States, the limit of acceptable milk is 750,000 cells/ml of milk. Canada and European Union have lower standards, which are 500,000 and 400,000 cells/ml of milk, respectively. The number of SCC in milk is usually lower during the winter (November – January) and higher during the summer (July – August) (Miller et al., 1999).

The presence of SCC is like a double-sided sword. Farmers get penalties when bulk tank milk SCC exceeds standards. In addition to the safety issue, using milk with a low level of SCC also results in a better yield of cheese. Apparently, the industry prefers to have milk with low SCC to provide high quality dairy products for the consumers. On the other hand, a higher level of SCC in milk is important to the resistance against mastitis. SCC higher than 600,000 cells/ml in milk was able to protect the mammary gland from *S. aureus* challenge (Postle et al., 1978). Herds with a lower bulk tank SCC showed an increased incidence of clinical mastitis (Miltenburg et al., 1996). When an intramammary infection occurs, more residential leukocytes are required to win the first battle with

bacteria and eliminate the inflammatory stimuli before the infection deteriorates. Otherwise, the inflammation augments, and more and more leukocytes, mostly neutrophils, have to be recruited from blood stream to participate in the battle.

1.5. Neutrophils

Neutrophils are derived from the bone marrow, and then migrate into the bloodstream where their half-life is about 10 hours. During this period, they circulate and patrol the body by sensing signals, so-called chemoattractants, from local inflammatory response. IL-8 and C5a are the most potential chemoattractants for neutrophils in the bovine mammary gland (Shuster et al., 1997; Lee and Zhao, 2000). There seems to be variations among neutrophils from different species (Styrt, 1989). For example, bovine neutrophils do not respond to formyl-methionyl-leucyl-phenylalanine (FMLP) (Forsell et al., 1985), which has been shown to activate human neutrophil (Young and Beswick, 1986; Willems et al., 1989; Carolan et al., 1997).

The multilobed and irregular nucleus of neutrophils makes them extremely deformable to squash into tiny openings, for example the tight junction between mammary epithelial cells (Paape et al., 1979, Lin et al., 1995). This may explain their prompt arrival when being called on duty. A rapid recruitment of neutrophils is crucial to the outcome of infectious diseases, including mastitis (Burvenich et al., 1994; Seiler et al., 2000; Yang et al., 2002). The increased severity and incidence of mastitis in cows during parturition and early lactation is associated with the inability of neutrophils migrating into the mammary gland in response to invading pathogens (Shuster et al., 1996). However, prolonged diapedesis of neutrophils through tight junctions between adjacent mammary secretory

cells may result in damage to mammary parenchymal tissue and decreases milk production (Capuco et al., 1986).

1.6. Bactericidal functions of Neutrophils

1.6.1. Migration

In order to eliminate invading bacteria, neutrophils must migrate from blood vessel to the inflammatory tissue space. This movement of neutrophils is stimulated by chemoattractants and modulated by specific cell adhesion molecules, including selectins and integrins, located on the surface of neutrophils, endothelium, epithelium, and extracellular matrix (Springer, 1995). In the bovine mammary gland, five biological barriers exist for diapedesis, including (1) endothelial monolayer of blood vessel (2) basal lamina surrounding the blood vessel (3) extracellular matrix (ECM) (4) basal lamina surrounding the alveoli, and (5) epithelial monolayer lining the mammary alveoli (Harmon and Heald, 1982). Therefore, the recruitment of neutrophils can be divided to three sequential steps, transendothelial migration, locomotion in ECM, and transepithelial migration.

Expression of adhesive receptors on cell membrane is upregulated when vascular endothelial cells and neutrophils receive inflammatory signals from the underlying tissue (Carlos and Harlan, 1990). The interaction between the neutrophil and the endothelial cell begins with a slow "tethering and rolling" movement, which is mediated by the selectin family (L-, P-, and E-selectin) and their sialylated oligosaccharide ligands (Springer, 1995). The purpose of this process is to slow down the velocity of neutrophils in bloodstream. The interaction is then taken over by a firm attachment, which is regulated

by neutrophil β_2 integrins (CD11a/CD18, CD11b/CD18, and CD11c/CD18) and their immunoglobulin superfamily ligands (ICAM-1, ICAM-2) on endothelial cells. This step results in the flattening of neutrophils on the endothelium and the initiation of moving through intercellular junctions.

When neutrophils reach the extravascular space, they interact with ligands on fibroblasts and ECM proteins, such as laminin, fibronectin, and collagen, and keep moving to the site of infection. Migration in ECM is modulated by β_2 (CD18) and β_1 (CD29) integrins, including very late activation antigen-4 (VLA-4), VLA-5, and VLA-6, (Pakianathan, 1995; Gao and Issekutz, 1997, Roussel and Gingras, 1997). After crossing ECM space, neutrophils contact the last barrier, epithelium. Neutrophil transepithelial migration is dependent on one of the β2 integrins, CD11b/CD18 (Smits et al., 1997), but not on CD11a/CD18 and CD11c/CD18 (Parkos et al., 1991; Colgan et al., 1995). Once neutrophils arrive at the milk cistern and encounter invading bacteria, the bactericidal functions are implemented.

1.6.2. Phagocytosis

When neutrophils influx into inflammatory sites, they phagocytose and kill bacteria. The initiation of phagocytosis is the recognition and attachment of invading bacteria, which is mediated by specific receptors and ligands. Traditionally, opsonins (antibodies, complement fragments) and their receptors (Fc receptors and complement receptors CR1, CR3) are considered as the major molecules orchestrating phagocytosis (Allen and Aderem, 1996). Indeed, the presence of opsonins significantly enhances the phagocytosis of bovine neutrophils (Paape et al., 1996). However, more and more cellular receptors

involved in phagocytosis have been identified, such as integrins (Blystone et al., 1994) and receptors recognizing bacterial surface components, mannose and β-glucan (Ezekovitz et al., 1990; Czop and Kay, 1991). Moreover, CD14, a putative receptor for LPS expressed on phagocytes, has been shown to bind to Gram-negative and Gram-positive bacteria (Jack et al., 1995; Dziarski et al., 1998). The ligation of phagocytic receptors and their ligands triggers the polymerization of actin at the site of contact, and then the pseudopods of neutrophils surround and engulf bacteria. The internalization of bacterial particles leads to the formation of "phagosome".

The signal transduction responsible for this actin-based mechanism is complicated and remains obscure. The polymerization of actin seems to be initiated by the phosphorylation of receptor-associated immunoreceptor tyrosine-based activating motifs (ITAMs), which is regulated by various protein tyrosine kinases (PTK) (Greenberg and Grinstein, 2002). In addition, protein kinase C (PKC) has been shown to be involved in phagocytosis (Celli and Finlay, 2002). Finally, the signal transduction result in the activation of Arp2/3, a complex of actin-binding proteins, which is required for the cytoskeletal alterations (May et al., 2000). After the internalization is completed, the process is taken over by a microtubule-based system which is responsible for the trafficking of intracellular lysosomes and the fusion of the lysosome with the phagosome (phagolysosome).

1.6.3. Respiratory burst

The killing of engulfed bacteria takes place inside the phagolysosome. This event involves a variety of toxic agents generated by the respiratory burst of neutrophils. The

respiratory burst is composed of a series of oxidative burst reactions initiated by NADPH oxidase, which transfers one electron across the membrane of phagolysosome to oxygen. This in turn leads to production of superoxide anion (O_2^-) and its downstream toxic products, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and hypochlorous acid (HOCl) (Clark, 1999). Among which, HOCl is the strongest bactericidal agent known to be produced by neutrophils (Klebanoff, 1968). There was a strong negative correlation between the respiratory burst activity of neutrophils and the severity of *E. coli* mastitis (Heyneman et al., 1990). It has been shown that 5 proteins are involved in the respiratory burst of neutrophils, two of which (a 22 kDa peptide and a 91 kDa glycoprotein) consist of cytochrome *b*, the membrane-bound catalytic component of NADPH oxidase (Parkos et al., 1987) and the other three (p47*phox*, p67*phox*, and Rac1 or Rac2) are located in cytoplasm (Kanegasaki, 1993). Extracellular release of superoxide anion may contribute to the tissue damage associated with inflammatory reactions.

1.6.4. Exocytosis

Bovine neutrophils contain three different types of granules (Sandgren, 1991), azurophilic, specific, and tertiary granules. The last one is larger, denser and more numerous than the other two types. These granules contain bactericidal substances such as myeloperoxidase (azurophilic), elastase (azurophilic), lysozyme (both azurophilic and specific), lactoferrin (specific), and gelatinase (tertiary). Upon neutrophil activation, intracellular granules fuse with plasma membrane and extracellularly release their content, a process named "exocytosis". These enzymes can not only facilitate elimination of invading bacteria but also accelerate neutrophil migration from microvessels toward

the inflamed sites by local degradation of the endothelial base membrane (Huber and Weiss, 1989). The presence of Ca^{2+} is crucial to trigger this function (Kuijpers et al., 1992; Quade and Roth, 1997). Inflammatory cytokines, such as IL-8, has been shown to induce exocytosis of neutrophils (Willems et al., 1989; Kurdowska et al., 1995).

2. *Escherichia coli* mastitis

Bovine mastitis induced by gram-negative rods, such as *E. coli*, *Klebsiella* and *Enterobacter*, is generally referred as coliform mastitis. Although coliform bacteria only account for less than 5 % of all cases of bovine mastitis, it has been reported that approximately 80 % of "clinical" mastitis cases are resulted from this family of pathogens (Eberhart, 1977; Paape et al., 1996). More importantly, coliform mastitis may cause death in severe cases. The incidence of coliform mastitis might have been underestimated due to its transient nature, usually less than 7 days. However, as a consequence of improved milking hygiene, the spreading of contagious pathogens has been minimized, which in turns has increased the percentage of environmental pathogens, including coliform bacteria (Blowey and Edmondson, 1995). Bovine coliform mastitis is most frequently caused by *E. coli*.

E. coli mastitis frequently occurs during early lactation, and the risk increases with parity (Barkema et al., 1998). This has been associated with low numbers of neutrophils in the circulation and impaired functions of neutrophils, especially during parturition (Kehrli et al., 1989; Heyneman et al., 1990). Moreover, newly calved cows showed different susceptibilities to *E. coli* mastitis and were classified into two groups, severe and moderate (Hill et al., 1979). It has been proposed that the difference was due to

different abilities of neutralizing and absorbing lipopolysaccharide (LPS), a virulent factor of *E. coli* bacteria, from the cows (Vandeputte-Van Messom et al., 1993).

2.1. Host responses to *E. coli* infections

2.1.1. Lipopolysaccharide

There is a general agreement that the pathogenesis of *E. coli* is mainly mediated by LPS, or endotoxin, a cell wall component of the outer membrane of Gram-negative bacteria. Intramammary administration of LPS was able to elicit clinical symptoms similar to those observed in clinical *E. coli* mastitis cases (Paape et al., 1974; Shuster et al., 1993). LPS is released when the membrane of bacteria loses its integrity, which could happen during proliferation, death, and being attacked by phagocytes and complements. Structurally, LPS is composed of a lipid portion (lipid A), a core oligosaccharide (core antigen), and repeated polysaccharide unites (O antigen). Lipid A is the toxic portion of LPS, which anchors this molecule to the out membrane of bacteria (Raetz, 1990). The core antigen is highly conserved among Gram-negative bacteria. In contrast, the O antigen is unique for different strains. Therefore, the vaccine against *E. coli* mastitis, J-5, was made by a mutant *E coli* strain with its polysaccharide units trimmed and allowed the core antigen to be exposed. This design favors the production of core antigen specific antibodies and provides a broader protection.

The molecular weight of a LPS monomer is about 10 kDa (Petsch and Anspach, 2000). However, it has been demonstrated that LPS molecules aggregate in forms as micelles and vesicles with diameters up to 0.1 μm (Seydel et al., 1993). Although the aggregated form represents the majority (> 99 %) of LPS isolated from *E. coli*, the

monomeric LPS has a stronger biological activity (Takayama et al., 1994). LPS is extremely stable and is able to induce strong biological responses at a very low concentration. The threshold of LPS in circulation to elicit the outbreak of inflammatory responses is 5 endotoxin units (EU, 1 EU ≅ 0.1 ng) per kg body weight in human (European Pharmacopoeia, 1997). However, LPS molecules isolated from different origins have been shown to have different biological activities *in vitro* (Zughaier et al., 1999). During *E. coli* mastitis, LPS is high in milk and is only sporadically detected in blood (Dosogne et al., 2002). However, excessive absorption of LPS into the circulation may induce deleterious inflammatory responses and cause death in severe cases, which is clinically referred as "septic shock". Several cellular receptors have been shown to react with LPS and activate resting leukocytes, including β_2 integrins (Ingalls et al., 1998), L-selectin (Malhotra et al., 1996), and CD14 (Wright et al., 1991). However, accumulated evidence indicated that CD14 is the main candidate responsible for initiation of LPS-induced inflammatory reactions.

2.1.2. CD14

CD14, a 53-55 kDa glycosylated phosphatidylinositol (GPI)-anchored protein expressed on monocytes/macrophages and neutrophils (Haziot et al., 1988; Wright et al., 1991; Paape et al., 1996), has been shown to interact with a variety of bacterial components and defined as a pattern recognition molecule (Pugin et al., 1994). Human CD14 gene is located on chromosome 5 and encodes a single 1.4 kb mRNA (Ferrero and Goyert, 1988), whereas bovine CD14 gene in macrophages possesses two mRNA transcripts, 1.5 and 3.1 kb, respectively (Yang et al., 1995). However, *in vitro* translation

of these two mRNA yielded a protein of 38-40 kDa, which is similar to the size of unglycosylated human CD14 (Yang et al., 1996a). Bovine CD14 gene, encoding 373 amino acids, was cloned and showed 61-73% homology to mouse, rabbit and human CD14 (Ikeda et al., 1997). Expression of membrane CD14 (mCD14) in monocytes/macrophages and neutrophils can be regulated by various mediators during infections. Cytokines, including TNF-α, IFN-γ, IL-2, and LPS have been demonstrated to upregulate CD14 expression (Landmann et al., 1991; Parsons et al., 1995; Landmann et al., 1996). The rapid expression of mCD14 upon stimulation may be associated with relocation of intracellular granules containing CD14 in neutrophils (Detmers et al., 1995; Paape et al., 1996; Rodeberg et al., 1997). In addition to phagocytes, CD14 was found to be expressed in human, but not bovine, mammary epithelial cells (Labéta et al., 2000).

Binding of mCD14 to LPS leads to activation of CD14-expressing cells and production of cytokines, including TNF-α, IL-1, IL-6, and IL-8 (Martin, 2000). The secretion of these cytokines plays a role in the pathogenesis of *E. coli* infections and the host defense. However, overwhelming production of TNF-α is the main causation of multiple organ failure and death as seen in "septic shock" (Waage et al., 1989). The importance of the binding between mCD14 and LPS has been demonstrated in many studies. Using monoclonal antibody (mAb) against CD14 was able to abrogate the binding of mCD14 to LPS and inhibit the activation of monocytes (Fenton and Golenbock, 1998). CD14-deficient mice have been shown to be more resistant to LPS challenge due to the inability of macrophages to produce TNF-α in response to LPS (Haziot et al., 1996; Moore et al., 2000). Furthermore, transgenic mice expressing human CD14 had an increased susceptibility to LPS shock (Ferrero et al., 1993). A recent study

reported that mCD14 also played a role in activation of human T cells and monocytes by *S. aureus* lipoteichoic acid (Ellingsen et al., 2002).

2.1.3. Signal transduction in LPS-activated leukocytes

Because the GPI anchor does not have a transmembrane and a cytoplasmic domain, it has been questioned how signals being transduced intracellularly. Recently, the Toll-like receptor (TLR) has been identified as a cell-surface co-receptor for CD14 and plays a role in the signal transduction of LPS-activated leukocytes (Chow et al., 1999). Toll is a transmembrane receptor involved in the anti-fungal activity of *Drosophila* and shares a similar cytoplasmic domain with mammalian IL-1 receptor (Lemaitre et al., 1996). Human homologues of Toll, TLRs (TLR-1 to -10), are expressed in a variety of cells, including endothelial cells, dendrite cells, and leukocytes (Rock et al., 1998). Among which, TLR-4 is well characterized as the co-receptor involved in the signal transduction induced by LPS. TLR-4 deficient mice showed a decreased sensitivity to LPS (Hoshino et al., 1999).

TLR-4-mediated signal transduction ultimately leads to activation of a transcription factor NF-κB, and this in turn triggers production of inflammatory cytokines. Under resting conditions, NF-κB is associated with an inhibitory IκB protein and is retained in the cytoplasm. The release of NF-κB into the nucleus requires the phosphorylation of IκB by IκB kinases (IKKs), including IKK α, IKK β, and IKK γ (Karin and Ben-Neriah, 2000). At least two adaptor proteins, Myd88 and TNF receptor-associated factor (TRAF), and other kinases, such as IL-1R-associated kinase (IRAK) and NF-κB-inducing kinase (NIK), are involved in the signal transduction (Takeuchi and Akira, 2001). In addition to

the pathway regulated by TLR-4, neutrophils can be activated by internalizing LPS and subsequent vesicular fusion (Detmers et al., 1996).

2.1.4. LPS-binding protein

The direct binding of LPS to mCD14 is minimal. However, the affinity of CD14 toward LPS was increased by > 1000 fold in the presence serum factors, such as LPS-binding protein (LBP), a 60 kDa acute phase protein in serum produced by hepatocytes (Hailman et al., 1994). However, human respiratory epithelial cells have recently been reported to produce LBP in response to inflammatory cytokines, including IL-1, IL-6 and TNF-α (Dentener et al., 2000). The concentration of LBP in serum is 2 – 20 μg/ml and may increase to 100 – 200 μg during an acute infection (Fenton and Golenbock, 1998). Bovine LBP has been shown to enhance the binding to LPS to human monocytes and removal of mCD14 abrogated this interaction (Horadagoda et al., 1995). LPS-induced inflammatory responses were dramatically suppressed in LBP-deficient mice (Wurfel et al., 1997). The ability of LBP to break down LPS micelles and make active LPS monomers available may explain these observations (Hailman et al., 1994). Moreover, this catalyzing effect has been shown to accelerate the binding of LPS to high-density lipoproteins (HDL), which results in the neutralization of LPS (Munford et al., 1981). LBP has also been indicated to be involved in LPS-mediated endothelial cell damage (Yang et al., 1996b) and CD14-independent phagocytosis of macrophages (Klein et al., 2000). Furthermore, LBP has been shown to play a role in the acquisition of LPS by soluble CD14 (Wurfel et al., 1995).

2.2. Soluble CD14

A soluble form of CD14 (sCD14) was found in normal serum, urine, milk, and the culture supernatant of human monocytes (Bazil et al., 1986; Labéta et al., 2000; Wang et al., 2002). Soluble CD14 has the same structure as mCD14, except the GPI anchor, and retains their ligand-binding capacity. Therefore, the molecular mass of sCD14 is slightly smaller than mCD14, which is about 48-53 kDa (Bazil and Strominger, 1991). Shedding from the membrane of mCD14-expressing cells has been described as the main source of sCD14 in body fluid. Cleavage of mCD14 is mediated by membrane-associated serine proteases, which are activated in monocytes stimulated by various stimuli, such as LPS (Bazil and Strominger, 1991). However, a larger form of sCD14, 56 kDa, was encoded by the same mRNA and released by a protease-independent mechanism (Bufler et al., 1995). The biological relevance between these two forms of sCD14 is still unclear. Bovine sCD14, a 46 kDa protein, was detected in milk whey by an anti-ovine CD14 mAb (Wang et al., 2002).

2.3. Biological functions of sCD14

Since the existence of sCD14 was discovered, the biological function of sCD14 has been extensively studied. Soluble CD14 was detected in human serum, ranging from 2 to 6 μg/ml, and the concentration was increased in patients with various diseases, including lupus erythematosus (Nockher et al., 1994), malaria (Wenisch et al., 1996), and tuberculosis (Juffermans et al., 1998). Markedly increased sCD14 was also detected in the bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (Martin et al., 1997) and the cerebrospinal fluid (CSF) from mice with bacterial

meningitis (Cauwels et al., 1999). Recruitment of leukocytes to the site of inflammation is usually associated with infectious diseases. Therefore, it has been postulated that the increase of sCD14 is attributed to newly recruited leukocytes which expressing mCD14 (Niki et al., 2000). Shedding of mCD14, the main source of sCD14, was augmented in LPS-stimulated neutrophils (Parsons et al., 1995). The biological relevance of this scenario is not well understood. However, binding of sCD14 to LPS has a number of beneficial effects in terms of minimizing deleterious inflammatory responses.

2.3.1. Inhibition of TNF-α production

Over produced TNF-α from LPS-activated monocytes/macrophages and neutrophils is responsible for the notorious "septic shock" which may lead to death in severe cases. This activation of leukocytes is mediated by mCD14, LBP, and TLR-4. Sharing identical ligand-binding sites, soluble CD14 has been shown to compete LPS molecules with mCD14, which avoids overwhelming activation of leukocytes and production of TNF-α. Recombinant human sCD14 (rhsCD14) was able to decrease the production of TNF-α by LPS-stimulated murine macrophages *in vitro* (Haziot et al., 1995). *In vivo*, rhsCD14 administered before or simultaneously with LPS significantly inhibited concentrations of TNF-α, IL-6 and IFN-γ in the blood of challenged mice (Stelter et al., 1998)

2.3.2. Formation of LPS/sCD14 complex

Formation of LPS/sCD14 complex is accelerated by LBP and is at least involved in two biological reactions. First, activation of CD14-negative cells. At the presence of serum, human endothelial and epithelial cells, which do not express mCD14, had an

enhanced secretion of IL-8 in response to LPS (Pugin et al., 1993). This response was abrogated by either immunodepletion of LBP or addition of mAb against sCD14. As a potential chemoattractant of phagocytes, an enhanced production of IL-8 might be crucial to the recruitment of neutrophils during the early stage of infections. This idea was supported by a recent study in which LPS/rbosCD14 complex demonstrated a similar effect on bovine mammary epithelial cells and sensitized the recruitment of neutrophils in bovine mammary glands challenged with a low does of LPS (Wang et al., 2002). Second, neutralization of LPS. The neutralization of LPS occurs when LPS monomers bind to HDL, which is catalyzed by LPB. However, the formation of LPS/sCD14 complex acts as a "shuttle" transferring LPS to HDL and accelerates the neutralization more than 30-fold (Wurfel et al., 1995).

2.3.3. Involvement in other biological reactions

In addition to regulating the host defense specific to LPS, sCD14 has been shown to be involved in many other biological responses. Soluble CD14 was described as a naturally occurring soluble B cell mitogen and increased IgG_1 production from human B cells with a concomitantly reduced IgE production (Arias et al., 2000; Filipp et al., 2001). This interaction is mediated by a region of sCD14 that is not involved in LPS recognition. Binding of sCD14 to *E. coli* bacteria and the peptidoglycan of Gram-positive bacteria was demonstrated (Jack et al., 1995; Dziarski et al., 1998). However, the biological relevance of this sCD14-bacteria binding remains to be addressed. Soluble CD14 has been indicated to modulate apoptosis. LPS-induced apoptosis of bovine endothelial cells was sCD14-dependent and was blocked by anti-CD14 mAb (Frey and Finlay, 1998).

2.4. Significance of sCD14 in milk

The presence of sCD14 in human milk was initially detected by Labéta et al (2000) and the concentration was up to 20-fold of that in human serum (2-6 μg/ml). The level of sCD14 was higher in colostrums and milk samples from early stage of lactation (< 7 days postparturition), averaged 67.09 ± 27.61 μg/ml. Soluble CD14 was identified in fresh bovine milk, but not in commercially bottled milk, and quantification was not carried out (Labéta et al., 2000; Wang et al., 2002). Considering the beneficial effects of sCD14, it has been proposed that the high content of sCD14 in milk, especially the colostrums, is important in protecting the neonatal intestine from bacterial colonization and in modulating immune responses of newborns (Jones et al. 2002). Moreover, a higher level of sCD14 in milk might be able to increase the resistance to mastitis. However, this hypothesis requires further investigations.

2.5. Therapeutic effects of sCD14

The therapeutic effect of sCD14 was best characterized in mice intraperitoneally challenged with a fatal dose of LPS. Recombinant hsCD14 administered before or simultaneously with LPS derived from *Salmonella abortus equi* (8 μg/gBW) dramatically increased the survival of challenged mice from 23-29 % to 68-79 % (Stelter et al., 1998). Similar results were demonstrated by mice challenged with *Salmonella minnesota* LPS (12 μg/gBW) (Haziot et al., 1995). Both studies indicated that an inhibited TNF-α production in serum was associated with the protective effect of sCD14. On the other hand, the effect of sCD14 administration on infections induced by live bacteria is not fully understood. In a mouse *Streptococcus pneumoniae* meningitis model, rhsCD14

increased growth of bacteria and the concentration of TNF-α in cerebrospinal fluid of challenged mice (Cauwels et al., 1999). It has been reported that monocytes deploy different intracellular pathways upon interacting with Gram-positive and Gram-negative bacteria through mCD14 (Rabehi et al., 2001). Therefore, sCD14 might play a different role in Gram-negative bacterial infections. Nevertheless, possible therapeutic effects of sCD14 on Gram-negative bacterial infections can be postulated based on following observations. CD14-deficient mice intraperitoneally challenged with *E. coli* (Haziot et al., 1996) or *Bacteroides fragilis* (Woltmann et al., 1999) had reduced dispersion of bacteria throughout the peritoneum. The authors proposed that reagents capable of blocking or neutralizing mCD14 on monocytes might be able to inhibit the growth of Gram-negative bacteria. In addition, a novel pathway, which is not mediated by CD14 and TLR-4, has been shown to efficiently attract neutrophils when triggered by LPS (Haziot et al., 2001). Both CD-14 and TLR-4 deficient mice intraperitoneally challenged with *E. coli* demonstrated an early recruitment of neutrophils in the peritoneal cavity and a faster clearance of bacteria in comparison with the control mice. As a matter of fact, activation of mCD14/TRL-4 might interfere with this pathway. Taken together, it is possible that administration of exogenous sCD14 can compete LPS with mCD14 and minimize activation of phagocytes. This in turn enhances the clearance of bacteria and reduces the severity of infections.

