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Bovine mammary interleukin-8 receptor expression and genetic association with Streptococcus uberis based mastitis.

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I am submitting herewith a thesis written by Lydia Jean Siebert entitled "Bovine mammary interleukin-8 receptor expression and genetic association with Streptococcus uberis based mastitis.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Bovine mammary interleukin-8 receptor expression and genetic association with *Streptococcus uberis* based mastitis.

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Dedication

This thesis is dedicated to my grandmothers, Mary Margaret Landers Siebert Calhoun and Mary Patricia Kilduff Ward. They were two of the strongest women I have ever known and taught me to be the same. They encouraged me from early on to pursue my dreams and never give up. Without their inspiring words and reassurances I would not have accomplished all that I have today.

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Abstract

Mastitis is an economically distressing disease in the dairy industry. Bacterial pathogens enter the gland and encounter cell types that release immune mediators including interleukin (IL)-8. IL-8 has two membrane bound receptors: CXCR1 and CXCR2. CXCR1 and CXCR2 are expressed on neutrophils and other cell types in other species but their expression is unknown in the bovine mammary gland. To test this, mammary tissue samples from six Holstein dairy cows were subject to dual immunofluorescence with bovine specific CXCR1 and CXCR2 antibodies and cell type markers. CXCR1 was expressed on alveolar epithelial cells, fibroblasts, and leukocytes. CXCR2 expression was identified on alveolar epithelial cells and leukocytes. Within the bovine CXCR1 sequence, key amino acids 122, 207, 245, 327, and 332 have the potential to influence receptor efficiency in ligand binding and/or downstream signaling effects. Previous studies have linked some of these SNPs with mastitic phenotypes. The three SNPs (VWHRR, VWHKH, and AWQRR) were representative of 99% of the population. To test the haplotype effect, forty Holstein dairy cows were haplotyped and experimentally challenged with *Streptococcus uberis*. Various infection responses were monitored until 28 days post challenge. All cows with the VWHRR x VWHRR (n=5) and AWQRR x VWHRR (n=6) haplotype combinations required antibiotic treatment to clear infection whereas only 33.33% of VWHRR x VWHKH cows (n=7) required antibiotic therapy (p=0.0153). Cows with a VWHRR homozygous haplotype displayed significantly higher milk (1.05 ± [plus or minus] 0.21) and mammary (1.23 ± [plus or minus] 0.17) scores and AWQRR x VWHRR cows had

significantly lower milk (0.17 ± [plus or minus] 0.18) and mammary (0.41 ± [plus or minus] 0.14) scores then cows of all other haplotype combinations (p=0.0263 and p=0.0161 respectively) indicating levels of inflammation differed among haplotype combinations. VWHRR homozygous cows also displayed significantly higher *S. uberis* bacteria counts (981.99 ± [plus or minus] 1947.05) then cows of any other haplotype combination (p=0.0348). Somatic cell counts (p=0.1399) and milk yield (p=0.6126) were not influenced by haplotype. Coupling this knowledge together indicates the critical role this receptor-ligand complex plays in a cow's ability to resist mastitis.

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Abbreviations and Symbols

AIPL	Animal Improvement Programs Laboratory
APC	Antigen presenting cell
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CNS	Coagulase-negative *Staphylococci*
CTAP	Connective tissue activating peptide
CXCL1	GRO-α, NAP-2
CXCL2	GRO-β, MIP-2
CXCL3	GRO-γ, MIP-2b
CXCL5	ENA-78
CXCL6	GCP-2
CXCL7	NAP-2, PPBP
CXCL8	IL-8, GCP-1, NAP-1, NCF
DHIA	Dairy Herd Information Association
DIM	Days in milk
ELR^+	Glutamic acid-leucine-arginine positive
ENA	Epithelial cell neutrophil-activating peptide
ETREC	East Tennessee Research and Education Center
GAR	DyLight 549 Goat-anti-rabbit IgG
GCP	Granulocyte chemotactic protein
GRO	Growth related oncogene
HAM	DyLight 488 Horse-anti-mouse IgG
HRP	Horseradish peroxidase

ICAUC	Institutional Care and Use Committee
IL	Interleukin
INF	Interferon
LPS	Lipopolysaccharide
MCERV	Microbiology across Campuses Educational and Research Venture
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NAP	Neutrophil-activating protein
NCF	Neutrophil chemotactic factor
NMC	National Mastitis Council
PAMP	Pathogen-associated molecular pattern
PBP	Platelet basic protein
PPBP	Pro-platelet basic protein
ROS	Reactive oxygen species
SCC	Somatic cell count
SCS	Somatic cell score
SNP	Single nucleotide polymorphism
SUAM	*Streptococcus uberis* adhesion molecule
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Chapter I: Introduction

Mastitis, an inflammation of the mammary gland, is an ongoing issue in the dairy industry that costs producers billions of dollars annually (NMC 2005). Bacteria most frequently cause mastitis. Once bacteria enter the gland, encountered cells generate danger signals and release cytokines; among them interleukin-8 (IL-8), growth related oncogene-γ (GRO-ɣ), and epithelial cell neutrophil-activating peptide-78 (ENA-78). Interleukin-8 is released by a variety of cell types in response to stressors such as: reactive oxygen species (ROS), bacterial fragments, and other pro-inflammatory cytokines (DeForge *et al.* 1993; Grob *et al.* 1990). Interleukin-8 is responsible for three key immune related functions: stimulating the migration of other cell types (Grob *et al.* 1990; Takata *et al.* 2004), regulation of host cell survival (Kettritz *et al.* 1998; Shamaladevi *et al.* 2009), and modulation of chemokine and cytokine profiles.

Moyes (2009) revealed *S. uberis* induced mastitis causes a 1054-fold increase in IL-8 gene transcription. Experimental challenge with *S. uberis, E. coli* and *Serratia marcescens* demonstrated increased concentrations of IL-8 in the milk in comparison to uninfected glands (Bannerman *et al.* 2004; Riollet *et al.* 2000; Shuster *et al.* 1997). Direct infusion of IL-8 into the mammary gland increased the number of leukocytes, antibodies and serum albumin in the milk suggesting IL-8 impacts several immune related functions in the gland (Watanabe *et al.* 2008).

Interleukin-8 has two specific, membrane-bound receptors, CXCR1 and CXCR2. CXCR1 is expressed on neutrophils, other immune cells (Del Rio *et al.* 2001; Grob *et al.* 1990; Holmes *et al.* 1991; Lee *et al.* 1992; Moser *et al.* 1991), human mammary stem cells, and breast cancer cells (Ben-Baruch 2003). CXCR2 is also expressed on

immune cells, especially neutrophils (Ahuja and Murphy 1996; Grob *et al.* 1990; Lee *et al.* 1992; Moser *et al.* 1991; Sprenger *et al.* 1994) as well as endothelial cells, and some epithelial cells (Wislez et al. 2006). In bovine mammary tissue, it is unknown which cell populations express these two receptors. Because of the increased concentrations of IL-8 observed in mastitic glands and IL-8's association with immune related functions, a more thorough understanding of the cell types expressing these receptors is critical for understanding mammary gland responses to infection.

The bovine CXCR1 gene has several single nucleotide polymorphisms (SNPs) that our lab has associated with mastitis (Youngerman et al. 2004). The key SNP demonstrating this association was at position +735, but several others also were identified (+365, +621, +980, +995) (Table 1). The +365 SNP, located on the first extracellular loop, involves a T to C switch, a valine to alanine amino acid change, has close proximity to the ligand binding site and the ability to affect ligand binding and affinity (Leong *et al.* 1994; Monteclaro and Charo 1996; Pighetti *et al.* 2012). The +621 SNP induces an early stop codon truncating the receptor and removing its ability to perform internal signaling upon ligand binding if it is expressed. The +735 SNP has been linked to increased cases of subclinical mastitis (Youngerman *et al.* 2004), decreased calcium signaling upon IL-8 binding (Rambeaud and Pighetti 2007), decreased neutrophil migration to the site of infection (Rambeaud and Pighetti 2005), decreased ROS generation from neutrophils to aid in bactericidal activities, and increased neutrophil survival (Rambeaud *et al.* 2006). The +980 and +995 SNPs are located towards the intracellular C-terminus of the receptor. Through specific

manipulation of the receptor sequence, the C-terminus including the +980 and +995 SNPs was identified as playing a key role in binding of intermediates, migration, and receptor internalization and polymorphisms in the nucleotide sequence could impact these functions (Raman *et al.* 2010). Based on the bovine CXCR1 SNPs, amino acid haplotypes were developed and three (VWHKH, VWHRR, AWQRR) were representative of more than 99% of the sample Holstein population (Pighetti *et al.* 2012). Cows with haplotypes that include fewer conserved amino acid changes may demonstrate decreased receptor functionality, making VWHRR the haplotype most likely to display the most decreased function. This suggests that examining CXCR1 haplotypes instead of the individual SNPs within the receptor's gene may provide a more comprehensive understanding of a cow's potential for fighting off mastitis and streamline efforts for genetic selection.

Based on IL-8 increases during multiple types of infection, known IL-8 receptors expressed on multiple cell types in other species, and the genetic association of CXCR1 SNPs to disease susceptibility, *I hypothesize that the IL-8 receptors, CXCR1 and CXCR2, will be present on more than just immune cells within the mammary gland and that cows with two copies of the specific CXCR1 haplotype, VWHRR, will display an increased susceptibility to developing mastitis following direct challenge.* The following two objectives will allow for the testing of the preliminary hypothesis:

1. Evaluate types of cells expressing CXCR1 and CXCR2 in bovine mammary tissues.

2. Determine the association of CXCR1 haplotype on resistance to direct *S. uberis* challenge.

Table 1: CXCR1 SNP summary and location.

SNP	Base Change	Amino Acid Change	Location
+365	T→A	V→A	1st intracellular loop
+621	G→A	W→Stop	2nd extracellular loop
+735	G→C	Q→H	3rd intracellular loop
+980	A→G	K→R	C-terminus
+995	A→G	H→R	C-terminus

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Chapter II: Literature Review

Mammary Gland

The mammary gland is a complex and essential organ to bovines and other mammals where it serves two main functions: to provide the offspring nutrition for development/growth and immunity. Offspring consuming milk secreted form the mammary gland are provided nutrition and immunity. The milk contains essential fatty acids, amino acids, vitamin and minerals needed for calf growth and development (Armstrong 1959). These nutrients are provided in a form that is highly digestible and absorbable to the still developing offspring. Secreted milk also contains antimicrobial factors, immunoglobulins, lysozyme, complement, lactoferrin, and leukocytes, which are crucial components for the development of both gut and systemic immunity (Gopal and Gill 2000; Larson *et al.* 1980; Oviedo-Boyso *et al.* 2007). These immune components are essential for bovine offspring survival because, unlike humans, dams do not pass antibodies to their offspring in utero (Larson *et al.* 1980). Furthermore, immune components and cells present within the milk also aid the mammary gland itself in fighting off invading pathogens (Bishop *et al.* 1976; Oviedo-Boyso *et al.* 2007; Reiter 1978; Reiter and Oram 1967).

The bovine udder is composed of four individual mammary glands separated by a layer of connective tissue lamellae (Dyce and Wensing 1971). Within the parenchyma of each gland exists the secretory units which are termed alveoli (Figure 1) (Dyce and Wensing 1971). The alveoli differ in size depending on their location in the gland, but are generally between 0.1 and 0.2 millimeters in diameter (Dyce and Wensing 1971). The alveoli include a layer of milk secreting epithelial cells, which will secrete milk into

the lumen of the alveoli (Figure 1). Connecting the alveoli are a series of small excretory ducts (Dyce and Wensing 1971). As you descend further down the gland, the ducts merge and increase in size until you reach lactiferous ducts (Dyce and Wensing 1971). The dozen or so lactiferous ducts then converge on the large cistern that connects to the ductus papillaris, or streak canal where milk is secreted (Dyce and Wensing 1971). The mammary gland's ductal and alveolar structures develop in a pad of adipose. Cellular signals that aid in mammary gland differentiation and development are provided by the collective stromal tissue which includes the adipose pad (Neville *et al.* 1998). By the time a cow reaches first parturition, the udder is fully developed with the mammary epithelium supported in stroma containing adipose cells (Figure 2) (Hovey *et al.* 1999; Neville *et al.* 1998). The stroma also contains other cell types including fibroblasts and immune cells that can aid in development and differentiation and supports vascularization which is a necessity for the mammary gland (Neville *et al.* 1998). The supply of blood to the udder comes from the mammary artery, which runs from the pudendal trunk, along the abdomen and then to the udder. From there, the blood flow continues in a series of smaller vessels to provide nutrients to the essential milk producing cells. Endothelial cells construct the blood vessels within the gland, and are present in high numbers because milk production requires a high level of vascularization (Dyce and Wensing 1971). Innervation of the bovine mammary gland is fairly uncomplicated. The iliohypogastric and ilioinguinal ventral branches innervate the cranial glands and the skin of the udder whereas the caudal glands are innervated by the mammary branches of the pudendal nerve. There is little innervation within the

gland itself with only sympathetic innervation to blood vessels and the teat sphincter (Peeters *et al.* 1949).

There are many cell types present within the mammary gland that reside within several tissue types (Table 2, Table 3). Epithelial cells are the primary component of the epithelium (epithelial cells, myoepithelial cells, and lumen) and comprise 70% of the cells in the parenchyma (Akers *et al.* 2006; Capuco *et al.* 1997). Epithelial cells synthesize and secrete milk (Bauman *et al.* 2006). Their main role is to remove nutrients from the neighboring blood capillaries and use the obtained nutrients to synthesize milk (Bauman *et al.* 2006). In order to facilitate these nutrient transfer roles, epithelial cells are polarized, meaning the basal side has a much different role than the apical side. The basal side, neighboring the basement membrane and capillaries, retrieves nutrients and the apical side, neighboring the alveolar lumen, synthesizes and secretes milk (Emerman and Pitelka 1977). The endoplasmic reticulum is linked to the synthesis of milk proteins and the golgi apparatus to the production of lactose and the secretion of milk components in secretory vesicles (Bauman *et al.* 2006). The number of mitochondria present within a given epithelial cell is in direct correlation with the energy needs of the cell during lactation (Veltri *et al.* 1990). Furthermore, because epithelial cells line all the ductal and alveolar structures, they are often the first cell type encountered by invading pathogens and as such help initiate the immune response (De Schepper *et al.* 2008).

