

**A SERIES OF *IN VITRO* STUDIES INVESTIGATING THE ROLE OF  
LACTOFERRIN IN CALF INNATE IMMUNITY**

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A DISSERTATION

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

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In Partial Fulfillment

Of the Requirements for the Degree

of

Doctor of Philosophy

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by

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## **DEDICATION PAGE**

I cannot emphasize enough the importance of the support and love that I have received from my family: Donat Aspinel Dawes, father (now deceased), Phyllis Winnifred Dawes (mother), Langston A. Dawes (brother), Clyne E. Dawes (brother), Lois A. Dawes (sister) and Cheryl I. Dawes (now deceased), uncles, aunts, cousins .... To you all: Your prayers, wake up calls, counseling, laughter, editing skills, visits and yes financial support have all made it possible for me to reach this juncture in my life. For these and so much more, I THANK YOU!

## **ACKNOWLEDGEMENTS**

It is very difficult to express my gratitude to each individual who contributed to the successful completion of my doctoral process and so lest I fail to recognize you by name, I would like to extend heartfelt thanks to those who cared in diverse ways for my experimental calves, those who helped with laboratory management and experimental techniques, and those whose concessions facilitated increased research time.

To the members of my doctoral committee: Doctors Jeffrey Lakritz, Jeff W. Tyler, Antoinette Marsh, Barry J. Steevens, Robert L. Larson, Daniel E. Hassett and Charles R. Brown, and supporting friends who tired with me through the entire process; to those who were recruited mid-stream, I will always be grateful for your kindness.

Dr. Lakritz thanks for enabling my entry into the graduate degree program shortly after beginning my Residency training. Your confidence in me, which never waned despite your transfer to The Ohio State University, was a constant motivator. Dr. Tyler, your ready guidance, constant concern and consideration were always comforting.

Thanks also to the members of the wider community for helping me to keep body and soul together, especially after the death of two of my ever supporting family members. To the Missouri United Methodist Church, the College of Veterinary Medicine and Choral Union **Thank you all!**  
Most of all, **Thanks to Almighty God!**

This work was supported by grants from the Department of Veterinary Medicine and Surgery and College of Veterinary Medicine, Committees on Research (COR), and USDA NRI-CGP agreement #2001-35204-10799. The authors wish to thank Aislinn N. Halaney, Mary Cockrell (UMC), and Andreas Nelsbach (Cell Signaling Technology, Inc.) for providing technical assistance.

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## **LIST OF ABBREVIATIONS**

bp - base pairs

dNTPs – deoxynucleotide triphosphates

EGTA - ethylene glycol-bis (beta-aminoethyl-ether)-N,N,N',N'- tetraacetic acid

g - gravity forces or the unit of measure for relative centrifugal force

ml(s) - milliliter(s)

MgCl<sub>2</sub> - magnesium chloride

min(s) - minute(s)

mM - millimolar

NaCl - sodium chloride

nM - nanomolar

ng- nanograms

Na<sub>3</sub>VO<sub>4</sub> – sodium orthovanadate

Tris - HCL – tris (hydroxymethyl) aminomethane hydrochloride

µg - micrograms

µl - microliter

U - units

**A series of *in vitro* studies investigating the role of lactoferrin in calf innate immunity**

**Maisie Dawes**

**Dr. Jeffrey Lakritz, Dissertation Supervisor**

**ABSTRACT**

The central hypothesis of the ensuing research is that bovine colostral lactoferrin (LF) will impact the health of cattle by improving innate cell function and reducing the effects of lipopolysaccharide (LPS) on the host immune responses. Lactoferrin will indirectly enhance innate immune function and modulate the host inflammatory responses. Our long-term goal is to improve productivity in the cattle industry by reducing morbidity and mortality among neonatal and juvenile calves. We hope that through the following investigations the dependence on conventional antibiotics will decrease in favor of this novel protein or its peptide derivatives.

Neonatal bovine mortality rates continue to impact the economic sustainability of the United States cattle industry. In 2001, the pre-weaning mortality rate among dairy heifers nationwide was 8.9%. Based on an individual cost of \$2,000.00 per pregnant heifer this translates into a potential loss \$800 million dollars to the dairy industry. Losses experienced in the feedlot cattle industry are equally high. Gram-negative infections contribute largely to cattle morbidity and mortality rates. Although some reports attribute the lethal effects of Gram-negative bacteria to endotoxemia and bacteremia the contribution of the host's innate immune

response to morbidity and mortality is critical to the pathophysiology of disease. Given its myriad functions: iron chelation, immune modulation, anti-inflammatory and anti-endotoxin properties, LF may prove useful in attenuating the systemic disease in the bovine.

## **CHAPTER 1**

### **I. INTRODUCTION - THE MAMMALIAN IMMUNE SYSTEM**

All higher organisms possess an immune system that protects the host from microbial pathogens (bacteria, viruses, fungi, parasites, allergens) and clears them from the body. The concept of immunity dates back to 400 BC, around the time of the Athenian plague and is later documented in Asian history refers to the efforts of the Chinese who encouraged the healthy to inhale powdered smallpox lesions in order to induce mild ‘immunizing’ small pox infections (Parham 2005, 1). However, it was apparently not until the early 1800 that this concept was scientifically formalized when Dr. Edward Jenner (1749-1823) of England produced the first experimental vaccine using a related cowpox virus to inoculate the healthy against the more deadly smallpox infections (Parham 2005, 2).

The host’s first barrier to infection is the epithelial layers of the skin, and the mucosal surfaces of the respiratory, urogenital and gastrointestinal tracts and eyes (Parham 2005, 7). Once these surfaces are breached, pathogen recognition and eventual elimination becomes the responsibility of the two major arms of the immune system: the innate immune system which responds immediately and nonspecifically to invasions, and the adaptive or acquired immune system that must be primed over time to ensure efficiency. Both systems consist of cellular and soluble factors. All cells originate in the bone

marrow while the soluble components originate from effector immune cells (antibodies and cytokines), visceral organs (the liver) or are from components of plasma.

Using a bovine calf model, three experiments were performed to evaluate the role of lactoferrin a non-heme associated pleiotropic glycoprotein on the immune cell function. Peripheral blood cells of the innate and adaptive immune systems were subjected to various treatments in order to evaluate the modulatory effects of this protein on parameters such as innate cellular killing mechanisms, cellular proliferation and cellular cytokine production in response to bacterial components and other mitogenic stimulants. A brief overview of the cellular and soluble components of innate and adaptive immunity is presented below, and will be followed by an introductory chapter encompassing the structure and properties of the protein under investigation.

### **THE INNATE IMMUNE SYSTEM**

The innate immune system consists of a battery of soluble plasma complement proteins as well as inflammatory cells, both of which participate in microbial killing. The cellular components of Innate Immunity originate in the bone marrow. These include macrophages and dendritic cells the first line of defense, as well as polymorphonuclear leukocytes (PMN's), natural killer cells and gamma delta T cells (Figure 1.1). In our experimental model, we focused on the roles of cells of the monocyte-macrophage system and polymorphonuclear leukocytes or neutrophils.

Microbial invasion of host tissues is first detected by resident macrophages- mature forms of circulating monocytes- that reside in tissues- and immature dendritic cells, which in this role, function as antigen presenting cells (Akira et al. 2006). Once microbial presence is detected through toll-like and other membrane-bound receptors, the cytoplasmic transcription factor, nuclear factor- κB (NF-κB) translocates to the nucleus and directs the transcription of proteins associated with the inflammatory response (e.g., cytokines, chemokines, prostanoids, leukotrienes, proteases and acute phase proteins). Sequential phosphorylation of members of families of mitogen activating protein kinases (MAPK) have been shown to modulate cytokine production (Bernt Van den Blink et al. 2001; Woo et al. 2004). These soluble proteins, namely IL-8 (or chemokine CXC8), TNF-α, IL-1, and IL-6 attract inflammatory cells to the sites of infection and induce changes in local blood flow, vascular size and permeability, and hence, contribute to the development of the hallmarks of inflammation - pain, heat, redness and swelling (Janeway 2005, 55). Inflammatory cells that infiltrate these sites include neutrophils and natural killer (NK) cells. Like neutrophils, macrophages also engulf microbes killing them in vesicles referred to as phagolysosomes, but are less efficient at this than PMN's. Unlike neutrophils, macrophages, process microbes for presentation to cells and are one of the professional antigen-presenting cells involved in adaptive immunity (Latimer 2003, 46).

Polymorphonuclear leukocytes (neutrophils) are the most abundant of the white blood cells and are rapidly recruited to sites of inflammation. These

granulocytes contain primary or azurophilic and specific or secondary cytoplasmic granules. Primary granules contain microbicidal contents such as myeloperoxidase, lysozyme, defensins, bacterial-permeability-inducing protein, and enzymes such as neutral proteases, acid hydrolases and elastases. The secondary granules contain enzymes such as collagenases and plasminogen activator and microbicidal products which include lactoferrin, lysozyme, cathelicidins, and alkaline phosphatase (present in cattle and horses *only*). These microbicidal products are released into phagolysosomes following phagocytosis of bacterial pathogens. Neutrophils are the primary source of the extracellular matrix protein, metalloproteinase-9 (Latimer 2003, 46; Lakritz et al. 2004c).

The complement system consists of over 20 different proteins. These proteins exist as zymogens or inactive proteases and either opsonize (coat) or neutralize and kill pathogens upon activation (Janeway 2005, 55; Matheswaran et al. 2003). They are primarily associated with plasma but also exist in tissues, the fluids of body compartments (pericardial, synovial and cerebrospinal fluids), lymph and colostrum (Matheswaran et al. 2003). Complement activation may occur by one of three ways and is dependent on the type of pathogen encountered (Janeway, 2005, 55; Rainard 2003) (Figure 1.2). The classical complement pathway is initiated when the first complement protein, C1q, binds to host C reactive protein bound to either antibody or bacterial polysaccharides (Janeway, 2005, 55).

The mannose-binding lectin pathway is initiated in the early stages of infection. Plasma concentrations of bacterial or viral mannose-containing carbohydrates and host lectins become elevated (Janeway, 2005, 55). The alternative complement pathway is initiated when the complement protein C3 is spontaneously activated to bind microbial surface structures, namely, polysaccharides (Abbas and Lichtman, 2003, 293).

### **THE ADAPTIVE IMMUNE SYSTEM**

The adaptive or acquired immune system so called because it develops subsequent to antigenic exposure. The primary function of this arm of mammalian immunity is immunologic memory (Pulendran and Ahmed 2006). Its primary function in disease resistance is pathogen elimination late in the infection phase and protection against subsequent infections (Akira et al. 2006). Initiation of acquired immune responses occurs in the T cell-rich regions of secondary lymphoid tissues (lymph nodes, spleen, tissue-specific mucosal lymphoid aggregates) where antigens are presented to mature naïve (cells which have never been exposed to antigens or microbial products) T and B lymphocytes which continuously circulate through the lymphatic and vascular systems (Pulendran and Ahmed 2006). Antigen presentation may be performed by activated mature dendritic cells, macrophages or B cells otherwise referred to as professional antigen-presenting cells (Janeway, 2005, 55). Major characteristics that differentiate this branch of the immune system from the innate system are:

1. Specificity: All effector cells are generated upon recognition of specific antigenic epitopes via antigen-specific receptors derived during cellular maturation in primary lymphoid organs.
2. Diversity: The adaptive immune system has evolved a mechanism whereby cells are able to respond to a myriad number of antigenic determinants. The diverse nature of the adaptive immune response includes the production of a variety of antibodies by host B cells, while T cell responses may be Th1, Th2 or T regulatory cell-dependent.
3. Memory: After each exposure, effector cells further differentiate into quiescent memory cells, to provide enhanced immune protection at the time of subsequent exposure.
4. Non-reactivity to Self: During early development, B and T lymphocytes bearing receptors for self antigens are eliminated from the host (Clonal Deletion/Tolerance).
5. Specialization: Immune cells and effector molecules bear receptors that are specific for certain pathogenic epitopes. Consequently, immunity to one pathogen does not afford immunity to another (Pulendran and Ahmed, 2006; Janeway 2005, 55; Parham 2005, 238).

Dendritic cells are highly specialized and are the most important of the antigen-presenting cells (Pulendran and Ahmed 2006). Like phagocytes of the innate immune system, they recognize microbes or microbial components through pattern recognition receptors. The initial activation of these cells occurs

early at infection sites as part of the innate immune response. Later they migrate to peripheral lymphoid organs where they further mature and upregulate the expression of the co-stimulatory and adhesion molecules necessary to facilitate T cell interaction, activation and clonal expansion (Pulendran and Ahmed 2006).

## **MAMMARY GLAND PHYSIOLOGY AND THE NEONATE**

The mammary gland is a complex organ with two distinct phases of mammary growth; that occurring during the pre-gestational period and the other which occurs between lactation cycles (Gabai 2003). Regardless of the period of mammogenesis, mammary gland health involves the interaction between anatomical, cellular and soluble factors of innate and adaptive immune mechanisms (Rainard 2003; Sordillo, Shaefer-Weaver, and DeRosa 1997).

Colostrogenesis, the transfer of immunoglobulins into mammary secretions prior to parturition (Barrington et al. 1999) marks a period during which immunogenic components are concentrated in the mammary gland (Sordillo, Shaefer-Weaver, and DeRosa 1997). In the bovine, colostrogenesis begins several weeks before parturition and peaks 1 to 3 days prior to then (Weaver et al. 2000). IgG<sub>1</sub> the primary immunoglobulin (Ig) found in colostrum is transferred from serum to the lacteal secretion along with IgA, IgM and IgG<sub>2</sub>, by a receptor-mediated mechanism (Weaver et al. 2000). Colostrum also contains high concentrations and measurable amounts of lactoferrin, lysozyme, lactoperoxidase, interleukins, interferons, TNF- $\alpha$ , leukocytes and complement (Hitoki, Yamanaka et al. 2003; Sordillo, Shaefer-Weaver, and DeRosa 1997; Smith and Schanbacher 1977).

Together these components contribute to mammary gland health as well as serve as the source of passive immunity for the agammaglobulinemic neonatal calf (Sordillo, Shaefer-Weaver, and DeRosa 1997).

Lactoferrin serves several roles during the early 'dry off' period when the animal is no longer being milked. These include iron sequestration from pathogens including Gram-negative bacteria, inhibition of bacterial colonization and promotion of phagocytic killing by enhancing complement deposition on bacteria (Sordillo, Shaefer-Weaver, and DeRosa 1997; Kai et al. 2002). In fact, since an increase in lacteal LF concentration is often observed in the face of mammary gland inflammation/infection, LF has been termed an acute phase protein of the mammary gland (Zheng et al. 2005). In *in vitro* studies, scientists demonstrated concentration-dependent inhibition by citrate on LF effects in bacterial cultures (Smith and Schanbacher 1977). Citrate, a buffer produced by epithelial cells, chelates iron into a form that is readily usable by bacteria (Sordillo, Shaefer-Weaver, and DeRosa 1997). During the periparturient period, the time near calving, milk citrate concentrations are elevated over that of lactoferrin (Smith and Schanbacher 1977). The increased incidence of mastitis observed during this time has been attributed to this change in milk composition (Smith and Schanbacher 1977). Lactoferrin has been shown to modulate lymphocyte and other cellular responses both in the mammary gland and *in vitro*, thus warranting its further investigation in the neonate (Smith and Schanbacher 1977; Dawes et al. 2002; Baker, Baker, and Kidd 2002). Major cell types present in lacteal secretions include macrophages, PMNs, NK cells B and T lymphocytes

and epithelial cells or lactocytes (Smith and Schanbacher 1977). Given its pleiotropic roles in mammary gland physiology and health, implications of its clinical significance in experimental Gram-negative infections in other species and its beneficial effects on innate cellular function, lactoferrin may prove beneficial in protecting the bovine neonate from the local and systemic effects of coliform bacteria and modifying the neonate's immune responsiveness to endotoxin (Smith and Schanbacher 1977; Debbabi et al. 1998; Szcister-Ciesielska et al. 1995).

### **COMMON BACTERIAL PATHOGENS OF THE BOVINE**

Health is the state where positive host factors predominate over predisposing environmental factors (Figure 1.3). Common causes of mammalian infections include bacteria, viruses, parasites, protozoans and fungi. In cattle, Gram-negative bacteria predominate in disease states. In the neonate, coliforms (Enterotoxigenic *Escherichia coli* (ETEC); and *Salmonella* species) are commonly implicated in gastrointestinal disease; while *Mannheimia hemolytica* and *Pasteurella multocida* are commonly implicated secondary invaders in viral pneumonias. The marked susceptibility of bovine neonates to Gram-negative septicemia is supported by the 8.9% pre-weaning mortality currently observed among dairy heifers. The high mortality rate has been attributed to the lethal effects of lipopolysaccharide (LPS), the toxic moiety of the outer membrane of Gram-negative bacteria (Gerros et al. 1993; Moore et al. 1992).

Lipopolysaccharide is a negatively charged molecule with a molecular

weight of approximately 18,000 kilodaltons (kD). It is composed of four regions: the O-antigen which consists of repeating units with polysaccharide side chains, the highly variable outer core which is usually three sugars long with one or more covalently bound sugars as side chains, the less variable inner core which includes 3-deoxy-D manno-oct-2 ulosonic acid or (KDO) and the covalently-linked lipid A - a dimer of phosphorylated glucosamine sugars (Van Amersfoort, Van Berkel and Kuiper 2003) (Figure 1.4). Core saccharides include N-acetylglucosamine, glucose, galactose, heptose, phosphate and ethanolamine (Nau and Eiffert 2002). Lipid A is a potent stimulator of the host's innate and adaptive immune systems. It is the primary initiator of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 production (Amersfoort, Van Berkel and Kuiper 2003; Gerros et al. 1993; Moore and Morris 1992). Given that LF has iron-chelating ability, is bacteriostatic as well as bactericidal and taking into consideration its critical roles in the diseased and healthy mammary gland of cattle, we intend to investigate its potential roles in calf immunity.

## **DEVELOPMENT OF IMMUNITY IN THE CALF**

Ruminant placentation does not permit the transfer of maternal immunoglobulins to the fetus *in utero* (Weaver et al. 2000). Therefore, the bovine neonate's serum is essentially agammaglobulinemic at birth (Weaver et al. 2000). Disease resistance in the neonate is therefore dependent upon the passive immunity derived from colostral ingestion (Weaver et al. 2000; Kruse PE, 1983). Passive acquisition of maternal immunoglobulins is facilitated by the

ability of the neonatal enterocyte to non-selectively absorb immunoglobulins and other macromolecules during the first 24 to 36 hours after birth (Weaver et al. 2000; Kruse PE, 1983). Thereafter, transepithelial absorption of macromolecules is low (Kruse PE, 1983). Additionally, during this period proteolytic activity within the digestive tract is low and trypsin inhibitors present in colostrum limit the destruction of immunoglobulins and other biologically active macromolecules (Godlewski et al. 2005; Kruse 1983).

Once internalized by enterocytes, immunoglobulins are transcytosed into the lymphatic system and from there enter the bloodstream via the thoracic duct (Weaver et al. 2000; Kruse 1983). In nature, the calf normally stands within 30 minutes of birth and consumes its first colostral meal shortly thereafter. In dairy-based colostrum management programs where calves are immediately removed from their dams, the recommended optimal time to colostral ingestion is 8 to 12 hours after birth (Weaver et al. 2000). Forced-feeding colostrum by an esophageal feeder is recommended (Weaver et al. 2000; Molla 1978). While the exact mechanism of ‘closure’ still remains to be determined, it appears to be initiated by colostrum ingestion, is believed to be under some endocrine influence and is coordinated by digestive enzyme development (Kruse 1983; Stott et al. 1979). The highly vacuolated immature enterocyte population undergoes marked proliferation and is replaced by a mature microvillus surface devoid of pinocytotic capability (Godlewski et al. 2005; Weaver et al. 2000; Kaup et al. 1996; Kruse 1983). Time of ingestion, quality and quantity of colostrum ingested, method of colostral administration, fetal stress and acid: base status of the calf

can influence the passive transfer of colostral immunoglobulins (Weaver et al. 2000; Kruse 1983; Stott et al. 1979; and Molla 1978). Adequate transfer of colostral immunoglobulin in the calf is represented by a serum IgG concentration of 1,000 mg/dl or greater. This correlates with a serum total protein concentration greater than or equal to 5.2 g/dl (Tyler et al. 1998). Since the neonatal calf has had limited exposure to environmental antigens, pathogen elimination during the precolostral post natal period is dependent upon innate immune mechanisms, primarily phagocytic leukocytes and complement proteins (Osburn 1981).

When compared with adults, neutrophils as well as key components of the complement system function much less efficiently in neonatal calves (Mueller et al. 1983; Zinkl and Kabbur 1997). Not only are circulating levels of the C3 complement protein low but neutrophil Fc-receptor expression and bactericidal activity are less efficient, resulting in inherent susceptibility to bacterial infections (Mueller et al. 1983 ; Osburn 1981; Zinkl and Kabbur, 1997). Additionally, owing to limited antigenic recognition, the immune system of the bovine neonate is characterized as having limited ability to recognize and target lipopolysaccharide (LPS) prior to 4 weeks of age (Mueller et al. 1983; Osburn 1981). The bovine neonatal immune system has been shown to consist of a high proportion of naïve T cells with the ability to suppress immunoglobulin production, en utero antibody production as early as 150 days of gestation, has been reported (Nonnemecke et al. 2005; Smith 2002, 707). In a previous study researchers reported endogenous IgG<sub>1</sub> production, 3 weeks after birth (Devery, Davis and Larson 1979). However,

the rate of production was minimal (1g/day) and is invariably dependent on antigenic exposure (Devery, Davis and Larson 1979). The bovine neonate also has a high proportion of antigen-presenting cells with defective co-stimulatory function, and depressed T helper cell 1(Th1) cytokine production (Nonnecke et al. 2005).

Combined, these factors result in neonatal calves being highly susceptible to Gram-negative, intracellular bacterial and viral infections.

Bovine colostrum contains nutrients such as vitamins and essential fatty acids, as well as non-nutrient components including immunoglobulins (IgG<sub>1</sub>), complement, leukocytes, lysozyme, tri-peptide growth factors and anti-oxidants which have demonstrated roles in innate immunity (Blum 2006; Matheswaran et al. 2003; Weaver et al. 2000; Lindmark-Måansson and Åkesson 2000; Migliore-Samour et al. 1992); peptides such transforming growth factor- beta (TGF-β), insulin-like growth factor-1 (IGF-1) and cytokines.

While colostrum-replete calves are less likely to develop neonatal respiratory and enteric disease, failure of passive transfer does not guarantee mortality in affected calves (Weaver et al. 2000; Tyler et al. 1998). However, some studies have demonstrated that the disadvantages associated with failure of passive transfer often persist later on in life (DeNise et al. 1989; Robison, Scott, and DeNise 1988; Tyler et al. 1998). For instance, calves with failure of passive immunity, or those which have failed to ingest an adequate volume of colostrum within the appropriate time-frame (24 hour serum IgG concentration < 5.2 g/dl), have significantly increased relative risk of mortality- a risk that persists

at least until 10 weeks of age (Tyler et al. 1998). Additionally, decreased productivity and longevity has been observed throughout the lifetime of affected animals within the herd (DeNise et al. 1989). In the 2001 National Animal Health Monitoring System (NHAMS) survey of pre-weaning mortality rates among live heifer births, 8.9 % of deaths were attributed to factors related to impaired host immunity ("AABP, ADSA, AFIA and, USDA" 2001). Failure of passive transfer also contributes to survivors' reduced productivity and longevity in the herd (DeNise et al. 1989; Robison et al. 1988).

Lactoferrin (LF) and lactoperoxidase are also present in colostrum. Both proteins may also play important roles in promoting calf innate immune responses. Lactoferrin is of particular interest for several reasons:

1. During the dry-off period, LF expression is up regulated in the non-lactating bovine mammary gland (Schanbacher et al. 1993; Sordillo et al. 1993; Smith and Todhunter 1982). Compared to maximal concentrations during the lactating period (0.35 mg/ml), LF concentrations during the involuting period achieve a maximum of 100 mg/ml 21 to 30 days into involution (Smith and Todhunter 1982; Sordillo et al. 1997)
2. Lactoferrin plays a crucial role in iron sequestration from bacteria, thus limiting bacterial proliferation (Valenti and Antonini 2005). Scientists demonstrated the failed induction of mastitis following the infusion of *Escherichia coli* (*E. coli*) into the non-lactating udder while disease developed after a similar infusion was administered into the udders of

- lactating cows (Van Hooijdonk, Kussendrager, and Steijns 2000; Smith and Schanbacher 1977).
3. Using a chimeric bovine lactoferrin construct to transfect mouse mammary tissue, scientists demonstrated up-regulation of LF expression following LPS stimulation (Zheng et al. 2005). Also the addition of *Staphylococcus aureus* and LPS to a bovine mammary cell culture system (separate experiments) led to increased LF production by bovine cells (Zheng et al. 2005).
  4. Based on the hydrophobic nature of portions of this molecule, LF binds molecules such as LPS, with high affinity (Baveye et al. 2000a; Lee et al. 1998).

During inflammation, mature milk lactoferrin concentrations increase from 0.35mg/ml to greater than 1 mg/ml (Smith and Todhunter 1982). Substantial data also indicates that LF also modulates mammary leukocyte function (Kai et al. 2002; Smith and Schanbacher 1977; Smith and Todhunter 1982).