2.6. Studying *E. coli* mastitis in a mouse model

The studying of *E. coli* mastitis in a mouse model was initially introduced decades ago (Chandler, 1970). A mouse mammary gland has only one teat canal and is

functionally and anatomically independent from the others, which is similar to the bovine mammary gland. In addition to the structural similarity of the mammary gland between cows and mice, using a mouse model has other advantages, such as minimized quantity of reagents needed, reduced management and high cost associated with dairy cattle. Mouse mastitis had been induced by either direct injection of bacteria with needles through the skin and into the gland, or by exposure of teats that had their teat-ends removed to a suspension of bacteria (Chandler, 1970; Anderson, 1979). However, in order to avoid damage to mammary tissue and teats, an improved infusion method using an ultra-fine (< 75 μm in diameter) micropipette was developed recently (Nguyen et al., in press). Acquisition of this new technique would be helpful to carry out preliminary experiments for studying bovine mastitis.

3. Bovine Mastitis Caused by *Staphylococcus aureus*

Staphylococcus aureus, a contagious, gram-positive coccus, is the most frequently isolated pathogen from cases of mastitis (Schukken et al., 1993; Barkema et al., 1998). It accounts for 19 to 40% of intramammary infection (IMI) cases, usually subclinical, and costs approximately 35% of the economic loss due to mastitis (Fox and Hancock, 1989). In Canada, it has been estimated that *S. aureus* infection may be present in as many as 90% of Ontario dairy farms (Kelton, 1999). As a contagious pathogen, *S. aureus* presents in infected quarters or the skin of udders, and the transmission between quarters or cows mainly occurs during milking (Bramley and Dodd, 1984). Two protective strategies are currently applied to control *S. aureus* IMI, milking hygiene and antibiotic treatment. However, in comparison with other pathogens, the curing-rate of *S. aureus* infections is

relatively poor. This could be resulted from the ability of *S. aureus* to locate intracellularly in cells such as epithelial cells and macrophages, and survive from antibiotic treatments (Almeida et al., 1996; Hébert et al., 2000). Moreover, drug-resistant staphylococcus has been generated as a consequence of abused antibiotic administration. Currently, complete eradication of *S. aureus* is not possible.

3.1. Pathogenesis of *Staphylococcus aureus*

3.1.1. Adherence and internalization

In comparison with *E. coli*, the pathogenesis of *S. aureus* is considerably complicated, which makes the infections difficult to be eliminated. The versatility of virulent factors is different from one species to another. After entering the mammary gland, the adhesion of *S. aureus* to epithelial cells is the first step of pathogenesis during mastitis (Frost, 1975; Wanasinghe, 1981). Adherence was also proved to be highly correlated to internalization which makes *S. aureus* resistant to the natural immune system and antibiotic treatments (Hensen et al., 2000). In addition to adherence, internalization of *S. aureus* to bovine epithelial cells requires a cytoskeleton rearrangement leading to engulfment (Almeida et al., 1996). Moreover, the signal transduction of epithelial cells is involved in the cellular uptake of *S. aureus*. Genistein, an inhibitor of protein tyrosine kinase (PTK), inhibited 95% of the internalization of *S. aureus* by a bovine mammary epithelial line (MAC-T) (Dziewanowska et al., 1999). In addition to epithelial cells, *S. aureus* adheres to extracellular matrix (ECM) as well. In fact, it seems that *S. aureus* has higher affinity for ECM components than for epithelial cells (Cifrian et al., 1994).

S. aureus produces several extracellular proteins to enhance adherence, such as clumping factor (Clf), coagulase, extracellular fibrinogen binding protein (Efb), and extracellular adherence protein (Eap) (Palma et al., 1999). Moreover, a group of bacterial proteins, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), mediate the binding of *S. aureus* to fibronectin, fibrinogen, collagen, laminin, and vitronectin in ECM (Patti et al., 1994). Among which, fibronectin-binding proteins (FnBPs) have been extensively studied. Strains of *S. aureus* deficient in expressing FnBPs showed impaired ability to adhere to and invade bovine mammary cells (Lammers et al., 1999). On human cells, expression of bacterial FnBPs has been proven to be critical to *S. aureus* adherence and internalization. Two strains of noninvasive gram-positive bacteria were transformed with plasmids coding FnBPs, and acquired the ability of invasion (Sinha et al., 2000). Fibronectin acts as a bridge between FnBPs and host cells. The putative epithelial receptor for fibronectin is $\alpha_5\beta_1$ integrin which recognizes the tripeptide sequence Arg-Gly-Asp (RGD) as a ligand (Sinha et al., 1999).

Integrins are transmembrane glycoproteins that consist of two protein subunits, a variable, larger α subunit and a common, smaller β subunit, noncovalently associated with each other (Anderson and Springer, 1987). At least sixteen α subunits and eight β subunits have been characterized, which in various combinations make as many as 21 different known integrins (Springer, 1994). Some of them only bind to a single target receptor but others may recognize multiple target receptors. Initially, β_2 integrins were found on leukocytes, and they play a role in leukocyte extravasation. Members of the β_1(CD29)-integrin, VLA-4 ($\alpha_4\beta_1$), VLA-5 ($\alpha_5\beta_1$), and VLA-6 ($\alpha_6\beta_1$), are well studied on leukocytes, and appear to bind to extracellular collagen, fibronectin, and laminin,

respectively (Gao and Issekutz, 1997; Roussel and Gingras, 1997; Shang and Issekutz; 1997; Werr et al., 1998). Expression of β_1 integrins on bovine mammary epithelial cells has not been confirmed so far. However, mouse mammary epithelial cells plated on tissue culture plastic were shown to up-regulate both mRNA and protein levels of β_1 integrin subunits (Delcommence and Streuli, 1995). It is still unclear whether collagen and laminin act as the bridge between β_1 integrins and their bacterial ligands, like collagen binding protein (CnBP), and mediate the adhesion. If it is the case, blocking the binding site of β_1 integrin should have a boarder inhibitory effect on *S. aureus* attachment.

3.1.2. Exotoxins and enzymes

S. aureus produces four haemolytic toxins, α, β, γ, and δ. Among which, α and β toxins are able to damage host cell membrane and promote the subsequent adherence (Cifrian et al., 1996a). α Toxin is a cytolysin that pouches pores on cell membranes and leads to cell death (Bhakdi et al., 1993), and has an inhibitory effect on proliferation of a bovine mammary epithelial cell line (MAC-T) (Matthews et al., 1994). On the other hand, β toxin, a sphingomyelinase generally produced by 75-100 % of staphylococcal strains, altars cell permeability and surface charge (Low and Freer, 1977; Poutrel and Ducelliez, 1979). Antibodies against α and β toxins reduced the adherence of *S. aureus* to bovine mammary epithelial cells (Cifrian et al., 1996b). Neutralization of these toxins may decrease the damage of mammary epithelium and lead to less exposure of ECM for bacterial binding. A number of extracellular enzymes and cell membrane components from *S. aureus*, such as coagulase, proteinase, staphylokinase, nuclease, lipase, catalase, and phosphatase, are also considered as virulent factors (Sutra and Poutrel, 1994).

Degradation of milk substrates by these enzymes provides required energy for bacteria metabolism (Anderson, 1976). Purified proteinases induced the detachment of bovine mammary epithelial cells *in vitro* (Zavizion et al., 1996). Moreover, coagulase alone has been shown to induce neutrophil migration and epithelial hyperplasia in mouse mammary glands (Anderson et al., 1982). However, the effect of coagulase on bovine mastitis has not been elucidated yet.

Expression of many of these staphylococcal exotoxins is modulated by a regulatory RNA molecule named "RNAIII". Two proteins, RNAIII-activating protein (RAP) and RNAIII-inhibiting protein (RIP), control the transcription of RNAIII (Balaban and Novick, 1995). RAP is continuously expressed by *S. aureus* and activates RNAIII only at a concentration threshold. Antibodies to RAP inhibited the activation of RNAIII, and in turn inhibited the synthesis of multiple staphylococcal exotoxins *in vitro* (Balaban et al. 1998). RIP, a 7-amino-acid peptide (YSPXTNF) produced by a non-pathogenic strain of *S. aureus* mutated by nitrosoguanidine competes with RAP for activation of RANIII and plays a suppressive role in production of virulent factors (Balaban and Novick, 1995). This suggests that all the virulent factors regulated by RNAIII transcription might be inhibited by blocking the autoinducers (RAP) or supplying RIP. A murine model of cutaneous infection was used to test whether immunization with RAP and administration of RIP can inhibit *S. aureus* infection. Compared to the control (100% infected), 72% of the mice immunized with RAP were not infected, and 90% of the mice remained free of disease when challenged with RIP pre-treated *S. aureus* (Balaban et al., 1998).

RAP and RIP reduced bacterial virulence by interfering with the signal transduction pathway that leads to production of toxins. In other words, they decrease the incidence

and severity of staphylococcus infections without interfering with bacteria growth. Therefore, *S. aureus* has less selective pressure to survive and mutate, which is beneficial to control this pathogen. In combination with currently available vaccines, antimicrobial therapies and natural host immune system, this new technology could be a potential approach to prevent bovine mastitis caused by *S. aureus*.

3.1.3. Interference with host immune system

The most crucial defense against *S. aureus* in the mammary gland is the phagocytosis of neutrophils. *S. aureus* failed to induce mastitis when milk somatic cell counts (SCC), dominantly neutrophils, was more than 6 X 10^5 (normally < 2 X 10^5) / ml (Postle et al., 1978). Another study also indicated that a higher pre-challenge SCC decreased the risk of *S. aureus* infection (Schukken et al., 2000). Neutrophils recognize the invading bacteria better through opsonization which is mediated by antibodies and C3b complement component (Peterson et al., 1978). To circumvent this fetal attack, a capsular polysaccharide (CP) is developed by *S. aureus* to interfere opsonization and phagocytosis. This makes *S. aureus* more resistant to the host immune system (Barrio et al., 2000, Hensen et al., 2000). It has been estimated that 94 to 100% of *S. aureus* isolated from mastitic cows are encapsulated (Norcross and Opdebeeck, 1983). Analysis of strains isolated from human infections showed that the capsules can be categorized into at least 11 different serotypes, and 70 to 80% of them belonged to T5 and T8 (Sompolinsky et al., 1985).

The capsular polysaccharides are carbohydrate polymers composed of repeating saccharide units. In the case of T5 and T8, each unit contains three monosaccharides: 2-

acetamido-2-deoxy-D-mannuronic acid, 2-acetamido-2-deoxy-L-fucose, and 2-acetamido-2-deoxy-D-fucose (Fournier et al., 1984; Moreau et al., 1990). They are different in the glycosidic linkage and the site of O-acetylation at the uronic acid moiety (Moreau et al., 1990). In contrast to the result from human, these two serotypes only accounted for 41% (T5 = 18%, T8 = 23%) of the strains isolated from cows with mastitis in the United States (Guidry et al., 1997). In a later report of Guidry et al. (1998), they further identified the rest of 59% belonged to another serotype, T336. Moreover, the distribution of serotypes varies geographically. In comparison with the distribution in the United States, percentage of serotypes from European samples were, T5 = 34%, T8 = 34%, T336 = 30%, and nontypable = 2% (Guidry et al., 1998).

One of the previous studies indicated that CP seems to impair bacterial adherence (Cifrian et al., 1994). However, the three serotypes, T5, T8, and T336, showed different abilities to adhere to bovine mammary cells (O'Brien et al., 2000). T8, expressing a flaccid type of CP, has larger exposure of cell wall and adheres to epithelial cells better than the other two isotypes. Furthermore, a bacterial surface protein, protein A, can bind to the Fc fragment of IgG, especially IgG_2 (Lindmark et al., 1983). This binding event in turn interferes the recognition of neutrophils toward the bacteria-antibody complex.

3.2. Vaccines against *S. aureus*

The attempts of developing vaccines to prevent *S. aureus* infections were emerged in late 19th century (reviewed by Anderson, 1978). Vaccines composed of virulent factors of *S. aureus* have been developed and evaluated. Due to the high cost of experimental cows, some of these studies only recorded the immunological and physiological responses after

vaccination without bacterial challenge. Others evaluated the efficacy of vaccines by either experimental challenges or field trials. A well-organized field trial is cost-, labor-, and time-consuming. On the other hand, the number of animals is usually limited in an experimental challenge, and this might cause statistical biases.

After reviewing the attempts implemented in the last two decades, as listed in **Table 1**, it can be concluded that the most of the early vaccines have either received poor responses or decreased prevalence and severity of mastitis only. Although some of the recent studies somehow showed positive results, the prevention of new IMIs caused by *S. aureus* has never been achieved efficiently. Rainard and Poutrel (1991) proposed three reasons to explain these results: (1) poor understanding of the immune system in the mammary gland (2) vaccines prepared *in vitro* did not contain the antigens expressed *in vivo* (3) inappropriate immunization schedules.

Table 1. Summary of recent evaluations of *S. aureus* vaccines

Antigen	Adjuvant	Type and Cow # (Vaccinated/Control)	Results	Reference
Killed Bacteria CP Extraction	N/A	Field trial (169/154)	Decreased IMI** Decreased SCC** Decreased loss of milk**	Yoshida et al., 1984
Protein A	FCA[1st], FICA[2nd]	Challenge (10/10)	Decreased IMI [NS] Decreased SCC** Increased milk yield [NS]	Pankey et al., 1985
Killed Bacteria Toxoids	FICA	Field trial (54/54)	Decreased IMI [NS] Decreased SCC [NS] Decreased severity of IMI**	Nordhaug et al., 1994
Killed Bacteria Toxoids	DXS+FICA	Field trial (887/932)	Decreased IMI [NS]	Watson et al., 1996
Killed Bacteria CP extraction	ALUM	Field trial (20/10)	Decreased IMI** Decreased SCC [NS]	Giraudo et al., 1997

Killed Bacteria CP Extraction	ALUM	Field trial (84/84)	Decreased IMI** Decreased SCC**	Calzolari et al., 1997
Bacterin	ALUM	Field trial (35/35)	Decreased IMI** Decreased SCC ^{NS}	Nickerson et al., 1999
FnBP	N/A	Challenge (60/60)	Decreased IMI**	Nickerson, 1999

NS=Non-significant **=P<0.05 **ALUM**=Aluminum Hydroxyl **FCA**=Freund's Complete Adjuvant **FICA**=Freund's Incomplete Adjuvant **DXS**=Dextran Sulfate

It has been suggested that the using capsular polysaccharides (CP) as antigens is essential to develop a successful vaccine against *S. aureus* (Relyveld, 1984). However, capsular polysaccharides are poorly immunogenic (Poolman, 1990). Injection of pure T5 CP failed to provoke an immune response in the milk, but CP (T5)-protein carrier conjugate did enhance antibody response (Gilbert et al., 1994). Based on these explorations, a new trivalent whole-cell vaccine was developed and currently under evaluation.

Phagocytosis is a critical function of macrophages and neutrophils to clear *S. aureus*, and is highly relied on the presence of opsonic antibodies in the milk (mainly IgG and IgM). Higher concentrations of serum antibodies are probably a prerequisite for more antibodies to be in the milk. However, enhanced antibody production is regulated by immune responses. Understanding the immune responses after vaccination and the transfer of antibodies from the blood to the mammary gland are undoubtedly pivotal to overcome this barrier and maximize the efficacy of vaccines for mastitis.

3.3. Kinetics of vaccination

3.3.1. Immune cells

The most important immune cells involved in vaccination are lymphocytes and antigen presenting cells (APCs). Lymphocytes matured in bone marrow and thymus are referred as B and T cells, respectively. Each of these B cells carries a membrane-bound antibody (immunoglobulin), which is composed of two heavy chains and two light chains. When the membrane-bound antibody encounters its specific antigen, B cells differentiate into B memory cells and plasma cells. The later is responsible for the subsequent antibody secretion (humoral response) in response to the cytokines produced by a group of T cells, T helper cells. On the other hand, T cells further develop to three categories, T suppressor cells, T helper cells (CD4+), and T cytotoxic Cells (CD8+), depending on the molecule (CD4 or CD8) associated with TCR (T cell receptor). T suppressor cells, not surprisingly, inhibit the activity of other immune cells in a negative-feedback fashion. However, the mechanism is poorly understood.

T cells can only recognize antigens through "antigen presentation" which is processed by antigen presenting cells (APCs) (Neefjes and Momburg, 1993). In other words, antigens must be degraded intracellularly into small fragments and combined with MHC (major histocompatibility complex) molecules in APCs. There are two different MHC molecules, class I MHC and class II MHC. Class I MHC molecules can be found in all of nucleated cells and complex with endogenous antigens. T cytotoxic cells (CD8+) react with this complex and, under the influence of cytokines produced by activated T helper cells, differentiate into cytotoxic T lymphocytes (CTLs) and T memory cells (cell-mediated or inflammatory response). CTLs are capable of eliminating altered self-cells,

like tumor cells, virus or intracellular pathogen-infected, and even cells of a foreign graft, by releasing hole-pouching "perforin" and several proteases called "granzymes". Alternatively, the elimination can be initiated by the interaction of Fas ligand on CTLs with the Fas receptor on the target cells. Both mechanisms activate the Caspase cascades and in turn induce the apoptosis of self-altered cells.

Class II MHC molecules only present in professional APCs, including macrophages, B cells, and dendritic cells. T helper cells react specifically to antigen-class II MHC molecule complex. This recognition, together with expression of co-stimulatory molecules on B cells (B7 family, CD40), leads to cytokine production from T helper cell, which is essential to activating B cell, T cytotoxic cells, macrophages, and other cells involved in the immune response. Different cytokines produced by T helper cells result in different types of immune response (Th1 or Th2) and antibody secretion.

3.3.2. Th1 and Th2 responses

T helper cells (CD4+) are further divided into two subsets, Th1 and Th2, depending on their functions and the cytokines they produce (Kim et al., 1985; Mosmann et al., 1986). Both of them are derived from the same precursor population of naïve T cells (Nakamura et al., 1997), and then differentiate into either one of the two types under the regulation of many factors, most importantly the cytokine environment. Interleukin-12 and IFN-γ released from other immune cells, such as nature killers (NKs) and macrophages, are considered to be the major cytokines promoting Th1-type responses. Interleukin-4 (IL-4) produced by basophils or mast cells is critical for Th2-type responses (Scott, 1993). It is also noteworthy that IL-2 is required for development of T helper

cells, but itself can not determine which type to be. Generally speaking, cell-mediated responses are triggered by Th1 cells restrictedly and humoral responses can be associated with both Th1 and Th2 (Constant and Bottomly, 1997).

Th1 cells were shown to produce IL-12, IFN-γ, and TNF-α, whereas IL-4, IL-5, and IL-10 are the main cytokines secreted by Th2 cells. Existence of a third population of T helper cells, assigned as Th0, was later reported (Firestein et al., 1989). However, this issue remains controversial. Th0 cells were found to have overlapping cytokine profiles (IFN-γ, IL-4, IL-10...) with the other two subsets, and some of them are actually antagonistic to each other (Paliard et al., 1988). For example, IFN-γ prevents development of IL-4-producing Th2 cells, whereas IFN-γ-producing Th1 cells being suppressed by IL-4. Because of this paradox, it was also proposed that Th0 cells are just mixed populations of CD4+ T cells (Openshaw et al., 1995).

3.3.3. Antibody secretion

An antibody molecule (immunoglobulins) is composed of four peptide chains, two heavy and two light chains, which are combined by disulfide bonds. The number and positions of these bonds are different from one isotype to another. The portion of antibody binding to antigens is called "Fab fragment". The other portion, Fc fragment, is usually recognized by phagocytic cells, like neutrophils. The isotype of an antibody is determined by five different heavy chains, γ, μ, α, ε, and δ, presenting for IgG, IgM, IgA, IgE, and IgD, respectively. The most abundant antibody (about 80%), IgG, can be further categorized into several isotypes, depending on the subclasses of γ heavy chain (IgG_1, IgG_2a, and IgG_2b in bovine species) (Butler et al., 1987). Initially, naïve B cells only

carry IgM and IgD on the surface. After antigen activation, B cells are able to synthesize all kinds of antibody. This isotype switching, without changing the antigen-specificity, is regulated by cytokines and B cell activators which result in DNA recombination of genes coding the heavy chains (Stavnezer, 1996). The production of antibodies is low and lagged at the initial vaccination (the primary response). As the number of memory lymphocytes is increased, the following boosters (the secondary response) usually induce higher and faster antibody production.

The four major isotypes of bovine immunoglobulins are IgG_1, IgG_2, IgM, and IgA. Binding of the antibody to its specific antigen can not only facilitate phagocytosis, but also slightly inhibit bacterial adherence to epithelial cells (Olmsted and Norcross, 1992). IgG_1 is the most abundant isotype and has a positive effect on macrophage phagocytosis (McGuire et al., 1979). Although milk macrophages are the predominant cell type in normal milk, they are usually sluggish and less effective than neutrophils in eliminating bacteria. Due to the lack of Fc fragment receptors on bovine neutrophils, IgG_1 does not show opsonic effects on neutrophil phagocytosis (Guidry et al., 1993). Receptors for the Fc fragment of IgG_2 and IgM have been identified on bovine neutrophils (Howard et al., 1980; Grewal et al., 1978), and IgM seems to be the most effective opsonin for neutrophils (Guidry et al., 1993). Because production of IgM is relatively low and transient, its effectiveness is limited. Under this circumstance, opsonic IgG_2 is expected to protect the immunized cows from invading bacteria. Nevertheless, other isotypes may still participate in neutralizing toxins and preventing adherence (Colditz and Watson, 1985; Norcross, 1991).

After vaccination, the cytokines released by T cells determine which isotype(s) of antibody to be produced by B cells. The major cytokine in Th2-type immune responses, IL-4, increases the generation IgG_1 and IgE, but suppresses that of IgG_2. In contrast, IFN-γ secreted in Th1-type immune responses strongly enhances the production of IgG_2 and decreases production of IgG_1 and IgE. Apparently, Th1-type immune responses are desired in immunized cows in terms of enhancing phagocytosis. The shift between Th1- and Th2-type immunity can be directly regulated by the adjuvant of a vaccine.

3.4. Adjuvants

An adjuvant is usually defined as materials included in the formulation of vaccines to increase the immunogenicity of antigens. The advantage of using adjuvants was discovered over 70 years ago (Glenny et al., 1926). When antigens were injected alone, the antibody response was very short. However, by mixing antigens with adjuvants, the response was prolonged significantly. Chedid (1985) proposed that adjuvants could increase the immunogenicity through the following concepts (1) formation of a depot of antigen at the site of injection for a longer period of release of the antigen (2) enhancement of presenting antigen to APCs (3) induction of desired cytokines which activate specific lymphocytes. The type of Th response (Th1 or Th2) can be directly altered by the adjuvant used, which is very crucial to the efficacy of a vaccine. However, some adjuvants may be toxic to the host.

The most common adjuvants used in veterinary vaccines are aluminum hydroxide (ALUM), dextran sulfate (DXS), Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FICA). ALUM is probably the safest adjuvant so far, but it only

stimulates weak Th2 response (Raz et al., 1996). FIA is an oil-in-water emulsion, and also generates only Th2 immune response (Forsthuber et al., 1996). FCA (FIA with dead mycobacteria) and DXS, on the other hand, do induce Th1-dominated response to the antigen (Watson, 1992; Forsthuber et al., 1996). However, the drawbacks of these adjuvants are their concomitant inflammatory side effects. Recombinant bovine cytokines, for example IFN-γ, were applied as adjuvants, but the results were not attractive (Pighetti and Sordillo, 1996).

Appropriately selected adjuvants not only enhance immune responses, but also determine the isotype of IgG produced. As mentioned previously, CPs are low in immunogenicity and T-cell independent. Therefore, there is no cell-mediated immunity, no memory response, and the antibody responses are short (Mond et al., 1995). This issue has been circumvented by immunizing CPs with protein carriers (or whole-cell vaccines). CP-protein conjugation improves the immunogenicity presumably due to the assistance from T cells activated by the antigenic carrier protein. However, a more effective adjuvant is required to maximize the desired immune responses.

4. Objectives of this research

Based on the literature review, we came up with different approaches to reduce bovine mastitis caused by *E. coli* and *S. aureus*. For *E. coli* mastitis, the approach is focused on the preventive effect of sCD14 administration. To avoid unnecessary expense, it would be a good idea to examine the effect of rbosCD14 administration on experimentally induced *E. coli* mastitis in a mouse model and to characterize endogenous sCD14 in bovine milk in advance. Therefore, our first three objectives were

(1) to investigate the effect of rbosCD14 administration on experimentally induced *E. coli* mastitis in a mouse model.

(2) to characterize factors affecting the level of sCD14 in milk, including stage of lactation, milk SCC, presence of bacteria or LPS-induced inflammation.

(3) to examine the outcome and possibly involved mechanisms of bovine mastitis induced by *E. coli*, with or without the presence of exogenous rbosCD14.