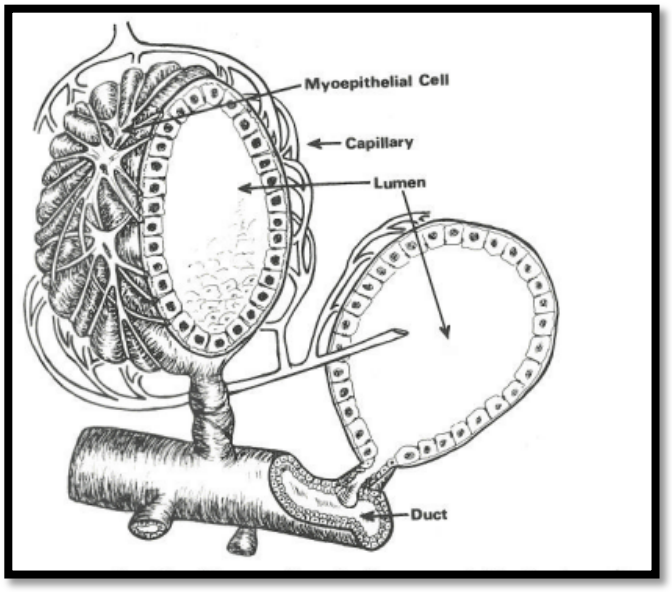

Figure 1: Diagram of an alveolus in a lactating mammary gland with surrounding myoepithelial cells and blood vessels. (Anderson *et al.* 1985)

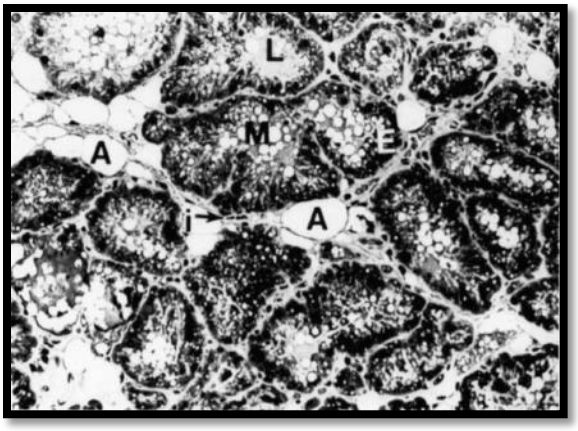

Figure 2: Developed murine mammary gland cross-section displaying epithelial cell (E) lined lumens (L) containing milk (M) surrounded by supporting adipocytes (A) (Jensen *et al.* 1991).

Table 2: Tissue types and structures present in bovine lactating gland (Akers *et al.* 2006).

Tissue Type	Percentage
Parenchyma	30
Lumen	55
Stroma	15

Table 3: Approximate cell types present in bovine lactating mammary parenchyma with ranges dependent upon gland region (Capuco *et al.* 1997).

Cell Type	Percentage
Endothelial cells	6.3 – 6.4
Epithelial cells	70.7 – 69.7
Fibroblasts	12.6 – 14.3
Leukocytes	4.5 – 5.4
Myoepithelial cells	5

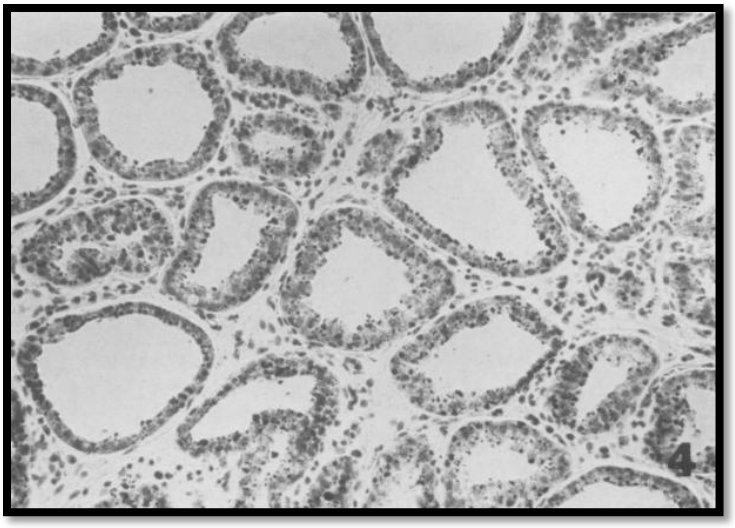

Figure 3: Cross-section of a fully developed mammary gland characterized by completely developed alveoli with maximized lumen area and minimized stromal area (Howe *et al.* 1975).

Myoepithelial cells comprise about five percent of the (Table 2, Table 3) mammary parenchyma (Akers *et al.* 2006; Capuco *et al.* 1997) and are seen in tandem with epithelial cells where they lie between epithelial cells and the basement membrane. They attach to both the basement membrane and epithelial cells by hemidesmosomes and desmosomes, respectively (Glukhova *et al.* 1995). Myoepithelial cells are associated with both alveolar and ductal epithelial cells. Those associated with alveoli are discontinuous and form a basket-like network around the alveoli but still allow some alveolar epithelial cells direct contact to the basement membrane. Those associated with ductal epithelial cells form a continuous layer that runs parallel to the long axis of the duct (Emerman and Vogl 1986; Gusterson *et al.* 1982). Myoepithelial cells are responsible for the contraction of epithelial cells and the subsequent release of milk into the lumen of the alveoli and ducts (Bissell *et al.* 2003). This action is completed by the actin and myosin that fills the cytoplasm of myoepithelial cells and is induced by oxytocin release associated with the initiation of milking stimulus (Gorewit *et al.* 1983; Lefcourt and Akers 1983). Lastly, myoepithelial cells play a great role in the production of basement membrane components that include fibronectin, collagen IV, bioactive laminins, and nidogen (Gudjonsson *et al.* 2002; Gusterson *et al.* 1982; Warburton *et al.* 1982).

Fibroblasts are typically associated with the stroma portion of the parenchyma (Table 2), comprise 12.6 – 14.3% (Table 3) of the mammary gland, and have been linked to a variety of essential functions (Akers *et al.* 2006; Capuco *et al.* 1997). They synthesize both collagen and extracellular matrix, which are major components of

connective tissues (Grinnell 2003). They also play a critical role in wound healing. Fibroblasts are recruited to wound sites by chemoattractant factors and are then responsible for generating new extracellular matrix needed for cell ingrowth (Singer and Clark 1999). Fibroblasts are intricately associated with the mammary stromal tissue (Hovey *et al.* 1999). They are known to release one or more diffusible factors that preferentially select for epithelial cell growth and differentiation as well as alveolar morphogenesis in the mammary gland (Darcy *et al.* 2000).

In the mammary gland, there are also lymphatic ducts that connect lymph nodes to the mammary gland and the blood supply. Lymphatic ducts carry a yellow liquid, lymph, which drains into veins. Lymph is primarily comprised of waste being funneled away from tissues and lymphocytes, a type of leukocyte (UK 2012). Leukocytes or white blood cells are needed for defense of the mammary gland (Paape *et al.* 2002a). Shafer-Weaver *et al.* (1996) observed several populations of leukocytes in a healthy mammary gland (Table 4). Cells expressing CD2 account for the largest fraction (53%) of leukocytes in the mammary gland. They include all cells in T-cell lineage and natural killer cells. This fraction can be broken down further into T-helper cells that are CD4 positive, T-cytotoxic/suppressor cells that are CD8 positive, and γδ T-cells, each represents approximately 13, 31, and 9% of the leukocytes in the gland respectively. B-cells, those responsible for specific antibody presentation and secretion, account for 35% of the leukocytes in the gland. Cells expressing CD5 include: some T-cells, some B-cells, and T-cell precursors, thymocytes that together represent 41% of the leukocytes present. Thus the majority of the leukocytes present in the mammary gland

are lymphocytes. Another significant proportion (43%) of the leukocytes present are antigen presenting cells (APC), which express major histocompatibility complex II (MHC II). Antigen presenting cells consist of B-cells, macrophages and dendritic cells. Macrophages and dendritic cells along with neutrophils, and some B-cells also express CD11, and these cells comprise 18% of the leukocytes in the gland. Another marker, CD172a is unique to monocytes and granulocytes (neutrophils, basophils, and eosinophils), accounts for 25% of the leukocyte population, despite the attempted exclusion of these cell types using a ficoll gradient. Lastly, non-T-cells, non-B-cells were identified via WC2 expression and represented only 4% of leukocytes in the mammary gland (Shafer-Weaver *et al.* 1996).

The Shafer-Weaver study specifically looked at mononuclear cell populations and exempted granulocyte populations, making it necessary to evaluate the leukocyte populations within the mammary gland further. Nickerson and Heald (1982) observed leukocytes present within both healthy and *S. aureus* infected quarters including populations of granulocytes (Table 5). They discovered that within the healthy mammary quarter lymphocytes were the dominant population comprising 69.06% of the leukocytes, which is congruent with the Shafter-Weaver et al. (1996). Antibody secreting plasma cells made up the next largest fraction representing 13.58% of the leukocytes. The last fraction is made up of mast cells, monocytes/macrophages, and neutrophils and these are responsible for only 10.19%, 1.89% and 0.04% of the leukocytes in the healthy quarter respectively. When a quarter is infected with *S. aureus*, the percentages of cells shift from primarily lymphocytes with minimal

granulocytes to approximately half lymphocytes and half granulocytes. Lymphocytes and plasma cells still represent a large fraction at 40.5% and 17.38% respectively. However, neutrophils increase dramatically to account for 32.9% of leukocytes in the infected quarter. The percent of monocytes/macrophages also increases to 5.39%, but the percent of mast cells decreases to 3.83%. These results indicate that when the gland is healthy lymphocytes provide the primary defense mechanism, but under an infection state, granulocytes, especially neutrophils, become primarily responsible for gland defense (Nickerson and Heald 1982).

Bovine milk from a healthy gland, like the gland tissue itself, contains many leukocyte populations (Table 5). The predominant population is T-cells (88%) which includes CD4+ T-helper cells (20%), CD8+ T-cytotoxic/suppressor cells (50%), γδ T-cells (3%) and some natural killer cells. Unlike in the gland, B-cells do not comprise a significant portion of milk leukocytes, accounting for only 6%. The remaining group are CD45R cells, which represent some T and B-cells, macrophages, and neutrophils; and is responsible for 17% of milk leukocytes (Taylor *et al.* 1994). The CD45R group does not include all T-cells due to the fact that as T-cells mature and differentiate they shed their CD45 receptors (Mackay *et al.* 1990).

Table 4: Leukocyte populations present in a healthy, mid-lactation bovine mammary parenchyma following the use of a ficoll gradient to exclude the majority of granulocytes (Shafer-Weaver *et al.* 1996).

Leukocyte (Marker Used)	**Percent Cells Staining Positive**
B-cells (surface IgM)	35
T-cells & NK cells (CD2)	53
T-helper cells (CD4)	13
B-cells, T-cells, & Thymocytes (CD5)	41
T-cytotoxic cells (CD8)	31
DC cells, Monocytes, Macrophages, Neutrophils, and some B-cells (CD11)	18
Antigen-presenting cells (MHCII)	43
Monocyte/Granulocyte (CD172a)	25
γδ T-cells (WC1)	9
Non-T, B-cells (WC2)	4

Table 5: Leukocyte populations present in healthy and S. aureus infected mammary quarters (Nickerson and Heald 1982).

Leukocyte	**Percent Cells in Healthy Quarter**	**Percent Cells in *S. aureus* Infected Quarter**
Lymphocytes	69.06	40.50
Plasma cells	13.58	17.38
Neutrophils	0.04	32.90
Monocytes/Macrophages	1.89	5.39
Mast cells	10.19	3.83

Table 6: Leukocyte populations present in milk secretions from a healthy, lactating bovine mammary gland (Taylor *et al.* 1994).

Leukocyte (Marker Used)	Percent Cells Staining Positive
B-cells (WC3)	6
T-cells (CD2)	88
T-helper cells (CD4)	20
T-cytotoxic cells (CD8)	50
γδ T-cells (WC1)	3
Leukocytes (B-cells, subset of T-cells, Macrophages, Neutrophils) (CD45R)	17

T-cells are the predominate leukocyte present in both healthy mammary parenchymal tissue and milk (Shafer-Weaver *et al.* 1996; Taylor *et al.* 1994). T-cells play a role in eliciting an immune response against invading pathogens and token antigens (Kaufmann 1988). T-cells are subdivided into two categories, αβ T-cells and γδ T-cells (Pardoll *et al.* 1987). Those of the αβ subtype can be further divided into T-helper (CD4+) cells and T-cytotoxic (CD8+) cells (Zlotnik *et al.* 1992). Both T-helper cells and T-cytotoxic cells recognize token antigens in the context of MHC on APCs using a T-cell receptor (TCR) that has α and β chains and can be present in a variety of tissue types (Fowlkes and Pardoll 1989). Once a T-helper cell recognizes antigen it releases a plethora of cytokines that aid activating cytotoxic T-cells, dendritic cells and macrophages, as well as the maturation of B-cells into effector or plasma cells (Zlotnik *et al.* 1992). Cytotoxic T-cells are responsible for destroying infected and/or damaged cells. The infected and/or damaged cells are recognized by cytotoxic cell T-cell receptor (TCR) interaction with antigen that is presented by MHC I (Park *et al.* 1993). T-cells expressing the γδ TCR are typically less predominant than those expressing the αβ TCR (Hein and Mackay 1991), and unlike αβ T-cells do not always require antigen presentation via MHC II to recognize antigen (Mackay *et al.* 1988). The γδ T-cells are typically involved in recognizing antigens of a lipid nature or bacterial danger signals such as degrading cells (Mackay and Hein 1991).

B-cells are another cell type that is highly present within the mammary parenchyma (Shafer-Weaver *et al.* 1996). The primary role of B-cells is to make antibodies specific to one recognized antigen (Clark and Ledbetter 1994). B-cells can

also serve to present antigens via MHC II (Cassell and Schwartz 1994). Naïve B-cells circulate in healthy blood and tissue and once they are exposed to antigen, they become one of two distinct subclasses: a plasma cell or a memory cell. Plasma cells are responsible for the secretion of specific antibodies against a single antigen in order to tag the invading pathogen and make it more recognizable for the immune system to destroy, as well as prevent binding to host cells (Clark and Ledbetter 1994). Memory cells also are specific to a single antigen and are formed from a single activated B-cell during the initial infection. Memory cells live for long periods of time and allow for a more rapid response to specific antigen upon sequential infection (Clark and Ledbetter 1994).

Monocytes are critical to mammary gland health but are present in low numbers due to the fact that once they migrate into the tissue they differentiate into macrophages or dendritic cells. They are however, always present in the blood supply and upon activation are quickly recruited to the sites of infection. Monocytes migrate from the blood supply into tissues via adhesions to endothelial cells lining blood vessels neighboring infection sites. Selectins expressed on endothelial cells weakly bind glycoproteins on activated monocytes and allow them to roll across the vessel wall and slow enough to bind more strongly via surface integrins that have counter-receptors expressed on endothelial cells. After this tight bond is established monocytes enter tissue via extravasation (Imhof and Aurrand-Lions 2004). Once at the site of infection they differentiate into macrophages and dendritic cells that can both engulf and destroy pathogens via phagocytosis (Gordon and Taylor 2005). Monocytes also enter tissue in

the manner described above to replace resident macrophages as needed when there is not an infection present (Gordon and Taylor 2005).

Macrophages are considered the ever-present sentinels of the mammary gland. Their primary function is to maintain tissue homeostasis and defense through clearing senescent cells, repairing damage (Gordon 1986; Gordon 1998), phagocytizing pathogens, and subsequently killing pathogens to eliminate the threat they pose to the gland (Outteridge and Lee 1981). Macrophages also take on the role of antigen presenting, displaying antigens from phagocytized bacteria in conjunction with MHC II to make them more readily recognized by T-helper cells (Politis *et al.* 1992). Several studies have also demonstrated the importance of macrophages in perpetuating the immune response by the release of chemoattractants (interleukin-1 (IL-1) and ENA-78) that are important for neutrophil and T-cell responses (Allmann-Iselin *et al.* 1994; Splitter and Everlith 1989; Ziegler *et al.* 1984).