## **CHAPTER 2**

### **II. LITERATURE REVIEW -**

#### **LACTOFERRIN- INTRODUCTION**

Lactoferrin is an 80 kilo Dalton non-heme associated glycoprotein belonging to the transferrin gene family (Steijns and van Hooijdonk, 2000; van Berkel et al. 1997). Following its discovery in 1939 by Sorenson and Sorenson, lactoferrin was isolated from both human and bovine milk by Johansson et al in 1969 (Baker, Baker and Kidd 2002; Baker E. and Baker H, 2005; Weinberg 2001Johansson, 1969; Vogel et al. 2002). In addition to major iron-transporting protein in mammalian blood, serum transferrin, other members of this family have been identified in other species (Baker and Baker 2004; Ward et al. 2002; Baker, Baker and Kidd 2002).

All members of the transferrin gene family consist of 670 - 690 amino acid residues (Lönnardal and Iyer 1995). The amino acid residues of human LF demonstrate approximately 60% sequence identity with that of human serum transferrin (sTF) (Baker and Baker 2005). Similar findings have been documented for the lactoferrins of other species. This suggests evolutionary duplication of the TF gene with some amino acid sequence divergence resulting in variability in function (Baker and Baker 2005; Weinberg 2001). Lactoferrin consists of 689 (bovine) and 692 (human) amino acids and dependent on its source, may contain up to 5 glycan residues (Yoshida, Wei, and Funkunaga

2000; Lönnerdal and Iyer 1995). Except for the immunoglobulin heavy and light chains, LF is the only other glycosylated protein present in whey fractions extracted from the non-lactating gland, normal colostrum, milk, or mastitic milk secretions (Lönnerdal and Iyer 1995).

Lactoferrin or lactotransferrin takes on a characteristic red color during incubation with ferric iron. To date LF has only been found in mammalian hosts (Baker and Baker 2005). The predominant cell types involved in its synthesis are the myeloid series and secretory epithelia (Baynes and Bezwoda, 1994). Lactoferrin has been identified in uterine fluid, saliva, seminal fluid, tears and colostrum, respectively (Kikuchi et al. 2003; Baker et al. 1994; Baker and Baker 2005; Johansson 1969). It is also present in the secondary granules of polymorphonuclear leukocytes (PMN's) or neutrophils.

### **LACTOFERRIN GENE EXPRESSION**

Currently, the amino acid sequence for human, pig, horse, cow, buffalo, sheep, camel, goat and mouse lactoferrins are available in sequence databases (Baker and Baker 2005). All share greater than 60% homology, with 70% existing between the human and mouse forms, 69% between human and bovine and 63% between the bovine and mouse proteins (Baker and Baker 2004). The LF forms obtained from sheep, goat, buffalo and cows share greater than 90% sequence identity (Baker and Baker 2005).

Seventeen exons encode LF. Fifteen of these are conserved within the bovine, human, porcine and mouse genome (Teng 2002). When compared with

human LF, bovine, porcine and mouse LF contain one or two amino acids less at exons 2 and 11 (Teng 2002). Codon interruptions are also fairly conserved. Additionally a highly conserved noncanonical TATA box and SP1 binding site (positions -28 and -69 respectively) immediately upstream of the LF exon 1 of cattle, swine, mouse, rat, goat, buffalo and camel are also believed to regulate LF expression. However, despite these similarities, LF expression is species, tissue and cell type specific and is regulated through many signaling pathways (Teng 2002). When tissue samples obtained from a lactating cow were analyzed for lactoferrin mRNA expression, investigators found high levels of expression in the mammary gland and liver, while intestinal expression was moderate. Baseline mRNA expression in the lung, kidney and spleen were low (Zheng et al. 2005). The constitutive expression of lactoferrin may relate to its sharing several features associated with the promoters of housekeeping genes. For instance, the bovine lactoferrin promoter (4.4kb) demonstrates a high GC content, a noncanonical TATA box and multiple SP1/GC elements (Zheng et al. 2005). Binding sites generally believed to be engaged by the transcription factors NF- $\kappa$ B, AP-1, STAT3, STAT5, estrogen, progesterone and glucocorticoid receptors, have also been identified within the LF promoter (Zheng et al. 2005). Studies have shown that the proximal portion of the LF promoter is highly conserved across species (Zheng et al. 2005).

Although the role of an estrogen response module for LF gene expression is well established in humans and the mouse, a similar role has not been identified for either the bovine or any other species (Zheng et al. 2005). In the

mouse, LF expression can also be induced by retinoic acid (Teng 2002). During granulopoiesis LF is transcribed during both the myelocyte and metamyelocyte stages and is finally stored in the secondary neutrophilic granules (Ward, Paz and Conneely 2005; Teng 2002) (Table 2.1). Zheng et al recently established the presence of two LPS-responsive regions in the bovine LF promoter. Induction of LF promoter activity appears to be dose-dependent (Zheng et al. 2005). Although the transcription binding sites are yet to be determined, three potential LPS-responsive elements have been identified. These include one STAT3, four AP-1 and six NF- $\kappa$ B sites (Zheng et al. 2005). In the bovine, increased LF mRNA expression and protein production also occur in response to the increased intra-alveolar pressure associated with the termination of milking or milk stasis (Sordillo et al. 1977).

Lactoferrin expression in the human and mouse reproductive tract has been investigated. It is constitutively produced by the uterine mucosa; but expression was induced in mice following estrogen administration. Similarly LF concentration in myoepithelial cells fluctuates with circulating estrogen levels. Uterine LF gene expression increases during proestrus, peaks at estrus and declines to basal levels from metestrus through to diestrus (Teng 2002). In humans endometrial expression of LF increases during the proliferative phase and decreases during the luteal phase (Teng 2002). In the dog, although the site of production is yet to be determined lactoferrin seminal concentration is positively correlated with spermatogenesis. In humans, LF is produced in the

prostate and seminal vesicles, while the epididymis serves as the source of lactoferrin in horses and swine (Kikuchi et al. 2003).

### **ISOFORMS OF LACTOFERRIN**

Lactoferrin exists in two forms: hololactoferrin describes the iron-saturated or diferric form while the term apo-lactoferrin refers to iron-free lactoferrin. Apo-lactoferrin is more susceptible to proteolysis and its molecular conformation is characterized by lobes that are more 'open' (Baker et al. 1994; Lönnerdal and Iyer 1995). Apo-lactoferrin predominates in mammalian host secretions and varies across species (Kei-Ichi, Kawano and Yoo 1991).

For instance, of all the mammalian species, both human colostrum and milk contain the highest concentrations (Kei-Ichi, Kawano and Yoo 1991; Shanbacher et al. 1993). Also, when compared with cattle, high concentrations persist in human milk throughout lactation (Kei-Ichi, Kawano and Yoo 1991; Shanbacher et al. 1993).

Using the non-detergent Nonidet P-40, human, goat, sheep and bovine holo and apo - lactoferrin were subjected to pH titration. The curves generated by all types of lactoferrin samples were similar, demonstrating a highly positive isoelectric point ( $pI = 9$ ) (Kei-Ichi, Kawano and Yoo 1991). The three were also compared using circular dichroism (CD) spectrophotometry over 3 wavelength ranges: 200-250, 250 - 350 and 300 - 600nm (Kei-Ichi, Kawano and Yoo 1991). Despite differences in size, findings support that the gross conformation of the three are similar (Kei-Ichi, Kawano and Yoo 1991). Additionally, except for the

degree of glycosylation, carbohydrate composition of the three ruminant lactoferrin proteins, are identical. Size variability is has therefore been attributed to the degree of glycosylation and iron saturation (Yoshida et al. 2000; Kei-Ichi, Kawano and Yoo 1991). Ruminant LF is different from human LF in that the ruminant proteins contain less helical configurations. However, the secondary and tertiary structural profiles of both human and ruminant LF are very similar (Spik et al. 1994; Kei-Ichi, Kawano and Yoo 1991).

### **STRUCTURAL CHARACTERISTICS OF LACTOFERRIN**

Lactoferrin is comprised of a single polypeptide chain consisting of two homologous globular lobes linked by an extended  $\alpha$ -helix. Intra-molecular disulfide bonds exist (Lönnerdal and Iyer 1995). The polypeptide bears a two-fold internal repeat with each lobe bearing amino ( $\text{NH}_2$ ) and carboxyl ( $\text{COOH}$ ) terminal halves. Both the amino and carboxyl termini share approximately 40% amino acid sequence identity and both display identical iron-binding capabilities (Baker and Baker 2005; Zheng et al. 2005; Steijns and van Hooijdonk 2000; Lönnerdal and Iyer 1995). Each lobe consists of two  $\alpha$  (N1 and N2) and two  $\beta$  (C1 and C2) domains at each lobar terminal which contribute to the formation of a deep cleft, the site of ferric iron binding (Baker and Baker 2005). Both lobes simultaneously bind an anion- preferentially carbonate- while reversibly binding iron with high affinity (Steijns and van Hooijdonk 2000). The amino acid residues aspartate (Asp), tyrosine (Tyr) and histadine (His) are integrally involved in iron-binding (Baker et al. 1994; Steijns and van Hooijdonk 2000). Strong hydrogen

bonds are formed as a result of interactions between two oxygen atoms, two synergistically bound carbonate ( $\text{CO}_3^{2-}$ ) ions, and an arginine side chain at the N-terminus as well as a nearby threonine (Thr) side chain (Baker et al. 1994). A substantial water-filled cavity is created within the interdomain cleft.

Using site-directed mutagenesis, Ward et al characterized the crucial role played by tyrosine residues. Mutation of this amino acid from either lobe resulted in selective loss of iron-binding capability of the affected lobe (Ward et al. 1996). Through pH-dependent studies, they also demonstrated that iron-binding at the COOH terminal lobe, critically determines the pH-binding stability of iron by the amino terminal lobe (Ward et al. 1996). Potent iron-chelating ability of LF has also been attributed to the presence of a salt rather than a diliysine bridge between lysine (Lys) 301 and Glutamine (Glu) 216 in the N-lobe (Baker et al. 2002). Lactoferrin has 260 times greater iron-binding affinity than serum TF. Reportedly, affinity constants range from  $10^{20}$  to  $10^{22}$  (Baker and Baker 2005; Weinberg 2001).

Lactoferrin retains iron around pH 3-4. Domain opening is essential for iron release and is potentially influenced by three factors: Specific receptors may pry open the domains; a decrease in the ph of the environment; and reduction of the bound ferric iron to its more weakly-binding ferrous state (Baker, Baker, and Kidd 2002). The arginine- and lysine-rich  $\text{NH}_2$ - terminal which contributes to the protein's highly basic nature is also believed to play a contributory role (Lönnnerdal and Iyer 1995; Ward et al. 1996).

## **STRUCTURAL CHARACTERISTICS UNIQUE TO BOVINE LACTOFERRIN**

When compared with the lactoferrins derived from other mammalian species, bovine LF is often more potent in its effects than human, caprine and ovine lactoferrin (Steijns J. and van Hooijdonk A. 2000). This property has been attributed to its unique structural characteristics:

1. Two isoforms of bovine LF lactoferrin-a and lactoferrin-b were identified in colostrum, normal milk and secretions from the non-lactating mammary gland, using cation exchange chromatography (Wei et al. 2001; Yoshida et al. 2000). Both forms are distinct from that present in milk neutrophils (Hurley et al. 1993).
2. Lactoferrin-a accounts for 30% of the total lactoferrin and is present in higher concentrations in colostrum versus milk. Its molecular weight (84,000kD) is also higher than that of LF-b (80,000kD). The only observed difference between LF-a and -b is the presence of a glycan at Asp 281 (Van Veen Geerts, and Van Berkel 2004; Yoshida et al. 2000). Lactoferrin-a is glycosylated at five instead of four sites (as in LF-b). Due to the demonstrated complexity of the glycans found at two sites in both LF-a and -b, Wei and others suggest that glycan synthesis in the bovine mammary gland may be under hormonal control during colostrogenesis (Wei et al. 2001). In contrast, human LF

is only glycosylated at two of its potential four sites (Wei et al. 2001).

3. All the glycans of both isoforms of bovine LF possess highly heterogenous structures. This has been attributed to incomplete processing of the protein by exoglycosidases in the Golgi apparatus due to rapid transport of the protein through the Golgi apparatus (Wei et al. 2001).
4. Functionally, the bacteriostatic and bactericidal activities of LF-a are more potent than those of LF-b (Wei et al. 2001; Yoshida et al. 2000; Steijns and van Hooijdonk, 2000; Wei et al. 1999).
5. Given its location in the N-linked glycosylation sequence (Asp281-Lys282-Ser283), glycosylation at Asp 281 and Lys282 - the major proteolytic site - appears to protect bovine LF from proteolysis by pancreatic and gastric enzymes, trypsin and pepsin respectively (Van Veen Geerts, and Van Berkel 2004). Bovine LF-a, is 10-fold more resistant to tryptic cleavage than bovine LF-b (Van Veen Geerts, and Van Berkel 2004).
6. On the other hand, glycosylation does not contribute to the proteolytic resistance of human LF which is 100 times more resistant than bovine LF (Van Veen Geerts, and Van Berkel 2004).

Pepsin cleavage of bovine LF, results in the generation of lactoferricin B. Although alpha-helical in structure, in solution this peptide adopts a twisted beta-

pledged structure which contributes to its many proposed and proven functions (Vogel et al. 2002). Lactoferricin is able to bind to, spontaneously cross and destabilize the bilayer of bacterial membranes (Vogel et al. 2002). He et al (1995) also demonstrated the ability of LF to be spontaneously internalized by cells and directly act on nuclear DNA (He and Furmanski 1995).

### **FUNCTIONS OF LACTOFERRIN**

Since its discovery in human milk in 1939, lactoferrin has been identified as having important roles in innate immune responses. Among its many roles, lactoferrin has been reported to have antibacterial, anti-fungal, anti-tumor, anti-inflammatory, and immuno-modulatory properties (Vogel et al. 2002). Recently, LF has been noted to demonstrate proteolytic activity, inhibit bacterial biofilm production, and promote osteoblastic activity (Baker 2005; Naot et al. 2005; Rogan et al. 2004). The highly positive charge of LF makes it unique among the other transferrin-like proteins. The N- terminus incorporates the most positively charged region of this polypeptide and provides a site for heparin, glycosaminoglycan and potentially DNA-binding (Baker, Baker and, Kidd 2002). It may very likely also contribute to LF's ability to bind to the negatively-charged phospholipid groups of cellular membranes and thus modulate the activity of various cells (Baker, Baker, and Kidd, 2002). Contiguous with this N-terminal patch, is the bactericidal lactoferricin domain with its unique display of surface arginine residues- a feature that is unique to LF (Baker, Baker, and, Kidd 2002).

**Antibacterial Properties** - Since neutrophils, the primary source of serum LF, are the first to respond to monocyte: macrophage induced up-regulation of adhesion molecules and leukocyte chemotaxis, lactoferrin is rapidly mobilized to sites of infection (Valenti. et al. 2005). Iron availability is important for the growth of bacterial pathogens and has been strongly correlated with bacterial virulence. It is also required for several aspects of microbial metabolism (electron transport, cellular growth and division, and enzymatic pathways) (Arnold et al. 1982). These phenomena have been repeatedly demonstrated in pathologic situations where bacterial overgrowth occurs in the face of high serum iron concentrations (Arnold et al. 1982). Given its iron-withholding ability, LF was initially assigned its antibacterial role based on its iron-chelating ability (Valenti et al. 2005). In its apo- form, each molecule of lactoferrin is able to strongly and stably bind two ferric ions ( $\text{Fe}^{3+}$ ) - one at each of the lobar termini - thus sequestering iron.

When compared with sTF, LF releases iron in a single phase from both its N- and C-lobe around pH 3.0. Release of iron from serum TF is also pH - dependent, but is biphasic (Baker, Baker and, Kidd 2002). Iron-chelating mechanisms within the host are often challenged by bacteria either through the production of iron-chelating compounds such as siderophores by the removal of iron from oxidized heme (hemin), or through direct competition with host iron-binding glycoproteins through specific receptor binding- a feature of highly host-adapted bacteria (Valenti et al. 2005). However, given that Arnold et al were unable to reverse apo-lactoferrin-induced inhibition of bacterial growth by iron supplementation, they ventured to elucidate the mechanism of lactoferrin-

induced bacteriostasis involved. Using immunofluorescence, they demonstrated direct bacterial binding by LF (Arnold et al. 1982). Cultures died within an hour of lactoferrin-bacterial co-incubation and bacterial viability was not regained following the removal of all detectable surface-LF (Arnold et al. 1982). The investigators proposed inhibition of carbohydrate metabolism or transport. Lactic acid synthesis was also abolished within an hour of LF treatment (Arnold et al. 1981).

Lactoferrin is also able to inhibit bacterial adherence to and colonization of mucosal surfaces (Valenti et al. 2005). While it appears to be a dose dependent phenomenon, it is independent of the iron-scavenging ability of this monomeric glycoprotein (Kawasaki et al. 2000). In another study, Qui et al noted two specific mechanisms by which LF limited adherence to and hence colonization of the human respiratory epithelium by *H. influenzae*. Lactoferrin directly inhibits and degrades the non-pilus Hap protein that facilitates host cellular interaction and efficiently extracts the auto-secreted IgA1 protease pre-protein from the outer bacterial membrane, thus facilitating neutralization of bacterial protease by milk source IgA1 antibody (Qui et al. 1998). Several *in vivo* studies demonstrating the protective effects of LF against both Gram positive and Gram-negative infections have been performed in a variety of species (Bhimani R. et al. 1999; Qui et al. 1998; Ochoa et al. 2003; Kawasaki et al. 2000 and Lee et al. 1998). In their study Ochoa and others focused on the ability of LF to inhibit the type III secretion system which is used by numerous bacterial pathogens to introduce effector proteins into host cells. In the case of enteropathogenic

*Escherichia coli* (EPEC), the type III secretion system executes the pathognomonic attaching and effacing lesions (Ochoa et al. 2000). Inhibition of actin polymerization, the mechanism by which bacterial attachment is enhanced through microvilli destruction, and degradation of EspB, a key bacterial protein involved in EPEC-induced hemolysis were the identified modes of LF activity. This finding supports earlier claims of lactoferrin's ability to demonstrate serine protease-like activity. The addition of protease inhibitors to the assays proved confirmatory (Ochoa et al. 2003).

It has been shown that susceptibility to infections parallels evidence of low concentrations of lactoferrin in human polymorphonuclear neutrophils. In fact, human neutrophils devoid of LF lost their bactericidal ability (van Hooijdonk A. 2000). Decreased neutrophil function has also been linked to inhibition of endogenous lysozymal functions (van Hooijdonk A. 2000). Predisposing conditions include malnutrition, pre- or post-operative starvation, hepatic failure, iron saturation secondary to parenteral administration as well as diabetes-associated glucose-induced protein modification. In the bovine, stage of lactation is also critical. Citrate, an anion that is present in high concentrations in bovine milk (4-8mM), is able to counteract LF- iron-binding efficiency through non-synergistic binding. Relevance to immunity is borne out by evidence demonstrating the ability of citrate to inhibit the bacteriostatic properties of bovine LF *in vivo* (Brodie et al. 1994; Steijns and van Hooijdonk 2000).

In the early 1970's several studies demonstrated the lactoferrin's close association with lysozyme in human neutrophils. Lactoferrin is also is also

present within a class of lysosomal granules (Leffell and Spitznagel 1972). More recently, reports indicate that despite their independent roles, LF may synergize with lysozyme in mediating its antibacterial effects (Ellison and Giehl 1991). Both proteins are actively secreted by PMNs into the external environment early in the inflammatory response and are also present in equally high concentrations in secretions of the eyes, oropharynx, and mammary gland, respiratory and reproductive tracts (Ellison and Giehl 1991).

Timing of LF administration has also been evaluated. In mouse *in vivo* studies, intravenous LF was considered more effective against *Staphylococcus aureus* infections when administered prophylactically (Bhimani et al. 1999). Similar findings were observed in mice experimentally and systemically infected with a lethal dose of *E. coli* (Van Hooijdonk et al. 2000). In another study where LF was administered within the same time frame as intravenous infection, but as an oral prophylactic, its inhibitory effect was significant (Lee et al. 1998).

**Anti-toxin (Endo- and Exo-) Properties** - To date, the bactericidal effects of LF have either directly or indirectly been associated with its anti-endotoxin property. Two mechanisms have been proposed. In 1990, Ellison and others demonstrated that the bactericidal effects of LF on Gram-negative bacteria could be neutralized by calcium supplementation of growth media. Rossi P. et al later confirmed that like ethylenediaminetetraacetic acid (EDTA), LF has the ability to chelate calcium (Rossi et al. 2002). Elimination of the membrane-stabilizing effects of this mineral on the outer membrane of Gram-negative bacteria resulted in LPS release and subsequent death (Rossi et al. 2002). The second

mechanism functions through the highly charged and cationic surface of this molecule which facilitates its interaction with biological surfaces of both microbial and host origin (Valenti and Antonini 2005). With respect to Gram-negative bacteria, this interaction occurs primarily between the N-terminal of the protein - where there is a concentration of positive charges - and the porins present on the outer membrane of these species. Through direct contact with lipopolysaccharide, this toxic component of the bacterial outer membrane is released and quickly enhances bacterial susceptibility to osmotic shock and the lytic effects of host antibacterial molecules (Valenti and Antonini 2005). Interaction between lactoferrin and Gram positive bacteria is believed to occur through electrostatic interactions between the charged lipid matrix of the microbes and the positively charged residues of the LF N-terminus (Valenti and Antonini 2005).

With respect to killing of Gram-negative bacteria, LF's role has been attributed to its membrane destabilization effects and induced lipid A release (Vogel H., et al. 2002; Rossi P., et al. 2002; Valenti and Antonini 2005). Membrane destabilization results from calcium chelation, while LPS release is directly related to the binding of bacterial porins by the LF amino terminal (Smith K. and Schanbacher F., 1977; Vogel H., et al. 2002; Valenti and Antonini 2005; Tomita et al. 1991). Endotoxin limits permeability of the outer membrane of Gram-negative bacteria by noxious agents such as bile salts, lysozyme and many hydrophobic antimicrobial agents and is therefore critical for bacterial survival within the host (Wilson et al. 2002).

Lactoferrin also demonstrates immuno-regulatory functions (Steijns and van Hooijdonk 2000; van Hooijdonk, Kussendrager, and Steijns 2000). The LPS-neutralizing ability of LF has also been linked to functional N-terminal peptide fragments (Valenti and Antonini 2005; Tomita et al. 2002; Gifford, Hunter, and Vogel 2005; Japelj et al. 2005). Since lactoferrin binding of lipid A limits its interaction with host macrophages, the expression of cellular inflammatory cytokines is subsequently reduced (Legrand et al. 2004; Tomita et al. 1991; Elass-Rouchard et al. 1998). Lipid A is central to the development of endotoxic shock- the result of the combined effects of host-derived cytokines, complement and coagulation cascade components (Gerros et al. 1993; Moore and Morris 1992; Hurley 1995; Wilson et al. 2002).

**Anti-inflammatory Properties** - A major topic of debate over the years has been the question of whether lactoferrin truly functions in an anti-inflammatory role. Evidence pertaining to its pro-inflammatory nature and its ability to inhibit oxygen-centered free radical production, have both been documented (Britigan, Serody, and Cohen 1994). While LF synthesis in PMNs appears to be complete by the early band stage, there is evidence that it is synthesized *de novo* by neutrophils during inflammation (Baynes and Bezwoda, 1994; Steijns and van Hooijdonk, 2000).

Central to the inflammatory response is stimulation of the host's innate immune cells and the endothelium to secrete pro-inflammatory cytokines and up regulate the expression of adhesion molecules, respectively. The attraction of inflammatory cells to activation sites leads to the development of deleterious

effects. Lipopolysaccharide the non-proteinaceous element of the outer membrane of Gram-negative organisms is a potent stimulator of inflammation (Baveye et al. 2000a). Besides activation of complement, stimulation of host factors is mediated primarily through CD14, a 55kD glycoprotein which functions as a specific LPS receptor (Baveye et al. 2000a). Also known as the CD14 scavenger receptor, it exists in both soluble (sCD14) and membrane-bound (mCD14) forms. Serum concentrations of 2 to 6 µg/ml of the soluble fraction exist in humans. Membrane CD14 is constitutively expressed on the monocyte/macrophage cell membrane as a glycophosphatidylinositol-anchored protein and has been described on neutrophils as well (Baveye et al. 2000a; Parham 2005, 238).

Due to its amphiphilic nature, LPS forms aggregates in circulation and in the absence of lipopolysaccharide binding protein (LBP), an acute phase binding protein (Baveye et al. 2000a). Spontaneous diffusion of LPS monomers from these aggregates to mCD14 occurs very slowly. Consequently, when circulating LPS concentrations are low, LBP catalyzes the transfer of LPS monomers to this isoform of the LPS receptor, thus increasing host sensitivity (Baveye et al. 2000a). In the face of endotoxemia, this mechanism is bypassed by direct activation of the endothelium by sCD14-LPS complexes. Leukocyte infiltration is promoted and mechanisms that ultimately end in disseminated intravascular coagulation and endotoxic shock are subsequently activated (Baveye et al. 2000). Both in vitro and in vivo animal and human model studies have demonstrated the anti-inflammatory role of lactoferrin as it relates to the inhibition

of host cellular/LPS interactions. Lactoferrin binds the lipid A portion of LPS with high affinity. However, based on in vivo investigations, it has been reported that optimal inhibition requires 12 to 24 hours of LF pretreatment (Lee et al. 1998). Preferential binding of LF to porcine CD14-positive peripheral blood mononuclear cells was observed in one study (Lee et al. 1998). Interference with LBP-mediated binding of LPS to mCD14 by lactoferrin has been repeatedly demonstrated, resulting in marked reductions in the production of the major pro-inflammatory cytokines IL-1, IL-6 and tumor necrosis factor alpha (TNF- $\alpha$ ) (Baveye et al. 2000a). Others have also reported the suppression of interleukin 1 (IL-1), interleukin 2 (IL-2), and tumor necrosis factor (TNF) production in mixed lymphocyte cultures treated with LF even at concentrations lower than 10-8M (Crouch et al. 1992). Similar studies have been performed to evaluate lactoferricin effects. This glycan moiety has been shown to suppress the production of IL-6 and IL-8 by LPS stimulated human cells of the monocytic and neutrophilic cell lines, respectively (Harshrnjit et al. 2000).