On the other hand, the approach for reducing *S. aureus* mastitis is focused on a newly developed trivanlent vaccine. Since lymphocytes play a pivotal role in response to vaccination, understanding how the vaccine affects functions of lymphocytes is important to improving the efficacy of vaccination. Thus, the last objective of our study was

(4) to monitor the effect of a trivanlent vaccine on immunity-associated responses , including antibody production, neutropjhil phagocytosis, lymphocyte subpopulations, and IFN-γ mRNA expression.

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CHAPTER II. RECOMBINANT BOVINE SOLUBLE CD14 REDUCES SEVERITY OF EXPERIMENTAL *ESCHERICHIA COLI* MASTITIS IN MICE

Jai-Wei Lee[1], Max J. Paape[2], Xin Zhao[1]

[1]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada

[2]Immunology and Disease Resistance Laboratory, USDA-ARS, Beltsville, MD-20901, USA

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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 lines of evidence show 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-wk 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 hours after injection for mice injected with either LPS or LPS plus rbosCD14 was 30 and 72 %, respectively ($P < 0.01$). At 48 hours 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 hours 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*.

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 (Haziot et al., 1988; Pugin et al., 1993; Paape et al., 1996). In addition, a soluble form of CD14 (sCD14) has been found in normal serum, urine, and milk (Bazil et al., 1986; Labéta et al., 2000; Filipp et al., 2001; Wang et al., 2002). Binding of LPS to mCD14 is upregulated by LPS-binding protein (LBP), an acute phase protein released by the liver during inflammation (Hailman et al., 1994). 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 (Martin, 2000). However, overwhelming production of TNF-α is the main causation of multiple organ failure and death as seen in "septic shock" (Waage et al., 1989).

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 (Haziot et al., 1994). Mice intraperitoneally challenged with LPS plus rhsCD14 had decreased fatality and blood TNF-α level compared to mice that received LPS alone (Haziot et al., 1995; Stelter et al., 1998). Moreover, LBP/sCD14 complexes bind LPS and transport it to high-density lipoprotein (HDL) (Wurfel et al., 1995). This process leads to detoxification of LPS in plasma. Presumably, sCD14 competes with mCD14 to interact with LPS and prevent 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 (Pugin et al., 1993), 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 general agreement that LPS is the key molecule involved in pathogenesis of mastitis induced by Gram-negative bacteria (Carroll et al., 1964). LPS is widely used to simulate *E. coli* infection in studying bovine mastitis (Paape et al., 1974; Shuster et al., 1993). The concentration of sCD14 in milk increases following intramammary injection of LPS (Lee et al., 2002). Recombinant bovine sCD14 (rbosCD14), previously cloned and expressed in our laboratory, sensitized bovine mammary glands to LPS and resulted in recruitment of neutrophils (Wang et al., 2002). 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, 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.

Materials and methods

Peritoneal challenge with LPS

Eighty-one 8-week-old female BALB/cj mice (17-22 g) (Jackson Laboratory, Bar Harbor, ME) 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). 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 (Wang et al., 2002). 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 hours for 3 days.

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 hours 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) were heated by a Bunsen burner and pulled horizontally to reduce the diameter to less than 75 µm. The micopipettes 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 (Bramley, 1976), and had been used in studies of *E. coli* mastitis in cows (Long et al., 2001) and mice (Anderson, 1978). Before challenge exposure, a tube of brain-heart infusion broth (Becton Dickinson, Cockeysville, MD) was inoculated with frozen *E. coli* and incubated for 18 hours at 37 ^{o}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 hours 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 (Nguyen et al., in press). 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 (Kerr et al., 2000). At 24 hours 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 hours 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 incubated for 18 hours 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).

Statistical analysis

Analysis of survival rate of mice after LPS challenge was made using GENMOD of SAS (SAS/STAT User's Guide, 2000). Comparisons of bacterial counts and TNF-α concentrations after intramammary infection with *E. coli* was made using PROC MIXED.

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Results

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 hours. All deaths occurred between 12 to 48 hours 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 hours after challenge, whereas only 30% of rbosCD14-treaed mice died during the same period ($P < 0.01$).

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 hours after challenge. There was more visible swelling of 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). Bacterial diagnostic 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).

Discussion

Mastitis is a major infectious disease of dairy cows and accounts for an annual $1.8 billion economic loss to the dairy industry in the United States (National Mastitis Council, 1996). 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 (Glauser et al., 1991). It is estimated that 80 to 90% of all coliform cases of mastitis become clinical, and that 10% of these cases result in death (Smith et al., 1985). 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 (Chandler, 1970). 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 (Chandler, 1970; Anderson, 1979). 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 (Haziot et al., 1995; Stelter et al., 1998). 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 (Ikeda et al., 1997), 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 (Haziot et al., 1995; Stelter et al., 1998),

compared to our study (37% at 48 hours 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 (Stelter et al., 1998). Also, differences due to age of the mice, source of LPS (Zughaier et al., 1999) and concentration of sCD14 probably contributed to differences among the studies in mortality.

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 (Dosogne et al., 2002). 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 (Cauwels et al., 1999). 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 (Jack et al., 1995; Dziarski et al., 1998), and together with LBP increase phagocytosis of serum-opsonized *E. coli* (Schiff et al., 1997).. However, LBP-mediated phagocytosis of *E. coli* was only slightly decreased after removal of mCD14 from the surface of rat alveolar macrophages (Klein et al., 2000). Further, binding and uptake of *E. coli* was not decreased by macrophages isolated from CD14-deficient mice (Moore et al., 2000). 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* (Haziot et al., 1996) or *Bacteroides fragilis* (Woltmann et al., 1999) 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 (Porat et al., 1991; Luo et al., 1993). 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 (Pugin et al., 1993; Lee and Zhao, 2000; Wang et al., 2002). Activation of

epithelial cells by sCD14/LPS complexes and recruitment of leukocytes was verified in a recent study in our laboratory (Wang et al., 2002). 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 (Shuster et al., 1996). In a previous study, the leukocyte count in CSF of *S. pneumoniae* challenged mice did not increase after administration of rhsCD14 (Cauwels et al., 1999), 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.

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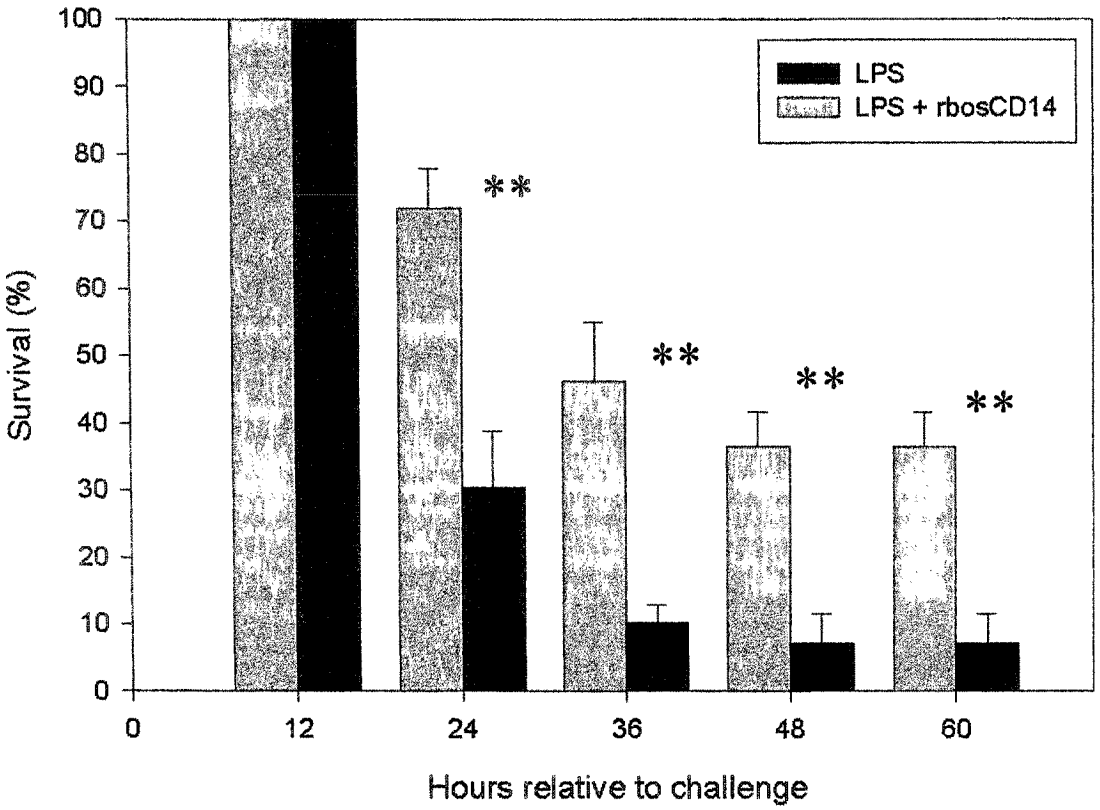

Figure 1. Effect of rbosCD14 on survival of mice injected intraperitoneally with LPS (8 μg/g of body weight, n = 41) or LPS plus rbosCD14 (6.8 μg/ g of body weight, n = 40). Results are presented as the mean and standard error of the mean from three experiments. * * ($P < 0.01$).

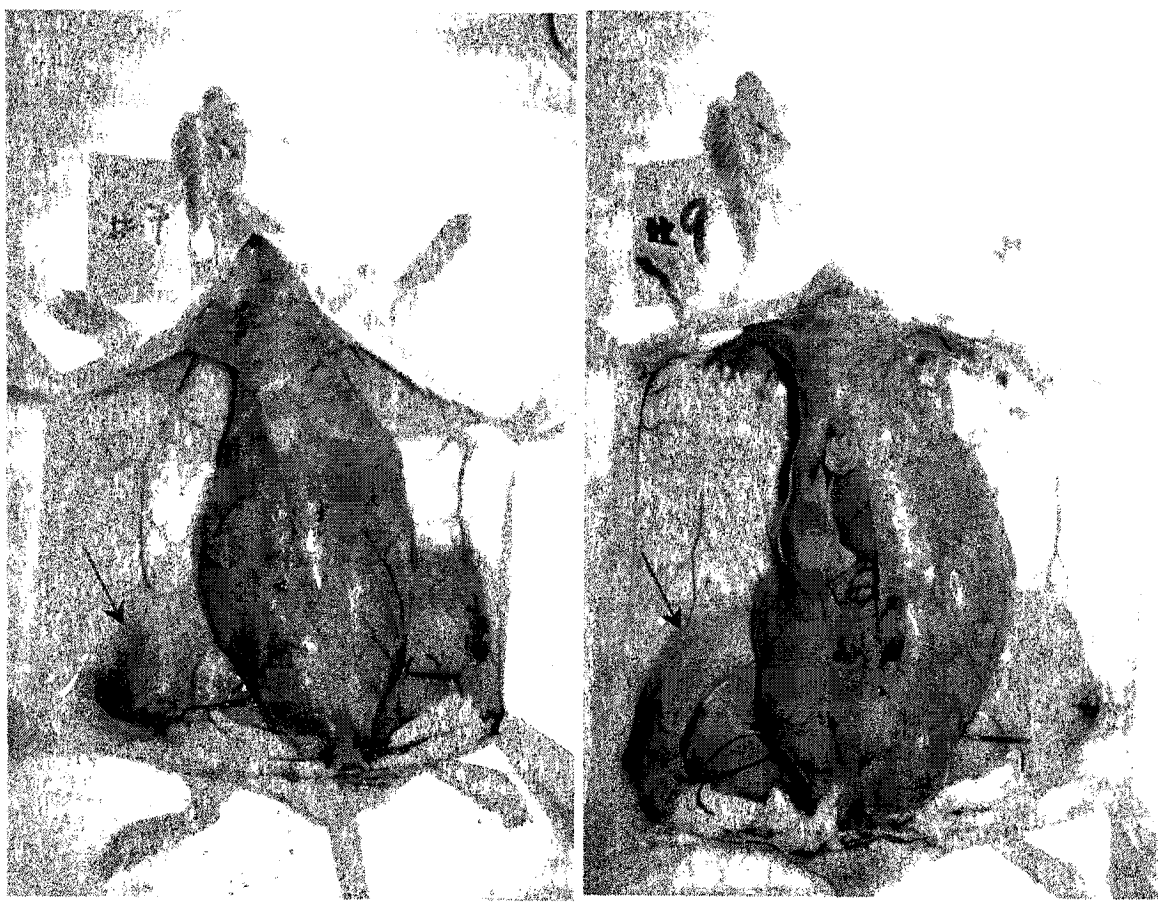

Figure 2. Appearance of abdominal mammary glands 24 hours after experimental challenge with *Escherichia coli* (10 to 20 CFU). The right abdominal glands (arrow) exhibited more severe clinical signs that included bloody exudation and dilated vessels compared to glands that received rbosCD14 (6 μg). Pictures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

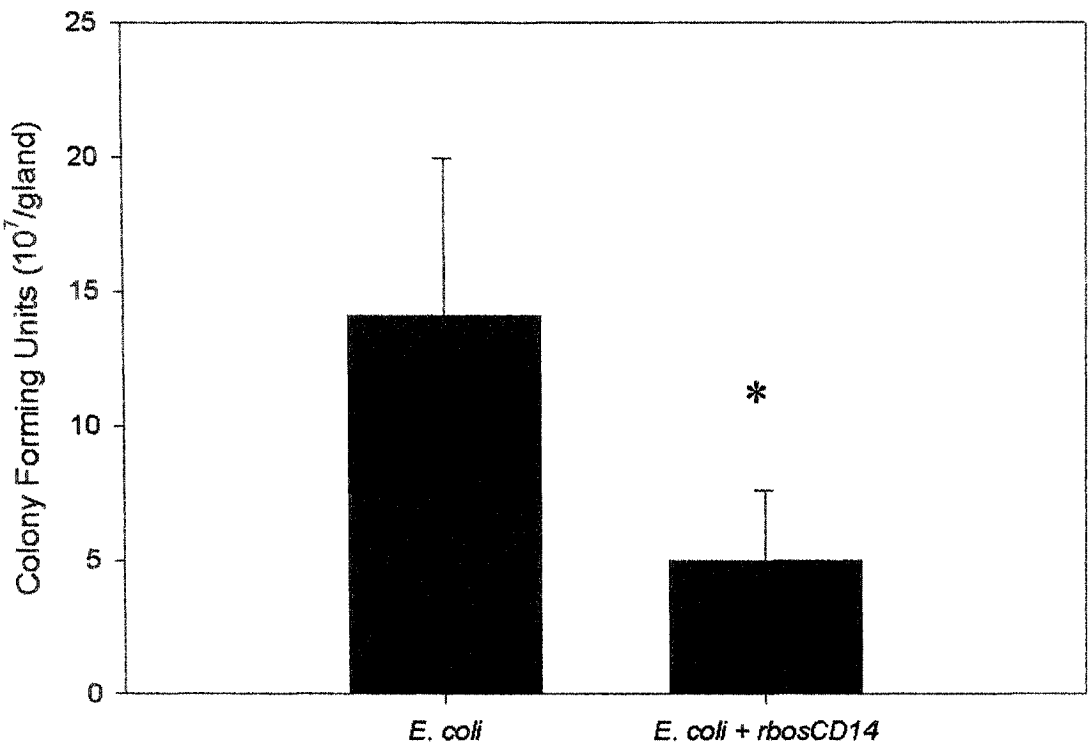

Figure 3. Effect of rbosCD14 on growth of *Escherichia coli* 24 hours after experimental infection of two abdominal mammary glands with either *E. coli* (10 to 20 CFU) or *E. coli* plus rbosCD14 (6 μg). Results are presented as the mean and standard error of the mean of 7 animals. * ($P < 0.05$).

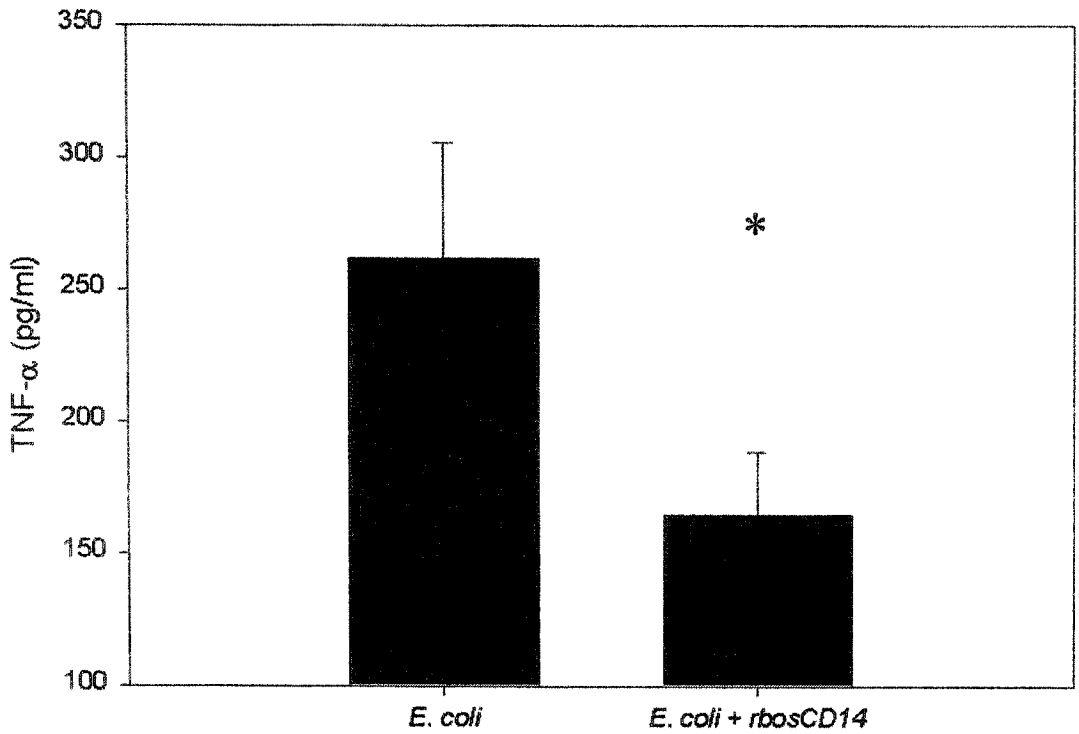

Figure 4. Concentration of TNF-α in mouse mammary glands homogenized in sterile PBS (1 ml PBS/100 mg of tissue) 24 hours after experimental infection of two abdominal mammary glands with either *E. coli* (10 to 20 CFU) or *E. coli* plus rbosCD14 (6 μg). Results are presented as the mean and standard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

CONNECTING STATEMENT I.

In Chapter II, the protective effect of rbosCD14 against Gram-negative bacteria infections was established. Administration of rbosCD14 significantly decreased the fatality of LPS-induced septic shock. In addition, by using a mouse *E. coli* mastitis model, rbosCD14 has been shown to reduce deleterious inflammatory responses, bacterial dissemination, and TNF-α production. This pioneering study confirmed the potential of using sCD14 to prevent bovine mastitis without costing a large amount of recombinant protein and associated expenses.

Before extending this experimental design to dairy cows, a better understanding about the endogenous sCD14 in bovine mammary glands is beneficial. In Chapter III, a competitive enzyme-linked immunosorbent assay was developed to quantify bovine sCD14. Factors affecting the level of sCD14 in milk, including stage of lactation, milk SCC, and presence of bacteria were characterized based on milk samples collected from 100 lactating cows. Moreover, the dynamics of sCD14 in milk during an intramammary LPS challenge was monitored.

CHAPTER III. ELEVATED MILK SOLUBLE CD14 IN BOVINE MAMMARY GLANDS CHALLENGED WITH *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE

J.-W. Lee[1], M. J. Paape[2], T. H. Elsasser[3], Xin Zhao[1]

[1]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec, H9X 3V9, Canada; and

[2]Immunology and Disease Resistance Laboratory and

[3]Growth Biology Laboratory, USDA-ARS, Beltsville, MD 20705

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Abstract

The purpose of this study was to determine whether soluble CD14 (sCD14) in milk was affected by stage of lactation, milk somatic cell count (SCC), presence of bacteria or lipopolysaccharide (LPS)-induced inflammation. Milk samples from 100 lactating cows (396 functional quarters) were assayed for sCD14 in milk to determine effects of stage of lactation, SCC and intramammary infection. The concentration of sCD14 was the highest in transitional milk (0-4 days postpartum). Most of the infected quarters (> 80%) were infected by coagulase-negative staphylococci and yeast. No difference was found between non-infected and infected quarters. One quarter of 6 healthy lactating cows was challenged with 100 μg lipopolysaccharide (LPS) in order to study the kinetics of sCD14 during an LPS-induced inflammation. Milk samples were collected at various intervals until 72 h after injection. Rectal temperature, milk tumor necrosis factor (TNF)-α, and interleukin-8 (IL-8) increased immediately after challenge. The increase in sCD14 paralleled the increase in SCC, peaked at 12 h and started to decline after 24 h. Serum leakage, as characterized by the level of bovine serum albumin (BSA) in milk, peaked at 4 h and then gradually decreased. All parameters remained at basal levels in control quarters throughout the study. *In vitro* experiments indicated that neutrophils released sCD14 in response to LPS, in a dose-dependent manner. The results indicate that the concentration of sCD14 was significantly increased in milk after LPS challenge. The increase was not likely due to serum leakage. Instead, infiltrated neutrophils might be the main source of increased sCD14 in milk during inflammation.

Introduction

Recognition of LPS by the innate immune system is critical to elicit inflammatory responses. The cellular receptor for interacting with LPS is CD14, a 53-55 kDa glycosylphosphotidylinositol (GPI)-anchored protein expressed on the membrane of monocytes/macrophages and neutrophils (Haziot et al., 1988; Wright et al., 1991; Paape et al., 1996). Although expression of CD14 on neutrophils is low (Ball et al., 1982), expression can be up-regulated by stimulation with cytokines (Buckle et al., 1990). A soluble form of CD14 (sCD14) was found in normal human serum, urine, and milk (Bazil et al., 1986; Labéta et al., 2000; Filipp et al., 2001). Shedding of mCD14 from monocytes and neutrophils results in sCD14 in body fluids (Bazil and Strominger, 1991; Haziot et al., 1993). The direct binding of LPS to CD14 is minimal and the presence of LPS-binding protein (LBP), an acute phase protein in serum, accelerates binding (Hailman et al., 1994). In response to LPS/LBP complexes, monocytes/macrophages release a spectrum of cytokines, including TNF-α, IL-1, IL-6, and IL-8, to initiate host defense (Martin, 2000). However, overwhelming production of TNF-α is the main causation of multiple organ failure and death as seen during "septic shock" (Waage et al., 1989).

Elevated serum concentration of sCD14 has been reported in patients with various diseases, including lupus erythematosus (Nockher et al., 1994), malaria (Wenisch et al., 1996), and tuberculosis (Juffermans et al., 1998). Increased sCD14 was also detected in the bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (Martin et al., 1997) and in cerebrospinal fluid (CSF) of patients with bacterial meningitis (Cauwels et al., 1999). The biological function of sCD14 has been extensively investigated. Soluble CD14 was shown to modulate humoral and cellular immune

responses by interacting with both B and T lymphocytes (Arias et al., 2000; Filipp et al., 2001). Recombinant human sCD14 was able to prevent LPS-induced septic shock and TNF-α production in mice (Haziot et al., 1994; Haziot et al., 1995; Stelter et al., 1998). Moreover, LBP has been shown to interact with sCD14 to rapidly acquire LPS and transport it to high-density lipoprotein (HDL) (Wurfel et al., 1995). This process leads to detoxification of LPS in plasma. Administration of recombinant bovine sCD14 (rbosCD14) decreased mortality of mice intraperitoneally challenged with LPS, and reduced severity of mastitis in mice after intramammary challenge with *Escherichia coli* (*E. coli*) (Lee et al., in press).

Soluble CD14 plays a crucial role in protecting the host from LPS challenge, or infection caused by Gram-negative bacteria. Mastitis caused by *E. coli* is a common management-related problem in dairy cattle, and causes a great deal of economic loss in the dairy industry. However, pathogenesis of *E. coli* and the response of the host immune system to this pathogen in the mammary gland are not well understood. There is general agreement that LPS, a cell wall component of Gram-negative bacteria, is the main pathogenic factor involved in infections by *E. coli* (Rietschel et al., 1994). Use of LPS to simulate *E. coli* intramammary infection is commonly used (Paape et al., 1974; Shuster et al., 1993). The mammary gland is a unique organ in terms of its biological function and structure. The existence of sCD14 in milk may protect dairy calves from gastrointestinal infections, and cows from mastitis. In the present study, we identified factors affecting concentration of sCD14 in milk, as well as the change in sCD14 in milk after intramammary challenge with LPS.

Material and methods

Animals and milk sample collection

Fore-milk samples were collected aseptically from 100 lactating Holstein cows at the Beltsville Agriculture Research Center before morning milking for determination of SCC, infection status and concentration of sCD14. Milk samples were categorized according to infection status (infected or non-infected), stage of lactation (transitional = 0-4 d, early = 5-99 d, mid = 100-200 d, or late = > 200 d), and level of SCC (low = < 250,000 cells/ml, medium = 250,000-750,000 cells/ml, or high = > 750,000 cells/ml). For endotoxin challenge, six clinically normal Holstein cows, in mid-lactation, were selected. Foremilk was aseptically collected 24 h before scheduled challenges. Selection criteria included noninfected mammary quarters and SCC less than 250,000 cells/ml milk. Use of animals for the LPS challenge study was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Intramammary challenge with LPS

One of the four mammary quarters was injected with 100 μg LPS (*E. coli* O111:B4) (Sigma, St. Louis, MO) in 10 ml sterile 0.85% normal saline immediately after the morning milking. One control quarter was injected with 10 ml of sterile saline. Rectal temperatures and foremilk samples were taken 0, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h relative to injection.