Dendritic cells share several similarities with macrophages. They too can phagocytize and kill invading pathogens as well as present antigens to T-helper cells via MHC II (Banchereau and Steinman 1998). Dendritic cells also stimulate clonal proliferation, differentiation into effector or memory cells, and the secretion of cytokines upon T-helper cell binding to MHC II (Paape *et al.* 2002b). They are also known to produce cytokines like tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12) to perpetuate the immune state, help recruit other immune cells such as neutrophils and macrophages, and enhance the cytotoxic abilities of natural killer cells and T-cytotoxic cells (Hope *et al.* 2003). Furthermore, dendritic cells play a crucial role in activating the

adaptive immune response. Once matured, dendritic cells can present antigen directly to T-helper cells which in turn interact with antigen primed B-cells to initiate the cascade that develops memory plasma B-cells (Steinman and Hemmi 2006). Dendritic cells can be found in alveolar structures, interalveolar spaces, and in the epithelium within the bovine mammary gland (Maxymiv *et al.* 2012). However, they are distinct from macrophage populations. Macrophages express MHC class II, $CD11c^{hi}$, and $CD14^{hi}$ whereas dendritic cells show MHC class II, $CD11c^{hi}$, $CD14^{lo}$, and CD205 marker expression. These differences in receptor expression make the two cell populations distinguishable via multicolor immunohistochemistry and flow cytometry assays (Maxymiv *et al.* 2012).

Neutrophils are cells that are recruited into tissues by the presence of infection. If no infection exists, their numbers will be relatively low, but if an infection is present, their numbers will show a marked increase. Neutrophils are recruited to the site of infection via chemoattractants and they enter tissues in much the same manner as described for monocytes (Burg and Pillinger 2001). More precise information about neutrophils is provided in the section titled 'Immune Mediators'.

Mastitis

Mastitis or mammary gland inflammation, an ongoing issue in the dairy industry, costs the dairy industry billions of dollars annually (NMC 2005). Losses come in a variety of forms, some of which include: discarded milk from infected cows, antibiotic therapies, replacement of culled cows, and extra labor costs for time spent treating and caring for mastitic cows (Akers and Nickerson 2011; Fetrow 2000; Jones *et al.* 1994;

Short and Lawlor 1992). However, the greatest loss is attributed to decreased milk production in cows that are subclinically and clinically infected (Akers and Nickerson 2011; Fetrow 2000; Jones *et al.* 1994; Short and Lawlor 1992). A number of factors can influence the probability or susceptibility of a cow contracting mastitis and includes: age, parity, stage of lactation, pathogen type, genetics, season, and climate (Hogan and Smith 1987; Oliver and Mitchell 1983; Ruegg 2003; Smith *et al.* 1985). Instance of mastitis increases with parity, the more offspring a cow has the more likely it becomes that she will contract mastitis (Ruegg 2003). Cows not actively being milked or in early lactation are more prone to developing mastitis. Cows in late stages of lactation have a significantly decreased instance of mastitis (Oliver and Mitchell 1983). Furthermore, warm temperatures and moist climates favor the growth of most pathogens, increasing their total numbers and thus the probability they will come into contact with a cow and cause mastitis (Hogan and Smith 1987). Because of the variety of factors, there is diversity in the instances mastitis across herds and breeds of dairy cattle. Herds are known to be have incidence of mastitis in 20-50% of their cows (Wilson *et al.* 1997) and breeds that have been subjected to selection methods for increased milk yield like Holstein dairy cows tend to be associated with increased mastitis (AIPL 2013). Furthermore, mastitis continues to be one of the primary reasons for decreased productive life (AIPL 2013).

Mastitis has two types of causes: non-infectious or infectious. Non-infectious causes are incidents such as blunt trauma to the udder. These traumas arise from kicks from another cow or collision with objects in the cows' housing environment. Infectious causes are much more diverse and caused by a microorganism entering the

gland and establishing infection. Some infectious agents are: algae, yeasts, and viruses, but the cases of mastitis that result from these infectious agents are a very small fraction compared to those cases that arise from bacteria.

Bacteria are present everywhere in the cows' environment. Making good management practices absolutely crucial to the control and prevention of mastitis (Gill *et al.* 1990; Jayarao and Wolfgang 2003; Ruegg 2003). During machine milking, bacteria present on or within the milking unit can easily be transferred into the teat end due to the relaxed state of the teat sphincter during the milking process (Hogan and Smith 1987). Bacteria can also be introduced during the administration of intramammary infusions, such as those used during the dry off (non-lactating) period (Hogan and Smith 1987). Another common means of entrance is directly from the environment. Soil in pastures, bedding, and pre/post milking sanitation processes all are possible sources of infection (Bushnell 1984; Hogan *et al.* 1989; Pankey and Drechsler 1993). Once the bacteria gain entrance to the mammary gland, they benefit in a variety of ways. The gland itself is protection from other environmental factors that could be detrimental to the bacteria. The gland is warm, providing the bacteria with a temperature closer to their optimum growth and replication temperatures. The milk present within the gland provides an energy source for bacterial growth. Thus, once bacteria are inside the mammary gland, they are in an environment that allows them to not only survive but potentially thrive.

Mastitis bacterial pathogens are highly prevalent and hundreds of causative species have been isolated. Mastitis has two basic classifications: clinical and subclinical. Clinical cases have symptoms such as inflammation (redness, heat,

swelling, and pain), dramatically increased somatic cell counts (SCC), atypical or decreased milk production, abnormal or lack of activity, fever and weight loss (Youngerman *et al.* 2004a). Subclinical cases produce less severe symptoms where visual changes in the udder are not observed, but somatic cell counts are still elevated, just not to the degree of a clinical incidence (Youngerman *et al.* 2004a). Subclinically infected cows cause the greatest loss of profit to farmers with a 10 to 12% decrease in milk production associated with a single subclinically infected quarter over the length of a single lactation (Akers and Nickerson 2011). The panel of symptoms observed with a particular case of mastitis can often be tied to the type of pathogen. Incidences of *E. coli* mastitis are typically of rapid onset with altered and decreased milk production and in some cases fever and lethargy and will clear in a few days (Smith and Hogan 1993). However, *S. aureus* mastitis typically start similarly to *E. coli* but lead to subclinical cases that show some alteration and reduction in milk production and can persist for the life of the cow (Sutra and Poutrel 1994).

These bacterial pathogens can be broken into two groups: environmental and contagious (Smith 1983). Environmental pathogens include a larger array of bacteria that are present everywhere in the surroundings of the dairy herd (Smith *et al.* 1985). Herds that demonstrate a pattern of increased clinical mastitis instance, particularly in spring and summer months when the weather is hot and wet, often indicate the establishment of environmental pathogens present in the mammary gland (Smith *et al.* 1985). Common environmental pathogens include *Streptococcus uberis*, *Streptococcus agalactiae Streptococcus dysgalactiae*, coliform bacteria (*Escherichia coli, Klebsiella* spp.), coagulase-negative *Staphylococci* (CNS), and *Enterobacter* spp. *(Hogan and*

Smith 1987; Jayarao and Wang 1999; Jayarao and Wolfgang 2003). Because of their prevalence, they are often used as models to study mastitis.

Streptococcus uberis is an environmental pathogen causing approximately 14-26% of all subclinical and clinical mastitis cases (Jayarao *et al.* 1999; Phuektes *et al.* 2001). It is the most commonly isolated pathogen during the dry period (Bramley 1984; Bramley and Dodd 1984; Oliver 1988). *In vitro* studies have indicated that within two hours after *S. uberis* exposure to epithelial cells, *S. uberis* closely associates with epithelial cell microvilli (Matthews *et al.* 1994). Further observation revealed pedestal formation indicating adherence of *S. uberis* with the epithelial cell membrane (Matthews *et al.* 1994). This adherence occurs between *S. uberis* and epithelial cells as well as extracellular matrix proteins (Almeida *et al.* 1996). Glycosaminoglycans facilitate adhesion (Almeida *et al.* 1999). Glycosaminoglycans are covalently bound to the abundant membrane proteins proteoglycans and often used by bacteria for attachment (Liang *et al.* 1992). Fang *et al.* (1999) identified a molecule specific to *S. uberis*, lactoferrin-binding molecule that aided in bacterial attachment. Lactoferrin, a milk protein, when added to *in vitro* mammary epithelial cell cultures facilitated the binding of *S. uberis* to epithelial cells (Fang and Oliver 1999). Mammary epithelial cells bind lactoferrin (Rejman *et al.* 1994), that in turn facilitates internalization of *S. uberis* into bound epithelial cells (Fang *et al.* 2000). This molecule was later re-named *S. uberis* adhesion molecule (SUAM) and the development of SUAM specific antibodies that decreased *S. uberis* adherence (Almeida *et al.* 2006). This evidence suggests that SUAM may be a novel candidate in the development of alternative treatments or preventative measures for *S. uberis* based mastitis.

Inflammatory Cytokines Linked to Infection

The innate immune system gives the mammary gland the ability to combat a variety of invading pathogens. Due to its relatively non-pathogen-specific methods of pathogen recognition, the innate system provides a rapid response even upon first exposure (Oviedo-Boyso *et al.* 2007). Initial recognition stems from pathogen-associated molecular patterns (PAMPs), which help to initiate the immune response. Bacterial cells are distinct from mammalian cells allowing for specific immune responses. Their cell walls are comprised of components like lipopolysaccharide (LPS), peptidoglycan and lipoteichoic acid that are considered PAMPs (Bannerman *et al.* 2004b; Han *et al.* 2003). PAMPs are recognized by a class of membrane-bound receptors termed toll-like receptors (TLRs) (Rosenberger and Finlay 2003). TLR4 binds bacterial components such as LPS, polypeptides and host associated compounds such as fibrinogen, heat-shock proteins, polypeptides that are released under stressful conditions (Beutler 2004; Takeuchi *et al.* 2000). Another TLR, TLR2 recognizes gram-positive associated molecules such as peptidoglycan and lipoteichoic acid (Takeuchi *et al.* 2000). TLR5 detects flagella that are characteristic of many species of motile bacteria and TLRs 3 and 9 bind dsRNA and dsDNA associated with endosomes respectively (Rosenberger and Finlay 2003; Wagner 2004). In addition to TLRs, chemical components of bacteria can be recognized by complement molecules, formylated peptide receptors, mannose-binding lectins, and ficolins (Akira and Takeda 2004; Fournier and Philpott 2005; Rooijakkers *et al.* 2005; Rosenberger and Finlay 2003). Once bacteria are recognized by these avenues, they induce the production of

immune mediators, especially cytokines, from local cell populations in the mammary gland (Bannerman *et al.* 2004b).

Cytokines are considered key immune mediators and some are associated with the inflammatory state, including TNF-α, IL-1, and interleukin-6 (IL-6). Bacteria enter the mammary gland and can interact with both epithelial cells and macrophages. The binding of bacterial PAMPs to TLRs induces production and secretion of TNF-α. TNF-α stimulates the recruitment and bactericidal activities of neutrophils, in addition to perpetuating the inflammatory state by aiding in the production of IL-1, IL-6, and arachidonic acid metabolites (Atkinson et al. 1990). Following experimental challenge with *S. uberis,* increases in milk TNF-α were observed approximately 72 h post infusion (Bannerman et al. 2004a). Furthermore, Moyes et al. (2009) demonstrated that during *S. uberis* mastitis, TNF-α transcripts were increased 45-fold, which most likely contributes to increased levels of TNF-α protein. TNF-α is the first cytokine to increase in milk following experimental challenge with *E. coli* and *Serratia marcescens*. Peak TNF-α concentrations were reached between 12 and 18 post bacterial inoculation and persisted for up to 48 h (Bannerman *et al.* 2004a; Bannerman *et al.* 2004b; Riollet *et al.* 2000; Shuster *et al.* 1997). Increased levels of TNF-α are also seen in sera and milk after infection with other coliforms including: *E. coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Nakajima *et al.* 1997). LPS injection into the gland has yielded conflicting results. After direct infusion of LPS into the gland, no significant increases in TNF-α were witnessed (Shuster *et al.* 1993). Contrastingly, increased TNF-α has been linked to endotoxic shock in acute cases of mastitis suggesting there

may be an intermediate process that could tie TNF-α and mastitis together (Havell 1989; Persson Waller *et al.* 2003; Ślebodziński *et al.* 2002).

Interleukin-1, like TNF-α, can perpetuate the inflammatory state and stimulate neutrophil migration and killing through stimulating the release of other mediators such as IL-8 (Bannerman *et al.* 2004b; Boulanger *et al.* 2003; Stein *et al.* 2003). Production of IL-1 is induced following recognition of bacterial presence by TLRs on macrophages and epithelial cells (Oviedo-Boyso *et al.* 2007; Yamanaka *et al.* 2000; Zhang and Issekutz 2002). IL-1 is known to be present in milk during the early stages of *S. uberis*, *E. coli*, and *S. aureus* infections (Bannerman *et al.* 2004a; Bannerman *et al.* 2004b; Oviedo-Boyso *et al.* 2007; Riollet *et al.* 2000; Yamanaka *et al.* 2000; Zhang and Issekutz 2002). Increases in concentrations of IL-1 begin shortly after increases in TNF-α (Riollet *et al.* 2000; Shuster *et al.* 1997). Following S. uberis mastitis, there is a 14-fold increase in IL-1 transcripts, which could contribute to increased levels of IL-1 protein (Moyes *et al.* 2009). LPS injection into the mammary gland produced increased IL-1 concentrations more rapidly, displaying increased levels of IL-1 as early as 2.5-4 h post injection (Shuster *et al.* 1993). Increased levels of IL-1 were not observed in milk following challenge with *Serratia marcescens* until 96 h post infusion of the bacteria (Bannerman *et al.* 2004a). Combined, these results suggest IL-1 plays a significant role in *S. uberis* and *E. coli* mastitis but not *Serratia marcescens* mastitis.

Interleukin-6 is synthesized primarily from macrophages and epithelial cells that have interacted with bacteria directly or with bacterial toxin via TLRs (Rainard and Riollet 2003; Zhang and Issekutz 2002). IL-6 is involved in the massive influx of

neutrophils into the mammary gland. IL-6 binding to endothelial cells induces the release of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) to recruit neutrophils and cause increased expression of adhesion molecules to aid in neutrophil extravasation (Romano *et al.* 1997). Moyes *et al.* (2009) demonstrated that during *S. uberis* mastitis, IL-6 transcripts were increased 430-fold, which may contribute to increased levels of IL-6 protein witnessed during mastitis incidence. IL-6 typically follows increased TNF-α and IL-1 concentrations at approximately 14 h post *E. coli* infection (Nakajima *et al.* 1997; Shuster *et al.* 1997). LPS injection resulted in a rapid increase in IL-6 concentrations occurring as early as 2.5 h (Shuster *et al.* 1993). Increased levels of IL-6 are also seen in sera and milk after infection with other coliforms including: *E. coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Nakajima *et al.* 1997). Together, this suggest that the degree of IL-6 response is dependent on the causative agent.

ELR$^+$ Chemokines

A family of glutamic acid-leucine-arginine$^+$ (ELR$^+$) chemokines includes: growth related oncogene (GRO)-α, GRO-β, GRO-γ, epithelial-derived neutrophil-activating peptide-78 (ENA-78), granulocyte chemotactic protein-2 (GCP-2), neutrophil-activating peptide-2 (NAP-2), and interleukin-8 (IL-8). The nomenclature of ELR$^+$ chemokines are summarized in Table 7. These chemokines share a C-X-C motif in their structures and are linked to both inflammatory and reparative functions (Miller and Krangel 1992; Oppenheim *et al.* 1991). An important three amino acid sequence, E-L-R, directly prior to the first cysteine residue of the C-X-C motif is linked to their ability to induce or inhibit

migration of neutrophils (Clark-Lewis *et al.* 1993; Clark-Lewis *et al.* 1995; Hébert *et al.* 1991).

Table 7: List of standardized and common names of ELR^+ chemokines.