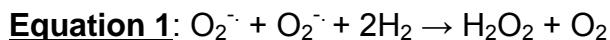
The effect of LF on sCD14-LPS- binding has also been investigated. Baveye et al reported a significant reduction in adhesion molecule expression by endothelial cells treated with human LF. Consequently, they concluded that this mechanism most likely contributes to the marked anti-inflammatory effects reported by Lee and others (Baveye et al. 2000a). Lactoferrin has also been proposed to have immuno-enhancing effects through its ability to enhance superoxide production through the chelation of iron, a catalyst of the Haber-

Weiss reaction. (Baynes and Bezwoda; Britigan et al. 1994; Tellado and Christou 1991)

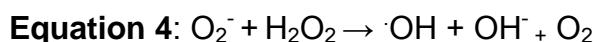
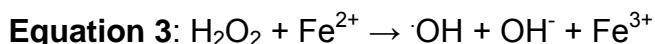
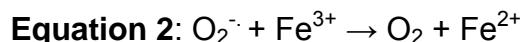
Normal host metabolic function involves tightly regulated biochemical reactions which result in the generation of H<sub>2</sub>O from molecular oxygen (O<sub>2</sub>), the terminal electron acceptor (Zinkl and Kabbur, 1997). Sequential reduction of O<sub>2</sub> yields intermediates such as the superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the hydroxyl ion (OH<sup>-</sup>), all of which are potentially toxic to biological systems (Zinkl and Kabbur, 1997). While most cells contain a variety of enzymatic and other mechanisms that limit free radical accumulation, these intermediates are utilized in host defense by the phagocytic cell lines (neutrophils, monocytes/macrophages, and eosinophils) (Zinkl and Kabbur, 1997).

Neutrophils exert their bactericidal function through phagocytosis and oxygen -dependent and/or oxygen-independent killing mechanisms. Oxygen dependent-mechanisms, otherwise referred to as respiratory burst, result in the generation of toxic oxygen metabolites (Davis et al. 1998; Zinkl and Kubbar, 1997). Following phagocytosis and cellular activation latent cytosolic and membrane associated components of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are assembled on the membrane of the phagosome to subsequently catalyze the membrane electron transport system (Davis et al. 1998; Underhill and Ozinsky 2002). NADPH oxidase is responsible for the transfer of electrons from NADPH to molecular oxygen, thus generating the superoxide anion (O<sub>2</sub><sup>-</sup>) [Figure 2.1] (Davis A. et al. 1998). O<sub>2</sub><sup>-</sup> is further

converted into other toxic reactive oxidants which generate secondary oxidative decarboxylation, deamination, peroxidation and halogenation reactions that culminate in the destruction of the offending bacteria (Zinkl and Kubbar, 1997). Since  $O_2^-$  is highly self-reactive at physiologic pH and automatically generates hydrogen peroxide ( $H_2O_2$ ) neighboring host cells also tend to be affected (Britigan, Serody, and Cohen 1994; Zinkl and Kubbar, 1997).



Although the reduction of  $H_2O_2$  to  $\cdot OH$  - the single electron reduction product of  $H_2O_2^-$  by the superoxide ion is a very slow one, it will proceed at higher rates in the presence of certain transition metal chelates such as some iron complexes. Consequently, in the resulting Haber- Weiss reaction,  $O_2^-$  acts as both the source of  $H_2O_2$ , as well as the reducing agent for the generation of  $Fe^{3+}$  from  $Fe^{2+}$  [Equations 2- 4] (Britigan, Serody, and Cohen 1994)



In light of the iron-chelating ability of lactoferrin, several studies have evaluated the catalytic effect of partially and fully iron - saturated lactoferrin on  $\cdot OH$  production by stimulated neutrophils or cell-free  $O_2/H_2O_2$  generating systems. The work of Ambruso and Johnston (1981) and that of others supported its proposed positive effect on the Haber-Weiss reaction. In retrospect, the  $\cdot OH$  - detection systems utilized in those studies have since then been classified as being unreliable indicators of hydroxyl ion generation (Britigan, Serody, and

Cohen 1994). Additionally, these earlier findings were refuted by several investigators who suggest that neither apo-lactoferrin nor iron-associated LF is efficient at catalyzing the Haber-Weiss reaction (Britigan, Serody, and Cohen 1994).

Using highly specific ·OH spin trapping techniques, others later compared this proposed function of lactoferrin with that of transferrin. In those experiments, both LF and transferrin were exposed to elastases, proteases, oxidants and LPS at physiologic concentrations. While cleavage of apo-transferrin negated its ability to prevent ·OH generation via the Haber-Weiss reaction, cleavage of apo-lactoferrin did not affect its ability to inhibit the reaction. Only a slight increase in ·OH generation was detected in holo- LF-treated assays. (Britigan, Serody, and Cohen 1994)

Two mechanisms of LPS-induced neutrophil “priming” have been proposed: 1. The LBP-CD14 pathway of the monocyte/macrophage activation system results in cytokine release (TNF- $\alpha$ , IL-6, IL-1) and neutrophil recruitment to inflammatory sites (Antal-Szalmás, 2000; Elass-Rochard et al. 1998). Since CD14 expression by neutrophils is also rapidly up regulated by tumor necrosis factor- $\alpha$ , granulocyte-colony stimulating factor (G-CSF), and LPS, priming directly through the LBP-CD14 pathway is also very likely (Antal-Szalmás, 2000 ; Britigan, Serody, and Cohen 1994 ; Wang et al. 1995). 2. Alternately, Baveye et al confirmed the role of L-selectin as the major LBP/CD14- independent LPS-binding site on neutrophils (Baveye et al. 2000b). In their study, they demonstrated a 55% reduction in LPS-induced neutrophil oxidative burst

following LF supplementation (Baveye et al. 2000b). Lactoferrin also demonstrated unique tryptase inhibition in a sheep model for asthma. Its role was mediated through LF-heparin- binding domains. LF inhibited both early and late-phase broncho-constriction (Elrod et al. 1997).

**Immunomodulatory Properties** - Lactoferrin affects both proliferation and differentiation of immune cells through its ability to modulate cellular signaling pathways. However, conflicting views regarding its effects on lymphocyte proliferation exist. While Esaguy and others report stimulatory effects, Richie et al and Ashorn et al suggest an inhibitory role. This protein has also been shown to promote the differentiation of both immature B and T lymphocytes by promoting preferential maturation of CD4<sup>-</sup> and CD8<sup>-</sup> cells to the T-helper cell line (CD4<sup>+</sup>) (Ward et al. 2002). Lactoferrin also up regulates the CD4 surface marker in the human Jurkat lymphocyte (T cell tumor) cell line through the mitogen-activated protein kinase signaling pathway (Dhennin-Duthille et al. 2000 ; Ward et al. 2002). Using a mouse model, Debbabi et al demonstrated the immunostimulating activity of LF on the mucosal immune system (Debbabi et al. 1998). Following interaction with its intestinal ligand post ingestion, high concentrations of LF-induced immunoglobulin A and G were detectable in the Peyer's patches, intestinal fluid and serum (Debbabi et al. 1998).

Both the iron-saturated and apo-forms of bovine LF can be immunogenic in mice. Following prolonged oral administration of bovine lactoferrin, specific A and G isotype antibodies could be detected in intestinal secretions as well as in

serum (Debbabi et al. 1998). Additionally, enzymatic degradation of apo-lactoferrin gives rise to immunogenic forms which are believed to be transported by enterocytes and M cells (Debbabi et al. 1998). Tomita et al (2002) proposed a mechanism for the immunomodulatory action of orally ingested LF. Since relatively minimal amounts of ingested LF is absorbed from the intestines in both human and animal models, it appears to act on the enterocytes and gut-associated lymphoid tissue (GALT) cells, through a receptor-mediated mechanism with resultant up-regulation of IL-8 production and possibly that of chemokines and other cytokines (Tomita et al. 2002).

Bovine LF was shown to stimulate murine lymphocyte proliferation and immunoglobulin production *in vitro* and is believed to supply the iron needed for cellular growth (Debbabi et al. 1998). Additionally, investigators demonstrated LF -enhanced proliferative responses by polyclonally activated lymphocytes as well as those activated by alloantigens. In the same study, they also characterized a LF receptor (LF-R) on all major lymphocyte subsets ( $\alpha\beta$  T cells, CD8 $^+$  T cells, CD4 $^+$  T cells and  $\gamma\delta$  T cells). However, compared to  $\alpha\beta$  T cells, expression by  $\gamma\delta$  cells was considered more likely. The expression of the LF-R appeared to be directly related to LF concentration (Mincheva-Nilsson, Hammarström, and Hammarström 1997). In *in vivo* assays, LF-R expression by decidual lymphocytes exceeded that of the transferrin receptor regardless of LF concentration. This was attributed to synergism between LF and transferrin in supporting the growth and proliferation of lymphocytes (Mincheva-Nilsson, Hammarström, and Hammarström 1997).

In earlier studies, Clevers et al showed that bovine neonates had the largest proportion of  $\gamma\delta$  T cells. Gamma delta T cells are believed to facilitate the primary responses of young immunologically naïve animals (Clevers et al. 1990). They accounted for 25% of the total peripheral blood mononuclear cell numbers (Clevers et al. 1990).

**Antiviral Properties** - Both bovine and human lactoferrin have demonstrated activity against an array of viruses including enveloped (herpes simplex viruses 1 and 2), non-enveloped (rotavirus, poliovirus and adenovirus) DNA and RNA viruses, as well as against viruses with different replication mechanisms (Valenti et al. 1998; Orsi 2004). Data support that the antiviral activity of LF is not associated with iron sequestration and in several studies, holo-LF isoforms demonstrated efficacy (Valenti et al. 1998; Orsi 2004). In fact, some studies demonstrate that apo-LF is less effective in its antiviral activity than the iron-saturated form. The higher inhibitory activity of diferric lactoferrin could be largely due to the conformational change resulting from metal ion saturation (Valenti et al. 1998; Orsi 2004).

It is generally believed that LF-induced inhibition occurs early in the infection phase and may involve direct binding of viral particles as well as competitive binding to host heparin sulfate and glycosaminoglycans, preferential binding sites of viral antigens. However, the mode of activity appears to be dependent on the strain of the virus as well as the phase of the viral infection (Jenssen 2005; Orsi 2004). While pre-incubation of adenovirus with bovine LF resulted in 95% inhibition of viral antigen synthesis, viral infectivity was only

inhibited by 60% when human epidermoid carcinoma laryngial (HEp-2) cells were pre-incubated with LF. This provided supporting evidence for previous inferences that the adenovirus utilizes receptors other than glycosaminoglycans for attachment to host cells (Pietrantoni A. et al. 2003). Other studies demonstrate that as with adenoviral infections, LF directly interacts with the viral particles of feline herpes virus (FHV-1), hepatitis C virus (HCV), and the human immunodeficiency virus (HIV). On the other hand, while competitive binding does not completely explain LF inhibition, infection by hepatitis B virus (HBV) and herpes simplex virus (HSV) is more effectively inhibited as a result of LF interaction at the cell surface (Jenssen , 2005 ). The antiviral activity of the lactoferricins has also been investigated with LFcinB being more efficacious. Antiviral characteristics have been demonstrated against HCV, human cytomegalovirus (HCVM), HIV, HSV-1 and HSV-2. LFcinH has only been shown to inhibit infections by HSV-1 and HSV-2 but to a lesser extent than has been exhibited by LFcinB (Jenssen, 2005).

**Anti-fungal Properties** - In 2000 Wakabayashi et al and Yamauchi and his group demonstrated the usefulness of bovine lactoferrin in clearing fungal infections in both humans and animals (Yamauchi et al. 2000). However, the mechanism of action was not determined. More recently (2005), investigators looked at the effect of orally administered bovine LF on host mechanisms involved in the elimination of *Trichophyton mentagrophytes* in guinea pigs. While, their findings were inconclusive they reported enhanced stimulation and *mentagrophytes*-killing ability by macrophages isolated from LF-treated guinea

pigs. They also noted the release of a macrophage-activating factor which appeared to function similarly to interferon- $\gamma$  (Wakabayashi et al. 2002). In another study, the killing of the dangerous opportunistic fungus *Candida albicans*, by LFcinB was investigated. At varying concentrations, LFcinB was lethal to the cultured colonies. Similar to the reports of Ellison and others (1990) regarding the bactericidal effect of the native protein, these investigators also noted that the lytic effects of LFcin were diminished following calcium supplementation. Magnesium supplementation also had a similar effect (Bellamy et al. 1993). Based on the profound ultrastructural changes that were observed, they proposed that LFcinB interacted directly with the cell membrane of the fungus (Bellamy et al. 1993).

**Anti-protozoal Properties** - Few studies have considered the role of lactoferrin as an anti-protozoal. While *Toxoplasma gondii* tachyzoites demonstrated sensitivity to the native protein, using an *in vitro* murine macrophage model, parasite infectivity was not affected (Orsi N., 2004) Intracellular development of the parasite was however inhibited. In another study, both oral and intraperitoneal administration of LFcinB induced resistance in mice. Similar findings were noted for *Eimeria stiedai* sporozoites (Orsi N., 2004) Contrasting results were noted with respect to *Trichomas vaginalis* and *Tritrichomonas foetus*, agents significant to human and bovine reproductive health, respectively. In both cases, the investigators note that LF served as a source of iron for these organisms rather than inhibited their growth (Orsi N., 2004).

In the ensuing chapters, the anti-inflammatory effects of LF will be further addressed in *in vivo* and *in vitro* calf models. These will serve as preliminary efforts towards additional *in vivo* investigations. Areas of focus will include prostanoid and cytokine elaboration and an attempt will be made to elucidate intracellular mechanisms involved.

## **ISOLATION AND PURIFICATION OF LACTOFERRIN / LACTOFERRICIN**

On an industrial scale, LF is currently being isolated and purified worldwide, from cheese whey and skim milk. Since the LF present in whey exists as a cationic protein, it is readily extracted through a cation-exchange resin, after which it is eluted using salt solutions. Following desalting and concentration procedures using ultrafiltration and difiltration membranes, the extract may either be pasteurized (heating at pH 4 to 90 - 100°C for 5- 10 min as well as the UHT method) and the purified powder ( $\geq 95\%$ ) is finally obtained by freeze-drying, or is alternatively, microfiltrated and spray-dried, respectively. The former method is preferred in order to inactivate bacteria as well as viruses such as the foot and mouth disease virus (Tomita et al. 2002). Fresh skimmed milk is digested under acidic conditions, using porcine pepsin. Following hydrolysis, the pepsin is inactivated and the reaction mixture filtered and concentrated by reverse osmosis. The final product obtained after pasteurization and freeze-drying, is lactoferricin powder of  $> 95\%$  purity (Tomita et al. 2002).

## **CHAPTER 3**

### **EFFECTS OF SUPPLEMENTAL LACTOFERRIN ON SERUM LACTOFERRIN AND IGG CONCENTRATIONS AND NEUTROPHIL OXIDATIVE METABOLISM IN HOLSTEIN CALVES**

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## ABSTRACT

Lactoferrin (LF) is an iron-binding protein present in both colostrum and secondary granules of polymorphonuclear neutrophils (PMNs). We hypothesized that supplemental Lf would enhance neutrophil function in neonatal calves. Newborn calves were assigned to receive colostrum (C), colostrum + LF (CLF 1g/kg), or milk replacer + LF (MRLF 1g/kg). Serum (LF and IgG) and whole blood (neutrophil isolation) samples were obtained prior to treatment (day 0), and at 24 hours, and 9 days of age. Serum IgG concentrations at 24 hours were: C =  $1911 \pm 994$  mg/dl (mean + SD); CLF =  $2181 \pm 625$  mg/dl; and MRLF = 0 mg/dl. Serum LF concentrations on day 0 were  $324 \pm 334$  ng/ml (range 0 - 863 ng/ml),  $135 \pm 158$  ng/ml (range 0 - 429 ng/ml) and  $318 \pm 337$  ng/ml (range 0 - 964 ng/ml) in C, CLF, and MRLF calves, respectively. Lactoferrin concentrations at 24 hours were significantly ( $P < 0.05$ ) higher:  $1564 \pm 1114$  ng/ml (range 335 - 3628 ng/ml);  $2237 \pm 936$  ng/ml (range 31 - 3287 ng/ml); and  $3189 \pm 926$  ng/ml (range 1736 - 4120 ng/ml) in C, CLF and MRLF calves, respectively.

Cytochrome C reduction in opsonized zymosan or phorbol ester -treated cells was not significantly affected by supplemental LF provided at birth. Orally supplemented LF is absorbed in calves, but does not alter PMN superoxide production and does not alter IgG absorption.

**Key words:** Opsonized zymosan (OP), Phorbol 12-myristate 13-acetate (PMA), Cytochrome C reduction

## INTRODUCTION

Lactoferrin (LF) is an iron-binding protein product of the transferrin gene family. Lactoferrin is a major constituent of the secondary granules of PMNs, and is also produced by epithelial cells including the mammary gland (Schanbacher, Goodman and Talhouk 1993). Higher concentrations of this protein are found in bovine colostrum than in milk (1 - 5 mg/ml versus 0.1 - 0.35 mg/ml) and is obtained by the neonate by ingesting colostrum (Smith and Schanbacher 1977). LF is stable when frozen (Lakritz et al. 2000a). It is absorbed by the neonate and is widely distributed in the body. The significance of LF in the health of the mammary gland has been investigated. There is an increase in the concentration of LF in milk (> 1 mg/ml) during mastitis and LF plays a prominent role in protecting the mammary gland from Gram-negative infection (Harmon, Schanbacher and Ferguson 1975; Schanbacher, Goodman and Talhouk 1993). Lactoferrin acts synergistically with IgG<sub>1</sub> to inhibit growth and colonization of the gut by *Escherichia coli* and *Klebsiella pneumoniae* (Sordillo, Shaefer-Weaver and DeRosa 1997). Lactoferrin also promotes the diapedesis to and retention of neutrophils at an inflammatory site, thus serving an auto-regulatory role (Smith and Schanbacher 1977). In *in vitro* studies, neutrophils obtained from calves fed heat-denatured colostrum, had decreased oxidative metabolism (Lakritz et al. 2000a) These calves had lower serum lactoferrin when compared to control calves receiving fresh frozen colostrum (Lakritz et al. 2000a) When bovine and ovine neutrophil (PMN) incubations were supplemented with

exogenous colostral lactoferrin (100, 265 and 530 µg LF/ml of media), oxidative burst was augmented (Fahey and McKelvey 1965; Lakritz et al. 2000a).

The primary goal of this study was to determine the effect of oral lactoferrin supplementation on serum lactoferrin concentration in calves. Additionally, we examined whether increases in serum LF concentrations were associated with augmented neutrophil superoxide production induced by two different stimuli. Finally the absorption of immunoglobulin G relative to LF absorption was also investigated.

## MATERIALS AND METHODS

**Animals** - Twenty-two Holstein bull calves were separated from their dams after birth prior to colostrum ingestion. They were sequentially assigned to treatment groups: calves were fed 4L of C (colostrum; n = 6), colostrum + LF 1 g/kg (CLF; n = 10) or milk replacer + LF 1 g/kg (MRLF; n = 6) within 4 hours of birth. Colostrum samples were pooled prior to administration. After 24 hours of age, calves were fed 2L of a commercial milk replacer (Land O'Lakes Inc., MN) twice daily until weaning. Fresh hay, calf starter and water were provided *ad libitum* from day 0. These studies were evaluated and approved by the University of Missouri-Columbia Animal Care and Use Committee.

**Handling and Administration of Initial Diets** - Containers of fresh-frozen colostrum were thawed in warm water. Where applicable, 1 g/kg body weight of purified bovine lactoferrin<sup>i</sup> was added to colostrum or milk replacer and the

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<sup>i</sup> Sigma-Aldrich Co, St Louis, MO

solution was homogenized using a commercial blender. Four liters (4L) of C, CLF or MRLF were administered to each calf by esophageal feeder<sup>ii</sup> within 4 hours of birth. Ten milliliter (10 ml) aliquots of each were retained and frozen at -20°C for later determination of LF and IgG concentration.

**Peripheral Blood Collection** - Peripheral blood was collected by jugular venipuncture into 10-milliliter (10 ml) serum clot tube<sup>iii</sup> and five 10-milliliter (10 ml) tubes containing Acid Citrate Dextrose (ACD)<sup>iv</sup> prior to feeding on day 0 (precolostral), and at 24 hours and 9 days of age. Additional serum samples were collected on days 30 and 60.

**Determination of Colostral/Treatment LF Concentration** - Treatment LF concentrations were determined using a two-antibody sandwich assay (ELISA<sup>v</sup>) as described (Harlow and Lane 1988). Colostrum samples were diluted 1:1000, using Dulbecco's Phosphate-Buffered Saline<sup>vi</sup> (PBS) with 3% bovine serum albumin<sup>vii</sup> (PBS-BSA). Briefly, plates were coated with goat anti-bovine lactoferrin<sup>viii</sup> (1 µg/well) for 2 hours, washed and blocked using 3% PBS-BSA with 0.02% sodium azide<sup>i</sup> overnight at 4°C. Aliquots of each of the calf treatments (100 µl) were placed into individual wells in duplicate. Standards (1000, 500, 250, 125, 62.5, 31.3, 15.63, and 7.81 ng of LF/ml) were prepared using known

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<sup>ii</sup> Reinheart Development Corp., Spencerville, IN

<sup>iii</sup> Monojet Sherwood Medical, St. Louis, MO

<sup>iv</sup> BD Vacutainer ACD Solution A, BD, Franklin Lakes, NJ

<sup>v</sup> Lactoferrin evaluation, Dynatech Immunolon Laboratories, Chantilly, VA

<sup>vi</sup> Dulbecco's PBS (1X) liquid, GIBCO BRL, Gaithersburg, MD

<sup>vii</sup> Fisher Biotech, Pittsburgh, PA

<sup>viii</sup> A10-126-A4 (affinity purified), Bethyl Laboratories Inc., Montgomery, TX

concentrations of bovine milk LF<sup>ix</sup>. Standard controls included 100 µL of PBS as a negative control. After 2 hours, the plates were washed and 100 µL of goat anti-bovine lactoferrin-horseradish peroxidase (HRP) conjugate<sup>x</sup> (1:2,500), were added to each well. Antibody bound to immobilized LF was detected by addition of 100 µL of ABTS<sup>xi</sup>. The amount of bound antibody conjugate was determined using an automated 96 well plate reader<sup>xii</sup> at 405 nm. Sample LF concentration was determined by comparing the sample absorbance to the linear regression of the standard concentrations versus absorbance.

**Determination of Serum LF Concentration** - Serum LF concentration was determined using an adaptation of the technique described above. Serum samples were diluted 1:100, using PBS<sup>-BSA</sup>.

**Determination of Serum IgG Concentration** - Initial screening of calves was performed using a temperature-sensitive refractometer<sup>xiii</sup>. All calves had failure of passive transfer (serum total protein concentration < 5.2 g/l). Serum IgG concentration was quantified using a previously described radial immunodiffusion (RID) assay (Dawes et al. 2002; Fahey and McKelvey 1965).

**Determination of Colostral/Treatment IgG Concentration** - Immunoglobulin G concentrations of colostrum and milk replacer were determined using an adaptation of the technique described above. Samples

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<sup>ix</sup> Bovine milk-protein reference calibrator, RC 10-125-3, Bethyl Laboratories Inc., Montgomery, TX

<sup>x</sup> A10-126P-3, Bethyl Laboratories Inc., Montgomery, TX

<sup>xi</sup> 2,2' - Azinobis (3-ethylbenzthiosoline-sulfonic acid) (ABTS), Kirkegaard Perry Laboratories Inc., Gaithersburg, MD

<sup>xii</sup> Labsystems Multiskan MS, FIN 00881, Helsinki, Finland

<sup>xiii</sup> Leica Inc., Buffalo, NY

were diluted 1:100 in barbital buffer. Five-microliter aliquots of the diluted colostrum or milk replacer were inoculated into each well of the assay plates.

**Polymorphonuclear Neutrophil Isolation** - Citrate blood samples from each calf were pooled and centrifuged (400 x g, 22°C, 10 min). Neutrophils were isolated from the red cell layer by cold lysis using previously described methods (Carlson and Kaneko 1973). The cells were further purified by differential centrifugation using ficoll-sodium diatrazoate<sup>xiv</sup> (Torre and Oliver 1988) Cell number was determined with a hemocytometer and viability by trypan blue exclusion. Purity of neutrophil preparation was determined by counting at least 200 cells/calf on Wright stained preparations. Typically, preparations with > 90% neutrophils with > 90% viability were utilized in the study.