Bacterial culture

Immediately after sample collection, each sample was plated in a 0.05-ml volume onto Trypticase soy blood agar. Diagnostic bacteriology was performed according to established guidelines (Harmon et al., 1990).

Sample preparation

An aliquot (2 ml) of the milk sample was removed for SCC. The remaining sample was decanted into 40 ml round-bottom centrifuge tubes and centrifuged at 46,000 × g for 30 min at 4°C. After centrifugation, the fat layer was removed and the skimmed milk was carefully decanted into new centrifuge tubes followed by centrifugation at 46,000 × g for 30 min at 4°C. The clear whey was removed and stored in aliquots at –20°C.

Determination of SCC

Samples were heated for 15 min at 60°C and maintained at 40°C until being counted (Fossomatic 90: Foss Electric, Hellerod, Denmark). The cell counter was calibrated every month with bovine milk somatic cell standards (Dairy Quality Control Institiute Services, Mountain View, MN). Duplicate counts were made on each milk sample.

Competitive enzyme-linked immunosorbent assay (ELISA) for Soluble CD14

Recombinant bovine sCD14 (rbosCD14) was generated by transfected insect sf/9 cells as described (Wang et al., 2002). Flat-bottom 96-well plates (Immulon 2, Dynatech, Chantilly, VA) were coated with 100 µl of rbosCD14 (5 µg/ml) in carbonate/bicarbonate buffer (pH 9.6) at 4°C, overnight. The plates were blocked with 2 % gelatin (Sigma) in

PBS containing 0.05 % Tween 20 (PBS-T), 200 µl per well, at 37°C for 1 h. After 3 washes with PBS-T, standards (rbosCD14 serially diluted with PBS-T containing 0.2 % gelatin (PBS-TG)) or samples diluted 50 fold with PBS-TG were mixed with equal volumes of mouse anti-ovine CD14 (Serotec, Raleigh, NC) in PBS-TG (1:500) and added to each well (100 µl) in triplicate. The antibody cross-reacts with bovine CD14 (Wang et al., 2002). Plates were placed on a shaker at room temperature for 1 h followed by 3 washes. Then 100 µl of horseradish peroxidase-conjugated goat anti-mouse Ig secondary antibody (Promega, Madison, WI) at 1:10,000 dilution in PBS-TG was added to each well and the plates were placed on a shaker at room temperature for 1h. After 3 washes, peroxidase activity was assayed by adding 100 µl of ABTS substrate solution (Kirkgaard & Perry Laboratories, Gaithersburg, MD) and allowed for color development in the dark for 15 min. The absorbance was determined by a microplate reader at 405 nm and the concentration of sCD14 was calculated by referring to a standard curve.

ELISA for bovine serum albumin (BSA)

Flat-bottom 96-well plates were coated with 1 µg/ml rabbit anti-BSA mAb (Bethyl, Montgomery, TX) in carbonate/bicarbonate buffer (pH 9.6) at 4°C, overnight. The plates were blocked with 2% gelatin in PBS-T, 200 µl per well, at 37°C for 1 h. After 3 washes, standards and diluted samples were added in triplicates (100 µl/well), and incubated at room temperature for 1 h. The plates were washed 3 times and 100 µl HRP-conjugated sheep anti-BSA mAb (Bethyl) at 1:5000 in PBS-TG was added per well. At the end of 1 h incubation at room temperature, plates were washed 3 times and 100 µl/well of ABTS substrate solution was added. After 15 min, absorbance was determined with a microplate

reader at 405 nm and the concentration of BSA was calculated by referring to a standard curve.

Cytokine detection

The concentration of TNF-α in milk was measured by specific double antibody radioimmunoassay (RIA) as described (Kenison et al., 1990). Milk IL-8 was determined using a commercially available human IL-8 enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), which has been shown to cross-react with bovine IL-8 (Shuster et al., 1997).

Neutrophil isolation

Bovine neutrophils were isolated from whole blood taken from healthy Holstein cows as described (Carlson and Kaneko, 1973) with slight modifications. Heparin-anticoagulated blood was mixed with an equal volume of Ca^{2+}-free Hank's balanced salt solution (HBSS, GIBCO/BRL, Gaithersburg, MD) and added onto 20 ml Ficoll-Paque solution (1.077 g/ml, Amersham Biosciences, Piscataway, NJ) in 50 ml tubes. After centrifugation at 1,500 × g for 40 min at 4°C, the plasma, buffy coat and upper portion of the red blood cell layer were aspirated and discarded. Neutrophils were isolated by hypotonic lysis of remaining red blood cells with Tris-NH_4Cl (0.017 M Tris base; 0.144 M NH_4Cl). Ice-cold HBSS was added to restore isotonicity. Cell suspension was centrifuged at 1,000 × g for 10 min at 4°C and resuspended in HBSS (10^7 cells/ml). This procedure usually produced a cell fraction containing >95 % determined by differential

cell counting of 100 cells. Viability was >99 % as determined by trypan blue exclusion.

In vitro stimulation of neutrophils

LPS was suspended in sterile HBSS made with non-pyrogenic water and serially diluted to desired concentrations. Isolated neutrophils, 3×10^6 cells, were incubated with 500 µl of various dilutions of LPS at 37°C with slight agitation. After incubation for 3 h, cells were spun down at 500 × g for 10 min. The supernatant was collected and stored at -20°C until assayed for sCD14.

Statistical analysis

The data were analyzed by least squares means using the general linear models procedure of SAS (SAS Inst. Inc., Cary, NC) according to the model

$$Y_{ijklmn} = \mu + Pi + Dj + Cij_k + Q_l + S_m + I_n + D*S_{jm} + e_{ijklmn}$$

where

Y_{ijklmn} = dependent variable "concentration of sCD14",

μ = mean,

P_i = effect of parity (1, 2, 3, 4, 5, 6),

D_j = effect of stage of lactation (transitional, early, mid, late),

C_k = effect of cow (1, 2, 3, ..., 100)

Q_l = effect of quarter (left front, left rear, right front, right rear),

S_m = effect of SCC (low, medium, high),

I_n = effect of infection status (infected, non-infected),

$D*S_{jm}$ = effect of interaction of stage of lactation by SCC,

e_{ijklmn} = residual effect.

The interaction of SCC by infection status was initially included in the model, and was removed due to lack of significance. For the other parameters, data are presented as mean ± SEM and compared to the mean observed prior to intramammary challenge using the MIXED procedure of SAS.

Results

Measurement of sCD14 in milk

Based on milk samples collected from 100 lactating cows (396 functional quarters), the effect of status of infection, SCC, and stage of lactation on the concentration of sCD14 in milk are shown (Table 1). The majority of the infections were caused by coagulase-negative staphylococci (78 %, n = 59 quarters). Remaining infections consisted of yeast (n = 5 quarters), *Staphylococcus aureus* (n = 5 quarters) and coliforms (n = 4 quarters). There was no difference ($P > 0.05$) in the concentration of sCD14 between infected and non-infected quarters or among infections caused by different pathogens. Transitional milk, 0-4 d in milk (DIM), had the highest concentration of sCD14 (11.44 ± 1.03 μg/ml) in comparison with other stages of lactation ($P < 0.05$).

Intramammary challenge with LPS

In order to investigate the kinetics of sCD14 in milk during an LPS-induced inflammation, 100 μg LPS was injected into left front quarters of 6 mid-lactation cows. Rectal temperature increased in all challenged cows and peaked (41.3 ± 0.3 °C) at 6 h

postinjection. (Figure. 1A). The response of TNF-α increased 2 h postinjection ($P < 0.05$). Maximal TNF-α activity (27.51 ± 6.31 ng/ml) occurred 8 h postinjection ($P < 0.01$). The concentration of interleukin-8 increased between 2 to 6 h (Fig. 1C). Increases in TNF-α and interleukin-8 were not observed in control quarters injected with saline ($P > 0.05$).

Milk SCC increased dramatically in challenged quarters, and remained stable in control quarters (Figure 2A). The cell count rapidly increased at 4 h ($P < 0.05$) after injection and reached its peak between 8 to 24 h after injection. To ensure that no new intramammary infections occurred during the study, milk samples were plated on blood agar plates every 24 h, and all quarters remained free of infection throughout the study.

The concentration of BSA in milk was measured as an indicator of the breakdown in the blood/milk barrier. Compared to the basal concentration, milk BSA increased at 2 h ($P < 0.01$), peaked at 4h ($P < 0.001$), and gradually declined starting at 8 h post-injection (Figure 2B). Variation in the concentration of BSA in milk among cows was very small before LPS injection (0.194 ± 0.01 mg/ml). However, the variation became quite large (4.36 ± 1.92 mg/ml) at the peak, indicating that the response to LPS was very different among cows.

The basal concentration of sCD14 averaged 4.61 ± 0.59 μg/ml. All LPS challenged quarters showed significantly elevated concentrations of sCD14 at 8 h postinjection ($P < 0.05$) (Figure 2C). Milk sCD14 reached the highest concentration at 12 h (21.49 ± 3.5 μg/ml, $P < 0.001$) and started to decline at 48 h after challenge.

In vitro stimulation of neutrophils with LPS

An *in vitro* assay was carried out to determine if bovine neutrophils release sCD14 extracellularly in response to LPS. The data showed that LPS was able to induce the release of sCD14 from neutrophils in a dose-dependent manner (Figure 3). Lower doses of LPS only slightly increased the release of sCD14 from neutrophils. However, when 100 and 250 μg/ml of LPS were used, the release of sCD14 was increased 2.9 and 5.5 – fold, respectively ($P < 0.01$).

Discussion

CD14 is involved in a spectrum of biological and immunological responses. It has been well documented that mCD14 expressed on leukocytes is the key molecule in recognizing invading pathogens and eliciting a cascade of inflammatory responses. On the other hand, sCD14 has been shown to prevent death induced by "septic shock", inhibit dissemination of pathogens, stimulate lymphocyte proliferation, and facilitate phagocytosis of bacteria. Recently, it has been proposed that sCD14 in human milk plays a role in breast-feeding associated benefits, such as reduced gastrointestinal infections in infants (Labéta et al., 2000). Because sCD14 isolated from mouse, human, and bovine, are all biological functional in terms of activating murine B cells (Filipp et al., 2001), consumption of cow milk containing sCD14 could have a similar beneficial effect. We therefore developed an ELISA assay to determine the concentration of sCD14 in normal bovine milk, and study the factors that might affect the level of sCD14 in milk.

Based on a herd screening of 100 lactating cows (396 functional quarters), the average concentration of sCD14 in milk, from 320 non-infected quarters, was 6.67 ± 0.44

μg/ml (Table 1). This was lower than the concentration of sCD14 in human milk (14.84 ± 6.40 μg/ml) (Labéta et al., 2000). A higher level of sCD14 in human milk was also reported within the first week postpartum, which is in agreement with our finding. The reason responsible for this observation is unclear. Teleologically, increased sCD14 and immunoglobulins in transitional milk are indispensable in protecting the newborns from infections. Serum sCD14 may be transported into milk as a consequence of increased permeability of epithelium during colostrumgenesis and higher SCC (Miller et al., 1993).

Surprisingly, infected quarters did not have a higher level of sCD14 (6.59 ± 0.45 μg/ml, n = 76). All infections were subclinical, and more than 80% of the infections were caused by coagulase-negative staphylococci (78 %, n = 59) and yeast (6 %, n = 5) which have been reported to have lower SCC in comparison with other pathogens (Gonzalez-Rodriguez et al., 1995). Moreover, direct binding of sCD14 to live bacteria has been demonstrated (Jack et al., 1995). However, how the binding affects the measurement of sCD14 in milk needs further investigation. For the infected quarters, it is possible that sCD14 may be associated with bacteria and cannot be detected in the whey samples due to their removal by centrifugation. This could explain why some of the infected quarters even had lower sCD14 in comparison with uninfected quarters of the same cow.

Elevated serum concentration of sCD14 has been reported in patients with various diseases, including lupus erythematosus (Nockher et al., 1994), malaria (Wenisch et al., 1996), and tuberculosis (Juffermans et al., 1998). Markedly increased sCD14 was also detected in the bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (Martin et al., 1997) and the CSF from mice with bacterial meningitis (Cauwels et al., 1999). However, this study shows for the first time that the level of

sCD14 was elevated in milk during an intramammary inflammation (Fig. 2C). Possible sources of sCD14 in milk could be blood serum leakage and cells in the mammary gland. According to our results, the concentration of BSA in milk, an indicator of serum leakage, peaked between 2 to 6 h after challenge (Figure 2B). One the other hand, sCD14 in milk did not increase until 8 h postinjection. These results ruled out the possibility that elevated sCD14 in milk was due to the breakdown of the blood/milk barrier. Human mammary epithelial cells have been shown to produce sCD14 (Labéta et al., 2000). However, we failed to detect cultured bovine mammary epithelial cell-derived sCD14 at both transcriptional and secretory levels (data not shown). On the other hand, the increase in sCD14 after LPS injection paralleled the increase in SCC (Figure 2A), which consists of mostly (95 %) neutrophils (Paape et al., 1979). We therefore tested whether bovine neutrophils released sCD14 in response to LPS *in vitro*. Although the data show that LPS did induce the release of sCD14 from bovine PMN (Figure 3), the required doses of LPS to elicit a significant response seem much higher than the concentration found during *E. coli* mastitis, as implicated in a previous study (Dosogne et al., 2002). The lack of LPS-binding protein (LBP) in the *in vitro* assay may have contributed to the insensitivity of neutrophils to the LPS. LBP has been shown to increase the affinity of mCD14 toward LPS by 1000 fold (Hailman et al., 1994). However, our results confirm that bovine PMN are capable of releasing sCD14 into milk. Previous findings also support neutrophils as the origin of sCD14 in milk. First, both *de novo* synthesis and membrane expression of CD14 were found to be upregulated in neutrophils isolated from mice with bacterial meningitis (Cauwels et al., 1999). Second, the level of sCD14 was found to be strongly related to the concentration of neutrophils in the BAL from patients with acute lung

injury (Martin et al., 1997). Third, expression of mCD14 on bovine neutrophils isolated from blood, normal milk, and milk from LPS-challenged quarters were 3, 68, and 5 %, respectively (Paape et al., 1996). This implies that agents in milk or the migration process can up-regulate expression of mCD14 on neutrophils, and that LPS challenge induces the release of CD14. In addition, intracellular CD14 has been reported to be stored in the granules of neutrophils (Detmers et al., 1995; Paape et al., 1996; Rodeberg et al., 1997). Therefore, expression of mCD14 can be augmented in activated neutrophils through degranulation, and this in turns leads to release of sCD14. A direct stimulation of LPS was not able to increase the surface expression of mCD14 in bovine neutrophils (data not shown). However, inflammatory cytokines, such as IL-1 and IL-8, are capable of inducing bovine neutrophil degranulation (Lee *et el.*, unpublished results) and might play a role in the release of sCD14.

In the present study, we demonstrate that sCD14 exists in normal bovine milk, and averages 6.67 ± 0.44 μg/ml. The concentration is highest within a few days of parturition. During an intramammary LPS challenge, the increase in sCD14 parallels the increase in SCC, but not the increase in BSA. *In vitro* stimulation with LPS indicated that neutrophils are capable of releasing sCD14 and might contribute to elevated sCD14 in milk after LPS challenge.

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Categories	sCD14 (μg/ml)
Status of intramammary infection	
Non-infected (n=320)	6.67 ± 0.44^{a}
Infected (n=76)	6.59 ± 0.45^{a}
Level of SCC	
Low, < 250,000 cells/ml	6.51 ± 0.46^{a}
Medium, 250,000 – 750,000 cells/ml	6.52 ± 0.46^{a}
High, > 750,000 cells/ml	6.86 ± 0.46^{a}
Stage of lactation	
Transitional, 0-4 d	11.44 ± 1.03^{a}
Early, 5-100 d	5.24 ± 0.53^{b}
Mid, 101-200 d	4.81 ± 0.50^{b}
Late, > 200 d	5.02 ± 0.42^{b}

[a,b,c]Means in the same category with different superscript statistically differ ($P < 0.05$)

Table 1. Effect of intramammary infection, SCC and stage of lactation on concentration of soluble CD14 in milk.

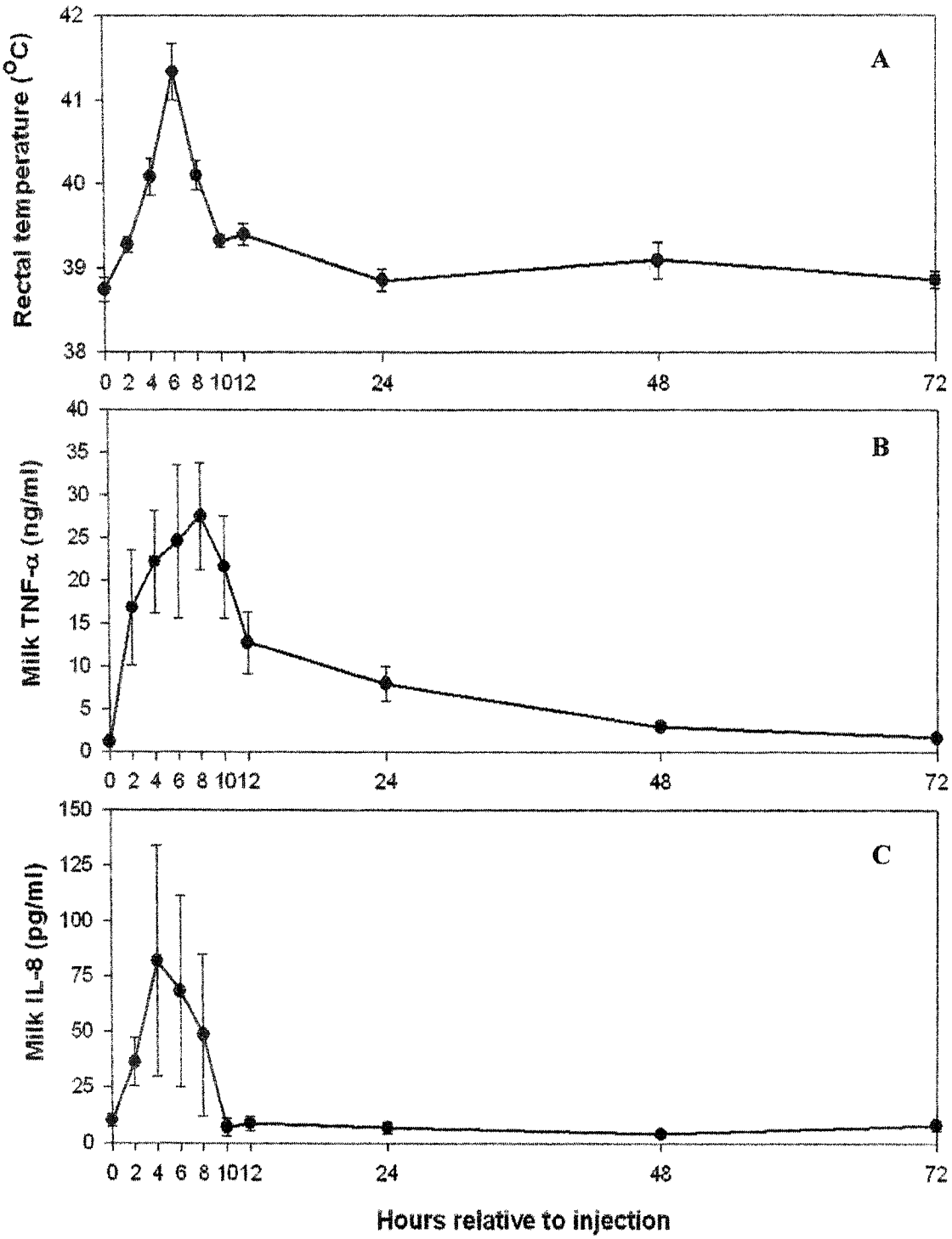

Figure 1. Rectal temperature (A), and concentration of TNF- α (B) and IL-8 (C) in milk during a LPS-induced intramammary inflammation. Left front quarters of six healthy cows were challenged with 100 μg LPS after milking (time = 0 h). Data are presented as the mean ± S.E.M.

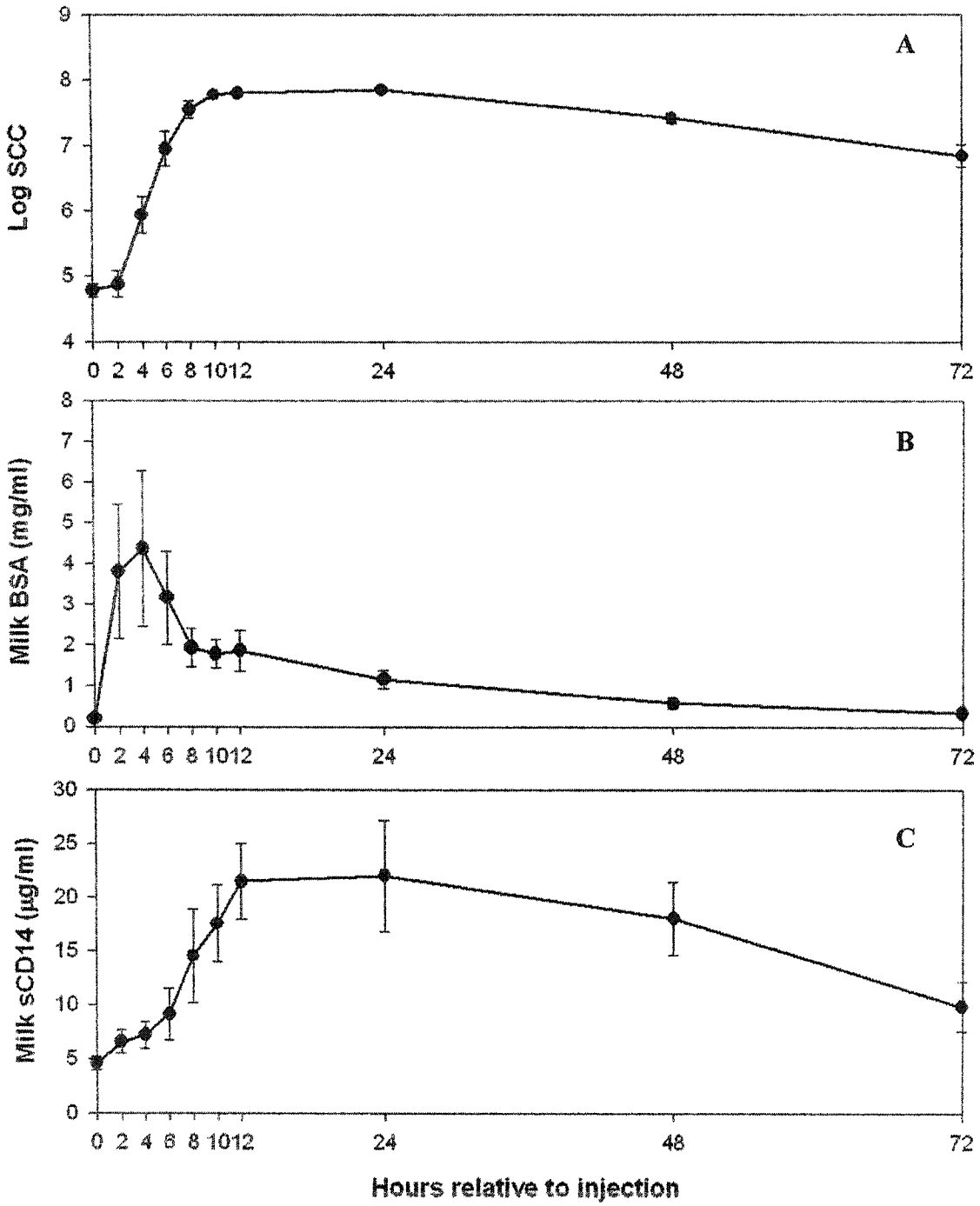

Figure 2. Concentration of somatic cells (A), BSA (B), and sCD14 (C) in milk during an LPS-induced intramammary inflammation. Left front quarters of six healthy cows were challenged with 100 µg LPS after milking (time = 0 h). Data are presented as the mean ± S.E.M.

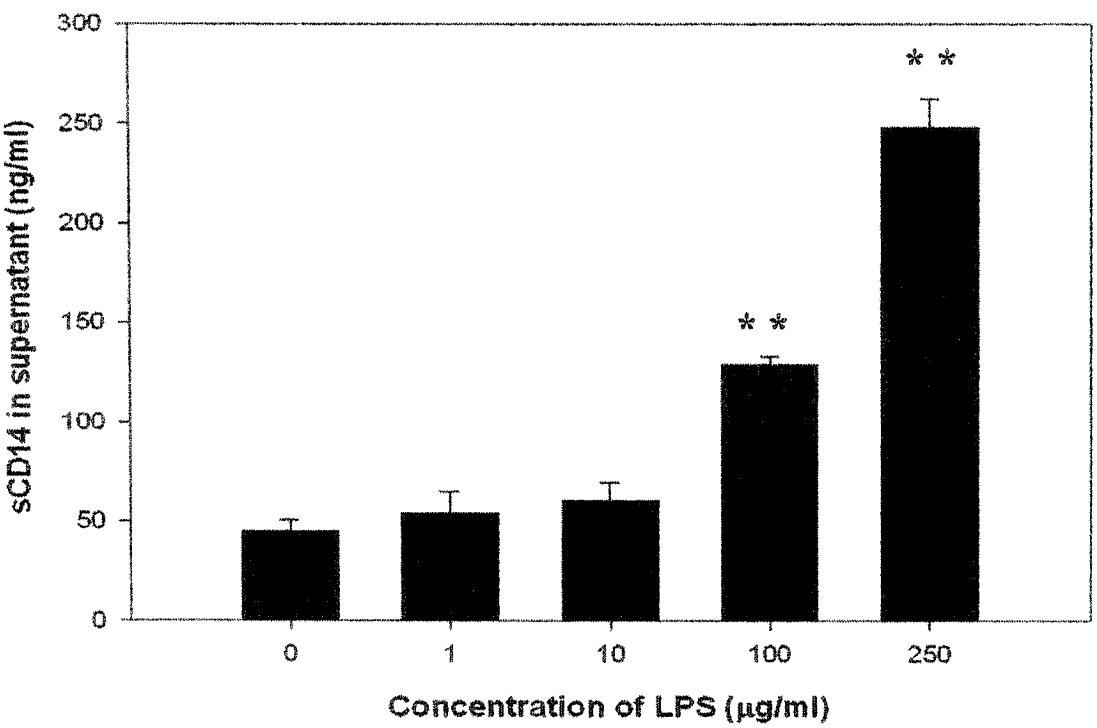

Figure 3. Effect of different concentrations of LPS on release of sCD14 from bovine neutrophils *in vitro*. Data are presented as the mean ± S.E.M. from three individual experiments. * * $P < 0.01$.