Standardized Name	Common Names
CXCL1	GRO-α, NAP-3
CXCL2	GRO-β, MIP-2
CXCL3	GRO-γ, MIP-2b
CXCL5	ENA-78
CXCL6	GCP-2
CXCL7	NAP-2, PPBP
CXCL8	IL-8, GCP-1, NAP-1, NCF

Growth Related Oncogenes

Growth related oncogene-α (GRO-α, NAP-3, CXCL1) is expressed by two cell types: epithelial cells, and macrophages. Bovine mammary epithelial cells secrete basal levels of GRO-α at all times (Lahouassa *et al.* 2007). *E. coli* stimulation increases GRO-α concentrations steadily until at least 24 h post exposure (Lahouassa *et al.* 2007). Upon stimulation with *S. aureus,* levels increase slightly by three h post exposure and return to basal levels by 24 h (Lahouassa *et al.* 2007). Unstimulated human mammary epithelial cells (Maheshwari *et al.* 2003), cultured human bronchial epithelial cells (Marshall *et al.* 2001) and human vaginal epithelial cells (Peterson *et al.* 2005) increase GRO-α production in response to infection. Upon stimulation of lung alveolar macrophages with *E. coli* endotoxin, GRO-α is released (Goodman *et al.* 1998). These indicate that GRO-α is released naturally and in response to a variety of bacterial stimuli.

Growth related oncogene-β (GRO-β, MIP-2, CXCL2) also is expressed by epithelial cells and macrophages. After stimulation with *E. coli*, cultured bovine mammary epithelial cells release GRO-β and continue for at least 24 h (Lahouassa *et al.* 2007). *S. aureus* stimulation resulted in increases similar to those observed by *E. coli* stimulation but did not surpass *E. coli* stimulation concentrations (Lahouassa *et al.* 2007). Similarly, GRO-β is released from unstimulated human mammary epithelial cells (Maheshwari *et al.* 2003), *S. aureus* stimulated human vaginal epithelial cells (Peterson *et al.* 2005), and *E. coli* endotoxin stimulated lung alveolar macrophages (Goodman *et al.* 1998).

Growth related oncogene-γ (GRO-γ, MIP-2b, CXCL3) is expressed by several cell types such as epithelial cells, macrophages, monocytes, and neutrophils. Bovine mammary epithelial cells constitutively express GRO-γ during uninfected and infected states (Rainard *et al.* 2008), as do human mammary epithelial cells (Maheshwari *et al.* 2003). *S. aureus* stimulated human vaginal epithelial cells also increase GRO-γ concentrations (Peterson *et al.* 2005). Furthermore, bacterial endotoxin and/or inflammatory cytokines, TNF-α and IL-1 stimulate GRO-γ release from lung alveolar macrophages, monocytes, and neutrophils (Goodman *et al.* 1998; Haskill *et al.* 1990; Iida and Grotendorst 1990).

The GRO chemokines have the ability to induce neutrophil migration (Geiser *et al.* 1993; Haskill *et al.* 1990; Lahouassa *et al.* 2007; Rainard *et al.* 2008; Youngs *et al.* 1997). Specifically, GRO-γ had been identified as the largest recruiter of neutrophils from bovine milk during both uninfected and infected states (Rainard *et al.* 2008). These chemokines also have been associated with events subsequent to neutrophil migration such as: neutrophil exocytosis, neutrophil shape changes, increases in intracellular Ca^{++} levels, and neutrophil respiratory burst (Ahuja and Murphy 1996; Geiser *et al.* 1993; Lahouassa *et al.* 2007; Rainard *et al.* 2008). GRO-γ is unique in possessing some effect on basophils, having the capability to induce chemotaxis and increases in intracellular Ca^{++} concentrations (Geiser *et al.* 1993). Overall, the GRO chemokines possess properties very similar to that of IL-8

Epithelial-derived Neutrophil-activating Peptide-78

Epithelial-derived neutrophil-activating peptide-78 (ENA-78, CXCL5), as its name suggests, was first discovered in an epithelial cell culture line (A549) (Walz *et al.* 1991), but also is released from macrophages and monocytes (Allmann-Iselin *et al.* 1994). Basal levels of ENA-78 are released from cultured epithelial cells (A549) at all times (Walz *et al.* 1991). Increased release of ENA-78 was not induced by LPS or other bacterial endotoxins, but it is massively induced by pro-inflammatory cytokines such as TNF-α and IL-1 (Walz *et al.* 1991).

Like its family members, ENA-78 is primarily known for its ability to attract neutrophils. In bovines, ENA-78 was first characterized from lung tissue (Allmann-Iselin *et al.* 1994). The potency of ENA-78 as a neutrophil chemoattractant was similar to that of IL-8 at most concentrations (0.1-100nmol/L) (Allmann-Iselin *et al.* 1994; Walz *et al.* 1991). However, IL-8's function was approximately ten times higher at the more elevated concentrations (100 and 1000nmol/L) (Allmann-Iselin et al. 1994; Walz et al. 1991). Bovine ENA-78 also was comparable to IL-8 at inducing neutrophil aggregation (Allmann-Iselin et al. 1994). However, ENA-78 activated the release of intracellular Ca^{++} more efficiently than low concentrations of IL-8 (1nmol/L) but comparable at higher concentrations (Allmann-Iselin et al. 1994; Walz et al. 1991). ENA-78 also mediates elastase release by neutrophils in a similar dose dependent manner (Walz et al. 1991). Lastly, ENA-78 was able to induce a shape change in neutrophils similar (i.e.: fast, prolonged, dose dependent) to IL-8 (Allmann-Iselin *et al.* 1994). All together, these data suggest ENA-78 has comparable capabilities to IL-8, but is not as potent.

Neutrophil-activating Peptide-2

Neutrophil-activating peptide-2 (NAP-2,PPBP, CXCL7) is a 70 amino acid cleavage product of the platelet α-granule component of platelet basic protein (PBP) and its precursor, connective tissue-activating peptide-III (CTAP-III) (Walz and Baggiolini 1989). The cleavage action of a monocyte released protease generates NAP-2 from its platelet derived precursor (Walz and Baggiolini 1990). The release of NAP-2 is often in response to stimuli such as *E. coli* LPS (Walz and Baggiolini 1989). Like its family members, NAP-2 can induce elastase release, increased levels of intracellular Ca^{++}, neutrophil chemotaxis, and neutrophil exocytosis (Loetscher *et al.* 1994; Walz *et al.* 1989). The NAP-2 induced changes were considered completely independent of its precursors, PBP and CTAP-III, because neither of the precursors were capable (Walz *et al.* 1989). NAP-2 also increases microvascular permeability to aid in neutrophil extravasation (Van Osselaer *et al.* 1991). However, NAP-2 is considered a less potent activator than IL-8 but still capable of recruiting neutrophils and aiding in their migration to the site of infection (Walz *et al.* 1989).

Interleukin-8

Interleukin-8 (IL-8, GCP-1, NAP-1, NCF, CXCL8) is an immune-related chemokine. Interleukin-8 is released in response to a variety of cell stressors such as reactive oxygen species, bacterial fragments, and pro-inflammatory cytokines (Baggiolini and Clark-Lewis 1992; Bautista and Spitzer 1990; DeForge et al. 1992; DeForge et al. 1993; Grob et al. 1990; Matsushima and Oppenheim 1989; Seitz et al. 1991). An activator responsible for IL-8 release is ROS. Reactive oxygen species

include superoxides, peroxides, and hydroxyl radicals. ROS are produced as intermediates or byproducts of normal cellular functions (Liu *et al.* 2002) including bacteria initiated leukocyte phagocytic processes (Bautista and Spitzer 1990). Superoxide anions (O_2^-) are converted to hydrogen peroxide (H_2O_2) which then can be broken down to a hydroxyl radical (OH·). Each by product has been associated with high levels of damage in surrounding tissues (Halliwell 1991). Increased concentrations of ROSs directly influence the production and release of IL-8 through the regulation of gene expression (DeForge *et al.* 1993). Furthermore, LPS stimulated whole blood treated with ROS scavengers leads to a significant reduction in the amount of IL-8 present within blood with no reduction in cell viability or the pro-inflammatory cytokines TNF-α, IL-1, or IL-6 (DeForge *et al.* 1992).

Bacterial fragments such as LPS, upon binding to TLR4 activate IL-8 release (Bautista and Spitzer 1990; Grob *et al.* 1990; Seitz *et al.* 1991). Pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, which are released in response to infection induce IL-8 release (Baggiolini and Clark-Lewis 1992; Grob *et al.* 1990; Matsushima and Oppenheim 1989; Seitz *et al.* 1991). All of these stressors (reactive oxygen species, bacterial fragments, and pro-inflammatory cytokines) are indicators of an inflammatory state, which provides a link between the inflammatory state and IL-8.

After IL-8 is released, it is responsible for key immune responses. Perhaps the most well documented function of IL-8 is its ability to stimulate migration of specific cell types. This ability allows recruitment of leukocytes, especially neutrophils, to the site of infection and allows the host to be more efficient in fighting off infection. Once bound to

the neutrophil, IL-8 induces three main changes in neutrophils. First, a conformational change allows adherence to endothelial cells which is essential for migration out of the blood vessel to infected tissues (Baggiolini and Clark-Lewis 1992). Second, exocytosis of soluble storage proteins from secretory vesicles and granules cause increased expression of adhesion molecules such as CD11b,c and CD18 that are essential for adhesion to endothelial cells (Baggiolini and Clark-Lewis 1992). Congruently, macrophages are activated to produce and release cytokines as well as enhance macrophage phagocytosis of IgG-opsonized bacteria (Soehnlein et al. 2008), and opsonization of pathogens like *Staphylococcus aureus* (Heinzelmann et al. 1998). Last, activation of NADPH-oxidase helps generate ROS used to combat foreign pathogens at the site of infection (Baggiolini and Clark-Lewis 1992).

Another function of IL-8 is the regulation of host cell survival, typically by prohibiting apoptosis and extending the life-span of neutrophils. This would allow them to fight infections longer (Kettritz *et al.* 1998; Shamaladevi *et al.* 2009). Interleukin-8 also modulates chemokine and cytokine profiles to aid in perpetuating the inflammatory state (Soehnlein *et al.* 2008). Interleukin-8 has two specific membrane bound receptors, CXCR1 and CXCR2, which are G-protein coupled receptors (Ahuja and Murphy 1996; Lee *et al.* 1992; Murphy 1994). Upon IL-8 binding to its receptors, it activates G-protein coupled responses. Downstream effects of G-protein signaling include two main signaling pathways: cyclic adenosine monophosphate (cAMP) and phosphatidylinositol. Both of these pathways are responsible for a wide variety of biological responses that range from hormone to inflammatory signaling (Baggiolini and

Clark-Lewis 1992). Furthermore, gene products influenced by *E. coli* based mastitis are those associated with G-protein coupled signaling (Buitenhuis *et al.* 2011; Mitterhuemer *et al.* 2010; Rinaldi *et al.* 2010). IL-8 is a key component of the immune response that amplifies the pro-inflammatory signals released from the site of infection.

Several different cell types including: epithelial cells, endothelial cells, fibroblasts, macrophages, dendritic cells, and neutrophils produce IL-8 in order for IL-8 to respond to stressors and induce the necessary downstream effects. Cultured primary mammary epithelial cells and MAC-T cells demonstrated IL-8 release in response to *E. coli* LPS, *E. coli,* and *S. aureus* stimulation (Boudjellab et al. 1998; Lahouassa et al. 2007). Further examination with the MAC-T cell line revealed LPS binding of TLR4 caused the release of TNF-α and IL-1, which causedIL-8 release (Boudjellab *et al.* 2000; Fitzgerald *et al.* 2007). The ability of epithelial cells to initiate the immune response is critical because they are often the first cell type encountered by invading bacteria. Cultured human endothelial cells (Strieter *et al.* 1989a) and fibroblasts (Strieter *et al.* 1989b) also release IL-8 in response to LPS, IL-1, and TNF-α stimulation. Bovine macrophages, the sentinels of the mammary gland, release IL-8 in response to a variety of pro-inflammatory cytokines, bacterial fragments and toxins, and phagocytosis (Craven 1983). Lastly, neutrophils, although commonly viewed as primarily responding to IL-8 released from other cells, can release IL-8 themselves in response to phagocytosis, LPS, and human/bovine lactoferrin. This perpetuated neutrophil recruitment to the site of infection via subsequent binding to the membrane bound receptors, CXCR1 and CXCR2, expressed on neutrophils (Bazzoni *et al.* 1991; Shinoda *et al.* 1996).

Upon infection within the mammary gland an increase in IL-8 was observed. Moyes *et al.* (2009) revealed that *S. uberis* induced mastitis caused a 1054-fold increase in IL-8 gene transcription, making it the most significantly altered gene in this study. Experimental intramammary challenge with *E. coli* also showed a significant increase in IL-8 mRNA production compared to non-challenged glands (Buitenhuis *et al.* 2011). A second *E. coli* study demonstrated IL-8 mRNA was up-regulated 120-fold in infected mammary tissue (Mitterhuemer *et al.* 2010). In similar findings, the IL-8 gene transcripts persisted for at least 24 h post *E. coli* infusion (Rinaldi *et al.* 2010).

The increase in gene transcripts most likely contributes to the increase in protein expression observed in following studies. Experimental challenge with *S. uberis* produced a sustained elevation in milk IL-8 levels for over 96 h post infusion (Bannerman *et al.* 2004a). The increased levels of IL-8 associated with *E. coli* mastitis follow the same pattern as *S. uberis*, with an initial surge in whey. IL-8 levels steadily decrease as the infection clears (Riollet *et al.* 2000; Shuster *et al.* 1997). In contrast, when *S. aureus* mastitis was experimentally induced, IL-8 was not detected in the whey. This pattern was consistent across six cows and suggests that pathogen specificity or pathogen action may play a significant role in the cytokines released (Riollet *et al.* 2000). *S. marcescens* intramammary challenge produced a rapid spike in IL-8 which tapered off after about 24 h (Bannerman *et al.* 2004a). Together, these data suggest IL-8 is a critical early indicator of an infection state within the mammary gland.

Watanabe at el. (2008) showed that injecting recombinant bovine IL-8 into the mammary gland produced a wide variety of effects. The most significant effects were a

dramatic increase in the number of somatic cells, specifically neutrophils, present in the mammary gland and in the milk, increased concentration of antibodies, and the amount serum albumin in milk in comparison to a gland not infused with IL-8. This suggests that IL-8 has the capability to alter the integrity of the milk-blood barrier allowing increased concentrations of somatic cells and serum proteins to enter the milk and dramatically increase the neutrophil population within the mammary gland (Watanabe *et al.* 2008).

CXCR1 and CXCR2

The CXCR1 and CXCR2 genes are located on chromosome 2q and each consists of a coding region of approximately 1000bp (Pighetti and Rambeaud 2006). The CXCR1 gene is located on chromosome 2q, on the opposite strand, and close to the q telomere, whereas the CXCR2 gene is located on chromosome 2q but closer to the centromere (Pighetti and Rambeaud 2006). In addition to their close proximity in the genome, the receptors' share a high degree of homology. The mRNA transcripts display 95% homology in their nucleotide sequences and the completed peptides share 84% homology, including 100% homology in the last, 50 amino acids of the C-terminus (Lahouassa *et al.* 2008; Pighetti and Rambeaud 2006).

Despite the homology between the two receptors, CXCR1 and CXCR2 display differential binding characteristics. CXCR1 has two ligands, IL-8 and the rarely seen granulocyte chemotactic protein-2 (GCP-2), whereas CXCR2 is more promiscuous. CXCR2 binds IL-8 with high affinity, like CXCR1, but it also binds GRO-α, β, γ, ENA-78, and NAP-2, which are all functionally similar to IL-8 (Ahuja and Murphy 1996; LaRosa *et*

al. 1992; Lee *et al.* 1992). Both receptors are part of the G-protein-linked seven transmembrane domain receptor family and possess the characteristic three intracellular loops (Murphy 1994). The extracellular amino-terminus of the receptor dictates ligand binding specificity (LaRosa *et al.* 1992). The intracellular carboxyl-terminus initiates receptor internalization and cellular signaling pathways via its coupled G-proteins (Ben-Baruch *et al.* 1995; Richardson *et al.* 2003).