**Stimulant Preparation** - Serum opsonized zymosan (OP) was prepared as previously described (Hasegawa et al. 1997; Higuchi et al. 1997). Phorbol myristate acetate (PMA) was dissolved in 1 ml of DMSO<sup>i</sup> to a final concentration of 1 mg/ml and stored at -20°C. Phorbol ester stock solutions were diluted to 1:100 (10 ng/ml) for use in the assay.

**Determination of PMN Superoxide Metabolism**- Two million ( $2 \times 10^6$ ) cells were stimulated with three OP and three PMA concentrations (OP10, 50, and 100 µg /ml; and PMA 30, 60, and 300 ng /ml). Superoxide production was performed as described (Markert, Andrews and, Babior 1984). Final concentrations of cytochrome C<sup>xv</sup> was 50 uM. Twenty units of superoxide

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<sup>xiv</sup> Accupaque (density 1.086), Accurate Chemical and Scientific Corp, Westbury, NY

<sup>xv</sup> Superoxide-free Horse heart cytochrome c, Sigma-Aldrich Co, St. Louis, MO

dismutase<sup>i</sup> (SOD) were added to each of the OP and PMA controls. After 20 minutes at 37°C, the supernatant was decanted into disposable cuvettes<sup>xvi</sup> and cytochrome C reduction was determined by scanning between 530 - 570 nm (Markert, Andrews and, Babior 1984)

*Statistical Analysis* - Serum IgG and LF concentrations prior to and 24 hours after treatment administration are reported as mean and standard deviation (SD). The effects of time, stimulant dose and treatment group on neutrophil superoxide production were determined using a nonparametric repeated measures analysis of variance procedure in which stimulant dose and time were treated as repeating variables. In this procedure superoxide production were ranked from smallest to largest response and rank was substituted for the superoxide production. Separate models were developed for assays using OP and PMA as stimulants. Statistical significance was set at  $P < 0.05$ . Serum IgG and LF concentrations were compared between the three experimental groups using paired t-tests. The differences were considered significant when the calculated  $P$  value was less than 0.05 ( $P < 0.05$ ). Statistical analyses were performed with the aid of a statistical software package<sup>xvii</sup>.

## RESULTS

*Treatments significantly alter serum LF concentrations* - Twenty-four hours after treatment, serum LF concentrations were significantly higher in CLF and MRLF compared to C ( $P < 0.001$ ) (Table 3.1). At two months (day 60), serum LF concentrations in all calves were within the range of pretreatment serum LF

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<sup>xvi</sup> Semi-UV cuvettes, model 14-385-935, Fisher Scientific, Pittsburgh, PA

<sup>xvii</sup> Current PC version, PC Statistical Analysis Software (SAS) program, SAS, Cary NC

concentrations. Lactoferrin concentrations in the treatments ranged from  $2.21 \pm 1.5$  g/l (range 0.82 - 4.4 g/l) in the colostrum group, to  $11.8 \pm 1.7$  g/l (range 9.3 - 12.9 g/l) in the colostrum + lactoferrin group; and  $11.6 \pm 0.95$  g/l (range 10.2 - 12.7 g/l) in the milk replacer + lactoferrin group (Table 3.1).

*Serum IgG concentration in calves post treatment* - Serum IgG concentration in all calves prior to treatment administration was 0 g/l (Table 3.2). Calves in C had serum IgG concentrations of  $19.1 \pm 9.4$  g/l 24 h after treatment, and  $20.5 \pm 9.8$  g/l on day 9. Calves in the CLF group had a mean serum IgG concentration of  $22 \pm 6.3$  g/l at 24 hours, while concentrations on day 9 were  $19.5 \pm 5.2$  g/l. Serum IgG concentrations in C and CLF calves were significantly higher ( $P < 0.001$ ) at 24 h than were concentrations in all calves at time = 0 and in MRLF calves at 24 h and 9 days. Twenty four hour serum IgG concentrations in C calves was not significantly different from either the day 9 concentrations (C) or from the 24 h and day 9 concentrations in CLF calves.

*LF did not alter superoxide production by calf neutrophils*- There was no association between treatment groups or sampling days and generation of the superoxide anion ( $O_2^-$ ) by OP-stimulated neutrophils. Rather, PMN superoxide activity was significantly dependent on OP-stimulant dose (Table 3;  $P < 0.001$ ). Following the stimulation of PMNs isolated from C calves, using opsonized zymosan at concentrations 50 µg/ml and 100 µg/ml, the resulting superoxide activity on day 0 was significantly greater than that measured when cells were incubated with OP at 10 µg/ml ( $10.8 \pm 4.0$  and  $13.2 \pm 5.6$ , respectively, versus  $4.3 \pm 0.8$ ) (Table 3). A similar trend was observed at 24 h and 9 days across all

treatments. Colostrum treatment group was not significantly associated with cytochrome C reduction in PMA stimulated cells. Neutrophil superoxide production was significantly dependent upon PMA-stimulant dose ( $P<0.02$ ) and sampling day ( $P<0.02$ ) (Table 3).

## DISCUSSION

Calves are at increased susceptibility to Gram-negative infections during the first few weeks of life, and affected neonates generally die from 3 days of age (Smith 2002, 352). This vulnerability is exacerbated by inability of the immune system of the calf to recognize endotoxin until 30 days of age (Osburn et al. 1974). Maternal cells, colostral proteins and innate immunity (neutrophils) that are acquired through colostral ingestion are therefore critical for neonatal survival. Neutrophil granules contain a number of biologically active compounds including lactoferrin (Swain et al. 2000; Zinkl and Kabbur 1997). The role of LF in bovine mammary health has been described Sordillo et al. 1997). Lactoferrin functions include iron chelation, LPS binding and modulation of superoxide production (Debbabi et al. 1998; Lee et al. 1998; Baynes and Bezwoda 1994; Britigan, Serody, and Cohen 1994; Tellado and Christou 1991). In addition LF becomes associated with cell surface receptors leading to some of its biological effects (Baynes and Bezwoda 1994; Britigan, Serody, and Cohen 1994; Lee et al. 1998; Swain, Jutila, and Quinn 2000). Neonatal calves have lower circulating concentrations of LF immediately post-partum when compared to those after ingestion of colostrum. Fresh colostrum contains immunoglobulins, functional

maternal leukocytes and LF (Riedel-Caspari and Schmidt 1990; Smith and Schanbacher 1977). Many dairy calves receive fresh-frozen colostrum that lack the presence of viable maternal leukocytes, however, LF is still present. Assessing the effects of colostral LF on cellular oxidative metabolism of colostral leukocytes may be of clinical significance.

Serum LF concentration was increased in all calves 24 hours after colostrum or LF supplemented product administration. Serum lactoferrin concentrations were significantly increased in calves provided supplemental lactoferrin when compared to calves receiving only colostrum (Table 1). Given the high concentration of proteins presented to the intestinal lumen of the CLF and MRLF calves, evidence of absorption of adequate amounts of IgG and LF underscores the absorptive capacity of the neonatal intestinal epithelium. Our data demonstrates that LF is absorbed from colostrum and supplemental LF significantly augments serum concentrations of LF.

Despite significant absorption, lactoferrin supplementation did not result in enhanced neutrophil superoxide production as was observed in *in vitro* studies (Lakritz et al. 2000; Wong et al. 1997). In both previously reported studies, augmentation of superoxide production following *in vitro* supplementation of LF occurred at concentrations ranging from 100 to 1000 µg /ml (Lakritz et al. 2000; Wong et al. 1997). This exceeds the concentrations achieved *in vivo* by 25 to 250 fold. Interestingly, elimination of LF from the serum of CLF and MRLF calves by day 9 appears to be occurring faster when compared to that of calves receiving colostrum alone. The estimated half life of the protein for each group

over the study period was 24.4 (colostrum alone), 12.2 (colostrum + 1 g/kg LF), and 16.6 (milk replacer + 1 g/kg LF) days. This decrease in half-life of LF in supplemented calves may be of clinical significance.

Our results suggest that the serum LF concentrations observed are not adequate to augment neutrophil superoxide production *in vitro*. One obvious pitfall to our assumption is that removal of serum components during cellular isolation may negate the effect of augmented serum LF concentrations when these cells are examined *in vitro*. We assumed some level of LF binding to neutrophils *in vivo*, would alter their response to *in vitro* stimulation. Circulating neutrophils are not likely to be primed or activated in healthy animals and circulating cells may not represent the entire functional capacity of neonatal neutrophils. Neutrophils from septic neonates, may function differently in the presence of elevated serum LF than the healthy animals studied here. Also, if one considers the great difference in functional capacity of circulating versus tissue neutrophils and relative LF concentrations in serum versus reported local tissue LF concentrations, the augmented serum LF is probably much lower than that present at local sites of inflammation.

The inability to augment neutrophil oxidative metabolism with *in vivo* LF supplementation does not preclude the importance of local lactoferrin concentrations such as those in the mammary gland, tears, other epithelial secretions, where measured concentrations appear far higher than in serum (Chantale et al. 2003; Gionfriddo et al. 2000). Higher local concentrations in the immediate vicinity of tissue cells (neutrophils) may modulate the inflammatory

response by increased superoxide production, sequestration of iron, binding locally produced bacterial products, or reducing cellular migration from the tissue (Wong et al. 1997). Higher local LF concentrations may function in an anti-inflammatory role by inhibiting the formation of toxic oxygen radicals in specific tissues, thus reducing tissue damage associated with cellular infiltrates (Britigan et al. 1994). One potential way to address this problem would be to determine tissue concentrations of LF achieved after *in vivo* oral supplementation with LF. Furthermore, assessing the superoxide production of tissue neutrophils (for example, milk or lung neutrophils) in the context of local LF concentrations achieved may provide a much different result than we obtained from relatively quiescent circulating neutrophils. Tissue cages may serve as a useful model to assess both local LF concentrations and superoxide production by elicited neutrophils.

This study has also ignored the potential antimicrobial activities of LF peptides (lactoferricins) formed by hydrolysis of lactoferrin in the gut (Tomita 1998, 189; Tomita and Bellamy et al. 1991). These peptides have marked activity against both Gram positive and Gram-negative bacterial species Tomita et al. 1991). They have also been shown to possess antioxidant activity by inhibiting the conversion of hydrogen peroxide to the hydroxyl ion via the Fenton reaction (Lindmark-Månsson and Åkesson, 2000). Assessing the concentrations of LF peptides absorbed from the gut after oral administration may prove to be of significance as it is not apparent from our study the extent to which these were formed and absorbed by the calves. Lactoferrin peptides would likely be

recognized poorly or not at all by the antibodies used in our ELISA or western blot assays.

Calves deprived of colostrum but given LF were hypogammaglobulinemic. IgG concentrations remained far below the required minimum of 10 g/l for adequate passive immunity. Following ingestion of treatments with immunoglobulin concentrations of  $133.7 \pm 21.3$  g/l and  $128.9 \pm 35.7$  g/l (CLF), both C and CLF calves had adequate transfer of passive immunity with serum IgG concentrations of  $19.1 \pm 9.4$  and  $22 \pm 6.3$  g/l at 24 h. By day 9, serum IgG concentrations were  $20.5 \pm 9.8$  in C calves and  $19.5 \pm 5.2$  in CLF calves. (Table 2) Colostrum-replete calves have fewer Ig-bearing lymphocytes in lymphoid organs and lower antibody production compared to colostrum deprived calves (Aldridge et al. 1998; Husband and Lascelles 1975). Colostrum-replete animals also demonstrate shorter serum half lives of inflammatory cytokines compared with colostrum deprived animals (Allen et al. 1993). Colostral LF absorbed at the time of birth may play a role in these immunologic changes due to cellular receptor mediated binding of lactoferrin (Gislason et al. 1995). Lactoferrin-mediated alteration in mammary T-cell phenotypes and lymphocyte proliferation has been proposed Aldridge et al. 1998; Archambault et al. 1988; Smith and Todhunter 1982).

Further examination of in vivo LF supplementation in calves should evaluate the contribution of orally administered LF on systemic availability of LF peptides, *in vivo* lymphocyte proliferation (antibody production; cell surface markers) and local or tissue LF levels in specific locations (lung; mucosal

immune tissues; tissue cage preparations). Conversely, evaluating cytokine/prostanoid production in LPS challenge of LF supplemented calves may be an alternative manner in which to evaluate systemic effects of oral LF.

## **CHAPTER 4**

### **IN VITRO EFFECTS OF LACTOFERRIN (LF) ON LIPOPOLYSACCHARIDE (LPS)-INDUCED PROLIFERATION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS.**

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## ABSTRACT

The objectives of this study were to define whether lactoferrin (LF) reduces lipopolysaccharide (LPS)-induced proliferation of bovine peripheral mononuclear cells (PBMC) *in vitro*. In parallel experiments, the effects of lactoferrin on prostaglandin (PGE<sub>2</sub>) production and cyclooxygenase-2 gene expression by LPS-stimulated PBMC were evaluated. Peripheral blood mononuclear cells were isolated from 2 to 11 month old Holstein bull calves (N=15) by density gradient centrifugation of blood obtained by jugular venipuncture. Proliferation assays using 4 x 10<sup>5</sup> cells/well in 96-well plates, were designed to allow addition of lactoferrin (200 ng/ml) and/or LPS (1 µg/ml) in a checkerboard fashion after which cells were cultured for 48 hours. Tritiated <sup>3</sup>[H]-thymidine<sup>xviii</sup> (1µC/well) was added to cultures 24 hours prior to harvesting. Cells were harvested on to filter mats and <sup>3</sup>[H]-incorporation was determined using a Wallac Microbeta workstation<sup>xix</sup>.

These experiments were repeated to determine whether iron supplementation (10 nM and 1mM Fe<sup>3+</sup>) would restore lymphocyte proliferation to lactoferrin-treated, LPS-induced cells. Prostaglandin (PGE<sub>2</sub>) production was determined in culture supernatants using a commercial enzyme immunoassay. Qualitative changes in inducible cyclooxygenase-2 (COX-2) mRNA expression

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<sup>xviii</sup> Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547  
<sup>xix</sup> Perkin Elmer, Shelton CT 06484-4794

were determined using RT-PCR. Transcripts were evaluated on 2% agarose ethidium bromide-stained gels.

$^3$ [H]-thymidine uptake was significantly less in cells co-cultured with LF and LPS compared with cells under LPS-only stimulation. Iron supplementation (1mM) significantly enhanced incorporation of tritium compared with all other treatments. Cellular proliferation was significantly less in cultures treated with lactoferrin followed by LPS-induction and in cells treated with lactoferrin supplemented with Fe and induced by LPS, in comparison with LPS-only or iron-only supplemented cells.

Prostaglandin E<sub>2</sub> production and cyclooxygenase-2 gene expression (COX-2) were evaluated in cultures without iron supplementation. LPS-stimulated cells pre-treated with LF produced significantly less PGE<sub>2</sub> than cells exposed to LPS alone. Similarly, COX-2 expression by LPS treated cells was also significantly reduced by LF pretreatment.

Our findings suggest that lactoferrin inhibits LPS - induced proliferation of bovine peripheral blood mononuclear cells. Prostaglandin E<sub>2</sub> production and COX-2 gene expression were reduced by prior incubation of LPS-stimulated mononuclear cells with lactoferrin. Lactoferrin pretreatment may be useful in attenuating LPS-mediated disease in young calves.

**Key words:** Lactoferrin (LF), Tritiated thymidine ( $^3$ [H]), cyclooxygenase-2, prostaglandin E<sub>2</sub>

## INTRODUCTION

Lactoferrin (LF) is a non-heme associated iron-binding glycoprotein of the serum transferrin gene family. Although it was discovered in 1939, it was not until the 1960's that Johansson et al isolated this 80 kD protein from both human and bovine milk (Vogel et al. 2002). Its role in innate immunity is well described and is presumed, was initially based on its close association with mucosal epithelia and their secretions (Steijns and van Hooijdonk 2000; Smith and Schanbacher 1977; Smith and Todhunter 1982; Sordillo, Schaefer-Weaver, and De-Rosa 1997). Lactoferrin has been demonstrated in colostrum, and other glandular secretions including saliva and tears. High concentrations are present in the secondary granules of polymorphonuclear leukocytes (PMN's). In disease, LF concentration becomes elevated in bovine mastitic secretions and is a direct result of increased secretion by mammary gland epithelia (Smith and Schanbacher 1977; Smith and Todhunter 1982; Sordillo, Schaefer-Weaver, and De-Rosa 1997; Harmon et al. 1975). In contrast, a direct link has been established between the degree of neutrophilia and elevations in serum LF. Lung, ocular, reproductive and other epithelia secretions also contribute (Steijns and van Hooijdonk 2000). Lactoferrin demonstrates specific anti-bacterial and anti-LPS properties (Steijns and van Hooijdonk 2000; Smith and Schanbacher 1977; Smith and Todhunter 1982; Sordillo, Schaefer-Weaver, and De-Rosa 1997). Lactoferrin chelates iron, creating an unfavorable environment for bacterial growth; specifically inhibits bacterial adherence to mucosal surfaces;

and is directly cytotoxic to bacteria (Ward, Paz, and Conneely 2005). Lactoferrin also demonstrates high binding affinity for the lipid A portion of LPS, ultimately limiting bacterial interaction with host cellular receptors such as the LPS monocyteoid CD14 scavenger receptor (Figures 4.1a and b). Given these properties, the role of LF in bovine immunity even at the neonatal stage, requires some focus.

Gram-negative infections predominate throughout the bovine life. Classically, these organisms possess a three-layered cell wall which consists of an inner and outer membrane. Lipopolysaccharide or endotoxin is present within the outer bacterial membrane and is of immunological significance. Not only is this component highly conserved among Gram-negative bacteria, but it initiates the generation of pro-inflammatory mediators such as interleukin (IL) -1, IL -6 and tumor necrosis factor (TNF) -  $\alpha$  (Rushworth et al. 2005; Nau and Eiffert 2002). Given the LPS and more specifically lipid A is targeted by LF, the potential for this iron-binding glycoprotein to direct host immune responses warrants its investigation in the bovine (Nau and Eiffert 2002). We hypothesized that LF would inhibit LPS-induced clonal expansion of mononuclear cells. The mechanisms targeted were iron sequestration and the production of growth-inducing and pro-inflammatory cytokines.

In addition to inducing the release of a wide spectrum of pro-inflammatory cytokines, LPS-induced cellular activation also initiates the metabolism of membrane phospholipids and ultimately, prostanoid release (Tilley, Coffman, and Koller 2001) (Figure 4.2). The immunomodulatory effects of LF have been

demonstrated and include altered cytokine production both *in vitro* and *in vivo* (Tilley, Coffman, and Koller 2001; Choe and Lee 1999). Other studies demonstrate modulatory effects on the expression and production of IL-2, IL-1 $\beta$ , granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-4 (Tilley, Coffman, and Koller 2001; Choe and Lee 1999).

The goal of this study was to determine the effects of LF on leukocyte proliferation associated with *in vitro* stimulation by LPS, as determined by  $^3\text{H}$  uptake and cytokine generation. In light of its iron-sequestering ability, cells were cultured in both iron-supplemented media and media devoid of iron-supplementation.

## MATERIALS AND METHODS

**Phase 1 Animals** - Seven (7) two-month old Holstein steers. Colostrum-replete calves were raised in the University of Missouri-Columbia Food Animal clinic. Calves were fed 2L of a commercial milk replacer diet (Land O'Lakes Inc. MN), twice per day until 8 weeks of age and were allowed free access to alfalfa hay and clean water. The diet was supplemented with a commercial calf starter ration.

**Phase 2 calves (n = 8)** were raised in like manner. At the age of 6 months, the diet which consisted of *ad libitum* mixed hay and fresh water was supplemented with a commercial mixed grain ration. Calves were kept on the study until 11 months of age. These studies were evaluated and approved by the University of Missouri-Columbia Animal Care and Use Committee.

**Mononuclear Cell Isolation** - Peripheral blood was collected by jugular venipuncture in 10-ml acid citrate dextrose (ratio 1:9 ACD: blood) blood tubes. Individual 10 ml blood tubes for each calf were later pooled and centrifuged<sup>xx</sup> in 50-mL conical polypropylene tubes<sup>xxi</sup> at 274 x g, 23°C for 15 minutes. Peripheral blood mononuclear cells were isolated using a previously reported technique (Carlson and Kaneko 1973). Once the buffy coat was harvested, cells were suspended in Hanks buffered salt solution (HBSS)<sup>xxii</sup>, and the targeted cells was obtained using differential centrifugation over an equal volume of histopaque<sup>xxiii</sup> in 15-ml polypropylene conical tubes<sup>xxiv</sup>. Samples were centrifuged for 30 minutes (342 x g, 23°C). Following cellular harvest at the histopaque/plasma interface, cells were washed in HBSS to remove the separating medium (285 x g, 23°C for 5 minutes). Residual red blood cells were lysed using either ammonium chloride (phase 1) or distilled water (phase 2) (Lakritz et al. 2000a). Isolated cells were re-suspended in 2.5 to 5 mls of HBSS and kept on ice during viability testing (0.1% trypan blue in PBS) and prior to culture (Carlson and Kaneko 1973). Typically, the viability of isolates ranged from 80 to 98 %. Cytospin preparations were obtained for differential determinations using Wright staining. Lymphocyte:monocyte ratios were typically 3.4:1.

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<sup>xx</sup> Beckman allegra™ 6R, Beckman Coulter

<sup>xxi</sup> Fisherbrand, # 14-375-150. Fisher Scientific, Swanee, GA

<sup>xxii</sup> GIBCO® Invitrogen cell culture supplements; Invitrogen Corporation, Carlsbad, California 92008

<sup>xxiii</sup> Histopaque (density 1.077), Accurate Chemical and Scientific Corp., Westbury, NY

<sup>xxiv</sup> Fisherbrand, # 05-527-45, Fisher Scientific, Sawnee, GA

### **Tritiated thymidine uptake by proliferating lymphocytes - Sterile**

twelve-well tissue culture plates were inoculated with a mixed population of 4 x 10<sup>6</sup> mononuclear cells in 2 mls of RPMI 1640<sup>xxii</sup> supplemented with 5-10% heat inactivated fetal bovine serum (FBS); MEM non-essential amino acids<sup>xxii</sup>, 2 mM L-glutamine<sup>xxii</sup>, 5 x 10<sup>2</sup> mM 2-mercaptoethanol<sup>i</sup> 50 U penicillin<sup>xxii</sup> and 50 µg streptomycin<sup>xxii</sup>, per ml. Treatments included control (CTRL), lactoferrin<sup>i</sup> only-treated cells (LF), LPS only-treated cells (LPS), LF + LPS-treated cells (LFLPS), Fe<sup>3+</sup>only-treated cells (Fe), lactoferrin + iron-treated cells (LFFe), iron + LPS - treated cells (LPSFe) and lactoferrin + iron + LPS-treated cells (LFLPSFe). Cells were incubated in a 37°C CO<sub>2</sub> humidified cell culture incubator<sup>xxv</sup> for 24 hours then re-evaluated for viability, prior to treatment. Cells in designated wells were pre-treated with LF (200 ng/ml) for one hour after which LPS [1µg/ml; strain O55:B5j] and Fe<sup>3+</sup> (10 nM and 1 mM) were added to respective wells. In iron-supplemented studies, culture plates were divided into equal halves. Iron-free studies were performed simultaneously with iron-supplemented cultures. Cells were maintained in culture for 72 hrs after which <sup>3</sup>[H]-thymidine<sup>i</sup> (1µCurie/well) was added to each well. At 24 hours, the contents of each well were thoroughly mixed by pipeting and 100 µl of media and cells from each well were transferred to 96-well tissue culture plates<sup>xxvi</sup> in triplicate. Cells were later harvested on to a filter mat<sup>xxvii</sup> using an automated cell harvester<sup>xxviii</sup>. Wells were not scraped prior

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<sup>xxv</sup> NuAire Laboratory Equipment Supply, Plymouth, MN

<sup>xxvi</sup> Flat bottomed Costar cell culture clusters, RK-01959-26, Cole-Parmer® Canada Inc. (formerly Labcor Inc)

<sup>xxvii</sup> Meltilex™ A,melt-on Scintilator sheets # 1450-441, Wallac Oy, Turku, Finland

<sup>xxviii</sup> TOMTEC 96-3M-402, 1000 Sherman Ave, Hamden, Connecticut

to transfer. Retained radioactivity, expressed as mean counts per minute, was determined using a Wallac Microbeta workstation<sup>xxix</sup>.