CONNECTING STATEMENT II

Results from Chapter III provided the basic information of endogenous sCD14 in bovine milk. The average concentration of milk sCD14, under normal conditions, was 6.67 ± 0.44 μg/ml and may increase up to 25 μg/ml during LPS-induced inflammation. Neutrophils recruited from the blood stream seemed to be the source of the increased sCD14 in milk during inflammation. In addition, the concentration of milk sCD14 was highest within a few days after parturition which indicating enriched sCD14 may play a role in protecting the newborns.

According to the results, we decided to examine the effect of administering 100 μg of rbosCD14 on intramammary LPS challenge and experimentally induced bovine *E. coli* mastitis. The challenge was performed in late-lactating cows after morning milking to minimize associated dilution factors. Although the protective effect of rbosCD14 has been demonstrated in a mouse *E. coli* mastitis model, due to the difficulty of collecting milk samples from mice, detailed cellular responses could not be monitored. This chapter undoubtedly provides additional information for the missing part.

CHAPTER IV. RECOMBINANT SOLUBLE CD14 REDUCES SEVERITY OF INTRAMAMMARY INFECTION BY *ESCHERICHIA COLI*

Jai-Wei Lee[1], Max J. Paape[2], Theodore H. Elsasser[3], and Xin Zhao[1]

[1]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada

[2]Immunology and Disease Resistance Laboratory, USDA-ARS, Beltsville, MD-20901, USA

[3]Growth Biology Laboratory, USDA-ARS, Beltsville, MD-20901, USA

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Abstract

The interaction among Gram-negative bacteria, the innate immune system, and soluble CD14 (sCD14) has not been well documented. Using a bovine intramammary *Escherichia coli* infection model, the effect of recombinant bovine soluble CD14 (rbosCD14) on milk somatic cell count (SCC), bacterial clearance and cytokine production was investigated. We first determined whether rbosCD14 would increase SCC during a lipopolysaccharide (LPS) challenge. Three quarters of each of 6 healthy lactating cows were injected with either 0.3 μg LPS, 0.3 μg LPS plus 100 μg rbosCD14 or saline. In comparison with quarters injected with LPS alone, the SCC was two fold higher ($P < 0.05$) in quarters injected with LPS plus rbosCD14 after challenge. We therefore hypothesized that when *E. coli* invade the mammary gland, sCD14 in milk would interact with LPS and rapidly recruit neutrophils from blood to eliminate bacteria before establishment of infection. To test this hypothesis, two quarters of each of 9 healthy cows were challenged with either 50 CFU *E. coli* plus saline or 50 CFU *E. coli* plus 100 μg rbosCD14. Quarters challenged with *E. coli* plus rbosCD14 had a more rapid recruitment of neutrophils, which was accompanied by a faster clearance of bacteria, reduced concentrations of TNF-α, IL-8 in milk and reduced clinical symptoms than challenged quarters injected with saline. Results indicate that increasing the concentration of sCD14 in milk might be a potential strategy to prevent or reduce severity of infection by coliform bacteria.

Introduction

Accumulated lines of evidence indicate that membrane CD14 (mCD14), a 53-55 kDa glycosylated phosphatidylinositol-anchored protein expressed on monocytes, macrophages and neutrophils is the cellular receptor for lipopolysaccharide (LPS) of Gram-negative bacteria (Haziot et al., 1988; Wright et al., 1991; Paape et al., 1996). A soluble form of CD14 (sCD14) is present in normal serum and milk (Bazil et al., 1986; Juffermans et al., 1998; Filipp et al., 2001; Wang et al., 2002). Shedding of mCD14 from the surface of leukocytes is the main source of sCD14 (Bazil and Strominger, 1991; Haziot et al., 1993). Patients with various infectious diseases or recovering from surgery have been shown to have an elevated sCD14 level in their plasma (Nockher et al., 1994; Wenisch et al., 1996; Juffermans et al., 1998; Niki et al., 2000). Increased sCD14 was also detected in the bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome (Martin et al., 1997), in cerebrospinal fluid (CSF) of patients with bacterial meningitis (Cauwels et al., 1999) and in milk from mammary glands challenged with LPS (J.-W. Lee, X. Zhao, and M. J. Paape, FASEB J., Abstracts Part I, 519.1, 2002).

The increase in sCD14 was suggested to be associated with the accumulation of neutrophils at inflammatory sites (Martin et al., 1997; Cauwels et al., 1999). However, the biological significance of this response has not been fully elucidated. The affinity of LPS for mCD14 is upregulated by LPS-binding protein (LBP), an acute phase protein released by the liver during inflammation (Hailman et al., 1994). In response to LPS/LBP complexes, monocytes and macrophages release a spectrum of cytokines, that include TNF-α, IL-1, IL-6 and IL-8 to initiate the immune response (Martin, 2000). However,

overwhelming production of TNF-α is responsible for deleterious inflammatory reactions and death caused by "septic shock" (Waage et al., 1989). Administration of sCD14 has been demonstrated to inhibit LPS-induced TNF-α production and decrease fatality in LPS challenged mice (Haziot et al., 1994; Haziot et al., 1995; Stelter et al., 1998). Moreover, acquisition of LBP by sCD14 has been shown to transport LPS to high-density lipoprotein (HDL) and lead to detoxification of LPS in plasma (Wurfel et al., 1995). Presumably, sCD14 competes with mCD14 for LPS to prevent activation of CD14-expressing immune cells. Moreover, enriched sCD14 in milk has been reported to act as a B cell mitogen and play a role in breast-feeding associated benefits, such as reduced gastrointestinal infections in infants (Labéta et al., 2000; Filipp et al., 2001). Taken together, it is postulated that increased concentration of sCD14 in body fluid may contribute to protection against infection by Gram-negative bacteria.

Escherichai coli is a common mastitis pathogen of dairy cows, and mastitis caused by *E. coli* represents a large economic loss to the dairy industry. We previously demonstrated that recombinant bovine (rbos) CD14 was able to reduce the severity of intramammary infection by *E. coli* in a mouse mastitis model (Lee et al., in press). However, cellular responses, such as recruitment of milk SCC induced by sCD14, were not monitored due to difficulty in collecting milk samples from mice. Because of the ease in obtaining milk samples from dairy cows, a bovine *E. coli* mastitis model is better suited for studying the role sCD14 plays in the pathogenesis of Gram-negative bacteria.

The objective of this study was to determine if intramammary administration of rbosCD14 to lactating dairy cows was able to reduce severity of infection after intramammary challenge with *E. coli*.

Materials and methods

Animals

Clinically normal Holstein cows in late-lactation, were selected for study. Selection criteria included noninfected mammary quarters with SCC less than 200,000/ml of milk at the time of LPS or bacterial challenge exposure. Use of animals for this investigation was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Intramammary challenge with LPS

Immediately after the morning milking, three quarters of each of six cows were injected with either 0.3 μg LPS (*E. coli* O111:B4, Sigma, St. Louis, MO, USA), 0.3 μg LPS plus 100 μg rbosCD14 or 0.3 μg LPS plus an equal volume (10 ml) of non-pyrogenic saline (Bio Whittaker, Walkersville, MD, USA). Milk samples were collected every 12 h after injection. The rbosCD14 was produced in a baculovirus expression system as described (Wang et al., 2002). Briefly, rbosCD14, with a deletion of 15 amino acids at the C-terminal end, was generated by insect sf/9 cells infected with a recombinant virus containing the gene. Endotoxin contamination of rbosCD14 was determined by a limulus ameobocyte lysate (LAL) assay (Biowhittaker, Walkersville, MD) and was found to contain less than 0.1 ng of endotoxin in 100 μg rbosCD14.

Preparation of bacteria

The organism used was a serum-resistant *E. coli,* strain P-4, serotype O32:H37, originally isolated from a clinical case of bovine mastitis (Bramley, 1976). This strain has been used in studies of coliform mastitis in cows (Long et al., 2001) and

mice (Anderson, 1978). Before challenge exposure, a tube of brain-heart infusion broth (Baltimore Biological laboratories, Division of Becton, Dickinson and Co., 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 at 37°C for 18 h. 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 transmittance of 80% at 610 nm (approximately 10^8 CFU/ml). Serial dilutions were made in non-pyrogenic 0.01 M phosphate buffered 0.85% saline, pH 7.4 (PBS) to approximately 1000 CFU/ml. The actual number of CFU injected (between 40 to 70 CFU) was confirmed by spreading 50 µl of the inoculum onto a blood agar plate and counting the number of CFU after overnight incubation at 37°C. The inoculum was further diluted to 100 CFU/ml and kept on ice until time of intramammary injection.

Intramammary challenge exposure

Three quarters of each of nine cows were injected with either 50 CFU of *E. coli* and 10 ml of saline, 50 CFU of *E. coli* and 100 µg rbosCD14 in 10 ml of saline or 100 µg rbosCD14 in 10 ml of saline. Bacterial challenge exposure in each cow was performed immediately after the morning milking, by injecting 10 ml of non-pyrogenic saline with or without 100 µg rbosCD14 followed by injection of a 0.5 ml volume of the prepared

inculum (100 CFU/ml) into the gland sinus. Milk samples were collected aseptically at 0, 6, 12, 16, 20, 24, 48, and 72 h relative to challenge.

Bacteriology

Bacteriology was carried out by spreading 40 µl of the diluted milk sample onto 5% blood agar plates. After 18 h of incubation at 37°C, the number of CFU on each plate was numerated and multiplied by the dilution factor. Gram-staining and observation of colony morphology were also conducted for identification of *E. coli*.

Determination of SCC

A 2 ml aliquot of milk was removed and heated for 15 min at 60°C and maintained at 40°C until being counted (Fossomatic 90; Foss Electric, HellerNd, Denmark). The cell counter was calibrated four times a year with bovine milk somatic cell standards (Dairy Quality Control Institute Services, Mountain View, MN, USA). Duplicate counts were made on each milk sample.

Cytokine detection

Milk samples were poured into 40 ml round-bottom centrifuge tubes and centrifuged at 46,000 × g for 30 min at 4°C. After centrifugation, the fat layer was removed and the skimmed milk was carefully decanted into new centrifuge tubes followed by centrifugation at 46,000 × g for 30 min at 4°C. The clear whey was collected and stored in aliquots at –20°C.

The concentration of TNF-α in milk was measured by specific double antibody radioimmunoassay as described (Kenison et al., 1990). Milk IL-8 was determined by using a commercially available human IL-8 enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA), which cross-reacts with bovine IL-8 (shuster et al., 1997).

Statistical analysis

Comparison of the trends in SCC after LPS injection was conducted using PROC MIXED. Data analysis of infection rate of quarters after *E. coli* challenge was performed using GENMOD of SAS (SAS/STAT User's Guide, 2000). Changes in the concentrations of CFU, SCC, Il-8 and TNF-α after intramammary challenge with *E. coli* was made using PROC MIXED.

Results

Effect of rbosCD14 on milk SCC after intramammary injection of LPS

Milk SCC in quarters injected with LPS plus 100 μg rbosCD14 was higher than in quarters injected with LPS and saline and averaged 16.1 ± 1.9 and 7.6 ± 1.5 × 10^6/ml, respectively (Fig. 1). However, due to the large variation in SCC among cows, the difference was only significant ($P < 0.05$) at 24 h after injection. Counts for saline injected quarters remained unchanged throughout the study.

Effect of rbosCD14 on clinical symptoms, SCC and intramammary infection after experimental challenge with *E. coli*

Quarters injected with rbosCD14 and challenged with *E. coli* had reduced clinical symptoms, such as mammary swelling and abnormal milk, compared to *E. coli* challenged quarters injected with saline, where clinical symptoms became noticeable at 12 to 24 h (Fig. 2). Only 2 of the 9 rbosCD14 injected quarters challenged with *E. coli* showed moderate swelling. All of the challenged quarters injected with saline exhibited severe swelling and abnormal milk.

Milk SCC for quarters challenged with *E. coli* and injected with either rbosCD14 or saline increased 6 and 16 h after challenge, respectively ($P < 0.05$) (Fig. 3). At 6 h SCC for rbosCD14 and saline injected quarters averaged 6.74 ± 2.04 and $0.51 \pm 0.08 \times 10^6$/ml milk ($P < 0.05$), respectively. There was no change in milk SCC throughout the study for control quarters injected with rbosCD14.

The average CFU in milk from quarters challenged with *E. coli* and injected with rbosCD14 was approximately 10 times less than CFU in milk from quarters challenged with *E. coli* and injected with saline (Fig. 4A). However, because of the large variation in CFU in milk among animals, differences were not statistically significant ($P > 0.05$). Administration of rbosCD14 facilitated clearance of *E. coli* from the gland (Fig. 4B). At 24 h after bacterial challenge, only 2 of 9 quarters (22%) challenged with *E. coli* and injected with rbosCD14 remained infected. For challenged quarters injected with saline 6 of 9 quarters (67%) remained infected.

The concentration of TNF-α in milk was lower in quarters challenged with *E. coli* and injected with rbosCD14 than challenged quarters injected with saline (Fig. 5A).

Although production of TNF-α was not completely inhibited in quarters injected with rbosCD14, the peak concentration at 16 h was less than quarters injected with saline, averaging 3.25 ± 0.80 and 0.80 ± 0.21 ng/ml of milk, respectively ($P < 0.05$).

Concentrations of IL-8 in milk from challenged quarters injected with saline were higher ($P < 0.05$) than challenged quarters injected with rbosCD14 (Fig. 5B). Concentrations of IL-8 in milk from challenged quarters injected with saline or rbosCD14 peaked at 16 h and averaged 266.21 ± 71.75 and 99.20 ± 56.84 pg/ml of milk ($P < 0.05$), respectively.

Discussion

Lipopolysaccharide is the major molecule responsible for the pathogenesis of infections caused by Gram-negative bacteria. Since the discovery that CD14 is the cellular receptor for LPS, numerous studies have been carried out to better understand how CD14 interacts with LPS to modulate immune responses. Recombinant human sCD14 (rhsCD14) has been shown to reduce mortality in mice challenged with LPS, that was attributed to reduced production of circulating TNF-α (Haziot et al., 1995; Stelter et al., 1998). In our laboratory, rbosCD14 was cloned and produced using sf-9 cells in a baculovirus expression system (Wang et al., 2002). Its protective effect against infection by *E. coli* was demonstrated in a mouse mastitis model (Lee et al., in press). However, due to difficulty in collecting milk samples from mice, we were not able to address mechanisms that contributed to the protective role of rbosCD14. In the present study, a bovine mastitis model was used that allowed for the collection of frequent milk samples.

This model enabled us to elucidate the protective effect of sCD14 following experimentally induced *E. coli* intramammary infection.

Neutrophils serve as the first immunological defense against invading bacteria. An early recruitment of neutrophils is crucial to clearance of bacteria and resolution of infection (Burvenich et al., 1994; Shuster et al., 1996; Seiler et al., 2000). In noninfected bovine mammary glands, the predominate cell types are macrophages (35-79 %) and neutrophils (3-26%) (Lee et al., 1980; Miller et al., 1993). After intramammary injection of either LPS or *E. coli*, the percentage of neutrophils in milk increase to greater than 95% (Paape et al., 1974; Shuster et al., 1996). Similar results were reported in mice intraperitoneally challenged with *Salmonella* (Yang et al., 2002). In the present study, intramammary injection of LPS together with rbosCD14 resulted in a two-fold increase in milk SCC compared to quarters injected with LPS and saline. This is in agreement with one of our previous studies demonstrating that rbosCD14 sensitizes the mammary gland to LPS in terms of recruiting SCC (Wang et al., 2002). Based on this result, a second study was initiated to determine if rbosCD14 could induce a similar effect and reduce severity of infection after intramammary injection of *E. coli*. We were able to demonstrate that intramammary administration of 100 μg rbosCD14 together with 50 CFU of *E. coli* initiated an early increase of milk SCC and a rapid clearance of bacteria. As a result, clinical symptoms, numbers of bacteria, milk SCC and peak production of the inflammatory cytokines TNF-α and IL-8 were reduced in rbosCD14 treated mammary glands.

In the absence of rbosCD14, inoculation of 50 CFU *E. coli* did not significantly increase SCC until 16 h after the challenge. One the other hand, quarters

received 100 μg rbosCD14 upon challenge showed significantly elevated SCC after 6 h. The protective effect associated with this early recruitment of neutrophils was supported by results from bacteriology. At 24 h after the challenge, only 2 out of 9 (22 %) quarters were still infected, in comparison with 6 out of 9 (67 %) in quarters challenged with *E. coli* only. Since addition of rbosCD14 into milk inoculated with *E. coli* did not change the rate of growth (data not shown), newly recruited neutrophils induced by rbosCD14 may have contributed to reduction in bacterial load.

The interaction among CD14, bacteria, and neutrophils has been investigated by means of three different approaches. First, using genetically knock-out animals. CD14-deficient mice were shown to have a delayed neutrophil influx in response to a peritoneal *Salmonella* challenge, which is associated with a reduced level of TNF-α (Yang et al., 2002). Contrarily, recruitment of neutrophils and bacterial clearance were enhanced in CD14 knock-out mice intraperitoneally challenged with *E. coli* (Haziot et al., 1996; Haziot et al., 2001) or *Bacteroides fragilis* (Woltmann et al., 1999). Second, blockade of CD14. Blockade of CD14 with anti-CD14 mAb in rabbits with *E. coli* pneumonia did not alter TNF-α, IL-8, and number of leukocytes in plasma and BAL. Although deleterious systemic responses were prevented, the dissemination of bacteria was increased by the blockade of CD14 (Frevert et al., 2000). Third, using exogenous sCD14. We have recently shown that administration of rbosCD14 was able to reduce clinical signs, bacterial load, and TNF-α production in a mouse *E. coli* mastitis model (Lee et al., in press). Conversely, in a mouse meningitis model, providing rhsCD14 increased both growth of *Streptococcus pneumoniae* and concentration of TNF-α in cerebrospinal fluid (CSF) of challenged mice (Cauwels et al., 1999). In contrast to our finding, leukocyte

counts in the CSF of these mice did not increase by administration of rhsCD14, thus the clearance of bacteria may have been hampered. It has been reported that monocytes deploy different intracellular pathways upon interacting with Gram-positive and Gram-negative bacteria through mCD14 (Rabehi et al., 2001). For example, blocking the p38 mitogen-activated protein (MAP) pathway only inhibited TNF-α production in monocytes stimulated with LPS, but not *Staphylococcus aureus*. Moreover, challenge with *S. aureus* increased the serum TNF-α level in CD14-deficient mice in comparison with control mice (Haziot et al., 1999). Therefore, the protective effect of sCD14 might be specific to Gram-negative bacteria.

In theory, using CD14-deficient mice and blockade of CD14 by mAb are similar in terms of removing or attenuating the cascade initiated by both mCD14 and sCD14. Administration of sCD14 minimizes the activation mediated by mCD14 by shifting immune responses to be sCD14-mediated. It is known that sCD14/LPS complexes are able to activate epithelial cells *in vitro* to secrete IL-8, a potent chemoattractant of neutrophils (Pugin et al., 1993; Lee and Zhao, 2000; Wang et al., 2002). However, in our study, the production of IL-8 was decreased in quarters challenged with *E. coli* and injected with rbosCD14. In addition to IL-8, important chemoattractants for bovine neutrophils include IL-1, IL-2, IL-6, leukotriene B_4 and complement components C5a and C3a (Paape et al., 2002). After intramammary challenge with *E.* coli the increase in C5a preceded that of IL-8, IL-1 and IL-6 (Shuster et al., 1997). Thus, C5a might be involved in the more rapid milk SCC in challenged quarters injected with rbosCD14. In addition, a novel pathway which is not mediated by CD14 and TLR-4, has been shown to efficiently attract neutrophils when triggered by LPS (Haziot et al., 2001). Both CD-14

and TLR-4 deficient mice intraperitoneally challenged with *E. coli* resulted in an early recruitment of neutrophils and a rapid clearance of bacteria compared with control mice. The authors suggested that activation of mCD14/TRL-4 actually interferes with this pathway. Therefore, it is possible that binding of LPS by exogenous rbosCD14 minimized activation of mCD14/TRL-4 on leukocytes, which had a positive effect on this novel pathway. Further investigation is required to identify the agent responsible for the prompt increase of milk SCC observed in these quarters.

The role of TNF-α in regulating immune responses is a double-edged sword and remains controversial. Too high a concentration of TNF-α leads to deleterious inflammatory reactions and death as seen in "septic shock" (Waage et al., 1989). On the other hand, reduction of TNF-α has been shown to be responsible for impaired neutrophil influx and bacteria clearance (yang et al., 2002). Although TNF-α is not a potential chemoattractant for neutrophils, it is capable of priming neutrophils to have enhanced expression of adhesion molecules that result in increased migration (Ferrante et al., 1988; Drost and MacNee, 2002). Nevertheless, the early milk SCC increase in mammary glands challenged with *E. coli* and rbosCD14 in the present study was not attributed to production of TNF-α.

In summary, intramammary injection of rbosCD14 together with *E. coli* initiated a more rapid increase in milk SCC that resulted in reduced numbers of *E. coli*, lower concentrations of TNF-α and IL-8 and a decreased inflammatory response when compared to *E. coli* and saline injected mammary glands. Results suggest that an early recruitment of neutrophils is critical to clearance of *E. coli* and contribute to the

protective effect rbosCD14. This strategy may be fundamental to minimizing the impact of infections caused by Gram-negative bacteria.

Acknowledgments

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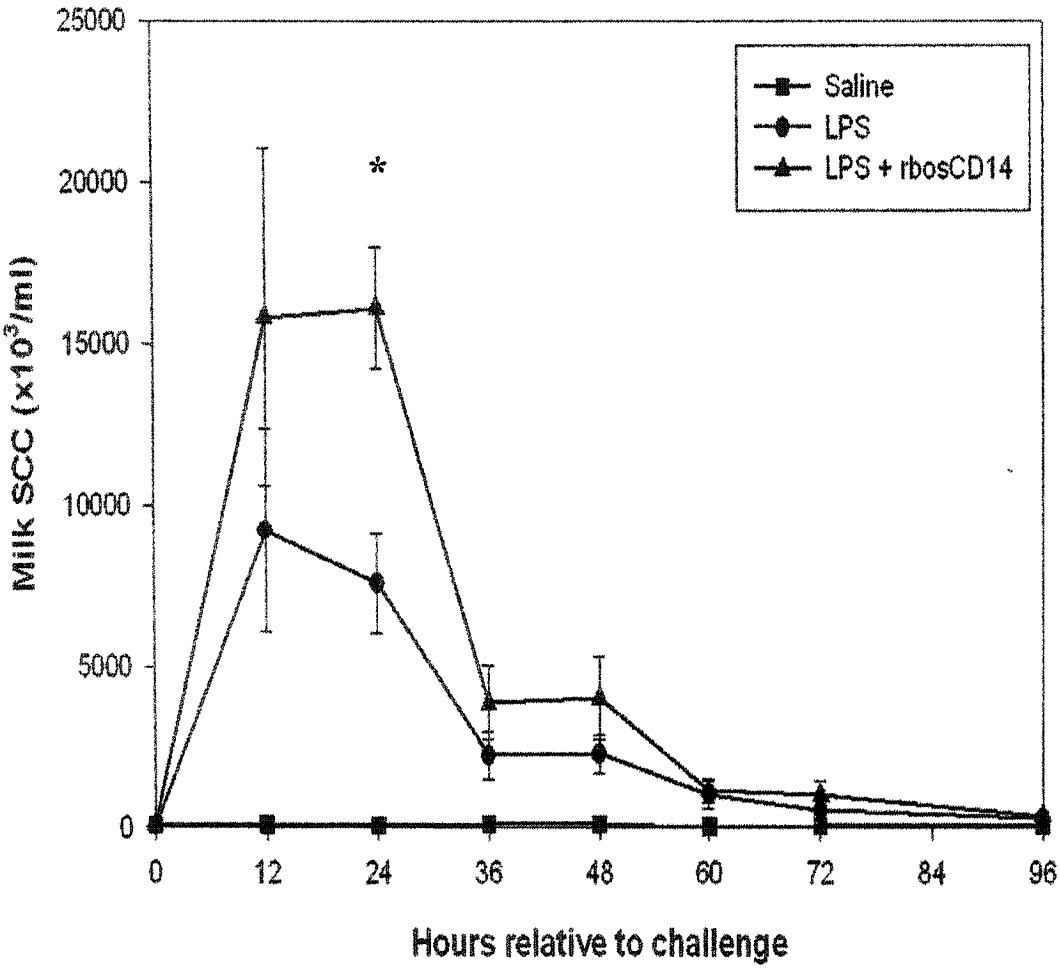

Figure 1. Milk somatic cell counts during a LPS induced intramammary challenge. Three glands of each cow were challenged with either saline (■), 0.3 μg LPS plus saline (●), or 0.3 μg LPS plus 100 μg rbosCD14 (▲). Data are presented as the means ± standard errors of the means of six cows. *, $P < 0.05$.