CXCR1 and CXCR2 receptors are expressed on neutrophils (Ahuja and Murphy 1996; Grob *et al.* 1990; Lee *et al.* 1992; Li and Gordon 2001; Paape *et al.* 2002a; Sprenger *et al.* 1994). The expression of CXCR1 has also been documented on other immune related cells including: dendritic cells (Sallusto *et al.* 1998), natural killer cells (Berahovich *et al.* 2006), CD4+ T-cells (Gasser *et al.* 2006), and CD8+ T-cells (Takata *et al.* 2004). Less has been documented about their expression on non-immune cell populations. However, CXCR1 has been shown to be expressed on human mammary stem cells (Ben-Baruch 2003) and endothelial cells (Murdoch *et al.* 1999), as well as several cancer cell types including: breast cancer cells (Ben-Baruch 2003; Charafe-Jauffret *et al.* 2009), prostate cancer cells (Shamaladevi *et al.* 2009), and malignant melanoma cells (Gabellini *et al.* 2009). The other receptor, CXCR2 is expressed on endothelial cells and some epithelial cells (Dwinell *et al.* 1999; Miller *et al.* 1998; Wislez *et al.* 2006). In bovine mammary tissue, it is unknown which cell populations express these two receptors and thus which cell types can respond to IL-8 and its functionally similar family members.

These receptors have been studied in a variety of species. The bovine CXCR1 and CXCR2 genes have significant amounts of synergy with the corresponding genes in other species including humans and mice (Grosse *et al.* 1999; Lahouassa *et al.* 2008; Pighetti and Rambeaud 2006; Sonstegard *et al.* 1997). Within the human CXCR1 gene, several SNPs have been identified and some have been associated with susceptibility to inflammatory diseases (Kato *et al.* 2000; Renzoni *et al.* 2000). A goal of the cattle industries is to improve general herd health using genetic selection processes. In efforts to identify potential marker candidates, studies hunted for SNPs within immune related genes, including the genes for CXCR1 and CXCR2. A study by Grosse *et al.* (1999) identified four key SNPs in the CXCR1 gene. These SNPs were originally reported in CXCR2 but, close homology between the sequences of the bovine CXCR1 and CXCR2 receptors caused confusion with the original annotation within public databases (Lahouassa *et al.* 2008; Pighetti and Rambeaud 2006). The majority of papers concerning bovine CXCR1 that were published prior to the discovery of the incorrect annotation in 2006 refer to CXCR2 when they are actually CXCR1. Further examination of these SNPs revealed their presence within both Angus and Herford breeds as well as their crossbreeds (Heaton *et al.* 2001). Upon examining these same SNP profiles in Jersey and Holstein dairy cattle breeds, all four SNPs were observed and a fifth SNP was identified (Youngerman *et al.* 2004a). Leyva-Baca *et al.* (2008) also looked at CXCR1 SNPs (c.-20-1648, c.-20-1586, c.+291, and c.+735) and the relationship to mastitis in Holstein dairy cows. Of these identified SNPs, only c.-20-1586 showed an association with high somatic cell scores (SCS) and was limited to the first and second lactations (Leyva-Baca *et al.* 2008).

Our lab further examined the CXCR1 gene in dairy cattle and determined an association between mastitis and certain SNPs in the bovine CXCR1 gene. Originally, five SNPs were identified: +612, +684, +777, +858, and +861 (Youngerman *et al.* 2004b). However, all were synonymous substitutions except +777, which induced a glutamine to histidine change (Youngerman *et al.* 2004b). Subsequent studies discovered the +777 SNP was linked to increased subclinical and clinical cases of mastitis (Youngerman *et al.* 2004a). These initial studies led to a more comprehensive examination of polymorphisms in the CXCR1 gene and the naming conventions changed to reflect the Human Genome Variation Society Guidelines (Table 8). The study discovered a total of 11 polymorphisms within the coding region: +291, +365, +570, +621, +735 (formerly +777), +816 (formerly +858), +819 (formerly +861), +980, +995, +1008, and +1068 (Pighetti *et al.* 2012). Of these, six are synonymous, four (+365, +735, +980, +995) induced amino acid changes and one (+621) induced an early stop codon (Pighetti *et al.* 2012). The +365 SNP is located on the first intracellular loop and has close proximity to the G-protein coupled receptor that allows for the signal cascade (Leong *et al.* 1994). Similarly, the +735 SNP is located on the third extracellular loop and has been linked to a variety of downstream effects of IL-8 binding that have been studied by our lab (Rambeaud *et al.* 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005; Youngerman *et al.* 2004a).

Table 8: A partial list of bovine CXCR1 SNPs indicating current and previous naming conventions (Pighetti *et al.* 2012).

Standardized SNP Position[a]	**SNP Position in Prior Publications**
c.-20-1648	-1830
c.-20-1586	-1768
c.+291	+344
c.+365	
c.+621	
c.+735	+777
c.+816	+858
c.+819	+861
c.+980	
c.+995	

[a] Based on Human Genome Variation Society Guidelines in reference to the cDNA and the position relative to the transcription start site within the genome.

The +735 SNP involves a G to C switch on the gene that results in a glutamine to histidine change in the amino acid structure of the CXCR1 receptor. Cows with the CC genotype have displayed increased cases of subclinical mastitis (Youngerman *et al.* 2004a), decreased calcium signaling upon IL-8 binding (Rambeaud and Pighetti 2007), decreased neutrophil migration (Rambeaud and Pighetti 2005), ROS generation from neutrophils to aid in bactericidal activities (Rambeaud *et al.* 2006) and increased neutrophil survival (Rambeaud *et al.* 2006). Other studies have demonstrated only a tendency for the +735 SNP to be associated with SCS that are typically seen in conjunction with incidences of subclinical mastitis (Beecher *et al.* 2010; Goertz *et al.* 2009; Leyva-Baca *et al.* 2008). These differences are most likely due to sample size, since the Youngerman (2004a) study had a small sample size (n=88) and the Beecher (2010), Goertz (2009), and Leyva-Baca (2008) studies had large sample sizes (n=246, n=749, n=338 respectively). We hypothesize that cows with the CC genotype at CXCR1 c.+735 are at a disadvantage for fighting off infections due to the impaired immune functions. Cows that have the GG genotype displayed normal levels of neutrophil survival, migration, ROS generation and internal calcium signaling, and have been associated with a lower instance of subclinical mastitis (Youngerman *et al.* 2004a). Those cows expressing the heterozygous genotype (GC) display a mixture of these responses and thus have functions that lie between normal and impaired levels of ability (Rambeaud *et al.* 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005; Youngerman *et al.* 2004a).

SNPs +980 and +995 occur close to the C-terminus of the receptor (Pighetti *et al.* 2012). The +980 position has been shown to influence ligand binding, migration, as

well receptor internalization of the CXCR2 receptor (Raman *et al.* 2010). This information is relevant due to the fact that the C-termini of the bovine CXCR1 and CXCR2 receptors are virtually identical (Lahouassa *et al.* 2008). Lastly, the +621 SNP that induces an early stop codon is located within the second intracellular loop of the receptor. Regardless of whether this switch causes partial or no receptor expression, the +621 SNP has potential to influence function of the CXCR1 receptor.

Individual tagSNPs were combined to form amino acid based haplotypes (Table 9) (Pighetti *et al.* 2012). A tagSNP represents a group of SNPs in high linkage disequilibrium or non-random allele association, increasing the probability that SNPs will be inherited together. Three amino acid haplotypes (VWHKH, VWHRR, AWQRR) represented more than 99% of the sample bovine population (n=88) (Pighetti *et al.* 2012). Knowledge of the potential effects of each SNP mutation allows for the conclusion that VWHRR homozygous cows may be impaired in their abilities to effectively respond to bound ligand compared to the other haplotype combinations. The existence of three dominant haplotypes within the sample population suggests looking at haplotypes instead of individual SNPs within the receptor's gene may provide a more comprehensive understanding of the cow's potential to fight off mastitis. Haplotype selection may also streamline efforts for genetic selection by increasing the number of identifying SNPs could allow for more specific selection of cows with a better genetic background relative to CXCR1.

Table 9: Relationships among CXCR1 c.+735 allele, tagSNP haplotype, and amino acid haplotype (Pighetti *et al.* 2012).

+735 Allele (Former +777)	**tagSNP Haplotype**	**Nucleotide Haplotype**	**Amino Acid Haplotype**
735	+621+735+816+980	365-621-735-980-995	122-206-245-327-332
C	GCCA	TGCAA	VWHKH
G	AGCG	CAGGG	AX
C	GCAG	TGCGG	VWHRR
G	GGCG	CGGGG	AWQRR

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Chapter III: Interleukin-8 receptor expression in bovine mammary tissue.

Interleukin-8 receptor expression in bovine mammary tissue.

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Abstract

Mastitis is an ongoing issue in the dairy industry, which often results from a variety of immune related responses to bacteria in the mammary gland. Many immune responses are mediated by interleukin-8 (IL-8) and its receptors CXCR1 and CXCR2. Exposure of the mammary gland to bacteria causes the release of IL-8. Subsequent binding of IL-8 to its receptors induces immune responses related to host cell survival, migration and chemokine/cytokine production. These receptors are typically regarded as being present on immune cells with an emphasis on neutrophils. Within the bovine mammary gland we hypothesize CXCR1 expression on immune cells and epithelial cells and CXCR2 expression on immune cells, epithelial cells, and endothelial cells based on research conducted in other species. To evaluate CXCR1 and CXCR2 expression in bovine mammary gland we used dual immunofluorescence on mammary tissue sections from 6 Holstein dairy cows in early (5-6d days in milk (DIM)), mid (59-99d DIM), and late (>250d DIM) lactation. Results demonstrate CXCR1 and CXCR2 have different patterns of expression. We discovered both CXCR1 and CXCR2 expressed natively in the bovine mammary gland. CXCR1 is expressed by epithelial cells, some fibroblasts and 10-50% of leukocytes, whereas CXCR2 is present on epithelial cells and 5-30% of leukocytes. These results indicate that multiple cell types in the mammary gland express receptors for IL-8 and are capable of responding to IL-8 released in the environment. Greater knowledge of how these cell populations respond

to IL-8 binding its receptors within the mammary gland could create novel treatment targets for mastitis.

Introduction

Mastitis costs the dairy industry billions of dollars in lost profits annually (NMC 2005). Mastitis is typically caused by bacteria entering the mammary gland. Once within the gland, bacteria encounter a variety of cell types, mainly epithelial cells because they comprise about 70% of mammary gland parenchyma (Capuco *et al.* 1997). These cells can initiate the immune response when bacterial components such as lipopolysaccharide (LPS) bind pathogen recognition receptors such as TLR4 on the cell surface of encountered cells. Upon TLR4 engagement many proinflammatory cytokines are released including TNF-α and IL-1 (Beutler 2004; Takeuchi *et al.* 2000). Interaction of TNF-α and IL-1 with their specific receptors causes the subsequent release of a second round of immune mediators, which can include ELR^+ chemokines (Boudjellab *et al.* 2000; Fitzgerald *et al.* 2007).

The ELR^+ chemokines include GRO-α (CXCL1), GRO-β (CXCL2), GRO-γ (CXCL3), ENA-78 (CXCL5), GCP-1 (CXCL6), NAP-2 (CXCL7) and IL-8 (CXCL8) (Grob *et al.* 1990; Lahouassa *et al.* 2007; Rainard *et al.* 2008; Walz *et al.* 1991; Walz *et al.* 1989). The ELR^+ chemokines initiate several immune functions necessary for combating infection. This includes chemotaxis of neutrophils to the site of infection, increased expression of adhesion molecules on endothelial cells, neutrophil respiratory burst, and neutrophil shape change, which is needed for extravasation from blood into tissues (Allmann-Iselin *et al.* 1994; Baggiolini and Clark-Lewis 1992; Geiser *et al.* 1993;

Grob *et al.* 1990; Lahouassa *et al.* 2008; Loetscher *et al.* 1994). ELR$^+$ chemokines also mediate host cell survival allowing cells to live longer and fight off infections by decreasing spontaneous apoptosis (Kettritz *et al.* 1998). Lastly, chemokine/cytokine profiles are altered by ELR$^+$ chemokines. This aids in perpetuating the inflammatory state and the continued increase in neutrophil chemotaxis and survival (Soehnlein *et al.* 2008).

The ELR$^+$ chemokines bind two specific receptors, CXCR1 and CXCR2. The CXCR1 receptor binds both IL-8 and GCP-1 with high affinity, but GCP-1 is rarely observed *in vivo* (Ahuja and Murphy 1996). The CXCR2 receptor binds all ELR$^+$ chemokines mentioned in the prior paragraph, but has the highest affinity for IL-8 (Ahuja and Murphy 1996; Lee *et al.* 1992). These two receptors are expressed on neutrophils but the expression of these receptors on other cell types is less well studied (Ahuja and Murphy 1996; Grob *et al.* 1990; Lee *et al.* 1992; Li and Gordon 2001; Paape *et al.* 2002; Sprenger *et al.* 1994). In other species CXCR1 expression has been demonstrated on endothelial cells, mammary stem cells, dendritic cells, natural killer cells, CD4+ T-cells, CD8+ T-cells, and eosinophils. CXCR2 has more limited expression including endothelial cells, epithelial cells, and eosinophils (Ben-Baruch 2003; Berahovich *et al.* 2006; Gasser *et al.* 2006; Park *et al.* 2010; Petering *et al.* 1999; Sallusto *et al.* 1998; Takata *et al.* 2004). Furthermore, receptor expression has been tied to a variety of cancers (i.e.: malignant melanomas, prostate cancer, and breast cancer) where they contribute to prolonged cell survival and metastasizing (Ben-Baruch 2003; Gabellini *et al.* 2009; Shamaladevi *et al.* 2009).

In the bovine mammary gland, little is known about CXCR1 and CXCR2 expression. In this study we evaluated CXCR1 and CXCR2 expression in lactating bovine mammary tissue through the use of dual immunofluorescence with antibodies specific to bovine CXCR1, CXCR2 and cell type markers.

Materials and Methods

Animal and Tissue Selection

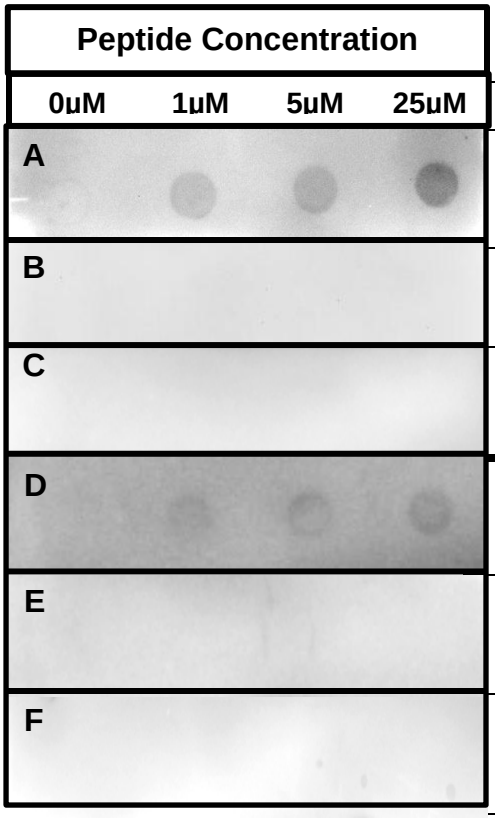
Tissue samples were obtained from n=6 Holstein dairy cows, two in early lactation (5-6d days DIM), two in mid lactation (59-99d DIM), and two in late lactation (248-337d DIM). From each cow, tissue samples were taken from a healthy quarter and a quarter infected with *Streptococcus uberis*, a common mastitis causing pathogen. Early lactation cow samples were obtained from cows sacrificed as part of a USDA-NIFA (2011-67015-30168) funded challenge study at East Tennessee Research and Education Center (ETREC; Knoxville, TN) and the mid and late lactation samples were obtained from our collaborator Dr. John Lippolis at the USDA-NADC (Ames, IA).