**COX-2 gene expression** - Except for the addition of iron, cells were cultured in vented 25 ml culture flasks under the same conditions as previously described. Following LPS-stimulation (24 hours) in the presence or absence of LF, culture media was aspirated and centrifuged (117 x g) for the collection of non-adherent cells. The resulting pellet was added to adherent cells inside each flask and subjected to lysis for total RNA isolation using a commercially available RNA extraction kit (TRIzol™ reagent)<sup>xxx</sup>. The optical density of aliquots of sample RNA diluted 1:100 with RNase-free water, was determined using spectrophotometry<sup>xxxi</sup> ( $OD_{260nm}/OD_{280nm}$  absorption ratio). Two micrograms (2 $\mu$ g) of RNA was used to generate complementary deoxyribonucleic acid (cDNA) in reverse transcriptase reactions<sup>xxxii</sup> (Nair et al. 1996). Polymerase chain reactions (PCR)<sup>xxxiii</sup> for determination of COX-2 expression were run in 50  $\mu$ L volumes using the following components per manufacturer's instructions: AmpliTaq Gold polymerase, 200mM dNTP's, 3mM MgCl<sub>2</sub>, 1X PCR buffer, DEPC<sup>xxxiv</sup>-treated nuclease-free water and forward and reverse primers (IDT, Coralville, IA) (Table 4.1). Primer sets, forward (5'- TCC AGA TCA CAT TTG ATT GAC A-3' and reverse 5'- TCT TTG ACT GTG GGA GGA TAC A-3' were generated based on previous reports (Cha et al. 2005). Gene transcripts (442 bp) were analyzed on

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<sup>xxix</sup> Wallac station

<sup>xxx</sup> GIBCO Life-Technologies, Carlsbad, CA

<sup>xxxi</sup> Molecular devices microplate spectrophotometer, Spectramax Plus 384, MN

<sup>xxxii</sup> Applied biosystems, Foster city, CA

<sup>xxxiv</sup> Diethyl pyrocarbonate, # D55758, Sigma-Aldrich, St. Louis, MO

ethidium bromide-stained 2% agarose gels<sup>xxxiv</sup>. Cytokine gene expression was normalized against the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH 468 bp)<sup>xxxv</sup> 2.6 picomoles of the forward primer: 5'- ATG CTG GTG CTG AGT ATG TAG TG-3' which corresponds to the nucleotide positions 229 to 252; and 3.5 picomoles of the reverse primer: 5'-ATC CAC AAC AGA CAC GTT GGG AG-3' which corresponds to the nucleotide positions 674 to 696 of the bovine mRNA sequence were added to PCR components as described above (Table 4.1). Three microliters of cDNA samples were amplified in PCR reactions for determination of COX-2 and GAPDH expression (Table 1). Reverse transcriptase -PCR COX-2 and GAPDH transcripts were simultaneously subjected to electrophoresis on 2% agarose gels (100V, 400 mAmps, 27 min). Stored images were analyzed by use of an image-analysis program<sup>xxxvi</sup>. Cyclooxygenase -2 expression was normalized to GAPDH and expressed as a percentage of control incubations.

**Prostanoid Production** - Culture supernatants were evaluated for prostaglandin E<sub>2</sub> production using a commercial competitive enzyme immunoassay<sup>xxxvii</sup>. According to the manufacturer's instructions, 50 µl of each diluted sample (1:100) was introduced into designated sample wells of 96- well plates coated with goat monoclonal anti-mouse IgG. All samples were assayed

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<sup>xxxiv</sup> Nu-Sieve 1:3 agarose, FMC Bioproducts, Rockland, ME

<sup>xxxv</sup> Accession NM U85042

<sup>xxxvi</sup> Image for Windows, version 4.0 3.2, Scion Corp, Frederick, MD

<sup>xxxvii</sup> ACE™ Prostaglandin E<sub>2</sub> EIA Monoclonal kit, Cat. No. 514010. Cayman Chemical Co. Ann Arbor, MI 48108

in triplicate while the standard PGE<sub>2</sub> curve was established using an 8 point serial dilution scale. Blank (BLK), total activity (TA), non-specific binding (NSB) and maximal binding (B0) wells were identified per protocol. Following the addition of each sample, 50 µl each of PGE<sub>2</sub>-acetylcholinesterase (AChE) conjugate or tracer<sup>xxxviii</sup> and PGE<sub>2</sub> monoclonal antibody (single concentration) were added to designated wells. Plates were covered with plastic film and left to incubate for 18 hours at 4°C. Wells were emptied and rinsed 5 times with wash buffer after which antibody binding was detected using 200 µl of AChE substratexxxix over a period of 60-90 minutes. Tracer binding is inversely proportional to the amount of sample PGE<sub>2</sub> and is signified by a distinct yellow color upon the addition of the Ellman's reagent. The assay specificity is 100% for PGE<sub>2</sub>, 18.7, 37.4 and 43% for PGE<sub>1</sub>, 8-iso PGE<sub>2</sub>, and PGE<sub>3</sub> respectively and <0.01 for thromboxane.

*Statistical Analyses* - Proliferation indices (PI) were log transformed then compared using a one-way analysis of variance (ANOVA), with the aid of a statistical software package<sup>xvii</sup>. Observed differences between treatments were considered significant when the calculated P value was less than 0.05 (P<0.05). Normally and Non-normally distributed data were evaluated using ANOVA & Kruskal-Wallace ANOVA on ranks, respectively. The effect of LF in the presence or absence of LPS was considered significant when P < 0.05.

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<sup>xxxviii</sup> EIA kit, catalogue # 414010

<sup>xxxix</sup> Ellman's reagent, item # 8, EIA kit, Cayman Chemical Co. Ann Arbor, MI

## RESULTS

*Effect of LF on Leukocyte Proliferation* - In phase1 of the experiment, pretreatment with lactoferrin, significantly reduced LPS-induced cell PI *in vitro* ( $P < 0.001$ ) (Figure 4.3). *In vitro* incorporation of label was also reduced in LFFE-treated assays ( $P < 0.05$ ) (Figure 4.3). Iron at the 10 nM Fe<sup>3+</sup> concentration did not significantly increase cellular proliferation above that of controls (results not shown). Incubation with iron only at the 1mM concentration resulted in a markedly elevated proliferation index (Figure 4.3). Lactoferrin significantly reduced the proliferation indices of iron supplemented and LPS-stimulated ( $P = 0.009$ ) cells (Figure 4.3).

*Lactoferrin inhibits COX-2 gene expression* - *In vitro* phase 2 experiments were performed without iron supplementation. Compared with LPS-only treated cells, COX-2 expression was significantly reduced by LF pretreatment ( $P = 0.026$ ) (Figure 4.4).

*Lactoferrin-treated PBMCs produce significantly less PGE<sub>2</sub>* - To examine the ability of lactoferrin to modulate the metabolism of membrane phospholipids, culture supernatants were evaluated for PGE<sub>2</sub> production. Prostaglandin E<sub>2</sub> concentration in treatment cultures was normalized to control incubations (no LF, no LPS) by calculating a stimulation index. Treatment stimulation indices were calculated by determining the ratio of sample to maximal tracer binding (Sample binding (B) / Maximal binding (B<sub>0</sub>) x 100 OR %B/B<sub>0</sub>). Values for diluted standard binding were determined in like manner. Each value was then plotted relative to standard concentrations, for identification of actual sample PGE<sub>2</sub> concentration.

Bovine PBMCs exposed to LPS after LF pretreatment, produced significantly less PGE<sub>2</sub> than cells exposed to LPS alone ( $P<0.05$ ). Lactoferrin-only treated cells also produced lower concentrations of PGE<sub>2</sub> (Figure 4.5).

## DISCUSSION

Findings from the current study support our hypothesis which states that LF would inhibit the clonal expansion of peripheral mononuclear cells under LPS stimulation. The demonstrated effects of lactoferrin in our *in vitro* assays suggest LF will inhibit LPS-stimulated leukocyte proliferation both in unsupplemented and iron -supplemented media. Expression of inducible cyclooxygenase -2 and subsequent generation of the eicosanoid prostaglandin E<sub>2</sub> were also inhibited in cultures pre-treated with LF and subjected to LPS stimulation.

Lipopolysaccharide (LPS) is well known for its mitogenic properties and the profound effects it exerts on both humoral and cellular immune responses. This component of the outer membrane of Gram-negative bacteria is composed of a somatic O region, a core R antigen and a lipid A moiety. While the former two antigens contribute to the molecule's immunogenicity, the lipid A moiety effects its pathogenicity (Wilson et al. 2002). Of primary concern is the activation of mononuclear phagocytes, through a membrane-bound complex composed of the CD14 LPS receptor, the toll-like receptor - 4 and the closely associated secreted MD-2 protein. This interaction is facilitated by the soluble acute phase protein, lipopolysaccharide-binding protein (LBP), and culminates into the development of the fatal septic shock syndrome, through the activation of the

transcription factor nuclear factor-kappa B (NF- $\kappa$ B) (Elass-Rochard et al. 1998). Septic shock results from changes induced by macrophage-generated cytokines, interleukin -1, -6, TNF- $\alpha$  and the eicosanoid mediators, prostaglandin E<sub>2</sub>, thromboxanes and the leukotrienes (Lohuis et al. 1988; Cullor 1992). These soluble proteins also play a key role in lymphocyte proliferation (Brock and Djeha 1997, 233).

The goal of the reported study was to investigate the ability of LF to alter LPS-induced proliferation of peripheral mononuclear blood cells using an *ex vivo* model. In order to achieve this goal, we attempted to mimic the *in vivo* scenario, bearing in mind several key factors: 1. Lymphocyte proliferation will occur subsequent to mitogenic stimulation; 2. Transferrin is required for optimal mitogen-induced lymphocyte proliferation *in vitro*; and 3. The acute phase reactant LBP, enhances LPS-induced activation of moncytoid cells, one of the host's major antigen presenting cells and the most efficient activator of differentiated effector T cells (Fenton and Golenbock 1998; Brock and Djeha 1997, 233). Assays therefore comprised of a mixed population of peripheral blood mononuclear cells (lymphocytes and cells of the monocytoid lineage) in RPMI growth media enriched with FBS - a natural source of transferrin and LBP (Gaston, Bacon and, Strober 1987; Mészáros, White and Parent 1995). Retention of tritiated thymidine was used as an indicator of cellular proliferation.

Lipopolysaccharide induction of *in vitro* COX-2 expression and PGE<sub>2</sub> production by bovine PBMCs, has also been reported (Lakritz et al. 2000b). This response occurs across species and has been associated with the hydrolysis of

arachidonic acid a constituent of membrane phospholipids (Curry Cogar and Cook 2005; Qi and Shelhamer 2005; Tilley, Coffman, and Koller 2001). Since the production of these pro-inflammatory mediators contribute greatly to the systemic inflammatory response observed during Gram-negative infections, we investigated the ability of lactoferrin to alter their expression and production by host cells, respectively.

Lactoferrin has been shown to modulate cellular proliferation (Brock and Djeha 1997, 233). In this study LF significantly reduced lymphocyte proliferation in all treatments, including assays devoid of LPS and supplemental iron, thus suggesting that demonstrated effects are occurring through mechanisms other than Lipid-A binding. In comparison with all other treatments, LF-only treated cells registered a significantly lower proliferation index. Lactoferrin has the ability to sequester iron, a required growth factor for all cellular mechanisms (Brock and Djeha 1997, 233). In comparison to its parent protein transferrin, LF is about 260 times more potent at iron chelation (Brock and Djeha 1997, 233; Monteiro and Winterbourn 1988). In light of the relatively high iron requirement for adequate hematology and tissue function, we also investigated LF effects in the presence of added iron (Brock and Djeha 1997, 233). Compared with controls, tritium-retained radioactivity was significantly increased in cells exposed to high levels of iron (1mM). Although this finding contrasts with that of Brock and others, Arnold et al also report a similar finding (Arnold et al. 1982; Brock and Djeha 1997, 233; Tomita et al. 1991). This could be attributed to oxidative damage and subsequent cellular repair at the molecular level, completion of additional rounds

of DNA replication, cellular division, or a combination of these factors (Arnold et al. 1982). In support of previous studies, LF addition significantly inhibited PBMC proliferation in iron-supplemented studies and likewise significantly decreased LPS-stimulated cellular proliferation. Cells stimulated by LPS, supplemented with 1mM ferric iron and co-incubated with LF also demonstrated significantly lower proliferation indices when compared with LPS and LPSFe cells. In their study of the antimicrobial effects of LF, Arnold et al investigated LF effects on cellular metabolism in *Streptococcus mutans* (Arnold et al. 1982). They report that pre-incubation of cells with apo-lactoferrin inhibited bacterial glucose metabolism - an iron-dependent mechanism. Although that parameter was not evaluated in the present study, the proposed phenomenon may have contributed to the present findings (Arnold et al. 1982).

Central to the known functions of LF is its ability to interact with key molecules including LPS, heparin, certain cellular receptors and glycosaminoglycans, an ubiquitous host polysaccharide and a major subunit of the cell wall of Gram-positive bacteria (Ward, Paz and Conneely 2005). Several *in vitro* and *in vivo* studies have investigated the effects of native LF or LF-derived peptides on endotoxin activity. Lactoferrin demonstrates high binding affinity to the lipid A portion of the endotoxin molecule (Legrand et al. 2004). While non-specific binding of LF to either host cells or pathogens is not unlikely owing to its highly charged surface, binding of the lipid A toxic moiety of endotoxin is particularly localized to the highly cationic basic-N-terminal region of the LF molecule (Ward, Paz and Conneely 2005).

The formation of the LF: lipid A complex bears physiologic significance in that it results in subsequent inhibition of host LBP-lipid A interaction. In the face of endotoxemia, this acute phase protein is released into circulation by host hepatocytes. Due to its amphiphilic nature, LPS forms aggregates in aqueous environments such as culture medium and blood (Baveye et al. 2000a). In the absence of LBP, spontaneous diffusion of LPS monomers from these aggregates to CD14 occurs very slowly. However, when LBP is present, it catalyzes the transfer of monomers from LPS aggregates to the 55 kD LPS or CD14 macrophage receptor and their binding rate accelerates dramatically (Baveye et al. 2000a). Thus interaction between LPS and LBP increases the sensitivity of host cells to the former (Baveye et al. 2000a). However, according to Baveye and earlier investigators, LBP is only essential for this role when circulating LPS concentration is low. Otherwise, LPS binds directly to sCD14 and initiates its inflammatory effects in this complex form (Baveye et al. 2000a).

The LPS receptor is constitutively expressed by monocytes-macrophages as well as neutrophils and exists in both the membrane-bound (mCD14) and soluble (sCD14) forms. In its former conformation, CD14 is anchored to the surface by a glycophosphatidylinositol (GPI) protein anchor (Baveye et al. 2000a). Since this anchor is devoid of both transmembrane and cytoplasmic domains, the activation of intracellular signaling pathways which initiate gene transcription, is facilitated by subsequent interactions with the closely-associated toll-like receptor 4 (TLR-4) and the soluble MD-2 protein (Akira, Uematsu, and Takeuchi 2006; Scherle et al. 1998). Resulting NF- $\kappa$ B intra-nuclear translocation

then directs the transcription and release of pro- inflammatory molecules such as IL-1 $\beta$  and COX-2 (Lee et al. 2004; Won et al. 2005). Previous *in vitro* studies suggest that the anti-inflammatory effects of LF may involve its intra-nuclear translocation and direct inhibition of NF- $\kappa$ B activation (Ward, Paz and Conneely 2005).

In the current study, LF pretreatment inhibited expression of the inducible pro- inflammatory COX-2 enzyme and hence limited prostaglandin production. The cyclooxygenases are dependent on substrates made available by phospholipases (Lakritz et al. 2000b). Phospholipase activation is induced by LPS (Olson and Brown 1986). Once arachidonic acid is liberated, it is initially metabolized into prostaglandin H (PGH) which is ultimately broken down into PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , the prostacyclins and thromboxanes (Lakritz et al. 2000b). The enzyme prostaglandin H synthase (PGHS) is required for the catabolism of PGH. The inducible form of PGHS is selectively up regulated in LPS-stimulated macrophages (Scherle et al. 1998). Although the current study lends support to previous reports which describe LF inhibition of LPS host leukocyte interactions, other mechanisms whereby inflammatory mediator expression is inhibited should not be disregarded without further study. Additional experiments in our lab have demonstrated altered *in vitro* expression of phosphorylated activating transcription factor-2 by LPS-stimulated PBMCs co-cultured with LF (Dawes 2006, 77). The exact mechanism of action is however still unknown.

In summary, the results of this study indicate that LF inhibits LPS - induced mononuclear cell proliferation. These findings also support the findings

of previous investigators (Brock and Djeha 1997, 233). This study also supports previously identified mechanisms namely, iron-scavenging ability, and inhibition of LPS-LBP-CD14 through high affinity Lipid A binding. Our results also confirm that iron may become a limiting factor in proliferation assays (Brock and Djeha 1997, 233). There is however the need to further quantify the iron-scavenging ability of lactoferrin as well as determine the effects of LPS stimulation on the concentration of other iron-binding and storage proteins in light of lactoferrin concentrations. Previous studies demonstrate that PGE<sub>2</sub> is the major prostanoid released into the bovine lung in response to LPS. Additionally, when alveolar macrophages were either stimulated with purified *Mannheimia hemolytica* leukotoxin or when calves were experimentally infected with this Gram-negative species, high levels of this eicosanoid was spontaneously released, suggesting stimulus-specific response (Lakritz et al. 2000b). Given the high frequency of failure of passive transfer in bovine neonates and the predominance of Gram-negative infections in cattle, future work will further explore the relationship between lactoferrin, cells of the innate immune system and cytokine production, as well as address the mechanism(s) of action that result in those changes.

## **CHAPTER 5**

### **AN IN VITRO STUDY OF THE EFFECTS OF LACTOFERRIN ON P38 MITOGEN ACTIVATED PROTEIN KINASE (MAPK) SIGNALING IN LPS- STIMULATED BOVINE PBMCS.**

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## ABSTRACT

The objectives of this study were to define the *in vitro* effect of supplemental lactoferrin on lipopolysaccharide (LPS)-induced mitogen-activated protein kinase signaling cascades. In related experiments, mRNA expression of pro-inflammatory IL-1 $\beta$  as well as of MMP-9 by LPS - stimulated cells after LF pretreatment were evaluated. This study utilized 8 Holstein steers (2-11 months of age) that were used as a source of peripheral blood mononuclear cells (PBMC). Cell cultures were performed in T-25 vented culture flasks in a 37°C CO<sub>2</sub> humidified cell culture incubator. Peripheral blood was obtained from steers by jugular venipuncture. Mononuclear cells were isolated by density gradient centrifugation. In *in vitro* p38 MAPK assays, 5 x 10<sup>6</sup> cells were initially cultured in fresh media prior to the addition of stimulants or supplements. Twenty-four hours later lactoferrin (LF) (200ng/ml) and/or lipopolysaccharide (LPS) (200 ng/ml) were added 1 hour apart. Culture proceeded for 18 hours after which the media was aspirated and adherent and floating cells were lysed using a commercially available cell lysis buffer. Lysates were stored at -80°C until used. This assay was performed using a non-radioactive p38 MAP kinase assay kit <sup>xl</sup>. Sample active p38 was selectively immunoprecipitated overnight in incubations containing 200  $\mu$ l of sample lysate (~ 200  $\mu$ g of protein) and 20 $\mu$ l of dually

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<sup>xl</sup> Non-radioactive IP-kinase assay, #9820, Cell signaling Technology, Inc, Danvers, MA 01923

phosphorylated mouse monoclonal antibody (Thr180/Tyr184)<sup>xli</sup>. The phosphorylation of recombinant human ATF 2 fusion protein (1 µg) was conducted in the presence of 200mM ATP and 1X kinase buffer. After brief incubation in a water bath (30°C) the reaction was stopped by addition of 3X sodium dodecyl sulfate (SDS) buffer mixed just prior to use with 150mM DTT. After being boiled for 5 minutes, samples were resolved on SDS-Page gels and transferred to nitrocellulose membrane. After blocking using nonfat dry milk, membranes were incubated overnight in primary antibody buffer. The following day, membranes were washed and incubated at room temperature with the secondary antibody for 1 hour. Phosphorylated proteins were detected by chemiluminescence and recorded by autoradiography.

Cells for total RNA isolation was carried out in parallel and RNA was isolated from cells using TRIzol™ reagent. Chloroform, isopropyl alcohol and 75% ethanol were utilized on TRIzol™ extracts previously frozen at -80°C, for phase separation, RNA precipitation and isolation respectively. Sample RNA concentration was determined spectrophotometrically. Two micrograms of each sample was used to generate single strand cDNA by reverse transcriptase. Polymerase chain reactions using 3 µl of the generated templates, as well as forward and reverse primers specific for bovine MMP-9, IL-1 beta and the housekeeping gene GAPDH, were performed. Polymerase chain reaction

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<sup>xli</sup> Primary antibody, immobilized phopho-p38 MAPK, #9219. Cell Signaling Technology, Inc., Danvers, MA

products were separated by electrophoresis in ethidium bromide-stained 2% agarose<sup>xxxiv</sup> gels and photographed using a gel imaging system.

Lactoferrin inhibited phosphorylation of ATF-2. MMP-9 expression, which is mediated by activation of proteins through the p38 MAPK signaling pathway, was enhanced subsequent to LPS stimulation of bovine PBMC. Pretreatment of cells with LF inhibited LPS-induced MMP-9 mRNA expression. Lactoferrin pretreatment of PBMC did not significantly alter LPS-induced expression of IL-1  $\beta$ . These findings in part support our hypothesis which states that LF will inhibit pro-inflammatory mediator expression through inhibition of p38 MAPK signaling. Our data suggest that lactoferrin interferes with LPS-induced p-38 activation of transcription factor ATF-2, in vitro. This attenuation in MAPK mediated phosphorylation was associated a decrease in protein expression as detected by western immunoblotting. Lactoferrin may be useful in attenuating the systemic effects of LPS-induced pro-inflammatory cytokine production by limiting signal transduction through MAP kinase cascades.

**Key words:** Activating transcription factor- 2 (ATF-2), p38 MAPK, Lactoferrin (LF), western immunoblotting

## INTRODUCTION

Macrophages serve the role of first line of defense in bacterial infections and are the primary source of inflammatory mediator production (Akira et al. 2006). While several external stimuli are capable of stimulating an inflammatory response activating these cells lipopolysaccharide (LPS) a critical component of the outer membrane of Gram-negative bacteria is well known for its mitogenic effects (Rushworth et al. 2005; Baveye et al. 2000a). We hypothesized that the anti-inflammatory effects of lactoferrin results from inhibition at the level of the intracellular p38 MAPK pathway. Lipopolysaccharide is a complex molecule and is well conserved among bacteria of this group and like other pathogen-associated molecular patterns (PAMPs), is non-specifically recognized by inflammatory cells (Akira et al. 2006). At physiologic concentrations, the acute phase lipopolysaccharide binding protein (LBP) facilitates host sensitization by catalyzing the transfer of LPS monomers from circulating aggregates to the membrane CD14 LPS receptor of phagocytes (Scherle et al. 1998; Rushworth et al. 2005). In endotoxemia, this role is usurped by soluble CD14 (Baveye et al. 2000a). Since the glycophosphatidylinositol-linked LPS receptor is devoid of both transmembrane and cytoplasmic domains, the mechanism by which the stimulatory response is transmitted was unknown until its interaction with toll-like and other membrane-bound pattern recognition receptors (PRRs) was elucidated (Scherle et al. 1998; Akira et al. 2006). Subsequent to the engagement of toll-like

4 receptors the adapter protein MYD88 becomes closely associated, resulting in the activation of several upstream kinases. Ultimately, pathways critical to the transcriptional regulation of cytokines become initiated through the activation of key factors such as the mitogen-activated protein (MAP) kinases and nuclear factor (NF)- $\kappa$ B (Billack et al. 2002; Ho et al. 2004) (Figure 5.1). The mitogen-activated protein kinase (MAPK) p38, one of the subgroups of MAPK, has been shown to be involved in the expression of genes involved in inflammation.<sup>7</sup> These include tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1beta (IL-1 $\beta$ ), inducible nitric oxide (NO), MMP-9 and cyclooxygenase-2 (COX-2), cytokines central to the inflammatory process (Kuldo et al. 2005; Spallarossa et al. 2005).

Previous investigators have demonstrated LPS -induced matrix metalloproteinase-9 (MMP-9) transcription (Lakritz et al. 2002c; Woo, Lim, and Kim 2004). Matrix metalloproteinase-9 is a member of the family of neutral endopeptidases which includes collagenases, gelatinases, metalloelastases, stromelysin and matrilysin (Woo, Lim, and Kim 2004). While they are well known for their roles in biological processes such as angiogenesis, embryogenesis, normal tissue remodeling and wound healing, the gelatinase MMP-9 (gelatinase B; 92-kD type IV collagenase) has been associated with chronic inflammatory conditions such as asthma and degrades the basement membrane components laminin and type IV collagen (Woo, Lim, and Kim 2004). Matrix metalloproteinase-9 is primarily neutrophils, with some production by macrophages in several species (Lakritz et al. 2002c). In normal and reparative states, gene expression and protein localization and activity are carefully

regulated to maintain a balance between degradation and repair (Bernt Van den Blink et al. 2001; Lakritz et al. 2002c; Woo, Lim, and Kim 2004). Gelatinase B expression is up regulated along with other inducible genes such as COX-2 and inducible nitric oxide synthase (iNOS), by cytokines released in response to LPS (Lakritz et al. 2002c). This occurs as a direct consequence of oxidant production by white blood cells and secondary to up regulated expression of cellular integrins (Lakritz et al. 2002c).

Interleukin 1- $\beta$ , a member of the family of IL-1 ligands, is a prominent fever-inducing cytokine of macrophage origin. Along with tumor necrosis factor alpha, it is one of the two important cytokines released during endotoxemia (Gerros et al. 1993). Attempts have been made to demonstrate the significance of p38 kinases in cytokine regulation in the macrophage. However, findings regarding the consequences of inhibiting phosphorylated p38 activity have to date been mixed. Blink et al. observed enhanced IL-6 and TNF- $\alpha$  production by L929 fibrosarcoma, 4-4 murine macrophage clones and peritoneal-derived macrophages pre-treated with SB203580, which inhibits the activity of phosphorylated p38 (Bernt Van den Blink et al. 2001). In contrast, cytokine production by non-transformed whole blood-derived cells was significantly less when compared with experimental controls (Bernt Van den Blink et al. 2001). In another study, inhibition of p38 kinases resulted in reduced IL-1- $\beta$  and TNF- $\alpha$  production in LPS-stimulated monocytes (Scherle et al. 1998). In the same study, Scherle et al also demonstrated decreased prostaglandin H synthase expression and hence decreased conversion of prostaglandin H into its

components, prostacyclin, thromboxanes, PGE<sub>2</sub> and prostaglandin F<sub>2α</sub> following inhibition of p38 signaling in cells of the monocyteoid lineage (Scherle et al. 1998).