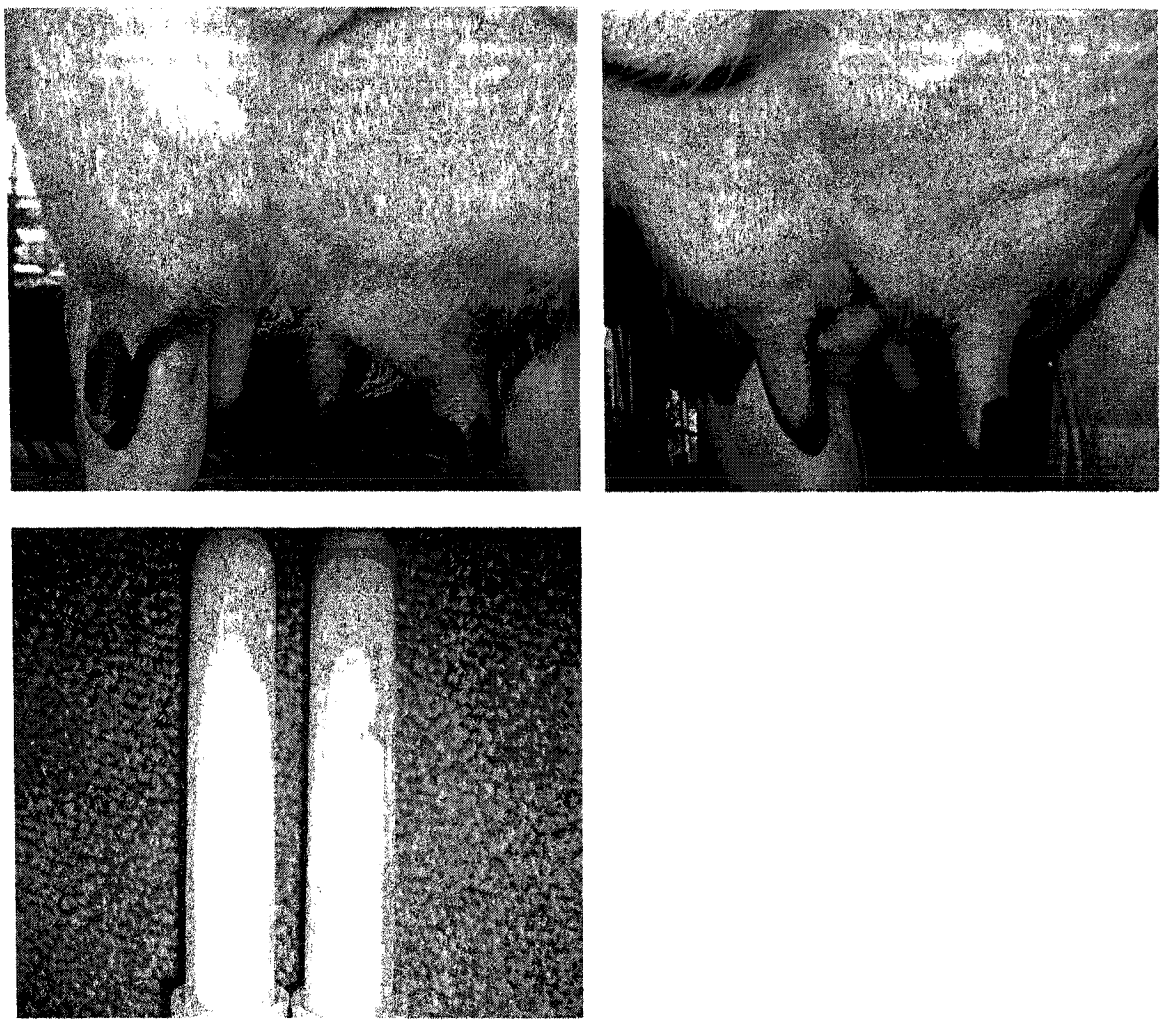

Figure 2. Appearance of mammary glands 24 h after experimental challenge with 50 CFU *E. coli* (A and B). The right glands exhibited more severe clinical symptoms that included swelling, stiffness, and redness compared to the left glands that received 100 μg rbosCD14. Pictures were randomly selected from among pictures of nine challenged animals. (C) Appearance of milk samples. The glands challenged with *E. coli* only produced yellowish and clumpy milk (right), in comparison with normal appearing milk produced by glands that received *E. coli* plus rbosCD14 (left).

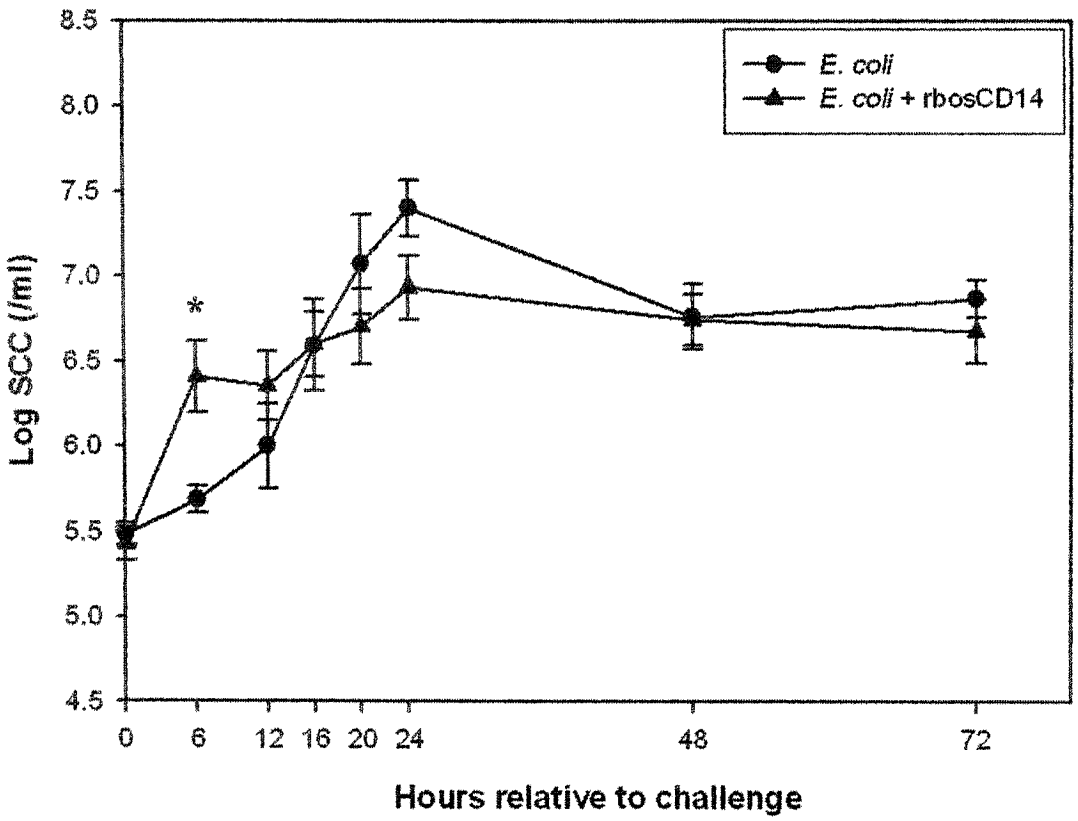

Figure 3. Milk somatic cell response during experimentally induced intramammary *E. coli* infection. Two glands of each cow were challenged with 50 CFU *E. coli* with (▲) or without (●) 100 μg rbosCD14. Data are presented as the means ± standard errors of the means of nine cows. *, $P < 0.05$.

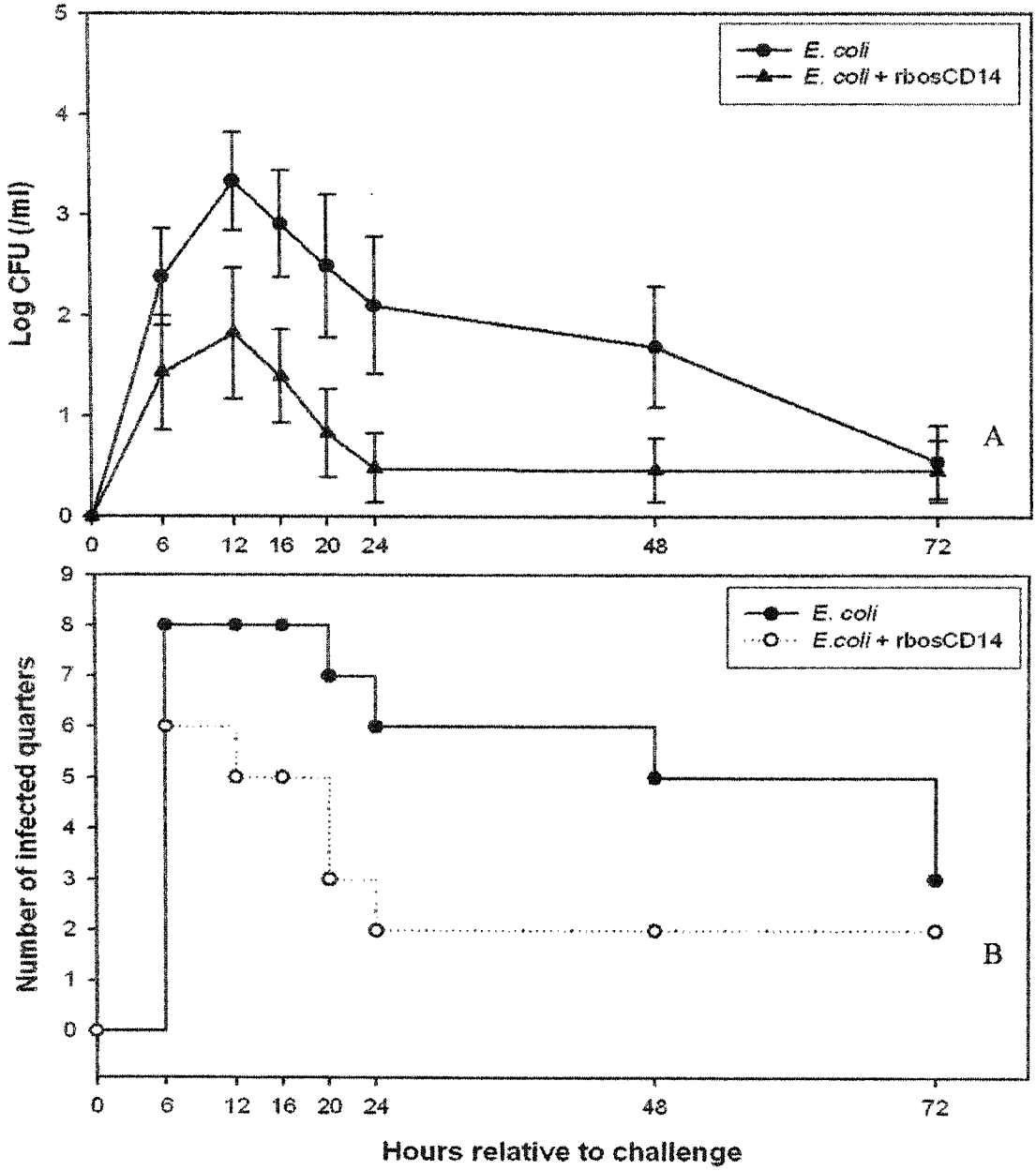

Figure 4. (A) Bacteria counts (CFU) in milk during an experimentally induced intramammary *E. coli* infection. Two glands of each cow were challenged with 50 CFU *E. coli*, with (▲) or without (●) 100 µg rbosCD14. Data are presented as the means ± standard errors of the means of nine cows. (B) The number of infected quarters after *E. coli* challenge, with (o) or without (●) 100 µg rbosCD14.

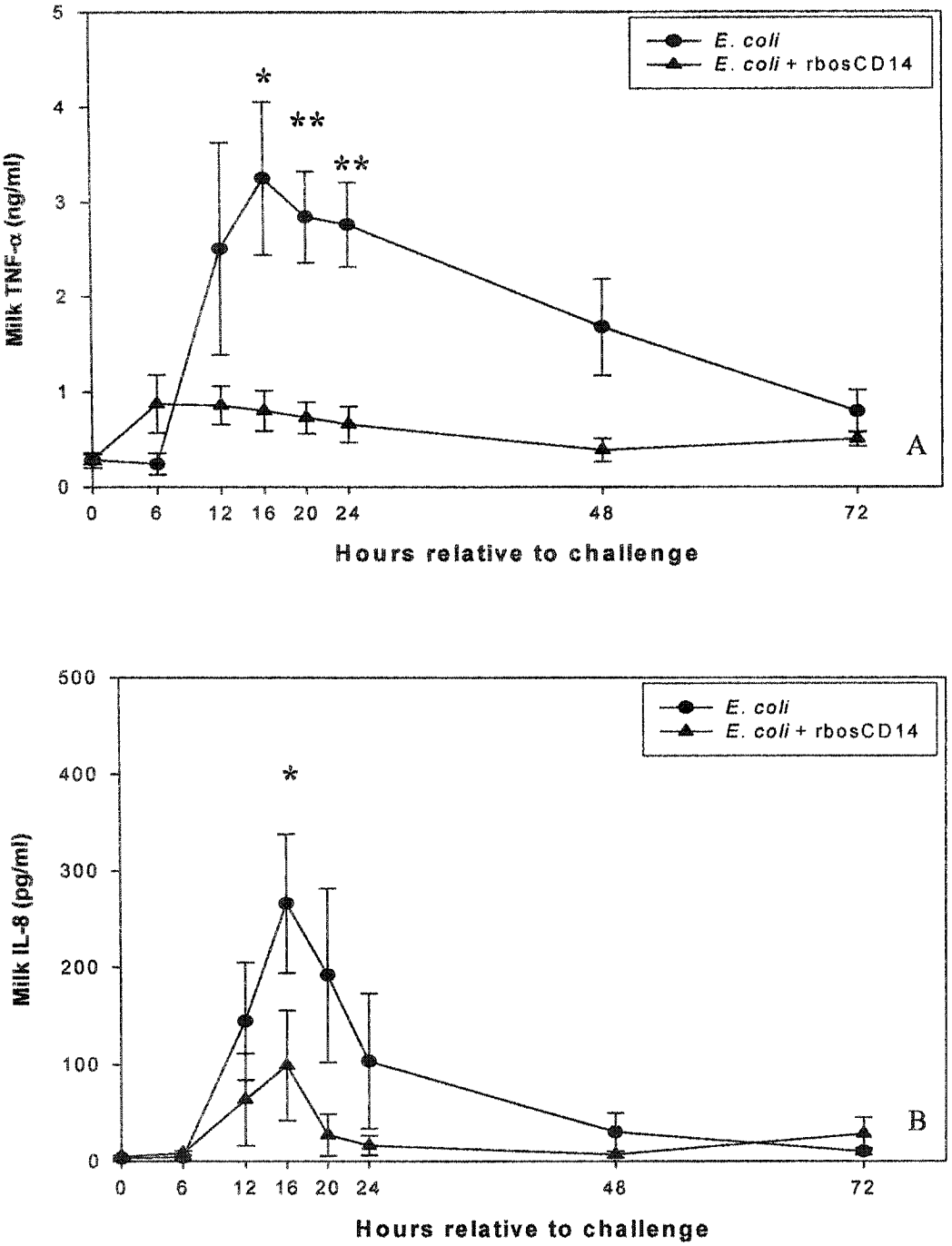

Figure 5. Concentration of TNF-α (A) and IL-8 (B) in milk during an experimentally induced intramammary *E. coli* infection. Two glands of each cow were challenged with 50 CFU *E. coli* with (▲) or without (●) 100 μg rbosCD14. Data are presented as the means ± standard errors of the means of nine cows. *, $P < 0.05$; **, $P < 0.01$.

CONNECTING STATEMENT III

It has been demonstrated in Chapter IV that upon exposure to LPS or *E. coli* bacteria, administration of exogenous sCD14 was able to sensitize bovine mammary glands in terms of recruiting leukocytes. The prompt recruitment of leukocytes, mostly neutrophils, resulted in mitigated clinical signs, faster clearance of bacteria, and reduced production of inflammatory cytokines, including TNF-α and IL-8. The results suggested that increasing the level of sCD14 in milk might be a potential strategy to prevent or reduce severity of bovine *E. coli* mastitis.

In addition to *E. coli*, *S. aureus* is also a major pathogen inducing bovine mastitis and is responsible for a great deal of economic losses. Three serotypes (T5, T8, T336) of *S. aureus* have been shown to comprise 100% of *S. aureus* mastitic isolates in the United States. Therefore, a novel trivalent vaccine, containing the three serotypes of *S. aureus*, has been developed by scientists in United States Department of Agriculture (USDA). In this study, effects of the trivalent vaccine, with or without adjuvants, were evaluated based on alteration of lymphocyte subpopulations, neutrophil phagocytosis, IFN-γ mRNA and antibody production. The information might be useful to understand immunological responses induced by the trivalent vaccine.

CHAPTER V. THE EFFECT OF A TRIVALENT VACCINE AGAINST *STAPHYLOCOCCUS AUREUS* MASTITIS ON LYMPHOCYTE SUBPOPULATIONS, NEUTROPHIL PHAGOCYTOSIS, IFN-γ AND ANTIBODY PRODUCTION

Jai-Wei Lee[1], Celia. N. O'Brien[2], Albert. J. Guidry[2], Kimberley. A. Shafer-Weaver[2], and X. Zhao[1]

[1]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada

[2]Immunology and Disease Resistance Laboratory, USDA-ARS, Beltsville, MD-20705, USA

Abstract

The effect of a novel trivalent (T5, T8, T336) vaccine against bovine *Staphylococcus aureus* mastitis on lymphocyte subpopulations, neutrophil phagocytosis, interferon (IFN)-γ and antibody production was evaluated. Twenty pregnant heifers were immunized with either trivalent, trivalent emulsified in Freund's incomplete adjuvant (FICA), trivalent with aluminum hydroxide (ALUM), or FICA alone, 30 days before the expected calving date followed by 2 boosts in a 2-week interval. Compared to the control, serum antigen-specific IgG_1 and IgG_2 were significantly increased in all the vaccinated groups before parturition and sustained until 3 weeks postpartum. Formulation with both adjuvants enhanced production of IgG_2, but not IgG_1. Nevertheless, IgG_1 was still the dominant isotype in serum. Immune sera which contained the highest titer of antibodies slightly increased neutrophil phagocytosis to the three serotypes of killed *S. aureus*, but most of the differences were not significant due to the large variation among cows. The effect of vaccination on lymphocyte subsets and IFN-γ mRNA expression was not persistent. The percentage of CD4+ lymphocyte was significantly higher in vaccinated groups than that of the control group 4 weeks after the primary immunization. In comparison with the control group, cows inoculated with trivalent vaccine and adjuvants had an increased percentage of CD8+ lymphocytes at two time points, 2 weeks before and after calving. IFN-γ mRNA expression was only increased in T8-stimulated mononuclear cells from cows immunized with trivalent or trivalent plus FICA after parturition. Our results indicated that the trivalent vaccine, with or without adjuvants, dominantly elicited Th2 type immune responses which are less effective in preventing *S. aureus* mastitis. A

formulation capable of inducing Th1 type responses will be required to improve the efficacy of the trivalent vaccine.

Introduction

Staphylococcus aureus, a contagious, gram-positive coccus, is the most frequently isolated pathogen from cases of mastitis (Schukken et al., 1993; Barkema et al., 1998). It accounts for 19 to 40% of intramammary infection (IMI) cases, usually subclinical, and costs approximately 35% of the economic loss due to mastitis (Fox and Hancock, 1989). In Canada, it has been estimated that *S. aureus* infection may be present in as many as 90% of Ontario dairy farms (Kelton, 1999). The curing rate of antibiotic treatment is low, and the infection often becomes chronic. This could be attributed to the ability of *S. aureus* to locate intracellularly, including epithelial cells and macrophages, and survive antibiotic treatments (Almeida et al., 1996; Hébert et al., 2000; Hensen et al., 2000). To date, an effective prevention of *S. aureus* mastitis has not been achieved. Dairy researchers have been trying to develop an effective vaccine to prevent *S. aureus* mastitis for the past several decades. Numerous strategies, including immunization with killed bacteria cells or their virulent factors, have been tried. However, these vaccines have shown poor responses. They have decreased prevalence and severity of mastitis only, but never efficiently prevented new intramammary infections (IMIs) caused by *S. aureus* (Pankey et al., 1985, Watson et al., 1996).

Phagocytes, mostly neutrophils, are recruited to sites of infections and play a crucial role in bacterial clearance. But *S. aureus* is able to produce capsular polysaccharides (CP) that masks cell wall components thus interfering with opsonization and phagocytosis of

phagocytes (Barrio et al., 2000). Thus making *S. aureus* more resistant to the host immune system (Barrio et al., 2000, Hensen et al., 2000). It has been estimated that 94 to 100% of *S. aureus* isolated from mastitic cows are encapsulated (Norcross and Opdebeeck, 1983). A total of 11 serotypes of *S. aureus* CP have been identified (Sompolinsky et al., 1985). The result of serotyping *S. aureus* isolated from 178 dairy farms in the United States indicated that only three serotypes, T5 (18 %), T8 (23 %), and T336 (59 %), were responsible for bovine *S. aureus* mastitis (Guidry et al., 1997; Guidry et al., 1998). In addition, the distribution of serotypes varies geographically (Tollersrud et al., 2000). In comparison with the distribution in the United States, percentage of serotypes from European samples were, T5 = 34%, T8 = 34%, T336 = 30%, and nontypable = 2% (Guidry et al., 1998).

It has been suggested that using CP as the antigen is essential to develop a successful vaccine against *S. aureus* (Relyveld, 1984). Theoretically, the interference of CP against phagocytosis can be overcome by the production of CP-specific antibodies. However, CP is categorized as a T-cell independent (TI) antigen, which is poorly immunogenic (Poolman, 1990). Injection of pure T5 CP failed to provoke an immune response in cows (Gilbert et al., 1994). Unlike protein-based antigens, T lymphocytes are not involved in the immune response to TI antigens. Two subsets of T lymphocytes, T helper cells (CD4+) and T cytotoxic cells (CD8+), modulate immune responses in different ways. CD4+ cells produce a variety of cytokines which lead to different types of immune responses (Th1 and Th2) and antibody production from B cells (Mosmann et al., 1986). Interferon (IFN)-γ and interleukin (IL)-4 are the key cytokines promoting Th1 and Th2 type immunity, respectively. Th2 type activates mainly humoral responses, including B

cells proliferation and antibody secretion. Th1 type may elicit both humoral (specific to IgG_2) and cytotoxic responses. Cytotoxic CD8+ cells eliminate altered self-cells, including intracellular pathogen-infected cells. This does not imply that T cells, or their cytokines, can not influence the immunogenicity of TI antigens. Using a CP-protein carrier conjugate has been shown to increase the production of CP-specific antibody (Fattom et al., 1993; Gilbert et al., 1994). Presumably, the protein antigen in conjugates activates T cells through a classical T-cell dependent pathway, and the cytokines produced by activated T cells augment anti-CP responses (Mond et al., 1995). Recently, the design of whole-cell vaccines has been shown to elicit stronger immune responses than CP-protein conjugates (Tollersrud et al., 2001). Therefore, a trivalent vaccine, containing the three dominant serotypes of *S. aureus*, has been developed by scientists in USDA.

Adjuvants also affect the efficacy of vaccines. Adjuvants increase the immunogenicity of antigens in three ways (1) formation of a depot of antigens at the site of infection, so the antigen can be released for a longer period, (2) enhancement of antigen presentation by antigen presenting cells (APC), and (3) induction of various cytokines which activate specific immune responses, for example, Th1 or Th2 responses. Various adjuvants have been shown to elicit different types of immune responses (Yip et al., 1999), different levels of antibody production and side effects (Stieneker et al., 1995). The most common adjuvants used in veterinary vaccines are aluminum hydroxide (ALUM) and Freud's incomplete adjuvant (FICA). Both adjuvants are inexpensive and have a good safety record (Cox and Coulter, 1997). In the present study, the trivalent vaccine was inoculated to pregnant heifers with or without FICA and ALUM. The effects

of different formulations were evaluated based on lymphocyte-associated immune functions, including alteration of lymphocyte subpopulations, production of IFN-γ mRNA, antigen-specific antibody secretion, and antibody-mediated phagocytosis.

Materials and methods

Preparation of vaccines

Three serotypes of *S. aureus*, T5, T8, and T336 (kindly supplied by A. Fattom, Nabi, Rockville, MD, USA), were grown in 10 ml of Columbia broth (T5 and T8) or tryptic soy broth (TSB) (T336) overnight at 37°C. Then 100 µl of T5 or T8 in Columbia broth were streaked on a Columbia agar plate (2 %) and incubated overnight at 37°C. Bacteria were harvested by washing the microorganisms off the plate with PBS. For T336, 100 µl suspension was inoculated to TSB in a 250 ml flask. Bacteria were killed by 1% formalin overnight at room temperature. Killed bacteria were washed twice with PBS and resuspended in 10 ml PBS. A small volume of the suspension was used to examine the viability and encapsulation of bacteria. Thereafter, the concentration of bacteria was determined by optical density (OD). Equal amounts of each serotype were mixed and emulsified 1:1 with PBS or the adjuvant, either FICA or ALUM. The final concentration of each serotype was 10^9/ml of vaccine.