Verification of Antibody Specificity

Dot Blot to Evaluate Specificity of CXCR1 and CXCR2 to Peptides

Antibodies were developed in rabbits against specific amino acid peptides of the N-termini of bovine CXCR1 (PTEDYDYSPCEISTET) and CXCR2 (MAETKFTSNIEGFC) which were conjugated to KLH to improve immunogenicity (21st Century Biochemicals, Inc., Marlboro, MA). These specific amino acid segments were synthesized (Peptide 2.0, Inc., Chantilly, VA and 21st Century Biochemicals, Inc., Marlboro, MA) and used to

verify antibody specificity between CXCR1 and CXCR2 on a PVDF membrane using a dot blot assay. From initial peptide stocks of 1mM, peptides were diluted 1:40 (25µM), 1:200 (5µM), and 1:1000 (1µM) in PBS and applied to the PVDF membrane using a vacuum. A control (no peptide) also was included. The membranes then were blocked using PBS + 1% non-fat dry milk + 0.1% Tween-20 for 20 min at room temperature. After a series of two washes in PBS, the desired primary antibody: rabbit anti-bovine CXCR1, CXCR2, or pre-immunization sera (1:1000) was applied to the membrane and allowed to incubate at room temperature for 30 min with shaking. The membrane was washed twice in PBST [PBS + 0.1% Tween-20] and once in PBS to remove excess primary antibody. Next, stabilized goat anti-rabbit HRP-conjugate secondary antibody (Pierce 34095, Rockford, IL; 1:5000) was incubated with the membrane for 30 min at room temperature with shaking. Lastly, after another two washes in PBST and one in PBS, the membrane was developed with a 1:1 solution of peroxide buffer and luminol/enhancer solution (Pierce 34095, Rockford, IL). Pictures were taken using Alpha Innotech Fluorchem imaging equipment and software. Various combinations of CXCR1 and CXCR2 antibodies and peptides were tested together using this method to ensure that cross-binding was not observed. Results indicate that cross-binding does not occur between antibody and pre-bleed combinations, CXCR1 antibody and CXCR2 peptide, or between CXCR2 antibody and CXCR1 peptide suggesting antibody specificity (Figure 4).



Peptide	Antibody	Expected Result
CXCR1	CXCR1	+
CXCR1	CXCR2	-
CXCR1	CXCR1 pre-bleed	-
CXCR2	CXCR2	+
CXCR2	CXCR1	-
CXCR2	CXCR2 pre-bleed	-

Figure 4: Dot blots indicating bovine CXCR1 and CXCR2 antibody specificity. (A) Specific CXCR1 antibody binds CXCR1 peptide with increasing concentrations of peptide. (B) CXCR2 antibody does not bind CXCR1 peptide. (C) CXCR1 peptide does not interact with CXCR1 pre-bleed sera. (D) Specific CXCR2 antibody binds CXCR2 peptide with increasing concentrations of peptide. (E) CXCR1 antibody does not bind CXCR2 peptide. (F) CXCR2 peptide does not interact with CXCR2 pre-bleed sera.

Bovine Mammary Gland Protein Lysate Preparation

Approximately 100mg of frozen tissue was lysed in modified RIPA buffer (50mM TrisHCl pH 7.4, 150mM NaCl, 0.1% TritonX100, 5mM EDTA supplemented with DTT (1mM) and inhibitors (Roche, 04693159001, Indianapolis, IN) by use of a rotor-stator homogenizer (3 bursts of 10 s) followed by sonication (3 bursts of 10 s) on ice. Resulting lysates were cleared by centrifugation at 15,000g for 3 min at 4°C and stored at -80^{o}C. Lysates from mouse liver and mammary tissue of all six cows were prepared. For each lysate, concentration was determined using a BCA Protein Assay (Pierce, Rockford, IL). (Performed by Leszek Wojakiewicz)

Western Blot

Twelve percent SDS-PAGE gels with electrophoretically resolved protein lysates (50µg per lane) were blotted onto PVDF membrane using Trans-Blot SD (Bio-Rad, Hercules, CA). MagicMark (Bio-Rad, Hercules, CA) was used as the molecular weight marker. Semi-dry blotting was performed at constant current of 60mA for 1 h in CAPS (for catode 1X CAPS + 1%SDS; for anode 1X CAPS +15% methanol) buffer system. Detection of both CXCR1 and CXCR2 was carried out using custom affinity purified polyclonal rabbit antibodies (IgG) raised against specific peptides described earlier (21st Century Biochemicals) with original concentrations of 0.019mM for anti-CXCR1 and 0.0098mM for anti-CXCR2. Prior to probing, blots were pre-blocked overnight at 4^{o}C in TBST buffer supplemented with 10% casein blocking reagent (Roche, 11921673001, Indianapolis, IN). Next they were incubated in TBST buffer with 5% casein blocking reagent and primary antibody at 1:200 dilution at room temperature on a rocking

platform, then washed three times for 15 min with cold TBST at room temperature on a rocking platform. This was followed by detection of primary antibody using a Femto detection kit (Pierce, 34095, Rockford, IL). Blots were incubated with goat anti-rabbit antibody conjugated to HRP (TBST, 5% casein blocking reagent, antibody at 1:10,000) for 45 min at room temperature on a rocking platform, washed three times for 15 min in cold TBST and incubated in 1mL of combined HRP substrate and enhancer for 1 min. Freshly air-dried blots were imaged on Alpha Innotech Fluorchem imaging equipment and software with exposure times ranging from 1-20 min. For experiments using primary antibodies pre-blocked with specific peptides, solutions containing those antibodies supplemented with 20µM specific peptide or equal volume of water, but without casein blocking reagent were pre-incubated 30 min at room temperature on a nutator, then supplemented with blocking reagent and used in protocol outlined above. (Performed by Leszek Wojakiewicz)

Preparation of Tissue Section Slides

Using plastic molds, approximately 1cm by 1cm pieces of mammary tissue stored at -80°C were embedded in optimal cutting temperature compound (OCT) using liquid nitrogen to the solidify mold. The tissue containing molds then were sliced to 6µm thick using a cryostat and placed on frost plus slides (Fisher 12-550-18, Pittsburg, PA). Slides were immediately fixed in -20°C acetone for 5 min and allowed to air dry. Slides were stored frozen at -80°C until time of staining.

Dual Immunofluorescence Staining

Slides were first brought to room temperature for 30 min, then fixed in -20°C acetone for 5 min, washed twice in PBST and then blocked using a sera blocking solution (PBS + 5% goat serum (Thermo Scientific, Rockford, IL) + 5% horse serum (Oxoid, Basingstoke, NH)) for 20 min with shaking. Next, slides were washed once in PBST and then incubated with the primary antibody combination (Table 10) overnight (15-18 hours) at 4°C with shaking in a mini-humidifier. Slides that received pre-immunization sera or no primary antibody also were included to serve as negative controls. Following overnight incubation, slides were washed three times in PBST and then incubated with secondary antibody combinations (Table 10) for 1 hour at room temperature, in the dark, with shaking in a mini-humidifier. Again, slides were washed three times in PBST and then counterstained for 5 min with 4,6-diamidino-2-phenylindole (DAPI) (300nM) (Invitrogen D3571, Grand Island, NY) at room temperature and washed twice more in PBST. A drop of mounting media (Vector H-1000, Burlingame, CA) was applied and a coverslip added followed by clear nail polish to seal the slide. Microscopy was performed using a Leica SP2 laser scanning confocal microscope and corresponding software. Images were taken of one area representative of the entire tissue section. Percentages of positive staining cells were determined by visual count of cells present within each image and averaged across all cows.

Table 10: Primary and secondary antibodies used for dual immunofluorescence.

Name	Cell Type	Source	Isotype	Species	Dilution
CD45 (CACTB51A)	Leukocytes	VMRD, Inc. 0491-0506	IgG_{2a}	Murine	1:500
Connexin-43 (2/Connexin-43)	Myoepithelial cells	BD Transduction Laboratories 610062	IgG_1	Murine	1:500
Pan-Cytokeratin (AE1/AE3)	Epithelial cells	Santa Cruz Biotechnology SC-81714	IgG_1	Murine	1:30
Vimentin (Vim3B4)	Fibroblasts	Dako M7020	IgG_{2a}	Murine	1:100
CXCR1	--	21st Century Biochemicals	Sera	Rabbit	1:1000
CXCR2	--	21st Century Biochemicals	Sera	Rabbit	1:1000
DyLight 549 Goat-anti-rabbit IgG (H+L) (GAR)	--	Vector DI-1549	IgG	Goat	1:1000
DyLight 488 Horse-anti-mouse IgG (H+L) (HAM)	--	Vector DI-2488	IgG	Horse	1:1000

Results

Western blots of mammary whole cell lysates from each cow were used to determine the specificity of bovine CXCR1 and CXCR2 antibodies. The CXCR1 blot displayed multiple positive staining bands in both bovine mammary gland and mouse liver (Figure 5 A). However, a band between 50 and 60kDa was present for bovine mammary gland but absent for mouse liver indicating CXCR1 specificity for this band in bovine mammary gland. The specific CXCR1 band between 50 and 60kDa was present across all cows with some variation in level of expression based on band density (Figure 5 B). The CXCR2 blot contained multiple bands but one band at approximately 60kDa was more dominant and observed for both bovine mammary gland and mouse liver lysates (Figure 6 A). Blocking of antibodies with CXCR2 peptide decreased or eliminated this band indicating this band was specific and cross-reacted with mouse CXCR2. The dominant CXCR2 band was present across all cows (Figure 6 B).

CXCR1 is expressed on mammary epithelial cells (Figure 7 C, D). CXCR1 staining (red) is concentrated basally on alveolar epithelial cells, and epithelial staining (green) is concentrated apically on alveolar epithelial cells. Dual expression is observed on alveolar epithelial cells from both uninfected and infected tissues (Figure 7 C, D). Myoepithelial cells, often observed as green punctate cells, did not express CXCR1 as indicated by Figure 7 E, F. CXCR1 is expressed on some fibroblasts in both infected and uninfected tissues (Figure 7 G, H), where dual expression is observed throughout the stromal regions between alveolar structures. Fibroblast expression of CXCR1 was not consistent across cows or infection status with 2/6 cows expressing CXCR1 on

fibroblasts in uninfected quarters and 4/6 cows expressing CXCR1 on fibroblasts in infected quarters (Table 11). Mammary leukocytes express CXCR1 with approximately 10-50% of leukocytes showing dual expression (Figure 7 I, J).

CXCR2 also is expressed on mammary epithelial cells (Figure 8 C, D). CXCR2 (red) is observed surrounding alveolar epithelial cells and concentrated to bright edges (Figure 8 C, D; Figure 9). The bright edges sometimes represent one alveolar epithelial cell and at other times represent a string of adjoining alveolar epithelial cells (Figure 9). The epithelial marker (green) demonstrates the same staining pattern. Overlaid images representing both markers display yellow staining indicating dual expression (Figure 8 C, D). Neither myoepithelial cells (Figure 8 E, F) nor fibroblasts (Figure 8 G, H) display CXCR2 in uninfected or infected gland tissue. Leukocytes express CXCR2 in both uninfected and infected quarters with approximately 5-30% of leukocytes demonstrating dual expression (Figure 8 I, J).

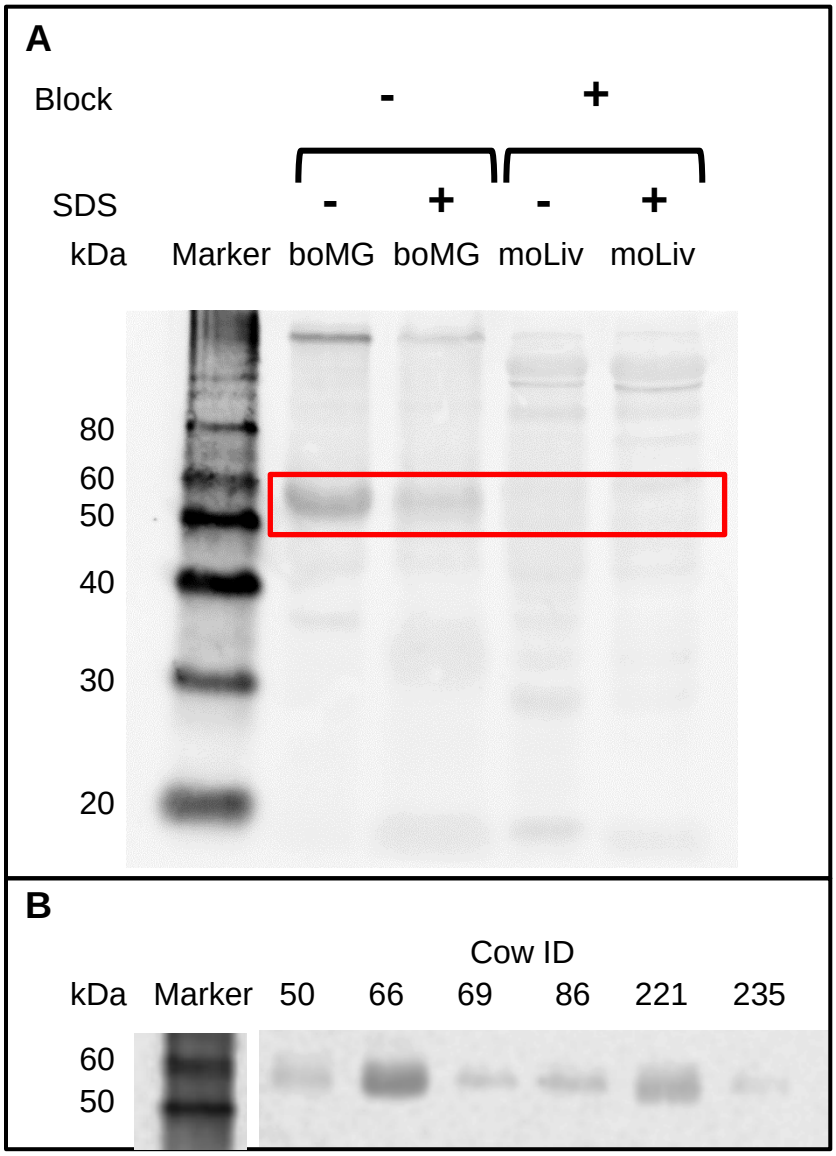


Figure 5: CXCR1 western blots results demonstrating specific binding of the bovine CXCR1 antibody for band between 50 and 60kDa. (A) Depicts duplicate lanes of bovine mammary whole cell lysate (boMG) or mouse liver whole cell lysate (moLiv). One boMG and one moLiv lysate was suspended in buffer with 1% SDS to ensure complete membrane breakdown and to optimized receptor binding. The boxed band between 50 and 60kDa present in boMG is absent from moLiv indicating specificity of CXCR1 for this band only in bovines. (B) Demonstrates band of interest between 50 and 60kDa is present in mammary whole cell lysates from each of the six cows used with some variation in level of expression based on band density.