In an earlier study in our lab, PGE<sub>2</sub> production by PBMCs pre-treated with LF prior to LPS stimulation was significantly less than that produced by cells incubated with LPS only (Dawes 2006). Enhanced COX- 2 expression and PGE<sub>2</sub> synthesis is consistently associated with enhanced IL-1β expression in several cell lines (Lee et al. 2004).

Given that there is overwhelming evidence supporting the role of lactoferrin in attenuating the production of inflammatory mediators, the goal of this study was to determine if that function could be associated with MAPK signaling and more specifically with the p38 MAPK pathway (Baker 2005; Baveye et al. 2000a; Baveye et al. 2000b; Baynes and Bezwoda 1994). To further elucidate its mechanism of action, we evaluated the modulatory effects of LF on the transcription of inducible pro- inflammatory cytokines.

## MATERIALS AND METHODS

**Animals** - Eight (8) Holstein steers were maintained from birth in well-ventilated stalls in the Food Animal clinic of the University of Missouri-Columbia Veterinary Medical Teaching Hospital. All animals were raised as colostrum-replete calves and were maintained on a commercial milk replacer diet (Land O'Lakes Inc., MN) until weaning, and were supplemented with a calf starter ration. From the age of weaning (2 months) to 11 months, animals were maintained on a diet consisting of *ad libitum* alfalfa, mixed or grass hay (dependent on the stage of growth) and water. Calf starter supplementation was

gradually switched to a commercial mixed cattle ration in am and pm feedings, beginning at the age of 6 months. This study was evaluated and approved by the University of Missouri-Columbia Animal Care and Use Committee.

**Mononuclear Cell Isolation** - Peripheral blood was collected by jugular venipuncture in 10-ml acid citrate dextrose (ratio 1:9 ACD: blood) blood tubes. All samples obtained from each calf at a single bleeding were later pooled and centrifuged in 50-mL conical polypropylene tubes<sup>xxi</sup> at 274 x g, 23°C for 15 minutes. Peripheral blood mononuclear cells were isolated from the buffy coat using the previously described differential centrifugation technique (Carlson and Kaneko 1973). Cells were suspended in Hanks buffered salt solution (HBSS)<sup>xxii</sup>, and layered over an equal volume of histopaque (density 1.077)<sup>xliii</sup> in 15-ml polypropylene conical tubes<sup>xliii</sup>. Samples were centrifuged for 30 minutes at 342 x g, 23°C thereby concentrating PBMCs at the histopaque/plasma interface. Cells were washed in HBSS to remove the separating medium (285 x g, 23°C for 5 minutes). Residual red blood cells were then removed by sterile hypotonic lysis (Lakritz et al. 2000a). Isolated PBMCs were re-suspended in 2.5 mls of HBSS and kept on ice prior to culture. Cells were evaluated for viability (typically 90 to 98 %) using trypan blue (0.1% in phosphate buffered saline [PBS]) exclusion (Carlson and Kaneko 1973). Cytospin preparations were obtained for differential cell determinations using Wright staining. The lymphocyte: monocyte ratio was typically 3.4:1. Five million cells were introduced into T-25 culture

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<sup>xlii</sup> Sigma Histopaque ©, # 1077. Sigma-Aldrich 3050 Spruce St., St. Louis, MO

<sup>xliii</sup> Nunc\* EZflip\* tubes # 12-565-802. Fisher Scientific, 3970 Johns Creek Court, Sawnee, GA

flasks containing sterile RPMI 1640 supplemented with 5-10% heat inactivated fetal bovine serum (FBS); MEM non-essential amino acids<sup>xxii</sup>, 2 mM L-glutamine<sup>xxii</sup>, 5 x 10<sup>2</sup> mM 2-mercaptoethanol<sup>i</sup>, 50 U penicillin<sup>xxii</sup> and 50 µg streptomycin<sup>xxii</sup> on a per ml basis.

**Preparation of monomeric LPS** - Monomeric LPS was prepared as previously described (Lakritz et al. 2002b). Briefly, in order to solubilize the LPS, 10 mg of O55:B5 was dissolved in 1 ml of FBS and incubated in a 37°C water bath for 1 hour in a sterile microcentrifuge tube (Lakritz et al. 2002b). After incubation, the microcentrifuge tube was placed in a beaker of warm water and sonicated (50 watts) for 10 min (Lakritz et al. 2002b). The solubilized LPS was returned to the water bath until aliquoted for storage at -80°C. Aliquots (10 mg/ml) were thawed on ice prior to use and diluted 1:100 in FBS for use in stimulation assays. Unused amounts of thawed LPS were discarded.

**Cell cultures and Lysate preparation** - Cells were plated at a concentration of 5 x 10<sup>6</sup> per five milliliters of media and grown overnight in a 37°C CO<sub>2</sub> humidified cell culture incubator. At 24 hours cell viability was re-evaluated, culture media was renewed and treatments were added. Treatments included control (CTRL), ethanol-extracted lactoferrin only-treated cells (LFex), non-extracted or native LF-only treated cells (LFnonX), LPS only-treated cells (LPS) and LFex + LPS-treated cells (LFexLPS) and LFnonx + LPS-treated cells (LFnonxLPS). Ethanol extraction of lactoferrin was performed in order to eliminate the possible existence of contaminating lipids as was previously

described (Cohen et al. 1992). Cells exposed to 25 µg/ml anisomycin<sup>xliv</sup> (an activator of p38 and c-Jun N-terminal kinases, a protein synthesis inhibitor as well as an antibiotic) served as positive controls (Ogawa et al. 2003). Cells pretreated with 50 mM SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)imidazole) (Calbiochem, La Jolla, CA) for 1 hour, as well as during kinase reactions served as negative controls<sup>xliv</sup>. Lactoferrin pretreatment (200 ng/ml) of designated cultures was performed one hour prior to LPS [1µg/ml; O55:B5<sup>i</sup>] addition.

Cells were cultured for eighteen hours after which the media was aspirated for centrifugation at 117 x g to obtain the cellular pellet. Adherent cells were harvested under non-denaturing conditions. Per manufacturer's recommendations the culture surface was washed with 1 ml of ice-cold Dulbecco's PBS<sup>xxii</sup>. All other reagents were either provided by or prepared according to the manufacturer. Adherent cells were then incubated on ice with 500 µL of a 1X cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin)<sup>xlv</sup>, 1 mM phenylmethylsulfonyl fluoride (PMSF)<sup>xlvi</sup> buffer solution for 5 minutes. Flasks were scraped<sup>xlvii</sup> to ensure complete removal of cells and the suspension was transferred to 1.5 microcentrifuge tubes for sonication<sup>xlviii</sup> (4x, 5 sec each on ice;

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<sup>xliv</sup> Personal communication Andreas Nelsbach, technical support, Cell Signaling Technology Inc.,

<sup>xlv</sup> 10X lysis buffer, # 93482, Cell Signaling Technology, Inc., Danvers, MA

<sup>xlvi</sup> # 93482 , Sigma-Aldrich 3050 Spruce St., St. Louis, MO

<sup>xlvii</sup> Fisherbrand\* cell scraper, # 08-773-3, Fisher Scientific, Sawnee, GA

<sup>xlviii</sup> Ultrasonic dismembranator, Model 100, Fisher Scientific, Sawnee, GA

setting 8). An equal volume of lysis buffer/PMSF solution was added to pelleted cells and transferred to the tubes containing the cellular suspension. The tubes were centrifuged (14,000 x g 10 minutes, 4°C) and the cell lysate (supernatant) was transferred to a new tube and stored at -80°C until the assay.

***In vitro* p38 MAPK assay** - This assay was performed using the p38 MAP kinase assay kit manufactured by Cell Signaling Technology, Inc. Two hundred microliters (~ 200 ng of protein) of each sample lysate was incubated overnight (gentle rocking, 4°C) with 20 µl of dually phosphorylated mouse monoclonal antibody conjugated to carbohydrates cross-linked to agarose hydrazide beads (Thr180/Tyr184)<sup>xli</sup>, to selectively immunoprecipitate sample active p38. Samples were centrifuged (14,000 x g, 30 sec, 4°C) and the immunoprecipitate was washed twice with 500 µL of ice-cold 1mM PMSF/1X lysis buffer<sup>xlv</sup> and twice with 500 µL of ice-cold 1X Kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>). Samples were kept on ice during washes and centrifuged after each wash (14,000 x g, 30 sec, and 4°C). Kinase reactions were performed in the presence of 200µM of Adenosine-5'-triphosphate (ATP)<sup>xlix</sup>, 1µg of ATF-2 fusion protein<sup>l</sup>, and 50 µL of 1X kinase buffer<sup>li</sup> for 30 minutes at 30°C. The reactions were terminated upon addition of 25 µL of 3X SDS buffer, supplemented just prior to use, with 150 mM dithiothreitol<sup>lii</sup>. Samples were boiled for 3 minutes, briefly

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<sup>xlix</sup> 10mM ATP, # 9804 (kit component), Cell Signaling Technology Inc., Danvers, CA

<sup>l</sup> Recombinant ATF-2 fusion protein, # 9224, Cell Signaling Technology Inc., Danvers, CA

<sup>li</sup> 10X kinase buffer, # 9802 (kit component), Cell Signaling Technology Inc., Danvers, CA

<sup>lii</sup> DTT #161-0610, Bio-Rad Laboratories, Hercules, CA

vortexed and centrifuged (14,000 x g, 30 sec, 4°C) then loaded on 4 -12 % SDS polyacrylamide gels<sup>lvi</sup> (30 µL; 200V, 37 min).

**Western Immunoblotting** - Following resolution on SDS PAGE gels, lysates were transferred to nitrocellulose paper (80V, 400 mAmps, 1.5 hr or 13V overnight). A pre-stained molecular weight marker was run in an adjacent lane to control for successful transfer. To confirm that an equivalent amount of protein was loaded in each lane, the membrane was stained in Ponceau stain (0.5% in 1% acetic acid) for 20 minutes then rinsed in distilled water to facilitate full visualization of protein bands prior to blocking. The membrane was rinsed in 1X tris buffered saline with tween-20 (TBS-tween) (0.1%) wash buffer with 0.2% BSA for 1 minute and subsequently blocked in 1X TBS-tween with 5 % w/v nonfat dry milk<sup>liv</sup> at room temperature for 1 hr. After three washes in wash buffer (15 ml, 5 min, 23°C, gentle rocking), the membrane was incubated overnight (gentle rocking) at 4°C in primary antibody<sup>lv</sup> (1:1000) dilution buffer to detect ATF-2 phosphorylation by upstream active p38 MAPK. The membrane was again rinsed thrice in wash buffer, and the secondary antibody incubation was performed (1 hr, 23°C, with gentle rocking) using Horseraddish peroxidase (HRP)-conjugated anti-rabbit IgG<sup>lvi</sup> (1:2000) and HRP-linked anti-biotin antibody (1:1000) to detect the biotinylated protein markers<sup>lvii</sup>. Following three additional

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<sup>lvi</sup> NuPage® Novex Bis-Tris gel, cat. no. NP0312BOX; Invitrogen Corporation, Carlsbad, California 92008

<sup>liv</sup> Carnation instant nonfat dried milk, Nestle-carnation, North York, ON, Canada

<sup>lv</sup> Phospho-ATF-2 (Thr71), # 9221; Cell signaling Technology, Inc, Danvers, MA

<sup>lvi</sup> Secondary conjugated antibody, # 7074, Cell signaling Technology, Inc, Danvers, MA

<sup>lvii</sup> Affinity-purified goat anti-biotin; # 7075, Cell signaling Technology, Inc, Danvers, MA

washes a chemiluminescent protocol using LumiGlo substrate<sup>lviii</sup> was performed in the dark, according to the manufacturer's instructions.

**Cytokine Expression using RT-PCR** - Cells were cultured in vented T-25 ml culture flasks as described above. Following LPS-stimulation (24 hours) in the presence or absence of LF, culture media was aspirated for collection of non-adherent cells by centrifugation (117 x g). The resulting pellet was added to adherent cells and subjected to lysis and total RNA isolation using a commercially available RNA extraction kit (TRIzol™ reagent)<sup>xxx</sup> per the manufacturer's recommendations. The total RNA pellet was air-dried, re-suspended in DEPC-treated water and incubated in a water bath (37°C for 20 minutes) to aid in complete dissolution. Spectrophotometric determination (OD<sub>260nm</sub>/OD<sub>280nm</sub> absorption ratio) of sample total RNA concentration was performed by measuring the optical density of aliquots diluted 1:100 with RNase-free water. A total of 2µg of RNA was used to generate cDNA in reverse transcriptase reactions (Nair et al. 1996). Polymerase chain reactions (PCR)<sup>xxxii</sup> were performed in a 50 µL reaction volume using the following components per manufacturer's instructions: AmpliTaq Gold polymerase, 200 mM dNTP's, 3mM MgCl<sub>2</sub>, 1X PCR buffer, DEPC<sup>xxxiii</sup>-treated nuclease-free water, 2.12 picomoles forward (IL-1β: 5'- AAA CAG ATG AAG AGC TGC ATC CAA -3'; and 2.12 picomoles reverse (IL-1β: 5'-CAA AGC TCA TGC AGA ACA CCA CTT-3') primers (IDT, Coralville, IA) (Table 5.1). Primers were generated using the

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<sup>lviii</sup> 0.5 mL 20X LumiGlo chemiluminescent reagent and 0.5 mL 20X peroxide, # 7003, Cell signaling Technology, Inc, Danvers, MA 01923 (mixed with 9.0 mL milli-Q water)

Vector NTI computer software program<sup>lxix</sup>, from complete IL-1 $\beta$ <sup>lx</sup> and MMP-9 mRNA<sup>lxii</sup> sequences published in the National Center for Biotechnology Information (NCBI) archives. The forward and reverse sequences (5' to 3') for IL-1 $\beta$  (394 bp) correspond to nucleotide positions 129 to 152 and 499 to 522 of the bovine sequence, respectively. Reactions for determination of MMP-9 expression (698 bp) were prepared as described earlier, with primer concentrations being 1.95 picomoles (forward) and 1.93 picomoles (reverse) (Table 5.1). The forward (5'-CGA CGA TGA AGA GTT GTG GT-3') and reverse (5'-GTA CAT GGG GTA CAT GAG CG-3') sequences (5' to 3') correspond to nucleotide positions 618 to 637 and 1256 to 1275 of the bovine sequence, respectively. Polymerase chain reaction (PCR) products were separated by electrophoresis in ethidium bromide-stained 2% agarose gels<sup>xxxiv</sup> and imaged on a gel imaging system<sup>lxii</sup>. Similarly, gene transcripts were generated for the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH 468bp)<sup>lxiii</sup> - 2.6 picomoles of the forward primer: 5'-ATG CTG GTG CTG AGT ATG TAG TG-3' which corresponds to the nucleotide positions 229 to 252; and 3.47 picomoles of the reverse primer: 5'-ATC CAC AAC AGA CAC GTT GGG AG-3' which corresponds to the nucleotide positions 674 to 696 of the bovine mRNA sequence (Table 5.1).

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<sup>lxix</sup> Vector NTI suite 6.0 for Windows NT, Informax Inc, Bethesda, MD

<sup>lx</sup> Accession M35589

<sup>lxii</sup> Accession NM 174744

<sup>lxiii</sup> Quantity One image analysis software; Personal Molecular Imager, BIO-RAD, Hercules CA

<sup>lxii</sup> Accession NM U85042

*Statistical Analysis* - IL-1 $\beta$  and MMP-9 transcripts were each simultaneously separated on individual gels with GAPDH transcripts to facilitate normalizing cytokine gene expression relative to the housekeeping gene. Messenger RNA expression of MMP-9, and IL-1 $\beta$  were expressed as a percentage of control incubations. Stored images were analyzed by use of an image-analysis program<sup>lxiv</sup>. Normally and non-normally distributed data were evaluated using ANOVA & Kruskal-Wallace ANOVA on ranks, respectively. The effect of LF in the presence or absence of LPS was considered significant when P < 0.05. Statistical analyses were performed with the aid of a statistical software package<sup>xvii</sup>.

## RESULTS

*Lactoferrin exhibits differential effects on cytokine mRNA expression by LPS-stimulated PBMCs* - Lactoferrin-inhibition of active p38 production was evaluated in light of pro-inflammatory, interleukin 1- $\beta$  and the gelatinase, matrix metalloproteinase-9 (MMP-9) expression using reverse transcriptase -PCR. Compared with the control cultures (no LF, no LPS), MMP-9 expression was elevated in cells stimulated with LPS (Figure 5.1). When compared with LPS-stimulated cells, MMP-9 expression by cells pretreated with LF prior to, LPS-stimulation (LFLPS cells) was significantly reduced (P <0.05) (Figure 5.2). Lactoferrin pretreatment did not significantly affect LPS-induced IL-1 $\beta$  expression by PBMCs (results not shown).

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<sup>lxiv</sup> Image for Windows, version 4.0 3.2, Scion Corp, Frederick, MD

*Lactoferrin alters ATF-2 protein expression by bovine PBMCs-* In a concurrently run study, we attempted to document LF-induced inhibition of phosphorylated p-38 phosphorylation of activating transcription factor (ATF)-2 by LPS-stimulated cells. Protein bands were transferred to nitrocellulose probed with antibodies and visualized by Western immunoblotting. Chemiluminescent detection facilitated the visualization of expressed proteins exposed to X-ray films (Figure 5.3). Immunologically reactive ATF-2 expression in LPS-treated cells was compatible with a prominent band at 40 kD. A clearly visible but less prominent band was present for anisomycin-treated cells. Lactoferrin inhibited the activity of dually phosphorylated p38 in our in vitro kinase assays. Detection of phosphorylated ATF-2 signal was altered in lysates generated from LF-pretreated LPS-stimulated PBMCs. Both LFnonX and LFex-only treated cells demonstrated similar results.

## DISCUSSION

The results of our study demonstrate that LF exerts differential effects on monocyteoid cytokine generation. While its effect on MMP-9 mRNA expression was inhibitory, we were unable to demonstrate any inhibitory effects on IL-1 beta gene expression. In the current study, we were able to demonstrate ATF-2 phosphorylation in both LPS-stimulated and anisomycin-treated cells. Compared with LPS-only treated cells, the expression of phosphorylated transcription factor ATF-2 by PBMCs pre-treated with LF prior to LPS exposure was altered. When compared with untreated controls, expression of phosphorylated ATF-2 was also affected by LF treatment.

Lymphocyte antigen recognition ultimately leads to the induction of new genes, a process which is facilitated by the activation of transcription factors, proteins that control the initiation of transcription by binding to regulatory sites in DNA (Janeway 2005, 222). The signal transduction p38 mitogen activating protein kinases may be activated by several stimuli including cytokines, extracellular stress and LPS of Gram-negative bacteria (Bernt Van den Blink et al. 2001; Sugino et al. 2000). Attempts have been made to elucidate their role in inflammation. Since Gram-negative infections predominate in both gastrointestinal and respiratory disease processes in cattle our model was considered ideal to perform these investigations (Lakritz et al. 2002c).

Matrix metalloproteinases are zinc-dependent enzymes which are responsible for the remodeling of host matrix in both physiological and disease states (Spallarossa et al. 2005). These zymogens are regulated at several levels

including transcription, secretion and activation (Spallarossa et al. 2005). Studies suggest that their activation is redox-dependent and occurs via MAPK signal transduction (Spallarossa et al. 2005; Kim Hyun-Seung et al. 2004). Of the MAPK family of proteins (p38 mitogen-activated protein kinases, the c-Jun amino-terminal kinases (JNKs) and the p42/44 or extracellular signal-regulated (ERK1 and 2) MAP kinases), prime responsibility of MMP-9 activation has been attributed to the p38 pathway (Spallarosa et al. 2005). The initiation of this activity has been linked to superoxide production (Woo, Lim, and Kim 2004; Spallarossa et al. 2005). While reports of the role of reactive oxygen species (ROS) in intracellular signaling are also increasing, this proposed mechanism contrasts with earlier proposals of a protein kinase C delta mediated mechanism (Spallarossa et al. 2005; Woo, Lim, and Kim 2004). Like phagocytosis, LPS-induced activation of moncytoid and other phagocytic cells, results in the generation of ROS (Thakur et al. 2006; Woo, Lim, and Kim 2004).

The effect of LF on the generation of ROS remains unclear. Some scientists have reported a direct link between LF or lactoferricin supplementation and enhanced *in vitro* superoxide production by phagocytic cells (Wakabayashi et al. 2003; Lakritz et al. 2000; Wong et al. 1997). Others have seen inhibitory to no effects on circulating neutrophils or have alternatively observed decreased production of the hydroxyl ion (Gutteridge et al. 1981; Britigan et al. 1989). Reports of enhanced phagocytic function inclusive of superoxide production by peritoneal macrophages following oral LF supplementation in guinea pigs and mice have also been made (Wakabayshi et al. 2002a, 2003b). While oral LF

supplementation did not affect PMN superoxide production in our earlier study, we were not able to rule out any effects played by the achieved serum LF concentrations.

In the current study, expression of phosphorylated ATF-2 was significantly reduced in cultures treated with SB203580. SB203580 a pyridinyl imidazole derivative, is a specific p38 MAPK inhibitor (Bernt Van den Blink et al. 2001). There have been no reports on its effects on ROS generation. Rather it directly prevents phosphorylation of downstream targets including the transcription factor ATF-2 through competitive binding against ATP (Bernt Van den Blink et al. 2001). Although LF pretreatment of LPS-stimulated PBMCs did not completely inhibit ATF-2 phosphorylation, expression of this transcription factor was qualitatively altered. Compared with untreated controls, expression of phosphorylated ATF-2 by LF-only treated cells was also reduced.

Interestingly, MMP-9 mRNA expression was also observed in cells devoid of LPS stimulation. Since MMP-9 expression must be induced, the observed expression by untreated control cells is most likely indicative of direct stimulation during cellular isolation and/or *in vitro* culture (Lakritz et al. 2002b). While one may argue that the effects of lactoferrin may solely be related to its ability to limit LPS activation of host cells, this would not account for its observed effects on MMP-9 and activated ATF-2 expression in the absence of LPS.

Previous reports of LF inhibition of mRNA IL-1 $\beta$  expression may be limited to expression at the tissue level. Additionally, inhibition studies by Lee et al. demonstrated correlation between IL-1 $\beta$  expression and activation of the

ubiquitous nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor (Lee et al. 2004). While some *in vitro* studies suggest the anti-inflammatory effects of LF may involve its intra-nuclear translocation and direct inhibition of NF- $\kappa$ B activation, this was not supported by findings of the current study (Crouch, Slater and Fletcher 1992).

Based on the reported results, it appears the anti-inflammatory properties of LF do in fact impact on p38 MAPK signaling within immune cells. However we were not able to establish the exact mechanism of its action or comment on dose-response effects. Despite this, given the high incidence of pulmonary disease in neonatal and juvenile cattle and the significance of MMP-9 to lung pathology, additional studies are warranted to further elucidate the potential role of LF in intracellular signaling and the mechanism of cytokine modulation, which unlike SB203580, appears to be outside of ATP antagonism.

## **SUMMARY DISCUSSION - WHERE DO WE GO FROM HERE?**

Since the initial discovery of LF, numerous efforts have been made to substantiate and or investigate the many putative roles of lactoferrin in modulating the host immune response. These named abilities have been based on several identified characteristics of this truly pleiotropic protein. Lactoferrin has the ability to sequester host iron from invading pathogens with high affinity reversibly so. Given its highly positive charge it is able to bind to several negatively charged surfaces including host components and cellular receptors, LPS, and directly to bacterial pathogens. Benefits arising from these interactions include inhibition of host: antigen interactions, modulation of host cellular response to antigenic stimulation and direct bactericidal activity.

We attempted to investigate the effects of a commercially available bovine clostral LF on the disposition of bovine immune cells (neutrophils and mononuclear cells) isolated from the peripheral blood of calves under *in vitro* stimulation. Despite positive results in previously performed purely *in vitro* experiments, findings from our first study, “Effects of Supplemental Lactoferrin on Serum Lactoferrin and IgG Concentrations and Neutrophil Oxidative Metabolism in Holstein Calves,” failed to support our hypothesis that oral supplementation of LF in neonatal (up to 2 weeks of age) calves on the day of birth would enhance superoxide production by neutrophils following *in vitro* stimulated using PMA and OP zymosan. Potential reasons for these findings center mainly on the removal of white cells from the peripheral blood for study *in vitro* and measurement of LF

alone, without measurement of potentially biologically active LF peptides.

Despite this, significant information was gleaned with respect to this species.

Neonatal calves are able to non-selectively absorb significant and measurable amounts of lactoferrin (from colostrum and supplemental sources) through the enterocyte during the early postnatal period. Interestingly, half life elimination of the protein appears to be source dependent with that from colostrum being more persistent in the serum. However, it would be difficult to draw any conclusions without performing true bioavailability studies using a bovine model. Now, given the outcome of the study some issues may be raised. Since lactoferrin is preformed within the specific granules of PMNs why would supplementation improve their function? Cellular uptake of LF is facilitated via membrane-associated receptors (Birgens HS et al. 1984). Within the host, lactoferrin concentrations become elevated in response to disease or/and inflammation (unregulated synthesis by mammary gland or other mucosal epithelia or neutrophil degranulation). The animals used in this study were in good health, and given that PMN function is maximal at the level of inflamed tissue, isolated neutrophils would not have been primed for activation. In a recent study, compared with the use of either treatment separately, activation of PMNs using a soluble ligand subsequent to LPS priming, resulted in a 3 to 6-fold increase in superoxide production (Sheppard et al. 2005). Our cells were not primed prior to activation. The lack of observed augmentation could be attributed to suboptimal function by calf neutrophils and the significantly lower serum LF concentrations achieved in this study compared with that which would have resulted at inflammatory sites.