Animals and immunization protocol

Twenty clinically healthy and pregnant heifers were selected and randomly assigned into four groups (1) FICA only (n = 5) (2) Trivalent only (T) (n = 6) (3) Trivalent + FICA (T+F) (n = 4) (4) Trivalent + ALUM (T+A) (n = 5). Heifers received the primary

immunization one month (D0) before the expected calving date followed by 2 boosts with the same formulation in a 2-week interval, D14 and D28, respectively. The immunization was administered intramuscularly (2 ml) and in the area of supramammary lymph node (2 ml). Use of animals for this study was approved by the Beltsville Agriculture Research Center's Animal Care and Use Committee.

Sampling of cows

Blood samples were collected from each heifer before immunization on D0, D14, and D28, as well as three times after calving, on C7, C14, and C21, respectively. Briefly, 100 ml of blood were collected from the jugular vein using two 60-ml syringes containing 6 ml of 40 mM EDTA (Sigma, St. Louis, MO). At each time point, blood samples were also taken from the tail vein, and serum was collected and stored at -20°C.

Isolation of mononuclear cells

Blood samples collected from the jugular vein were poured into 50 ml centrifuge tubes (Fisher) and centrifuged at 1,800 × g RPM for 30 minutes at 4°C. After centrifugation, 5 ml of buffy coat was removed and added to another 50 ml tube containing 20 ml HBSS (GIBCO/BRL). Thereafter, the mixture (25 ml) was carefully layered onto the surface of 12.5 ml ficoll-paque (Pharmacia) in a 50 ml tube. Mononuclear cells were separated from RBCs after centrifugation at 1,200 × g, 19°C, for 30 minutes. The mononuclear cell band was removed and mixed with HBSS to a volume of 45 ml, then centrifuged at 900 × g for 10 minutes, at 4°C. The pellet was resuspended in 10 ml HBSS and counted.

Determination of lymphocyte subpopulations

The determination of lymphocyte subpopulations was carried out as described (Shafer-Weaver et al., 1999). Briefly, mouse anti-bovine CD2 (αβ-T cells, 1:100), CD3 (T cell receptor, 1:100), CD4 (T Helper cells, 1:160), CD8 (T cytotoxic/suppressor cells, 1:400), MHC II (1:200), B cells (1:100), and CD18 (negative control, 1:100), monoclonal antibodies (VMRD, Pullman, WA) in 50 µl FACS solution (500 ml PBS, 10 ml FBS, 0.75 ml 20% Sodium Azide solution) were added to each well of round bottom 96-well plates. Isolated mononuclear cells, 2 X 10^6 in 50 µl FACS, were added to each well and incubated for 30 minutes at 4°C. Then the plates were centrifuged at 1,200 × g at 4°C for 5 minutes followed by washing with FACS (150 µl/well). The washing was repeated twice followed by adding 100 µl of FITC-conjugated goat-anti mouse secondary antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA). After another 30-minute incubation at 4°C, the plates were washed 3 times. The cells were resuspended in 150 µl FACS and placed in microtubes containing 350 µl FACS (final volume 500 µl). The percentage of each subpopulation was read by flow cytometry.

Quantification of IFN-γ mRNA

Production of IFN-γ was determined at the transcriptional level. Isolated mononuclear cells (5 X 10^6) were cultured with three CP antigens separately for 12 hours followed by centrifugation and the pellet was used for RNA extraction. The cells were lysed by 1 ml of TRIZOL reagent (GIBCO/BRL), and centrifuged after adding 0.2 ml chloroform (phase separation). The RNA, retained in the aqueous phase, was precipitated by mixing with equal volume of isopropanol and washed with 75% ethanol twice. Afterward, 20 µl

of DEPC-treated water were added to resolve the RNA, and the concentration of RNA was determined by the OD value at 260nm.

The expression of IFN-γ mRNA was quantified by competitive reverse transcriptase-polymerase chain reaction (RT-PCR) and RNA electrophoresis as described (Shafer-Weaver et al., 1999). Briefly, forward and reverse primers specific for bovine IFN-γ gene were designed (Forward 5'-AGCCAAATTGTCTCCTTCTACTTC-3'; Reverse 5'-CTGACTTCTCTTCCGCTTTCTG-3'). A recombinant RNA, contained the sequences to react with the primers, was previously synthesized and used as the internal standard to compete with IFN-γ mRNA. The internal standard generated a product larger than that of IFN-γ mRAN after RT-PCR (324 bp v.s. 261 bp). Adding known amount of internal standards (5×10^5 - 10^9 molecules) to the RT-PCR mixture resulted in visualization of two bands on agarose gel stained with ethidium bromide (Fig. 1). The amount of IFN-γ mRNA in samples was quantified by the standard curve based on the ratio of arbitrary densities of the internal standard and IFN-γ mRNA.

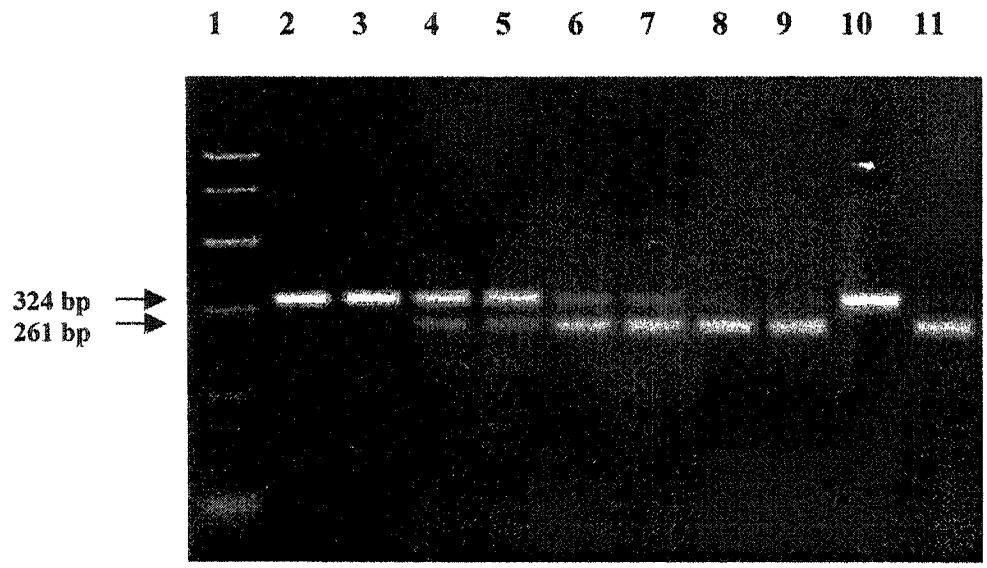

Figure 1. An example of the quantification of bovine IFN-γ mRNA by competitive RT-PCR. Various numbers of internal standard molecules were added to each lane, (2) 10^9 (3) 5×10^8 (4) 10^8 (5) 5×10^7 (6) 10^7 (7) 5×10^6 (8) 10^6 (9) 5×10^5, and competed with IFN-γ mRNA. Lane 10 and 11 were controls for the internal standard (10^7 molecules, no mRNA sample) and mRNA sample (no internal standard), respectively.

Detection of antigen-specific antibodies in serum

The antibody titer was measured by ELISA as described (O'Brien et al., 2000). Briefly, 96-well flat bottom plates were coated with either purified T5, T8, or T336 in PBS (1 μg/ml) and incubated overnight at 4°C. Then the coating solution was removed followed by blocking the plates with 1% chicken serum albumin in PBS at 22 °C for one hour. After washing the plates twice with PBS-T (containing 0.05% Tween 20), samples were diluted 500 times with PBS-T and added into the wells. The plates were incubated at 4°C overnight. Thereafter, the plates were washed three times with PBS, and incubated with rabbit anti-bovine IgG1, IgG2, or IgM, diluted in PBS containing 0.2% Tween 20 for 1 h at 37°C. After washing as above, alkaline-phosphatase labeled sheep anti-rabbit IgG (1:2000) was added to the wells and incubated for another one hour at 37°C. Substrate was added and the plates were read at 405 nm using a microtiter plate reader.

Neutrophil phagocytosis

Phagocytosis was determined using flow cytometry according to Saad and Hageltorn (1985). Briefly, blood neutrophils were isolated as described (Guidry et al., 1993). FITC-labeled bacteria (T5, T8, and T336) were slightly sonicated, and 5×10^7 bacteria in 50 μl were added to each vial containing 50 μl of HBSS-diluted sera (collected on either D0 or C7) pooled from each group. The vials were covered by foil and gently shaken at 37° for 30 minutes (opsonization). The concentration of isolated neutrophils was adjusted to 10 X 10^6/ml in HBSS, and 250 μl of the suspension (2.5×10^6 neutrophils) were added to each vial. All samples were incubated and rocked at 37°C for 30 minutes in dark. Thereafter, phagocytosis was ceased by adding 1 ml ice-cold NaCl (0.09%)-EDTA(0.04%). The

fluorescent intensity was read using a flow cytometry. The percentage of neutrophils with ingested FITC-labeled bacteria was determined after quenching extracellular fluorescence by adding 400 μl of 1% methylene blue. Samples containing neutrophils or bacteria only were used as controls.

Statistical analysis

Data on lymphocyte subpopulations and IFN-γ mRNA expression were analyzed using One-Way Analysis of Variance (ANOVA) with means comparisons of SAS (SAS Inst. Inc., Cary, NC). For the other parameters, comparisons were made by using the MIXED model procedure of SAS. Data were presented as least square means ± SEM. Differences were considered statistically significant when $P < 0.05$.

Results

Lymphocyte subpopulations

The effect of vaccination with different formulations on the expression of selected surface receptors of lymphocytes is illustrated in Table 1. Expression of CD2, a marker of αβ-T lymphocytes, on D14 was significantly ($P < 0.05$) lowered in the group receiving Trivalent only (T) than in comparison with groups immunized with the vaccine containing either one of the adjuvants (T+F or T+A). Vaccine formulations containing antigen with adjuvant also significantly increased the percentage of CD8+ lymphocytes at certain time points (T+F: D14 and C14; T+A: D14) when compared to trivalent only ($P < 0.05$). At D28, FICA only had a decreased percentage of CD4+ lymphocytes when compared to T and T+A. Expression of MHCII was the lowest in the group immunized

with T+F. No significant differences among the treatments were observed on expression of CD3 (T cell receptor), B cell receptor, and the negative control.

Interferon-γ mRNA transcript expression

Expression of IFN-γ mRNA from mononuclear cells in response to the three antigens (T5, T8, and T336) was assayed separately (Fig. 2). No significant differences among the treatments were found when stimulated by T5. However, mononuclear cells isolated from cows immunized with T and T+F had an enhanced IFN-γ mRNA expression in response to T8 and T336 within 2 weeks after parturition. On C7, the number of IFN-γ mRNA molecules, after logarithmical transformation, in 5 X 10^6 T8-stimulated mononuclear cells was significantly lower in the control group, 4.70 ± 0.19 vs 7.05 ± 0.79 (T) or 7.72 ± 0.14 (T+F) ($P < 0.05$). Similar results were observed on C14 as well. When stimulated with T336, the number of the control (5.34 ± 0.74) was significantly lower than that of T+F (7.45 ± 0.11) on C7 ($P < 0.05$).

Production of antigen-specific antibodies

In general, the vaccination significantly increased the titer of antigen-specific IgG_1 and IgG_2 in serum. The response of IgG_1 against T5 and T8 was rapid (Fig. 3). By D14, vaccinated groups showed an increase in IgG_1 to at least one of the three antigens. However, titers of IgG_1 to the three antigens were suppressed in cows immunized with T+F after calving in comparison with T and T+A. Antigen administered with ALUM or FICA did not further augment antigen-specific IgG_1 production in comparison with that of trivalent only. On the other hand, both adjuvants had a positive effect on the

production of IgG_2 (Fig. 4). T+F and T+A, but not T, had a significantly increase IgG_2 titer to at least one of the three antigens around parturition when compared to the control. In addition, the response of T8-specific IgG_1 and IgG_2 was stronger than those of T5- and T336-specific IgG_1 and IgG_2. None of the vaccine formulations enhanced the production of antigen-specific IgM (Fig. 5).

Neutrophil phagocytosis

To investigate if the sera from vaccinated cows could increase the phagocytosis of *S. aureus* by blood neutrophils, neutrophils were isolated from healthy cows and incubated with FITC-labeled *S. aureus* opsonized by pooled sera collected on D0 and C7. The C7 sera from cows immunized with T+F significantly increased the phagocytosis of T8 when compared to the sera from D0 (45.88 ± 12.33 vs 39.05 ± 12.33 %, $P < 0.05$) (Fig. 6). In fact, all immune sera, regardless of adjuvants, had an increased opsonic effect on neutrophils phagocytosis to T5 and T8 *S. aureus*. However, due to the large variation among cows, the differences were not statistically significant. The phagocytosis of T336 by neutrophils was not altered by vaccination.

Discussion

Staphylococcus aureus is the most frequently isolated pathogen from cases of bovine mastitis (Schukken et al., 1993; Barkema et al., 1998). After determining the three dominant serotypes of *S. aureus* responsible for bovine mastitis in the United States were T5 (18 %), T8 (23 %), and T336 (59 %) (Guidry et al., 1997; Guidry et al., 1998), a trivalent vaccine containing whole cells of these serotypes as antigens was developed to

prevent intramammary infections induced by *S. aureus*. However, due to the complicated pathogenesis of *S. aureus* and the poor immunogenicity of capsule polysaccharide (CP), the immunogenicity of this trivalent vaccine needs to be enhanced. The objective of this study was to investigate if the most common adjuvants, aluminum hydroxide (ALUM) and Freud's incomplete adjuvant (FICA), could promote lymphocyte-associated immune responses elicited by the vaccine.

It has been indicated that cows are more susceptible to mastitis during the postpartum (2 weeks after calving) period, which might be attributed to the suppressed immune responses of lymphocytes (Oliver and Sordillo, 1988). Thus, the primary vaccination and two boosts were immunized within a month prior to the expected calving date of each heifer. Theoretically, the maximal effect of vaccination could be reached during the postpartum period by following this immunization protocol. This was supported by the production of IgG_1 and igG_2 in serum. Nearly all the antigen-specific IgG_1 and IgG_2 reached the peak between D28 and C14. For all the three antigens, the response of IgG_1 was the strongest. IgM had a high preimmunization level for the antigens. Vaccination did not affect the production of IgM against *S. aureus* CP antigens, which was in agreement with an earlier study (O'Brien et al., 2000). Our data also showed that T8 is more immunostimulatory than T5 and T336 in terms of inducing antibody production. It has been suggested that the flaccid nature of T8 CP increases the exposure of the cell wall (O'Brien et al, 2000). Therefore, more protein antigens located on the cell wall of T8 *S. aureus* were available to augment the "conjugate" effect and subsequent humoral responses.

Vaccination did not significantly alter the subpopulations of lymphocytes. However, significant differences among formulations were observed sporadically at certain time points and might have biological relevance with the antibody production. For example, the trivalent vaccine, with or without adjuvant, increased the percentage of CD4+ lymphocytes on D28 when compared to the control (FICA only). Cytokines produced by CD4+ cells are important to the maturation of B cells which secrete antibodies. The increase of CD4+ cells coincided with the higher titers of IgG_1 and IgG_2 in serum observed from D28, indicating the requirement of CD4+ cells for IgG production. Moreover, addition of adjuvants significantly increased the percentages of αβ-T lymphocytes (on D14) and CD8+ lymphocytes (on D14 and C14) in comparison with cows immunized with the trivalent only. The percentage of αβ-T lymphocytes represents the subpopulations of both CD4+ and CD8+ lymphocytes. CD8+ cells are associated with the cytotoxic activity of T lymphocytes, which is triggered in Th1 type immune responses. The increased percentage of CD8+ cells suggested that formulation with adjuvants influenced a Th1 response. This is supported by the result that IgG_2 production, an indicator of Th1 type responses, was significantly increased in cows receiving vaccines emulsified with adjuvants. The expression of MHCII receptor was significantly lower in groups immunized with trivalent vaccines containing adjuvants. Although MHCII receptors are not required for the presentation of CP antigens, uptake of TI antigens has been shown to inhibit MHCII-restricted antigen presentation in macrophages (González-Fernández et al., 1997). It is possible that adjuvants increased the ingestion of CP antigens by antigen-presenting mononuclear cells, and this in turn decreased the

expression of MHCII receptors. Further investigations will be required to verify this hypothesis.

None of the formulations altered IFN-γ mRNA expression of mononuclear cells in response to purified T5 (Fig. 3). In comparison with the control, expression of T8 or T336-induced IFN-γ mRNA was significantly higher in mononuclear cells isolated from the group immunized with T or T+F within 2 weeks after parturition. However, the difference was not due to an upregulated IFN-γ expression in T and T+F, but a suppressed expression in the control group. Hyporesponsiveness of lymphocytes during the postpartum period has been reported previously (Harp and Nonnecke, 1986). Therefore, it would be more appropriate to interpret that the vaccination prevented the hyporesponsiveness of mononuclear cells during postpartum. The enhanced IFN-γ expression in response to T8 might partially contribute the higher titers of T8-specific IgG_2 in comparison with those of T5- and T336-specific IgG_2. IFN-γ is a critical cytokine shifting the immune response to Th1 type. Both ALUM and FICA have been shown to stimulate mainly Th2 type responses (Forsthuber et al., 1996). Therefore, it was not surprising to see the limited effect of these vaccines on the expression of IFN-γ.

Comparing opsonic effects of preimmunization sera and sera from C7 revealed that opsonization was slightly enhanced after vaccination. However, only the sera collected from cows immunized with T+F on C7 significantly increased the phagocytosis of neutrophils to T8 *S. aureus* (Fig. 4). Although the overall titer of antigen-specific antibodies was generally increased in the serum of cows immunized with vaccines, IgG_2, the most effective opsonin promoting neutrophils phagocytosis (Howard et al., 1980; Guidry et al., 1998), may not be produced sufficiently to enhance phagocytosis. However,

it has been shown that a minimum amount of specific IgG_2 antibody is required for promoting phagocytosis (O'Brien et al., 2000). Although production of IgG_2 was increased in vaccinated cows, the titer was still much lower than that of IgG_1 which is not opsonic for neutrophil phagocytosis (Guidry et al., 1993). In fact, IgG_1 was reported to inhibit neutrophil phagocytosis (Guidry et al., 1993).

Taken together, immunization of the novel trivalent vaccine, with or without adjuvants, increased the amount of antigen-specific antibodies in serum. However, their effects on other parameters, including alteration of lymphocyte subsets, expression of IFN-γ mRNA, and neutrophil phagocytosis were minimal. Neither ALUM nor FICA induced a persistent enhancement on these parameters. The failure could due to the Th2 type nature of both adjuvants. Nevertheless, adjuvants known to induce Th1 type immune responses, such as Freud's complete adjuvant (FCA), usually have undesirable side effects, such as tissue damages (Weeratna et al., 2000). Recently, bacterial DNA (CpG) has been indicated to induce strong Th1 type responses and IgG_2 production without concomitant side effects when used as the adjuvant (Chu et al., 1997; Chu et al., 2000; Weeratna et al., 2000). This approach may provoke Th1 type responses and improve the efficacy of this trivalent vaccine. Further research will be required to design an optimal formulation for preventing *S. aureus* mastitis.

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		Time					
Receptor	**Trt**	**D0**	**D14**	**D28**	**C7**	**C14**	**C21**
CD2	FICA	29.3±5.7	27.2±4.0^{ab}	29.4±4.1	29.4±10.4	25.9±3.9	27.6±6.7
	T	30.5±5.2	23.6±4.0^{b}	30.9±4.9	23.2±3.9	27.5±4.0	23.4±2.6
	T+F	34.7±5.2	37.0±3.5^{a}	36.0±4.2	33.3±5.7	34.6±5.7	30.5±2.7
	T+A	36.9±5.2	38.6±4.7^{a}	35.4±4.2	32.1±6.0	30.5±5.7	36.6±4.8
CD3	FICA	38.5±5.5	37.2±3.5	36.7±6.0	39.2±10.5	38.6±13.0	35.6±6.4
	T	38.9±5.5	30.9±3.6	36.9±3.2	32.4±3.5	34.8±6.0	31.1±4.6
	T+F	42.6±7.5	36.2±2.4	42.2±4.1	41.6±3.7	36.6±5.1	35.7±3.0
	T+A	43.5±3.7	42.0±4.1	43.6±6.4	36.5±5.3	36.0±4.0	42.5±7.8
CD4	FICA	10.7±3.9	15.1±1.0	8.6±3.0^{b}	8.7±5.6	11.2±5.3	15.4±6.8
	T	15.3±3.9	15.6±2.1	20.4±3.3^{a}	12.4±2.5	16.7±1.9	15.4±1.7
	T+F	20.1±4.8	16.6±1.2	17.7±0.2^{ab}	18.2±1.2	18.0±3.8	11.6±3.0
	T+A	11.9±5.3	20.4±6.0	20.4±2.7^{a}	18.8±3.7	12.6±4.0	15.6±7.3
CD8	FICA	8.3±2.6	8.9±2.3^{ab}	8.1±0.6	4.0±2.7	7.6±1.3^{ab}	8.2±2.3
	T	7.5±1.0	5.6±0.5^{b}	7.9±1.0	5.6±0.8	5.7±0.9^{b}	8.0±1.7
	T+F	12.3±3.5	11.2±1.6^{a}	2.5±4.4	10.4±3.5	12.2±1.6^{a}	7.0±2.0
	T+A	8.3±1.8	12.1±2.3^{a}	9.9±1.4	10.9±3.2	12.0±2.4^{ab}	15.5±3.1
MHCII	FICA	47.0±9.6	56.2±6.5^{a}	47.1±12.9	37.2±12.1	52.3±9.3	58.4±12.4
	T	36.4±9.2	48.3±7.2^{a}	37.3±9.5	44.5±8.6	50.6±9.9	59.1±8.7
	T+F	27.1±5.5	27.1±4.9^{b}	36.6±4.3	36.7±8.3	38.7±6.0	37.0±3.2
	T+A	37.6±8.7	37.9±6.4^{ab}	34.0±8.4	47.6±6.4	42.9±11.1	37.4±5.0
B cell	FICA	30.7±8.4	38.0±13.1	40.9±10.3	25.8±8.0	31.4±7.5	45.0±10.3
	T	28.7±9.8	35.4±8.9	27.4±7.7	30.8±7.4	39.5±10.7	51.1±12.7
	T+F	22.0±5.7	24.7±5.3	23.8±3.4	24.0±2.6	31.8±3.0	26.6±4.7
	T+A	24.2±5.4	26.6±4.5	26.0±5.1	31.8±6.4	30.2±6.0	25.8±3.2
NEG	FICA	1.8±0.6	1.5±0.4	2.2±0.5	0.9±0.4	1.5±1.0	1.1±0.2
	T	1.5±0.2	1.2±0.3	1.7±0.6	0.4±0.1	0.8±0.1	2.5±0.8
	T+F	0.9±0.2	1.6±0.6	0.8±0.2	2.9±1.6	3.7±1.0	1.3±0.7
	T+A	1.7±0.4	1.9±0.7	0.5±0.1	1.8±0.6	3.3±1.1	4.2±1.4

Table 1. Flow cytometric analysis of isolated peripheral blood mononuclear cells. Values are mean ± SEM of the percentage of positive-stained cells and are compared with other treatments at each time point. Values with different superscripts are significantly different at $P < 0.05$.