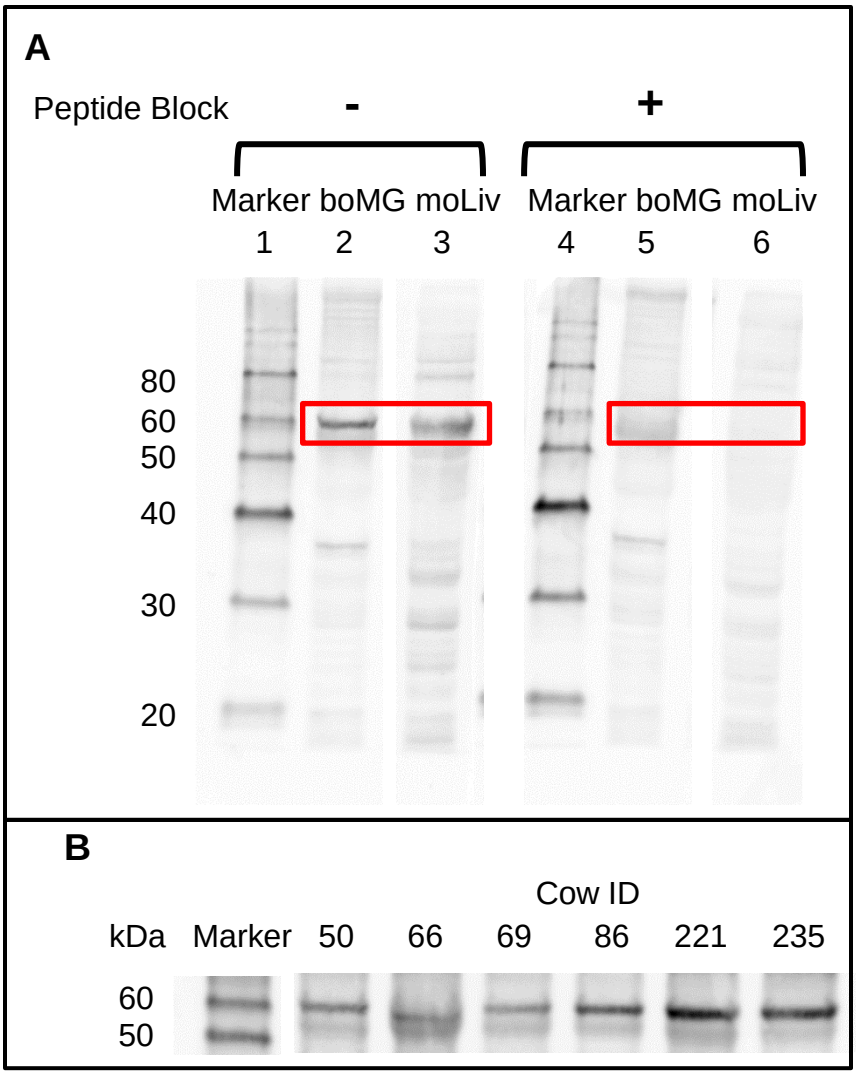


Figure 6: CXCR2 western blots results demonstrating specific binding of the bovine CXCR2 antibody for band at approximately 60kDa. (A) Depicts blots with lanes containing marker, bovine mammary whole cell lysate (boMG), or mouse liver whole cell lysate (moLiv). The boxed bands at approximately 60kDa in lanes 2 and 3 are removed via specific peptide blocking of antibody in lanes 5 and 6 and shows affinity for both bovine and mouse CXCR2. (B) Demonstrates band of interest at approximately 60kDa is present in mammary whole cell lysates from each of the six cows used.

Figure 7: CXCR1 expression in bovine mammary gland. Red corresponds to CXCR1 expression and green corresponds to cell specific markers. Yellow indicates dual expression of both red and green markers and is indicated by arrow heads (◄). Negative controls receiving only secondary antibody, (A) GAR and (B) HAM, demonstrated no staining. CXCR1 was expressed on alveolar epithelial cells in both (C) uninfected quarters and (D) infected quarters. CXCR1 was not observed on myoepithelial cells in (E) uninfected or (F) infected quarters. CXCR1 was observed on some fibroblasts in both (G) uninfected quarters and (H) infected quarters. Expression of CXCR1 was observed on leukocytes in both (I) uninfected quarters and (J) infected quarters.

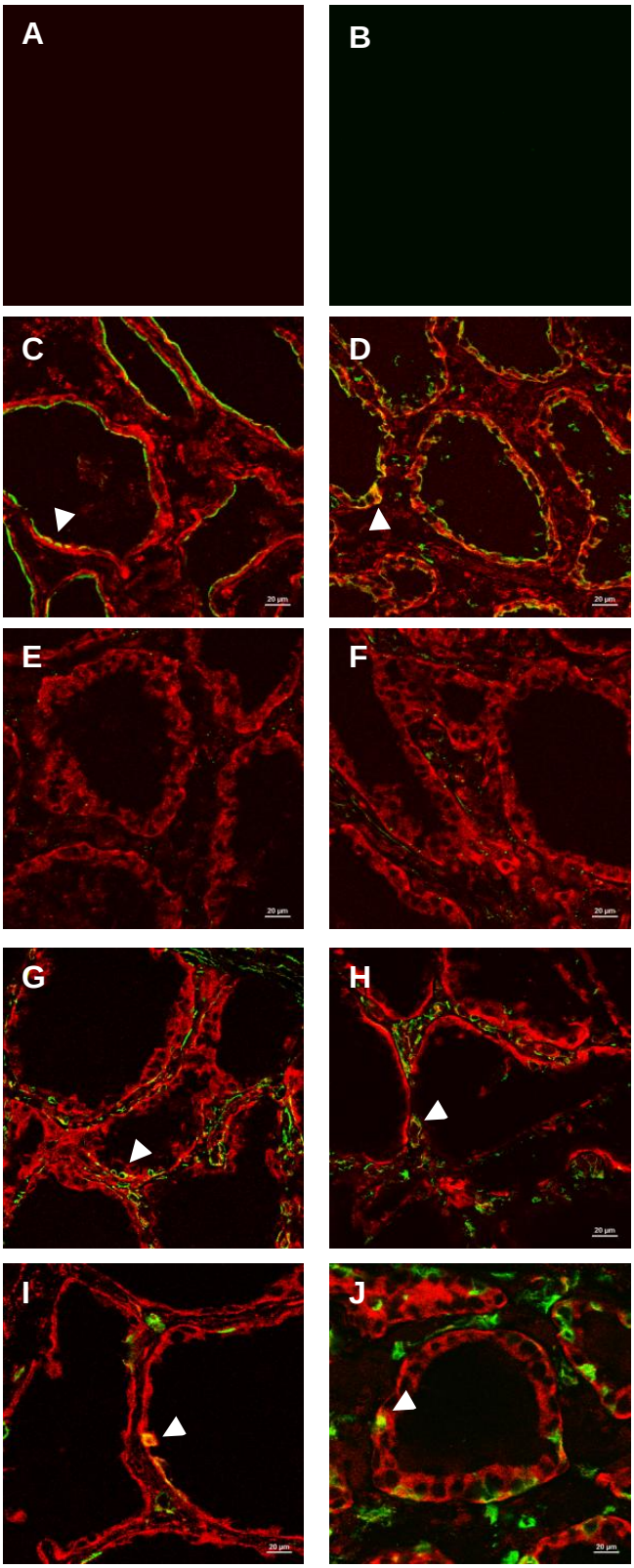


Figure 7: Continued

Table 11: Presence of CXCR1+ and CXCR2+ staining in specific cell types in lactating bovine mammary tissue.

IL-8 Receptor	Infection Status	Cell Type			
		Alveolar Epithelial Cells[1]	Myoepithelial Cells[1]	Fibroblasts[1]	Leukocytes (CD45) [1]
CXCR1	Uninfected	6/6	0/6	2/6	6/6
	Infected	6/6	0/6	4/6	6/6
CXCR2	Uninfected	6/6	0/6	0/6	6/6
	Infected	6/6	0/6	0/6	6/6

[1] Represents the number of cows that displayed positive staining for each cell type/receptor combination.

Figure 8: CXCR2 expression in mammary gland. Red corresponds to CXCR2 expression and green corresponds to cell specific markers. Yellow indicates dual expression of both red and green markers and is indicated by arrow heads (◀). Negative controls receiving only secondary antibody, (A) GAR and (B) HAM, demonstrated no staining. CXCR2 was expressed on alveolar epithelial cells in both (C) uninfected quarters and (D) infected quarters. CXCR2 was not observed on (E, F) myoepithelial cells or (G, H) fibroblasts in uninfected or infected quarters. Expression of CXCR2 was observed on leukocytes in both (I) uninfected quarters and (J) infected quarters.

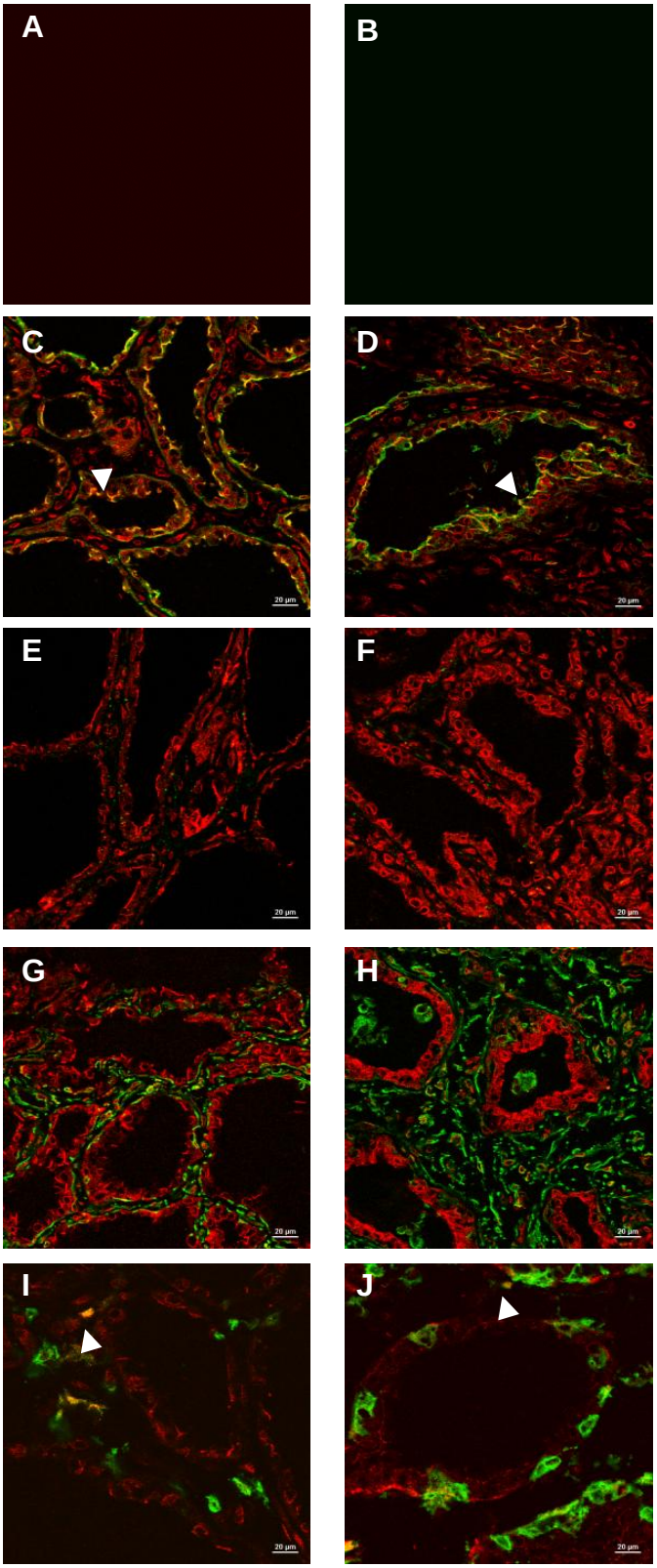


Figure 8: Continued

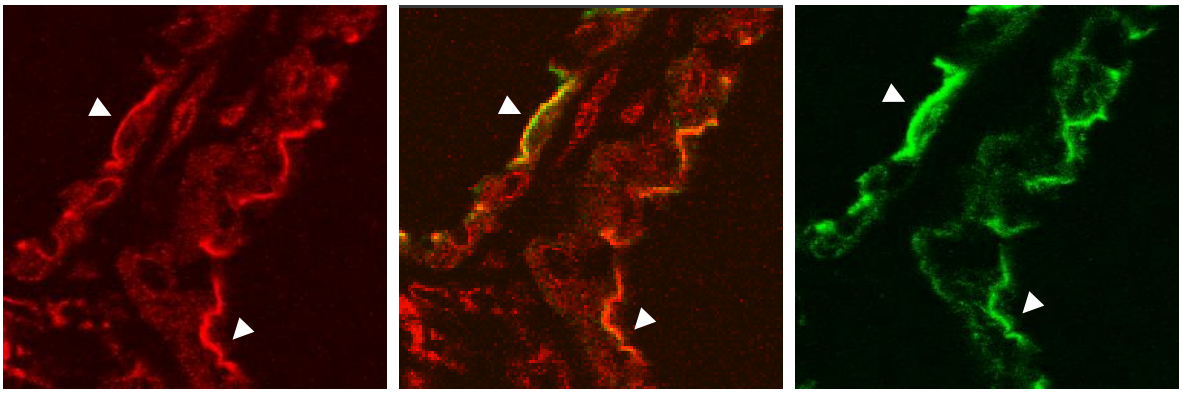


Figure 9: CXCR2 bright edge staining pattern on epithelial cells. Bright edges (◄) are observed in red CXCR2 stain (left), in green epithelial marker (right) and in composite, yellow images (middle). This pattern was consistent across all six cows and in both uninfected and infected glands.

Discussion

The ligands for CXCR1 and CXCR2 are present in the bovine mammary gland during both normal and disease states (Rainard et al. 2008). However, we do not know the ability of native bovine mammary gland cell populations to respond to these ligands. The objective of this study was to determine which cell populations can respond to these ligands by evaluating the expression of CXCR1 and CXCR2 on the native cell populations in the bovine mammary gland. Our results demonstrate alveolar epithelial cells express CXCR1 and CXCR2 in bovine mammary tissue. Expression of CXCR1 has not been demonstrated on these cells previously, but has been identified on human mammary stem cells(Ben-Baruch 2003). This suggests the potential for CXCR1 expression on differentiated mammary epithelial cells. CXCR2 expression previously has been demonstrated on lung alveolar epithelial cells in mice (Wislez *et al.* 2006). CXCR1 and CXCR2 also have been witnessed on mammary epithelial tumor cells (Miller *et al.* 1998). The expression of these receptors on these various tissue types allows them to respond to ligands released during and infection state.

The observation of CXCR1 and CXCR2 expression on bovine mammary epithelial cells suggests these cells are capable of responding to ligands in the mammary gland environment. For example, when bacteria enter the mammary gland, epithelial cells are typically the first cell type encountered and activate TLRs that initiate the immune response causing the subsequent release of inflammatory mediators, TNF-α, lL-1, IL-8,GRO-α, β, γ, ENA-78 and NAP-2 (Boudjellab *et al.* 1998; Boudjellab *et al.* 2000; Fitzgerald *et al.* 2007; Lahouassa *et al.* 2007; Rainard *et al.* 2008; Walz *et al.*

1991; Walz *et al.* 1989). Binding of IL-8 to CXCR2 has been associated with the ability of mammary epithelial cell tumors to invade neighboring tissues and metastasize suggesting a role of this receptor in epithelial cell migration (Nannuru *et al.* 2011). Furthermore, metastasized tumors release higher levels of IL-8 then primary tumors further supporting that the ligand/receptor complex has a strong role in metastasis or migration of mammary epithelial cells (De Larco *et al.* 2001). These data suggest the role of alveolar epithelial cells expressing CXCR1 and CXCR2 may be associated with increased survival and/or cell migration.