Also the concentrations achieved in earlier *in vitro* studies were in fact 25 to 250-fold higher (Lakritz et al. 2000a; Wong and Liu 1997). In other *in vitro* studies, LF has been shown to limit neutrophil oxidative burst. Evaluating this parameter in an endotoxemia model will prove enlightening.

Given the significance of Gram-negative infections in cattle, we proceeded to evaluate the responsiveness of PBMCs to LPS and the moldulatory effects of LF on identified cellular responses. While LPS is well known for its mitogenic effects, cellular exposure to LPS is also associated with the up-regulation of eicosanoid production, through the induction of enzymes involved in the release of arachidonic acid (Lakritz et al. 2000b). In experiment 2, “*In vitro* effects of lactoferrin (LF) on lipopolysaccharide (LPS)-induced proliferation of bovine peripheral blood mononuclear cells,” the observed reduction in LPS-stimulated cyclooxygenase-2 mRNA expression and the decreased production of PGE<sub>2</sub> by bovine PBMCs, strongly suggests that the most likely mechanism of action of LF in these assays was inhibition of lipid A: LBP binding. Measured endpoints were evaluated in separate experiments. Lactoferrin significantly inhibited proliferation indices of cells stimulated by LPS. This role is similar to one of the recognized functions of tilmicosin, a macrolide used for metaphylaxis (prophylactic treatment of animals at high risk of obtaining respiratory infections) as well as for the treatment of bovine respiratory disease in cattle (Lakritz et al. 2000b). The potential for the use of LF as adjunctive therapy therefore exists and may result in producers being provided with an option by which to reduce the impact of LPS on neonatal calf health. Given its current use as a topical antibiotic in the beef

(meat) industry and its worldwide use in human supplements, the potential for easy acceptance of lactoferrin in animal health is feasible.

The apparent enhanced uptake of tritiated thymidine by cells supplemented with iron in the absence of both LF and LPS was initially surprising. Since culture reagents were filter-sterilized and cultures were closely monitored for bacterial contamination, the possibility of H<sup>3</sup> incorporation by proliferating bacteria was considered less likely. Being a transition metal, iron is freely capable of donating and receiving electrons and when present in high concentrations, it catalyzes the transfer of electrons between the hydroxyl ion and hydrogen peroxide during lipid peroxidation events. It is therefore more likely that the observed increase in tritiated thymidine uptake was more closely related to the increased frequency of DNA strand breakage resulting from induced oxidative stress and subsequent DNA repair (Pendreno et al. 2005). The ability of LF to limit cellular damage in high iron states, speaks to its high-affinity iron-binding ability. Additionally, limiting iron availability via the Haber-Weiss pathway supports a previously-mentioned anti-inflammatory effect of this protein. Tissue damage that often accompanies infections in the host commonly results from the liberation of reactive oxygen species by activated phagocytes during pathogen elimination. Once produced, the superoxide ion is used for the generation of both the hydroxyl ion and hydrogen peroxide, potent tissue reactants. Binding of catalytic iron limits this reaction. Consequently, while limiting pathogen nutrient acquisition, lactoferrin is also protective to the host.

In our final experiment, "An *in vitro* study of Lipopolysaccharide-induced p38 mitogen activated protein kinase (MAPK) signaling pathway in lactoferrin-treated bovine PBMCs," we further attempted to investigate the potential intracellular transfer of LF and elucidate its activity on intracellular signaling pathways. We investigated the role of lactoferrin in limiting the expression of MMP-9, and IL-1 beta, two inducible pro-inflammatory cytokines. Compared with LPS -only treated cells, pretreatment of PBMCs with LF prior to LPS stimulation did not significantly alter IL-1 beta mRNA expression. MMP-9 expression was, however, significantly reduced. In light of the involvement of the MAP kinase pathway in cytokine production, we investigated the role of LF in p38 MAPK signaling and report altered expression of the ATF-2 transcription factor protein. Inhibition of the stress-associated expression of this transcription factor by untreated controls strongly supports that LF was functioning through mechanisms other than lipid A binding. This could be substantiated if similar inhibition is noted in assays in which cells are co-cultured with the p38 activator anisomycin. These studies will be performed in the near future.

While our findings largely confirm functions previously described in other species, the experiments we have performed serve as an excellent starting point and provide the basis for future *in vivo* bovine studies. It is noteworthy that our studies adhered to the widely agreed practice of LF pretreatment and we recognize this most likely represents a departure from field-associated infections. Since LF is acutely released in response to antigenic detection within the host,

treatment administration closer to the time of experimentally-induced disease may more closely mirror natural events and will be evaluated in the future.

## **VITA**

Dr. Maisie E. Dawes was born on June 17, 1969 in Mandeville, Manchester, Jamaica, West Indies. She is the last of five children (2 brothers and 2 sisters, 1 deceased). She received her primary through secondary school (high school and community college) education in the parishes of Manchester and Clarendon after which she journeyed to Portland, Jamaica where she graduated in 1990 with an Associate of Science degree in General Agriculture.

Dr. Dawes then spent two years in tenure with the Jamaica Agricultural Research Development Foundation in the capacity of Field Research Assistant in Moneague, St. Ann, establishing the project site for a Master of Science degree project, "Evaluating Cattle Growth on African Star Grass." She also assisted in sample collection and processing off site at the Bodles Agriculture Research Station, St. Catherine, Jamaica. Faced with the challenges of obtaining a student visa despite being accepted to Tuskegee University, Alabama, USA to pursue her bachelor of Science degree, Dr. Dawes worked for a semester at her alma mater, Knox Community College, Clarendon, in the capacity of farm

manager and later for another semester as science teacher at the now Christiana High School.

In the Fall of 1993, Dr. Dawes entered the portals of Tuskegee University where she completed her Bachelor of Science degree in Poultry and Animal Sciences after two years. She was awarded the Doctor of Veterinary degree in 1999 from the Tuskegee University School of Veterinary Medicine, and later journeyed to Columbia, Missouri where she successfully matched for an internship position in Food Animal Medicine. She has since then successfully completed a 3-year residency program, and her certification in Large Animal Internal Medicine (food animal focus), and is currently a clinical instructor with shared commitments in the Departments of Veterinary Medicine and Surgery and Veterinary Pathobiology in the College of Veterinary Medicine, University of Missouri-Columbia, Columbia, MO. Armed with her PhD, immunology focus, Dr. Dawes is eager to shoulder the challenges of pedagogy in the areas of immunology, food animal medicine and surgery and clinical instruction. She was elected Vice President of Sigma Xi UMC chapter in June 2005.

Since 1999 Dr. Dawes has enjoyed singing with the Chancel Choir of the Missouri United Methodist Church and the University-affiliated community Choir, Choral Union. She has recently begun making appearances with the church's drama team.

## APPENDIX

**Table 3.1.** Serum LF concentrations (ng/ml) in calves receiving colostrum (n = 6), colostrum + lactoferrin (n = 10) and milk replacer + lactoferrin (n = 6). Treatment lactoferrin concentrations (g/l) are listed in the far right column. LF was supplemented orally at 1 g/kg body weight.

Treatment group	Serum (ng/ml)					Colostrum/Treatment (g/l)
	Day 0	24 h	9 days	30 days	60 days	
Colostrum	324 ± 334 (0 - 863)	1565 ± 1114 <sup>a</sup> (335 - 3628)	1017 ± 886 (0 - 2743)	170 ± 162 (0 - 431)	271 ± 413 (0 - 1230)	2.21 ± 1.5 <sup>a</sup> (0.82 - 4.4)
Colostrum + LF	135 ± 158 (0 - 429)	2237 ± 936 <sup>b</sup> (31 - 3287)	682 ± 645 (38 - 1878)	461 ± 402 (0 - 1200)	84 ± 130 (0 - 326)	11.8 ± 1.7 <sup>b</sup> (9.3 - 12.9)
Milk replacer + LF	318 ± 337 (0 - 963.5)	3189 ± 926 <sup>b</sup> (1736 - 4120)	548 ± 726 (0 - 2164)	301 ± 376 (0 - 1048)	277 ± 372 (0 - 994)	11.6 ± 0.95 <sup>b</sup> (10.2 - 12.7)

Data in top line of each cell indicates mean ± 1 s.d.; data in bottom line of each cell indicates the range of the data points. Values with different superscripts are significantly different from each other (P < 0.05)

**Table 3.2.** Serum and colostrum concentrations of IgG in calves receiving colostrum, colostrum + 1 g/kg lactoferrin or milk replacer + 1 g/kg lactoferrin.

Treatment group	n	Treatment IgG g/l	Day 0 g/l	24 h g/l	9 days g/l
<b>Colostrum</b>	6	133.7 ± 21.3	0.0	19.1 ± 9.4	20.5 ± 9.8
<b>Colostrum + LF</b>	10	128.9 ± 35.7	0.0	22 ± 6.3	19.5 ± 5.2
<b>MR + LF</b>	6	0.0	0.0	0.0	0.0

Day 0 = blood obtained immediately after birth (precolostral); 24 h = blood obtained 24 hours after treatment administration; 9 days = 9 days of age. IgG concentrations less than zero Grams per liter are recorded as 0 g/l

**Table 3.3.** Superoxide production (as determined by cytochrome C reduction) by bovine neonatal neutrophils. Bovine peripheral blood neutrophils were added ( $2 \times 10^6$  cells/ml) and pre-warmed to 37°C, followed by incubation with the stimulus indicated, for 20 minutes.

	Day	OP Stimulus Concentration			PMA Stimulus Concentration		
		10 μg/ml	50 μg/ml	100 μg/ml	30 ng/ml	60 ng/ml	300 ng/ml
		nmoles O <sub>2</sub> <sup>-</sup> / 2 million cells/20 minutes			nmoles O <sub>2</sub> <sup>-</sup> / 2 million cells/20 minutes		
Colostrum	0	4.3 ± 0.8	11 ± 4	13 ± 6	23 ± 8	25 ± 7	24 ± 7
	24 h	3. ± 2	7 ± 5	12 ± 7	25.5 ± 16	26 ± 8.5	26 + 13.5
	9 days	3 ± 2	7.6 + 5	10 ± 6	23 ± 10	25.6 ± 8	26 ± 8
Colostrum + LF	0	4 ± 1.5	8.7 ± 3	12 ± 4	24 ± 4	25.6 ± 4	46 + 65
	24 h	3.8 ± 2	7 ± 4	10 ± 5	20 ± 8	21 ± 9	22 ± 6
	9 days	4 ± 1	7 ± 2	10 ± 3	20 ± 6	31 ± 29	23 ± 4
Milk replacer + LF	0	4 ± 2	9 ± 3.5	14 ± 5	28 ± 4	29 ± 5	29 ± 5
	24 h	6 ± 2	10 ± 4	14 ± 5	27 ± 3	27 ± 4	27 ± 3
	9 days	4 ± 1.5	21 ± 34	9 ± 3	24 ± 8	24 ± 6	26 ± 5

OP - opsonized zymosan; PMA = phorbol myristate acetate; values in cells indicate the mean ± s.d.

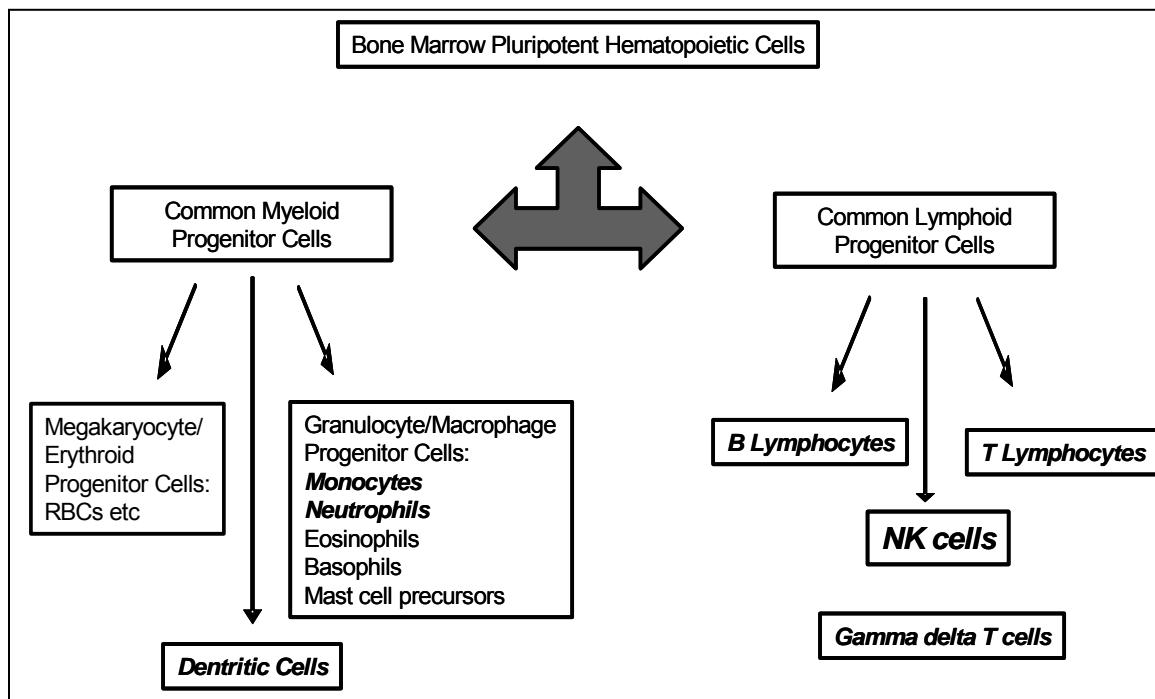
**Table 4.1:** Polymerase chain reaction amplification profiles used to detect pro-inflammatory cytokine and housekeeping gene expression in RNA isolated from bovine peripheral blood mononuclear cells.

Cytokine	Initial denaturation Temperature/time	Denaturation Temperature/time	Annealing Temperature/time	Extension Temperature/time	Elongation Temperature/time	Reaction Volume	No. of Cycles
COX-2 (442 bp)	94°C; 10 min.	94°C; 1 min.	55°C; 1 min.	72°C; 2 min.	72°C; 10 min.	50 µL	35
GAPDH (468 bp)	94°C; 10 min.	94°C; 1 min.	56°C; 1 min.	74°C; 2 min.	72°C; 7min.	50 µL	32

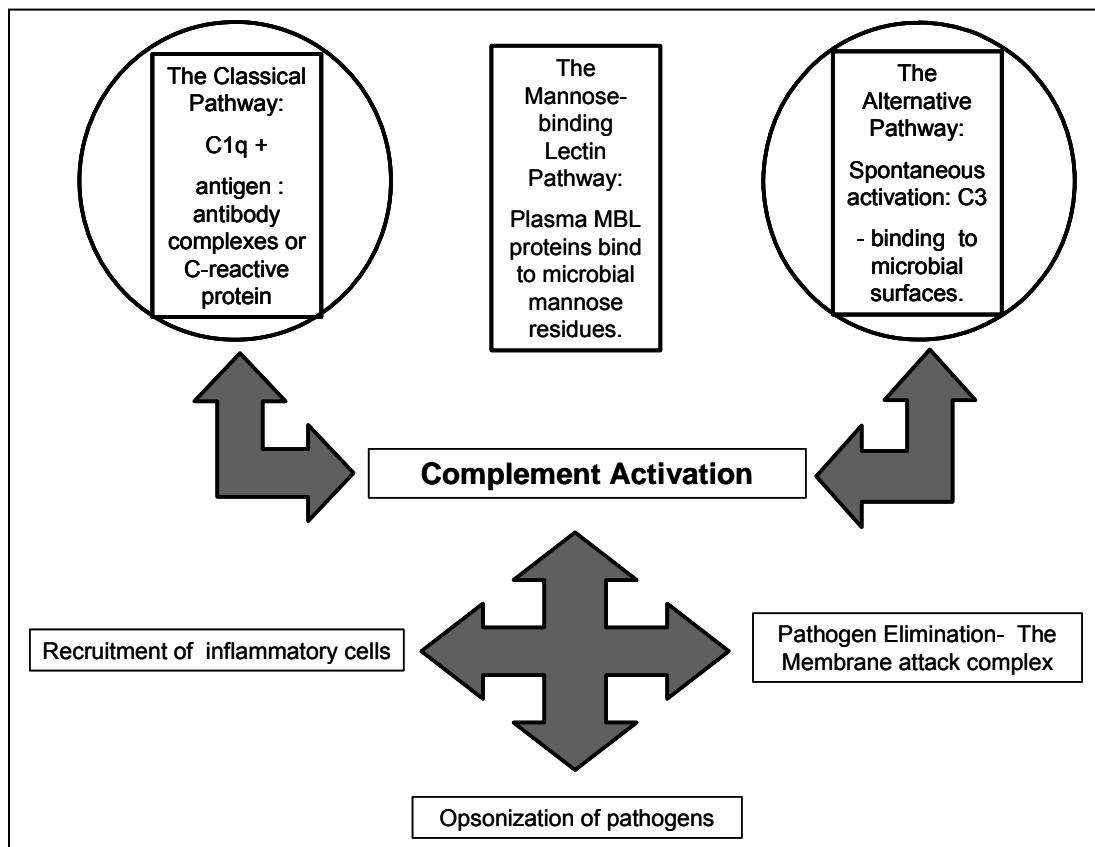
**Table 5.1.** Amplification profiles used in polymerase chain reaction assays to detect inflammatory mediator and housekeeping gene mRNA expression by bovine PBMCs *in vitro*.

Cytokine	Initial denaturation Temperature/time	Denaturation Temperature/time	Annealing Temperature/time	Extension Temperature/time	Elongation Temperature/time	Reaction Volume	No. of Cycles
IL-1β (394bp)	95°C; 10 min.	95°C; 1 min.	54°C; 1 min.	72°C; 1min.	72°C; 1 min.	50 µL	32
MMP-9 (658bp)	94°C; 3 min.	94°C; 1 min.	58°C; 1 min.	72°C; 2 min.	72°C; 7min.	50 µL	30
GAPDH (468 bp)	94°C; 10 min.	94°C; 1 min.	56°C; 1 min.	74°C; 2 min.	72°C; 7min.	50 µL	32

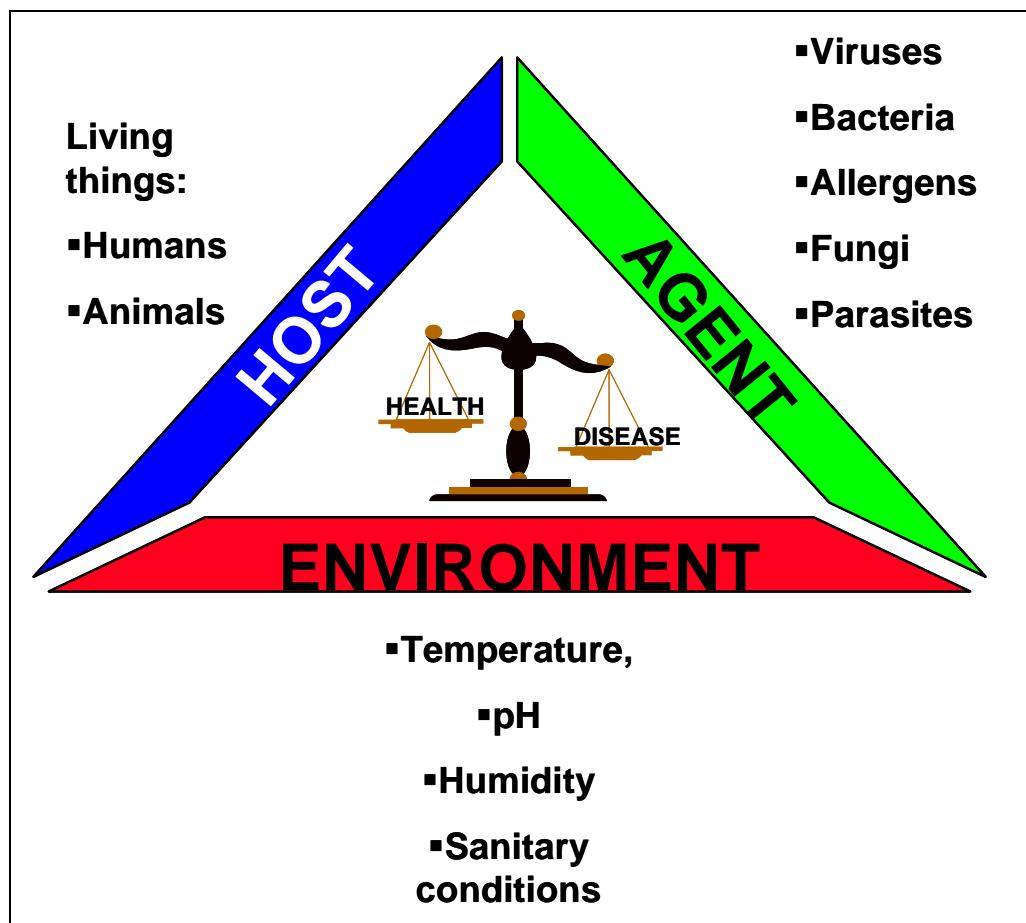
**Figure 1.1.** Cells of the immune system - role in immunity.



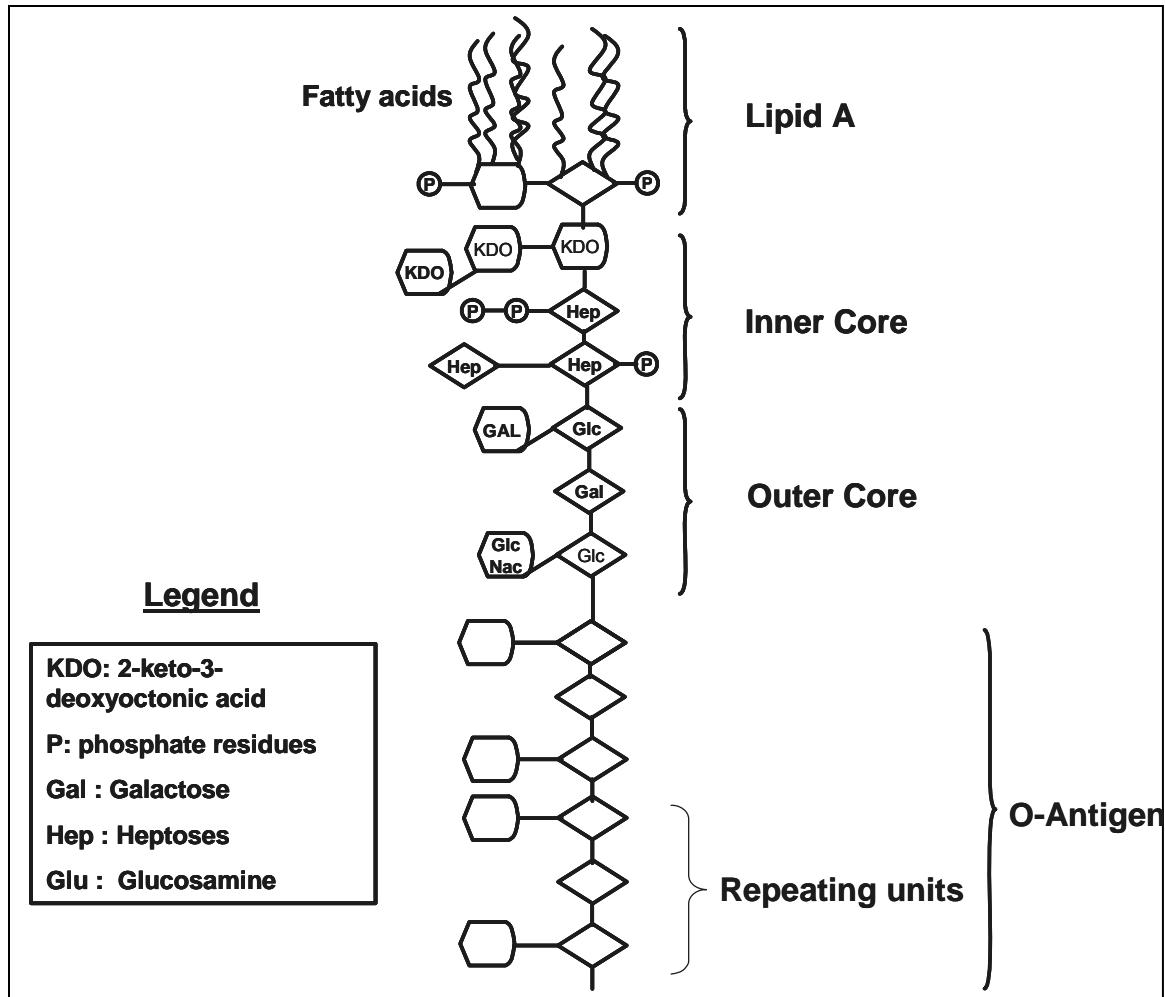
**Figure 1.2.** A schematic representation of the three pathways involved in complement activation.



**Figure 1.3.** The Epidemiological Triad depicts the potential interactions between the host, pathogens and environmental factors and their impact on health and disease in the host.