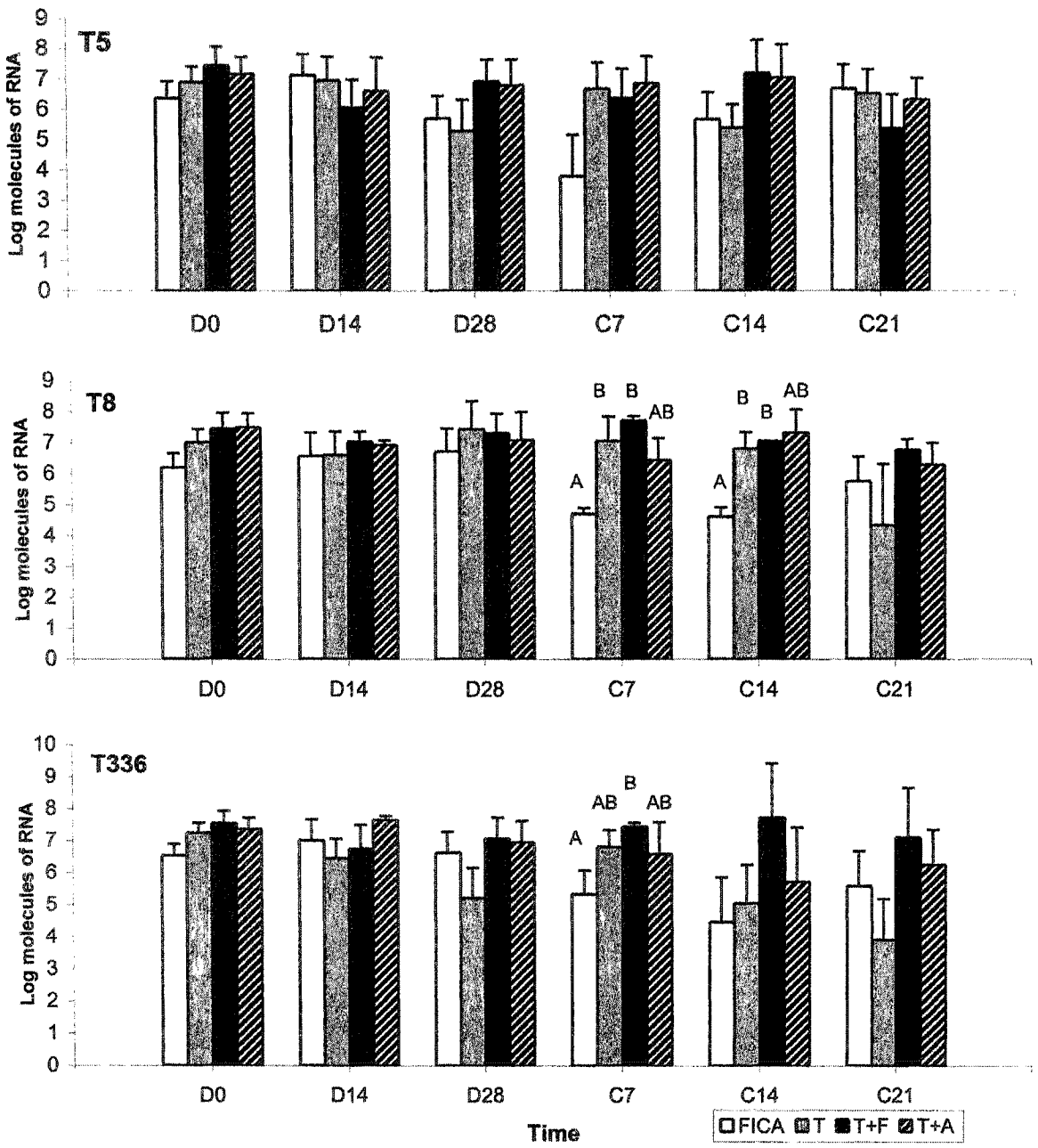

Figure 2. Quantification of IFN-γ mRNA using competitive RT-PCR. Isolated mononuclear cells were stimulated by purified T5, T8, and T336 antigen separately. The amount of IFN-γ mRNA molecules was logarithmically transformed and presented as mean ± SEM. Treatments with different letters at same time point are significantly ($P < 0.05$) different.

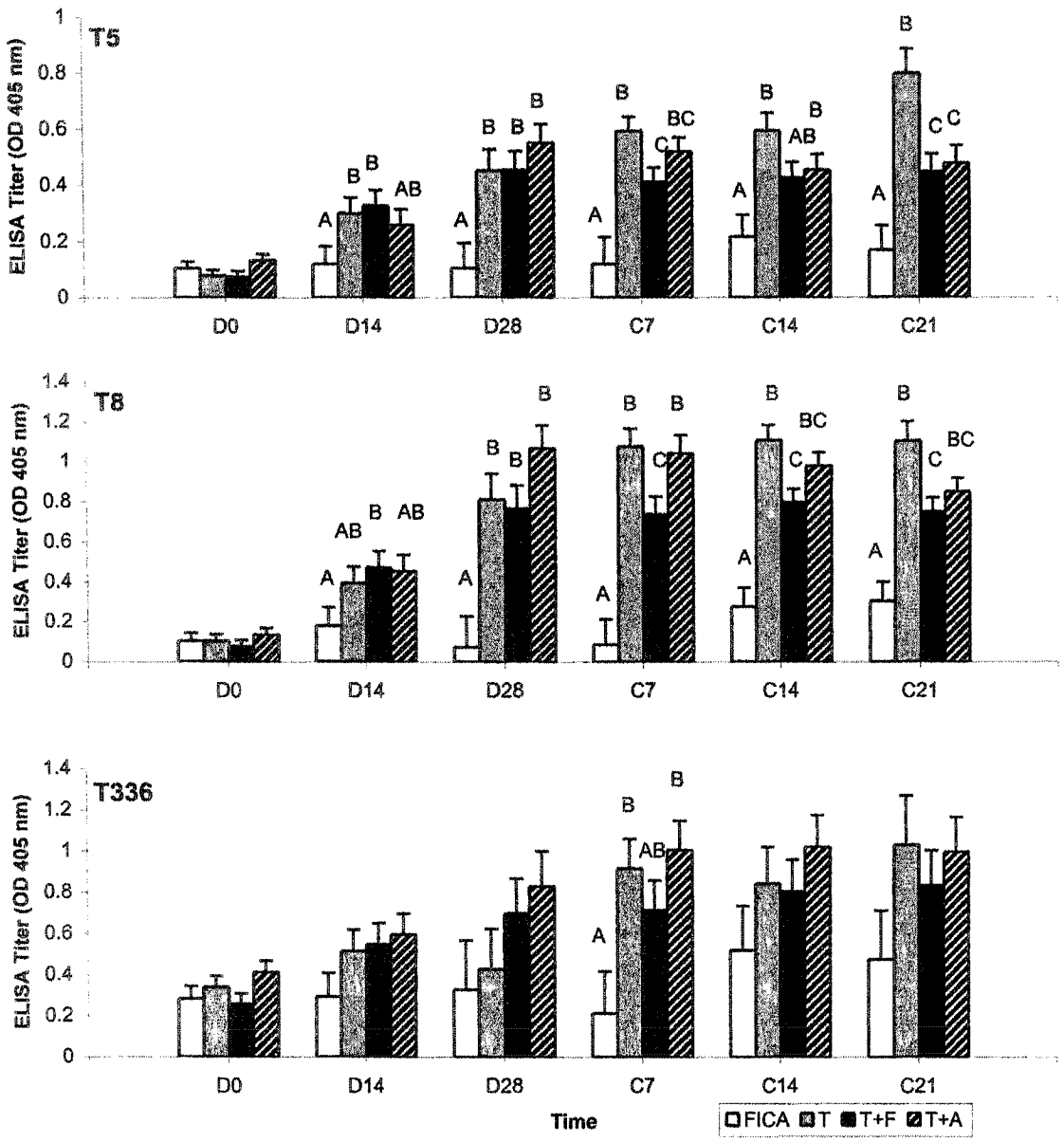

Figure 3. Antigen specific IgG_1 titers in sera as determined by ELISA. Serum samples were diluted 1/500. Data are expressed as least square mean ± SEM of the OD values read at 405 nm. Treatments with different letters at same time point are significantly ($P < 0.05$) different.

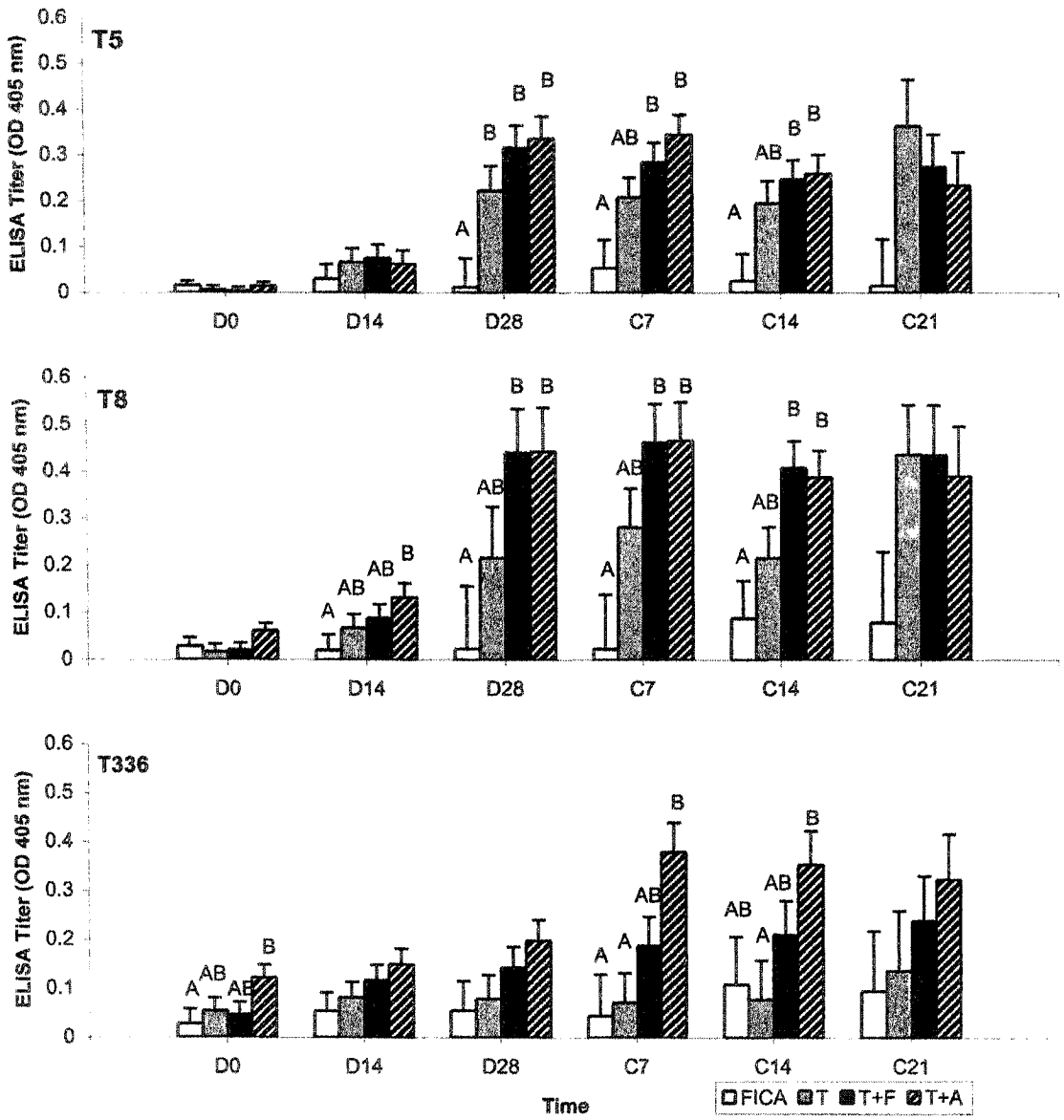

Figure 4. Antigen specific IgG_2 titers in sera as determined by ELISA. Serum samples were diluted 1/500. Data are expressed as least square mean ± SEM of the OD values read at 405 nm. Treatments with different letters at same time point are significantly ($P < 0.05$) different.

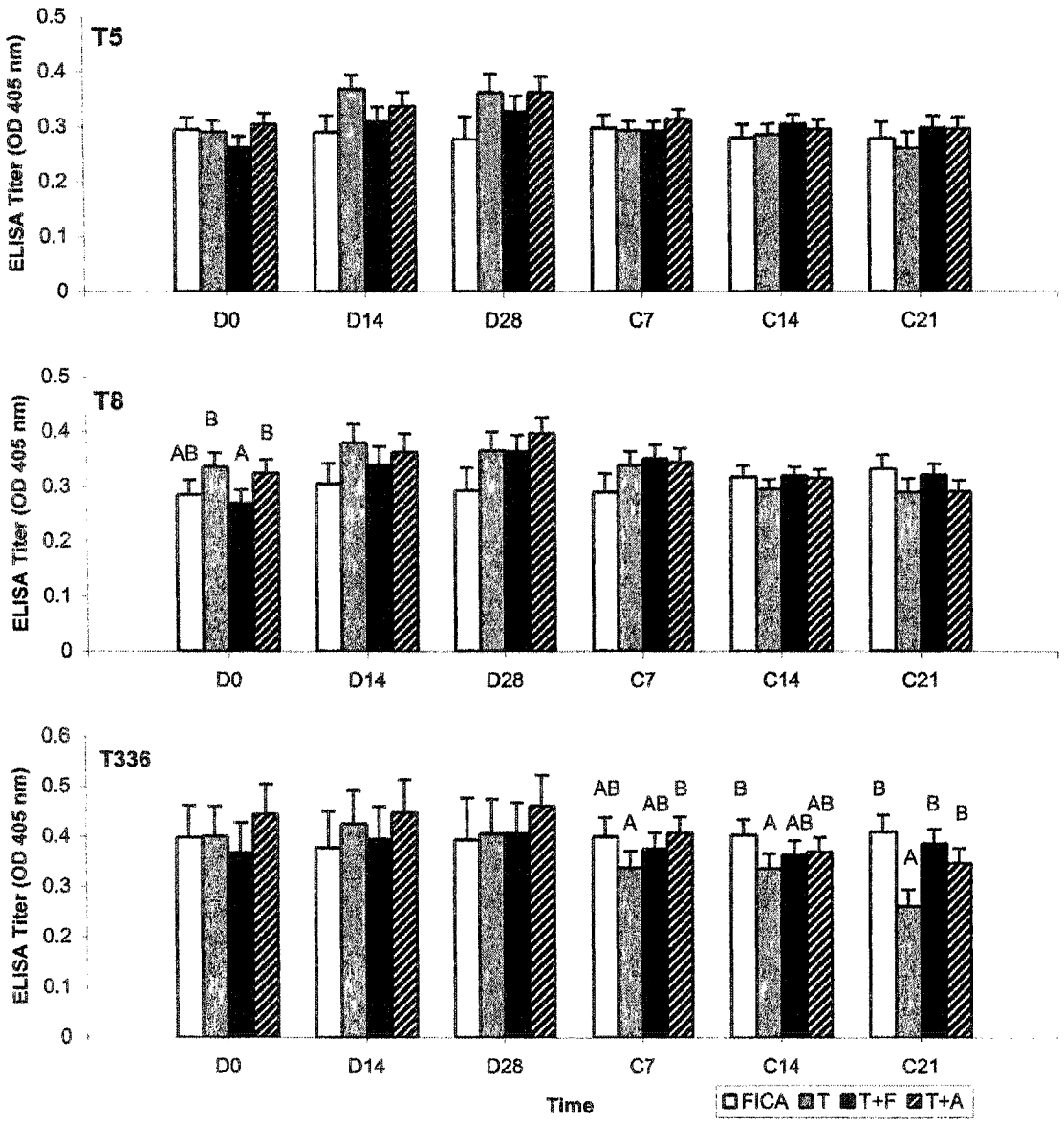

Figure 5. Antigen specific IgM titers in sera as determined by ELISA. Serum samples were diluted 1/500. Data are expressed as least square mean ± SEM of the OD values read at 405 nm. Treatments with different letters at same time point are significantly ($P < 0.05$) different.

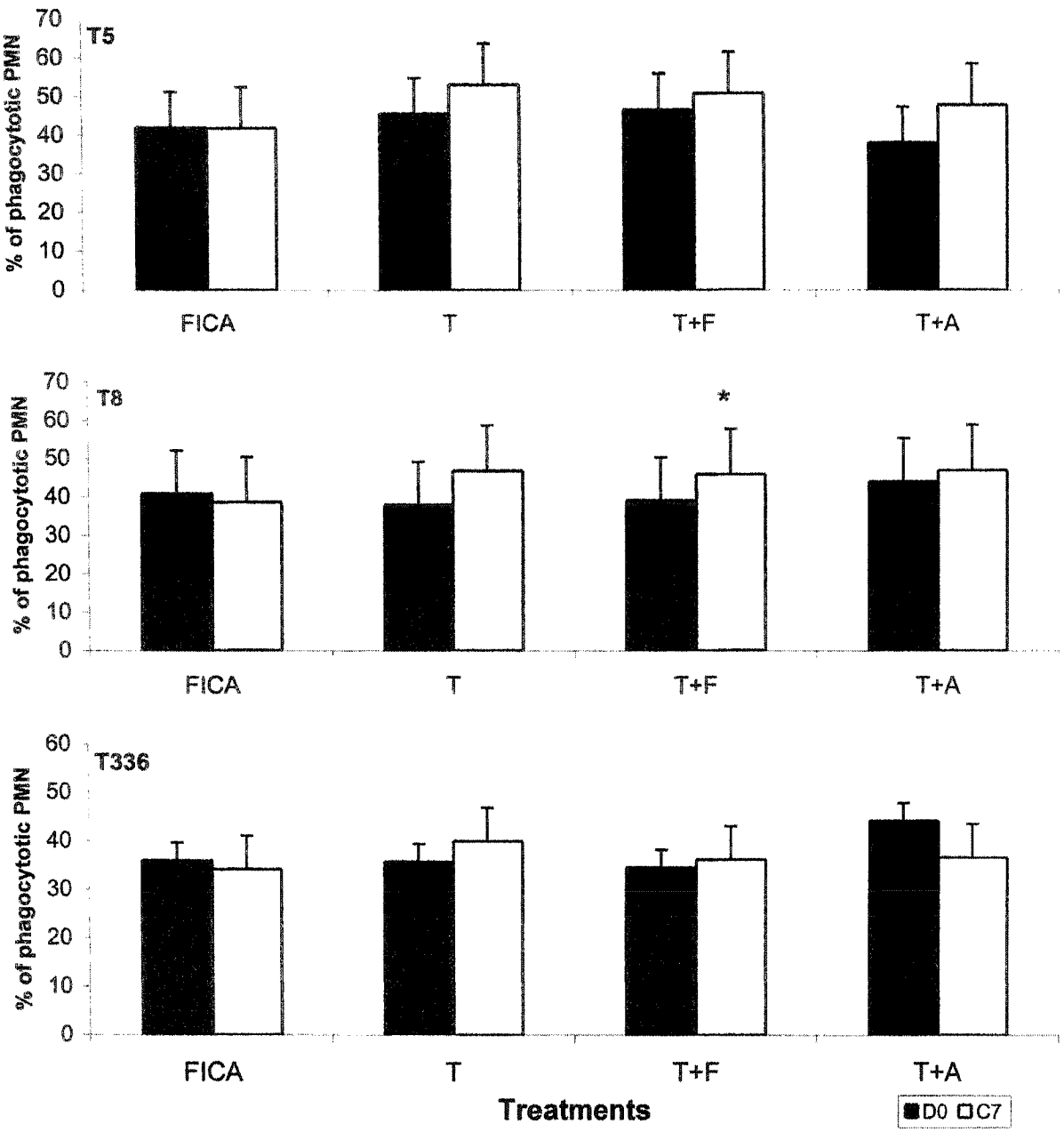

Figure 6. Phagocytosis of *S. aureus* by bovine neutrophils. Bovine neutrophils were incubated with FITC-labeled T5, T8, or T336 *S. aureus* opsonized by preimmunization (D0) and immune sera (C7). Values are least square mean ± SEM of the percentage of phagocytotic neutrophils. Comparisons were made between the value of D0 and the value of C7 for each treatment. *$P < 0.05$.

CHAPTER VI. CONCLUSIONS AND GENERAL DISCUSSION

E. coli and *S. aureus* are the most frequently isolated pathogens from cases of bovine mastitis. The two pathogens induce intramammary infections through different pathogenic pathways, which make *E. coli* mastitis different from *S. aureus* mastitis in many aspects. For example, the duration of *E. coli* mastitis is short, but it usually becomes clinical and may be fatal in severe cases. On the other hand, mastitis induced by *S. aureus* is more persistent and is usually subclinical and chronic. In addition, inflammatory cytokines, including TNF-α, IL-1, and IL-8 were detected in mammary glands infected by *E. coli*, but not *S. aureus* (Riollet et al., 2000). Therefore, application of controlling strategies specific to either one of the two pathogens might lead to a better protection against bovine mastitis. In this study, administration of sCD14 and immunization with a trivanlent vaccine, were carried out to reduce mastitis caused by *E. coli* and *S. aureus*, respectively.

Soluble CD14, originated from shedding of mCD14, has been shown to compete with mCD14 to react with LPS and mitigate the activation of mCD14-expressing leukocytes. The presence of sCD14 has been reported in a variety of body fluids, including milk. In our study, the concentration of endogenous sCD14 in normal milk was 6.67 ± 0.44 μg/ml, which was similar to the range of sCD14 in body fluids from other species. The concentration of sCD14 was the highest in milk produced within a few days postpartum. During an intramammary challenge with LPS, milk sCD14 was found to increase in parallel with the increase of SCC, but not with that of BSA, an indicator of serum leakage. Moreover, bovine neutrophils have been shown to release sCD14 in response to high doses of LPS *in vitro*. Taken together, recruited neutrophils might be the major

source of the increased sCD14 during inflammation in the mammary gland. The increased level of milk sCD14 during colostrumsgenesis and inflammation suggests that a high content of sCD14 in milk is important not only to the newborns, but also to the hosts.

The protective effect of sCD14 was first demonstrated in mice. Bovine sCD14 gene has been cloned and the recombinant protein (rbosCD14) has been expressed in insect sf/9 cells infected with recombinant virus containing the gene (Wang et al., 2002). Administration of rbosCD14 has been shown to decrease the fatality of LPS-induced septic shock and to reduce clinical signs, bacterial load, and TNF-α production of experimental *E. coli* mastitis. An overwhelming production of TNF-α from LPS-activated leukocytes is responsible for the deleterious inflammatory responses and fatality associated with septic shock. The results were in agreement with other studies which indicated that recombinant hsCD14 increased survival rates of LPS challenged mice by reducing TNF-α production (Haziot et al., 1995; Stelter et al., 1998). However, it was not possible to identify the mechanism contributed to the decreased dissemination of bacteria until the experiment was conducted in dairy cows.

Administration of rbosCD14 in experimental bovine *E. coli* mastitis demonstrated similar results to those from the mouse model. Quarters challenged with *E. coli* plus rbosCD14 had a faster clearance of bacteria, reduced clinical symptoms, and decreased concentrations of TNF-α, IL-8 in milk than challenged quarters injected with saline. More importantly, the response of SCC was significantly sensitized by rbosCD14. Recruited leukocytes, mostly neutrophils, in milk play a crucial role in defending the mammary glands from invading bacteria. Thus, it was concluded that the faster clearance

of bacteria was attributed to a prompt recruitment of neutrophils. It is known that sCD14/LPS complexes are able to activate epithelial cells *in vitro* to secrete IL-8, a potent chemoattractant of neutrophils (Pugin et al., 1993; Lee and Zhao, 2000; Wang et al., 2002). However, our results showed that neither IL-8 nor TNF-α were involved in the sCD14-mediated recruitment of neutrophils. Recently, a novel pathway which is not mediated by CD14 and TLR-4, has been shown to efficiently attract neutrophils when triggered by LPS (Haziot et al., 2001). The authors suggested that activation of mCD14/TRL-4 actually interferes with this pathway. Therefore, it is possible that binding of LPS by exogenous rbosCD14 minimized activation of mCD14/TRL-4 on leukocytes, which had a positive effect on this novel pathway. Further investigation is required to identify the agent responsible for the prompt increase of milk SCC observed in these quarters.

Our results were in contrast to those from an earlier study, in which the presence of excess mouse sCD14 increased growth of *Streptococcus pneumoniae* and concentration of TNF-α in cerebrospinal fluid (CSF) of challenged mice (Cauwels et al., 1999). It has been reported that monocytes deploy different intracellular pathways upon interacting with Gram-positive and Gram-negative bacteria through mCD14 (Rabehi et al., 2001). For example, blocking the p38 mitogen-activated protein (MAP) pathway only inhibited TNF-α production in monocytes stimulated with LPS, but not in monocytes stimulated with *Staphylococcus aureus*. Therefore, these discrepancies may be resulted from the fact that the protective effect of sCD14 is specific to Gram-negative bacteria.

The approach for controlling *S. aureus* mastitis was focused on evaluating a novel trivanlent (T5, T8, T336) whole cell vaccine in this study. The vaccine was prepared with

or without being emulsified with adjuvants, either FICA or ALUM, to examine whether adjuvants could increase the efficacy of the trivalent vaccine. Cows are more susceptible to mastitis during the postpartum (2 weeks after calving) period due to suppressed immune responses of lymphocytes. Thus, the primary vaccination was carried out a month prior to the expected calving date followed by two boosts in a 2-week interval, which made the immune response to reach its peak during the postpartum period. Our results showed that serum antibodies, including IgG_1 and IgG_2, were significantly increased by the trivalent vaccine. ALUM or FICA had a positive effect on production of IgG_2, but not IgG_1. However, production of IgG_2 was not strong enough to significantly enhance neutrophil phagocytosis. The failure of inducing strong Th1 type immune responses, as indicated by IFN-γ mRNA expression, may explain the limited response of IgG_2.

Our data also showed that T8 was more immunostimulatory than T5 and T336 in terms of inducing antibody secretion. It has been suggested that the flaccid nature of T8 CP increases the exposure of the cell wall (Cifrian et al, 1993). Therefore, more protein antigens located on the cell wall of T8 *S. aureus* were available to augment the "conjugate" effect and subsequent humeral responses. The results explained why the immune sera of T+F increased neutrophil phagocytosis to T8 *S. aureus* only. This could also be linked with the unsuppressed IFN-γ mRNA expression in T8-stimulated mononuclear cells. In general, immunization of the novel trivalent vaccine, with or without adjuvants, increased the amount of antigen-specific antibodies in serum. However, their effects on other parameters, including alteration of lymphocyte subsets, expression of IFN-γ mRNA, and neutrophil phagocytosis were minimal. Neither ALUM

nor FICA induced a persistent enhancement on these parameters. The failure could be resulted from the Th2 type nature of both adjuvants.

Taken together, the present study demonstrated that increasing sCD14 level in milk was able to sensitize the mammary gland in response to invading *E. coli* bacteria. A prompt recruitment of neutrophils during the early stage of infections contributed to a faster clearance of bacteria, as well as reduced clinical signs and cytokine production. This strategy may be applied to mitigate not only mastitis induced by *E. coli*, but also other infections caused by Gram-negative bacteria. On the other hand, a formulation capable of eliciting stronger Th1 type immune responses is required to improve the efficacy of the trivalent vaccine.

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