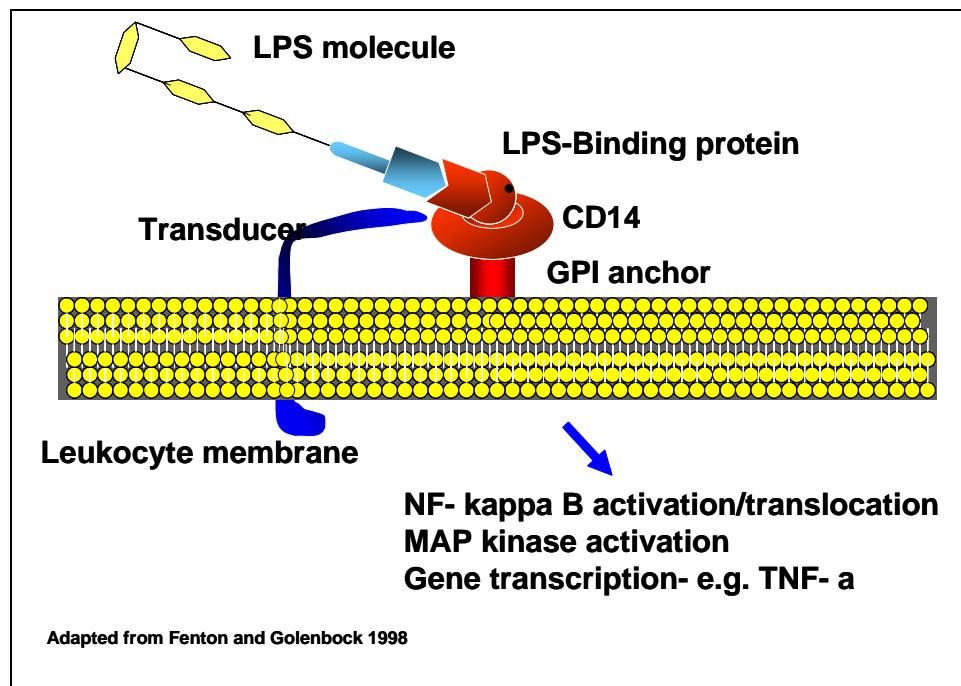


**Figure 1.4.** A schematic representation of Lipopolysaccharide (LPS) or endotoxin and its 4 components: Lipid A; the inner core; the outer core and the O antigen.

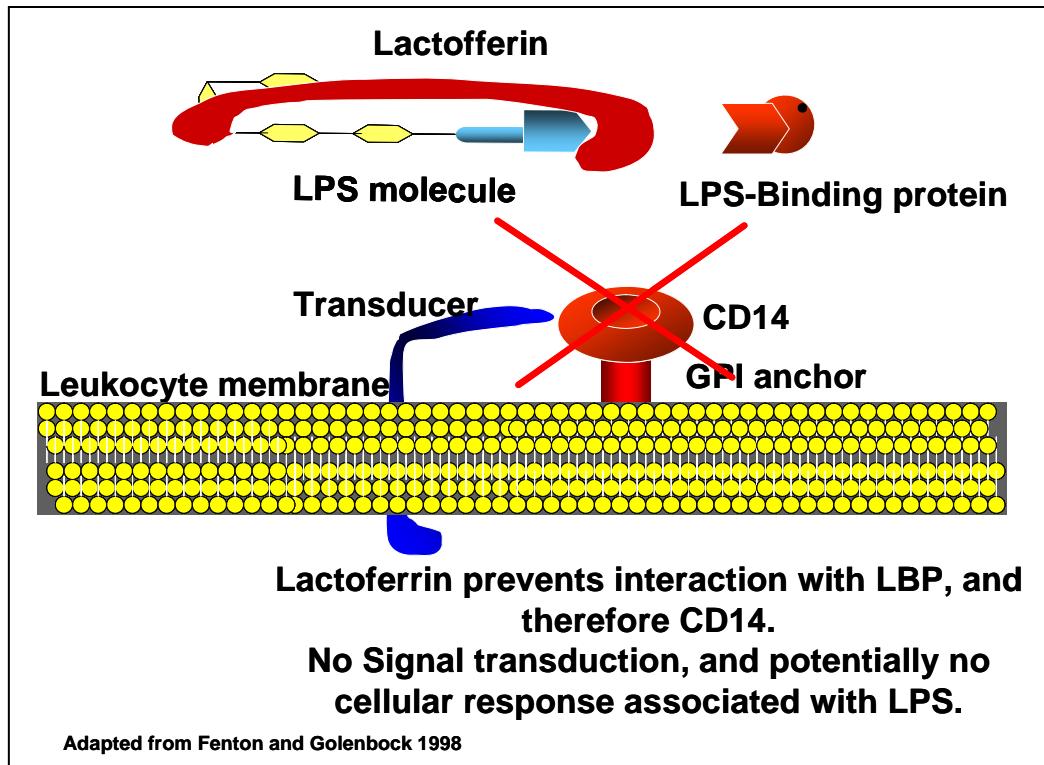


Adapted from Amersfoort, van Berkel and Kuiper 2003

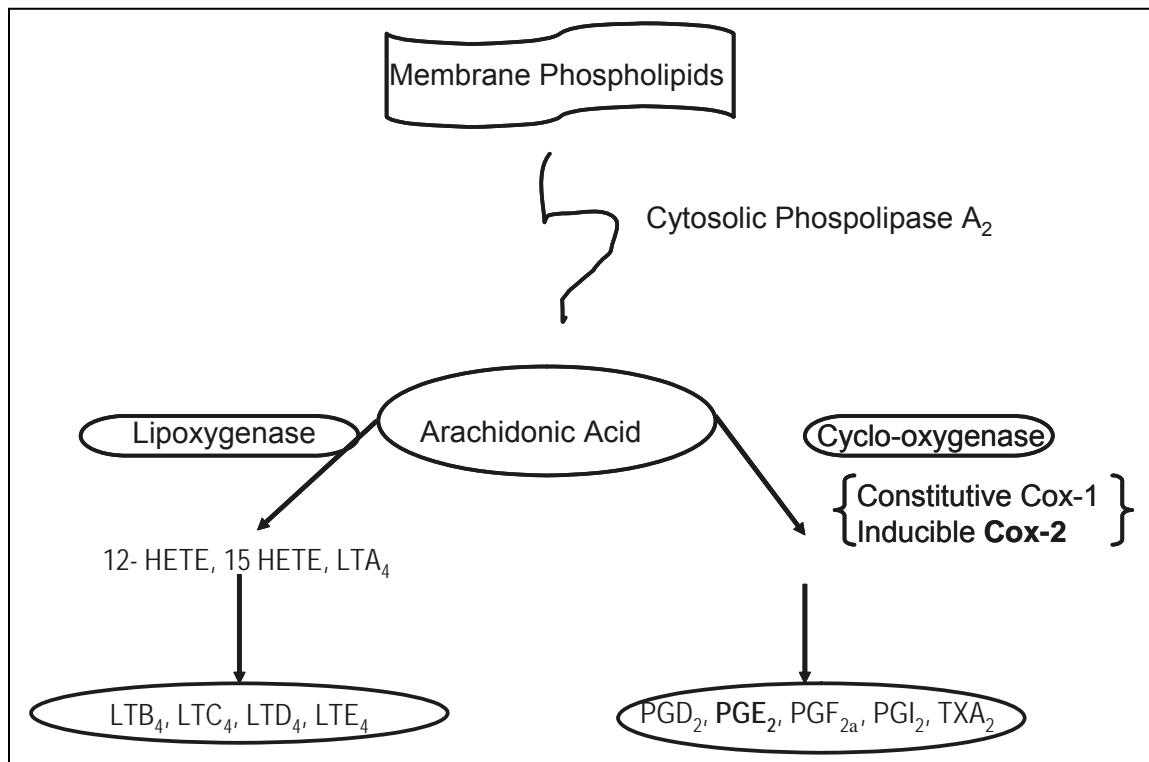
**Figure 4.1a.** Lipopolysaccharide:Lipopolysaccharide binding protein complex activates the host leukocyte through CD14, LPS receptor-binding. Activation of intracellular signaling pathways results in cytokine production.



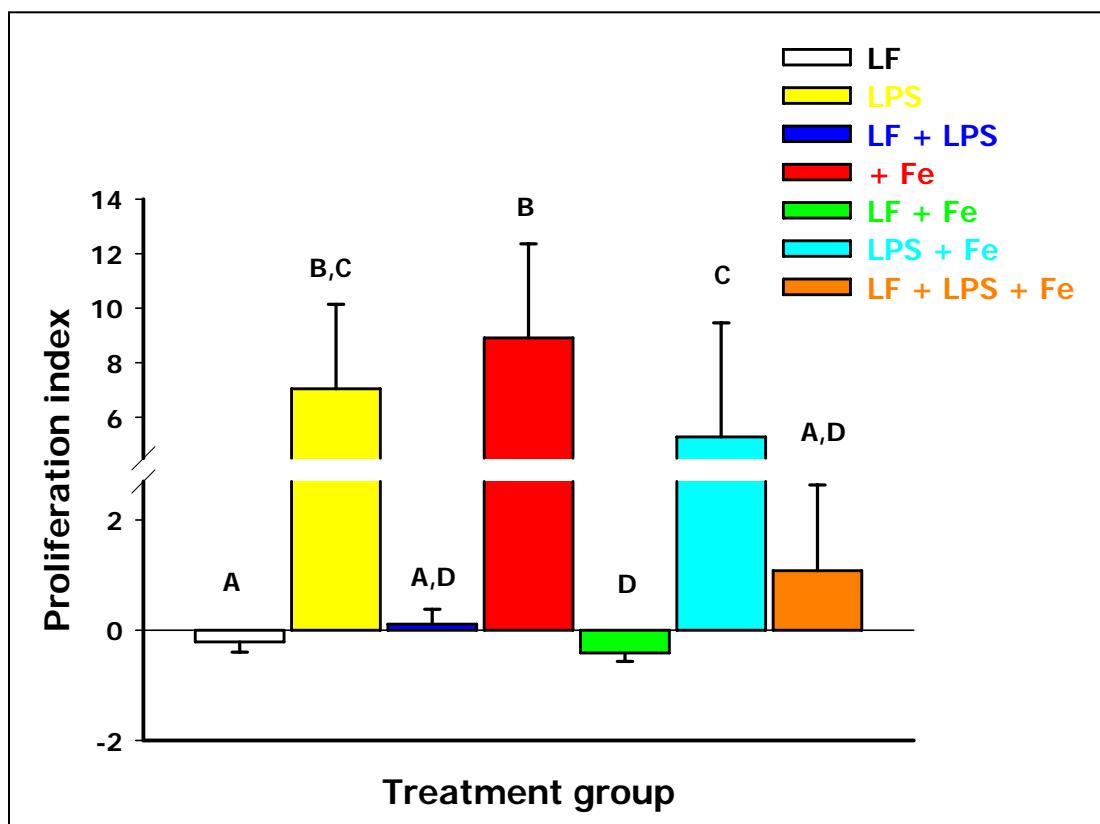
**Figure 4.1b.** Lactoferrin inhibits LPS:LBP binding to the leukocyte CD14, LPS receptor by binding to Lipid A.



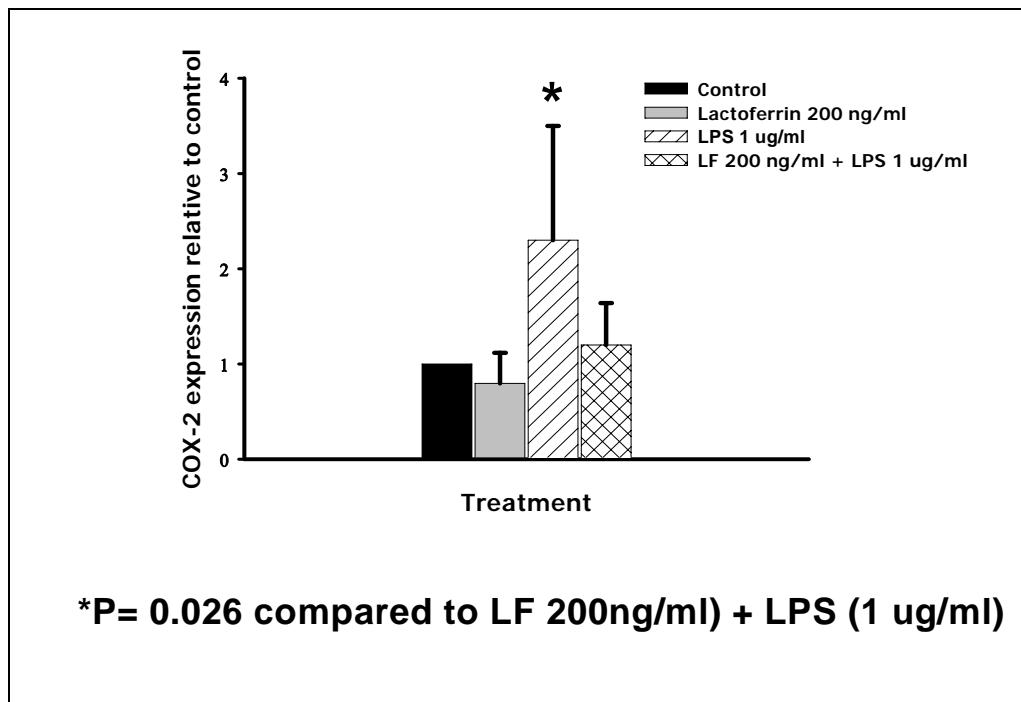
**Figure 4.2.** Diagrammatic representation of the Arachidonic Acid Pathway.



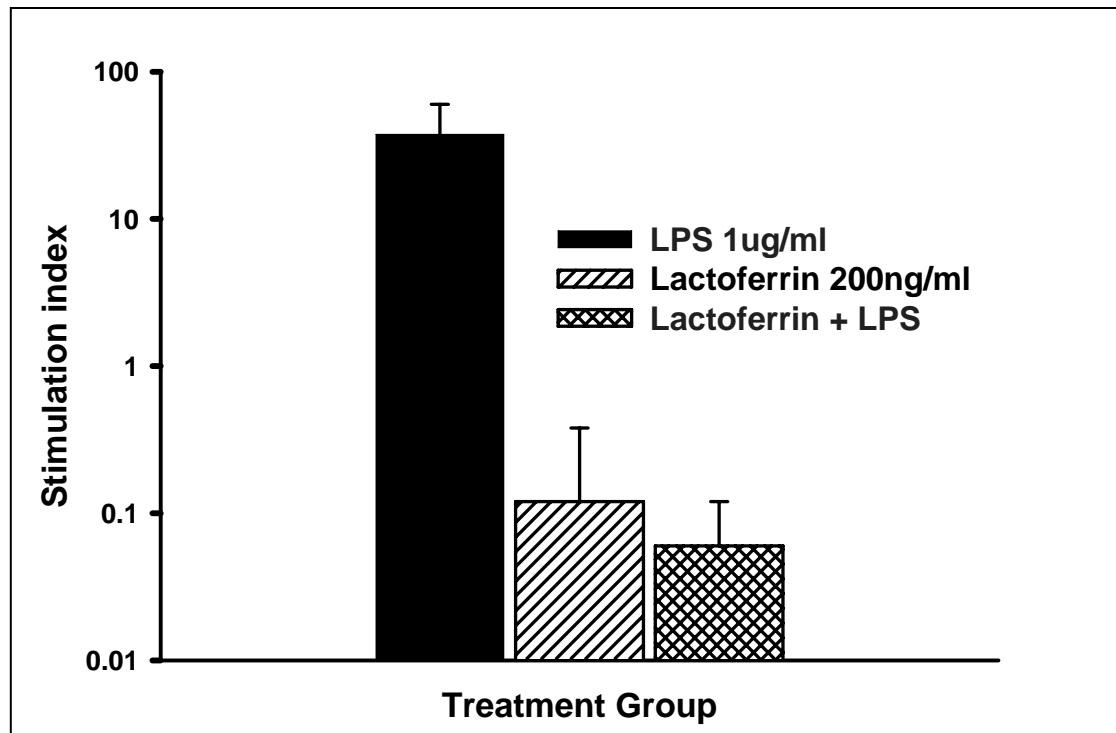
**Figure 4.3.** The effects of LF and exogenous  $\text{Fe}^{3+}$  (1mM) on LPS-induced lymphocyte proliferation index (PI). Proliferation indices were determined by expressing treatment CPM relative to controls. Bars bearing dissimilar nomenclature are significantly different ( $P < 0.05$ ).



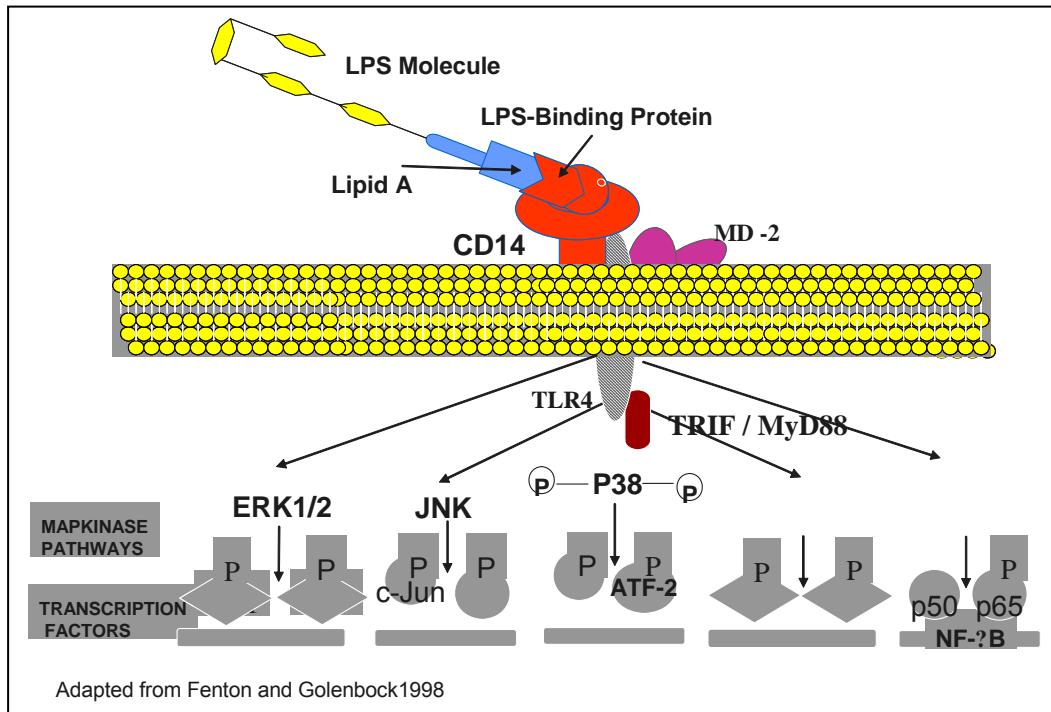
**Figure 4.4.** *In vitro* effects of LF on COX-2 gene expression relative to housekeeping gene glyceraldehyde-3 phosphate dehydrogenase GAPDH. This represents pooled data obtained from----- experiments.



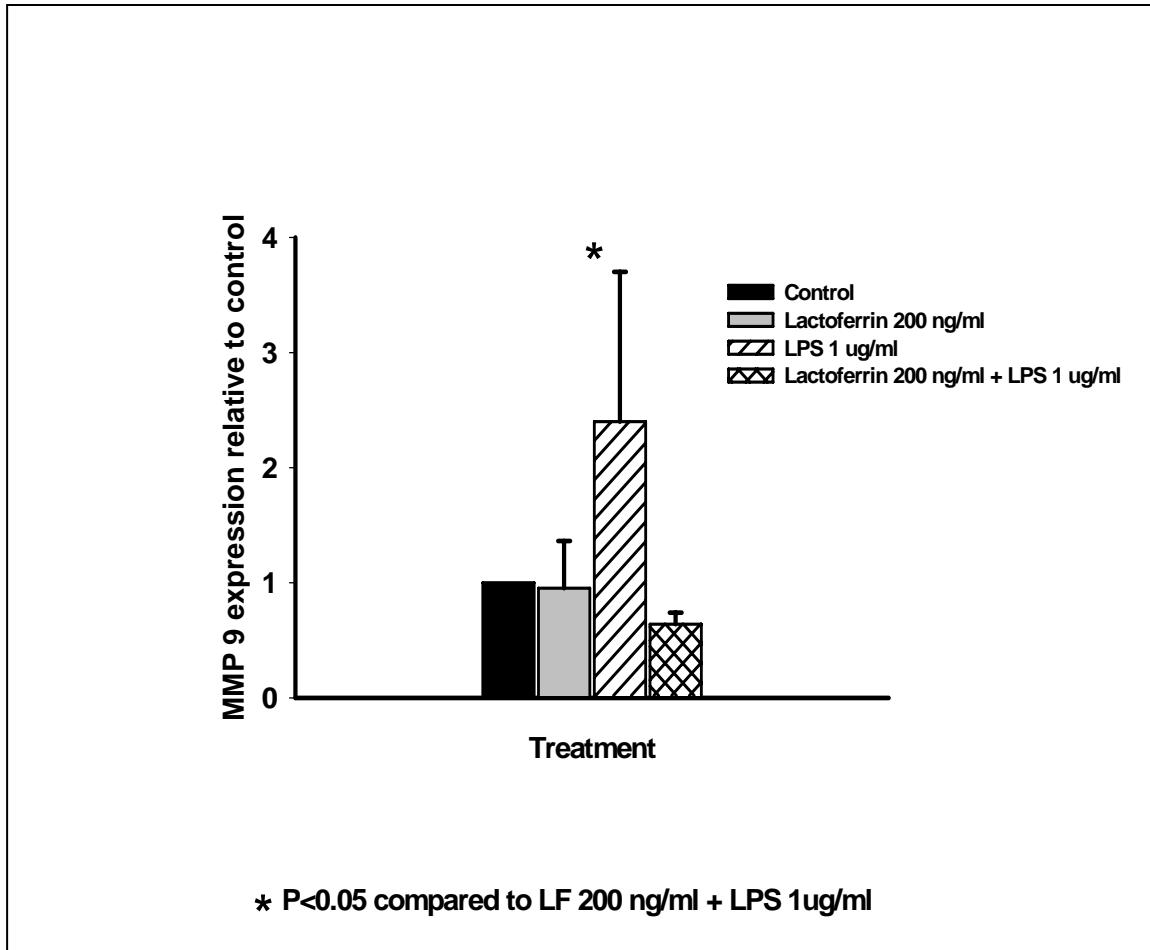
**Figure 4.5.** The *in vitro* effects of LF on LPS-induced PGE<sub>2</sub> production. Treatment stimulation indices are calculated relative to prostaglandin synthesis by untreated controls.



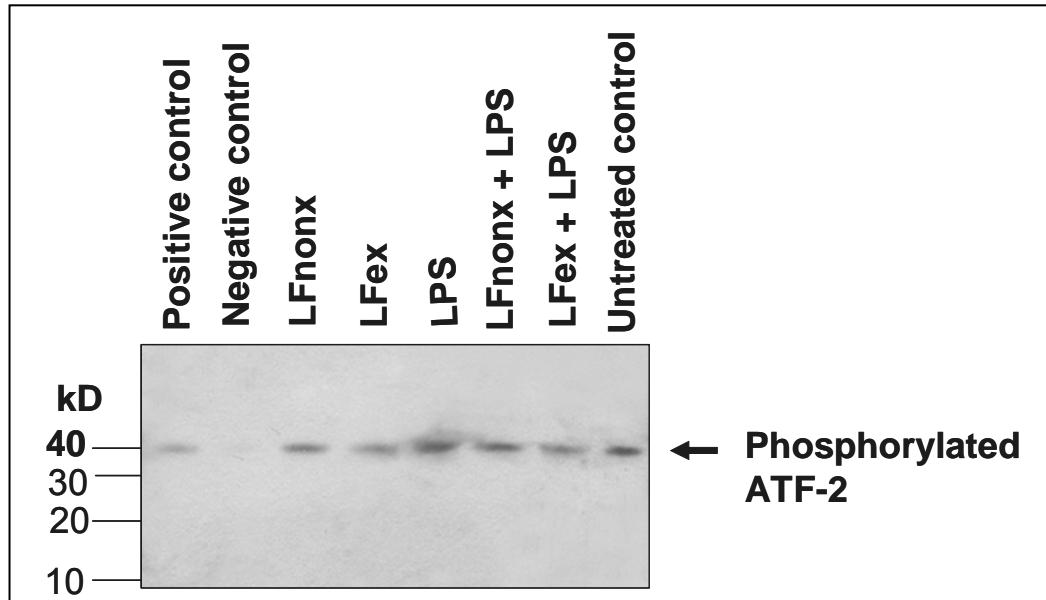
**Figure 5.1.** LPS activates intracellular pathways (e.g. p38 MAPK pathway) through toll-like receptor-4 mediated signaling.



**Figure 5.2** *In vitro* effects of LF on MMP-9 gene expression by LPS stimulated bovine PBMCs. Results are presented relative to housekeeping gene glyceraldehyde-3 phosphate dehydrogenase GAPDH



**Figure 5.3.** Western immunoblot showing LF-altered *in vitro* expression of phosphorylated activating transcription factor (ATF)-2 by phosphorylated p38.



LFex -LF (200ng/ml) after ethanol extraction and lyophilization; LFnonx- LF (200ng/ml) that has not been subjected to ethanol extraction; LPS- lypopolysaccharide, strain O55:B5 (200ng/ml); Untreated control - no LPS, no LF; negative control - 50 $\mu$ M SB203580; positive control - anisomycin-treated cells (25 $\mu$ g/mL; 25 mins prior to harvest).

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