Other evidence linking CXCR1 and CXCR2 expression on alveolar epithelial cells to migration was demonstrated by direct infusion of IL-8 into the bovine mammary gland. Direct injection of IL-8 into the gland caused a subsequent increase in serum proteins within the milk (Watanabe *et al.* 2008). Serum proteins in the milk suggests IL-8 in the gland alters the blood-milk barrier (Watanabe *et al.* 2008). The infection state further alters the blood-milk barrier. The breakdown of tight junctions, which are typically impermeable during lactation to separate milk from tissue, become leaky in response to TNF and interferon (IFN)-γ that are released in response to infection (Madara and Stafford 1989). Degradation of the tight junctions frees epithelial cells from their alveolar structure potentially allowing them to respond to released IL-8 by migrating. Given these pieces of evidence, future *in vitro* studies are needed in order to definitively conclude how bovine mammary epithelial cells respond to CXCR1 and CXCR2 binding ligand.

Basally concentrated CXCR1 (Figure 7 C, D) and apically concentrated CXCR2 (Figure 9) expression could be linked to ligand availability. GRO-γ is the predominant ELR^+ chemokine present in normal milk and is a ligand for only CXCR2 which we witnessed to be apically concentrated (Rainard *et al.* 2008). IL-8 has very low concentrations in the milk during an uninfected state (Rainard *et al.* 2008), suggesting basal secreted levels of IL-8 may be more concentrated in the parenchyma. The predominant ligand for CXCR1 is IL-8 and CXCR1 is which we observed to be basally concentrated. However, further studies focused on patterns of receptor expression on mammary epithelial cells could definitively decipher this relationship.

As previously stated, CXCR1 demonstrates apical concentration and CXCR2 demonstrates bright edge concentration. The pan-cytokeratin marker used to identify epithelial cells typically demonstrates luminal and bright edge concentration, thus co-expression is observed with CXCR2 (Figure 9) but not CXCR1 (Figure 7 C, D). The co-expression of the keratins with CXCR2 appears to be coincidental. Epithelial cell keratins are often concentrated to luminal edges in secretory structures or structures subjected to fluid pressures such as ducts and lumens (Schmid *et al.* 1983; Sun *et al.* 1979). It is unclear what the role of these luminal and bright edge concentrated keratins might play in the mammary gland and why CXCR1 does not co-express with them and CXCR2 does.

Some mammary fibroblasts express CXCR1 (Figure 7 G, H) but not CXCR2 (Figure 8 G, H). Fibroblasts' traditional role in inflammation has been to repair and restructure connective tissue (Singer and Clark 1999). In releasing immune related

peptides and cytokines, including IL-8, they can initiate the immune response to invading pathogens or inflammatory cytokines (Kandasamy *et al.* 2011; Strieter *et al.* 1989). The expression of CXCR1 on mammary fibroblasts most likely serves the purpose of increasing migration to the site of infection. Contrasting to our results, cultured human fibroblasts express CXCR2 but not CXCR1 suggesting expression on fibroblasts is species and/or tissue type specific (Moser *et al.* 1993). Future studies focused on the role of CXCR1 on mammary fibroblasts are needed to demonstrate the role that this receptor/ligand complex has in mastitis. However, we can speculate fibroblasts respond to IL-8 by migrating due to the constant remodeling within the mammary gland in response to an inflammatory state (Zhao and Lacasse 2008) and the role fibroblasts play in releasing immune peptides and cytokines (Kandasamy *et al.* 2011).

Leukocytes also express CXCR1 and CXCR2 in the bovine mammary gland. Our results show CXCR1 expression by approximately 10-50% of CD45+ leukocytes in the mammary gland and CXCR2 expression on approximately 5-30% of CD45+ leukocytes. As our interest was in overall cell populations that express CXCR1 and CXCR2, we did not evaluate individual leukocyte populations. However, based on prior research in other species and tissues, we predict neutrophils and T-cells are the predominant positive staining populations. These receptors are expressed on neutrophils (Ahuja and Murphy 1996; Grob *et al.* 1990; Lee *et al.* 1992; Li and Gordon 2001; Paape *et al.* 2002; Sprenger *et al.* 1994) and binding to its cognate ligand causes subsequent neutrophil migration to the site of infection and increased survival

(Baggiolini and Clark-Lewis 1992; Grob *et al.* 1990; Kettritz *et al.* 1998; Richardson *et al.* 2003). Several other cell types could be expressing these receptors. CXCR1 expression has been witnessed on human dendritic cells (Sallusto *et al.* 1998), natural killer cells (Berahovich *et al.* 2006), CD4+ T-cells (Gasser *et al.* 2006), CD8+ T-cells (Takata *et al.* 2004), and eosinophils (Petering *et al.* 1999), while CXCR2 expression has been observed on eosinophils (Petering *et al.* 1999) and would presumably influence their migration and survival. Prior studies have determined percentages of different leukocytes present within both uninfected and infected bovine mammary gland. Within a healthy gland, the majority are T-cells and antigen presenting cells (Nickerson and Heald 1982; Shafer-Weaver et al. 1996). Within an infected gland the number of granulocytes, especially neutrophils, increase and become the dominant leukocyte population present (Nickerson and Heald 1982). T-cells and antigen presenting cells have previously demonstrated CXCR1 expression and most likely account for the leukocytes expressing CXCR1 witnessed in healthy quarters. Granulocytes also express CXCR1 and most likely account for the CXCR1 expressing cell types in infected quarters, along with some T-cells and antigen presenting cells. Another large percentage of leukocytes in the mammary gland are B-cells (Shafer-Weaver *et al.* 1996). These cells, do not express CXCR1 and most likely account for the fraction of leukocytes not displaying CXCR1 expression. CXCR2 also was observed on leukocytes in our study. Previously CXCR2 has been witnessed on neutrophils and eosinophils, thus the CXCR2 expressing leukocytes are most likely these two cell types. Future studies examining leukocyte sub-populations will provide greater insight to which leukocytes are expressing CXCR1 and CXCR2 in the bovine mammary gland.

CXCR1 and CXCR2 expression were similar between uninfected and infected states indicating ligand presence determines if the downstream effects are observed. Basal levels of ELR^+ chemokines such as GRO-α, GRO-β, GRO-γ, and lL-8 are released at all times from mammary epithelial cells, but upon exposure to bacterial pathogens the levels increase at least 30-fold (Lahouassa *et al.* 2007; Maheshwari *et al.* 2003; Rainard *et al.* 2008). Secreted basal levels most likely contribute to remodeling of the mammary gland and removal of senescent and damaged cells. Recruitment of neutrophils and migration of local cell populations that play a large role in remodeling during lactation and the dry period (Capuco *et al.* 1997). This remodeling process is critical to maintaining lactation and efficiency of the bovine mammary gland and expression of CXCR1 and CXCR2 receptors could contribute to this process. In other species, CXCR1 and CXCR2 expression is typically linked to either neutrophils or tumor cells (Ben-Baruch 2003). However, unlike bovines, lactation is temporary. The ELR^+ chemokine/CXCR1/CXCR2 axis may facilitate the continual maintenance of the mammary gland required to perpetuate lactation in bovines. To properly confirm this tissue samples from a non-bred heifer, a non-lactating dry cow, and cows at various stages of lactation should be compared.

The results of this study indicate that CXCR1 and CXCR2 are expressed constitutively on bovine mammary leukocytes and alveolar epithelial cells, with CXCR1 expression on some fibroblasts. We can speculate that these receptors may serve to induce cell migration, increasing host cell survival and modifying local chemokine/cytokine profiles based on previous research. These processes are

essential to the routine maintenance and infection defense of the bovine mammary gland. However, further *in vitro* studies will be required to conclusively determine if these receptors allow mammary gland epithelial cells, fibroblasts and leukocytes to migrate, survive, and modify chemokine/cytokine.

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Chapter IV: Genetic variation in CXCR1 haplotypes linked to clearance of *Streptococcus uberis* infection in an experimental challenge model.

Genetic variation in CXCR1 haplotypes linked to clearance of *Streptococcus uberis* infection in an experimental challenge model.

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Abstract

Mastitis, an inflammation of the mammary gland, costs the dairy industry billions in lost profits annually. The prevalence and loss associated with mastitis has made new genetic selection methods a goal of research. Previous research has identified amino acid changes at positions 122, 207, 245, 327, and 332 in the IL-8 receptor CXCR1 that result in three dominant amino acid haplotypes: VWHKH, VWHRR, and AWQRR. We hypothesize different haplotypes will influence a cow's resistance or susceptibility to developing mastitis. To test this, Holstein dairy cows (n=40) were intramammarily challenged with *S. uberis* within three days post calving. All cows developed mastitis based on isolation of *S. uberis* from the challenged quarter at least twice. All cows (100%) with the VWHRR x VWHRR (n=5) and AWQRR x VWHRR (n=6) haplotype combinations required antibiotic treatment to clear infection and were significantly different from cows with a VWHRR x VWHKH (n=6) haplotype where only 33.33% required antibiotic treatment (p=0.0153). Cows with a VWHRR homozygous haplotype displayed significantly higher milk (1.05 ± 0.21) and mammary (1.23 ± 0.17) scores and AWQRR x VWHRR cows had significantly lower milk (0.17 ± 0.18) and mammary (0.41 ± 0.14) scores then cows of all other haplotype combinations (p=0.0263 and p=0.0161 respectively). Homozygotes for the VWHRR haplotype also displayed significantly higher *S. uberis* bacteria counts (981.99 ± 1947.05) then cows of any other haplotype combination (p=0.0348). Haplotype combination did not influence somatic cell counts

(p=0.1399) and milk yield (p=0.6126). This evidence suggests VWHRR x VWHRR and AWQRR x VWHRR cows are highly susceptible but VWHKH x VWHRR cows are less susceptible to *S. uberis* based mastitis. Furthermore, the genetic haplotype of the CXCR1 gene may have the ability to significantly influence the degree of inflammation observed and bacterial clearance without aid of antibiotic treatments, supporting that CXCR1 is critical for disease resistance.

Introduction

Mastitis is an ongoing issue in the dairy industry that costs producers billions in lost profits annually in the forms of lost milk, antibiotic therapies, and extra labor costs (NMC 2005). Improving overall herd quality through the use of genetic selection is a predominant goal in the dairy industry. In keeping with this goal, efforts have been made to identify single nucleotide polymorphisms (SNPs) within immune genes related to phenotypic immune functions to help in genetic selection. One such study by Grosse *et al.* (1999) surveyed a large array of bovine cytokines and cytokine receptors, among them the gene for CXCR1. CXCR1 is the receptor for the immune mediator interleukin-8 (IL-8). This receptor-ligand complex is responsible for several key immune responses including neutrophil migration, increased host cell survival, and the modification of chemokine/cytokine profiles (Grob *et al.* 1990; Kettritz *et al.* 1998; Soehnlein *et al.* 2008).

Four SNPs were identified in the bovine CXCR1 gene, three were synonymous and the fourth caused a glutamine to histidine point mutation at position 245 in the amino acid sequence of CXCR1 (Grosse *et al.* 1999). This SNP was published as

originating in CXCR2, but subsequent studies have revealed an improper annotation indicating the SNP is actually in the bovine CXCR1 gene (Lahouassa *et al.* 2008; Pighetti and Rambeaud 2006). Further examination of the CXCR1 gene revealed the presence of the same four SNPs (+612, +777, +858, and +861) in several beef and dairy breeds and a fifth SNP (+684) in Holstein and Jersey dairy breeds was identified (Heaton *et al.* 2001; Youngerman *et al.* 2004b).

More extensive studies evaluated the association of specific SNPs and haplotypes with mastitis. Youngerman *et al.* (2004b) identified SNPs at +612, +684, +777, +858, and +861 in the CXCR1 gene. Four of the SNPs were synonymous, but the +777 was linked to increased instance of subclinical and clinical mastitis (Youngerman *et al.* 2004a). Leyva-Baca *et al.* (2008) recognized four SNPs associated with the CXCR1 gene: -1830, -1768, +344, and +777. The +777 SNP corresponded to the previously identified amino acid change at position 245, but showed no correlation with increased somatic cell score (SCS), whereas the -1768 did, but only for the first and second lactations (Leyva-Baca *et al.* 2008). A more in depth study of the +777 SNP revealed associations with calcium signaling upon IL-8 binding (Rambeaud and Pighetti 2007), decreased neutrophil migration (Rambeaud and Pighetti 2005), decreased reactive oxygen species (ROS) generation from neutrophils to aid in bactericidal activities (Rambeaud *et al.* 2006), and increased neutrophil survival (Rambeaud *et al.* 2006).

A compilation of SNPs in the CXCR1 gene also resulted in a change in the naming convention of the SNPs to correlate with Human Genome Variation Society

Guidelines (Pighetti *et al.* 2012). A total of 11 polymorphisms were identified within the coding region of the bovine CXCR1 gene. Six SNPs were synonymous, four induced amino acid changes (+365, +735 (formerly +777), +980, and +995), and one (+621) induced an early stop codon (Pighetti et al. 2012). From the SNPs, amino acid haplotypes based on the positions were identified and three (VWHKH, VWHRR, AWQRR) were representative of more than 99% of the sample population (Table 12). The increase in specificity gained by combining individual SNPs could provide a more comprehensive and accurate means of genetic selection (Pighetti *et al.* 2012).

The majority of previous studies on CXCR1 SNPs have relied on field studies (Grosse et al. 1999; Heaton et al. 2001; Leyva-Baca et al. 2008; Pighetti et al. 2012; Youngerman et al. 2004a; Youngerman et al. 2004b). Field studies demonstrate that the CXCR1 SNPs can be linked to natural incidence of clinical mastitis and bacterial intramammary infection. However, studies have yet to determine if direct bacterial challenge can also be tied to CXCR1 SNPs. Based on the existing knowledge of each of the individual SNPs within the bovine CXCR1 gene, we suspect differences in a cow's resistance and susceptibility to mastitis. The goal of this study is evaluate the relationship between CXCR1 haplotype and a cow's susceptibility to and clearance of infection following direct experimental challenge with *Streptococcus uberis*.

Table 12: Relationships between CXCR1 tagSNP haplotype and amino acid haplotype (Pighetti *et al.* 2012).

tagSNP[i] Haplotype	Nucleotide Haplotype	Amino Acid Haplotype
+621+735+816+980	365-621-735-980-995	122-206-245-327-332
GCCA	TGCAA	VWHKH
AGCG	CAGGG	AX
GCAG	TGCGG	VWHRR
GGCG	CGGGG	AWQRR

[i] A tagSNP represents a group of SNPs in high linkage disequilibrium or non-random allele association, increasing the probability that SNPs will be inherited together

Materials and Methods

Animal Selection

This study was conducted using cows that were enrolled in a trial to test the effectiveness of a novel vaccine candidate against *S. uberis*, i.e.: *Streptococcus uberis* adhesion molecule (SUAM) (USDA-NIFA #2011-67015-30168). The forty Holstein dairy cows used were clinically healthy, pregnant, and at the end of their first or second lactation. Upon arrival at the ETREC Little River Dairy, the cows were allowed to acclimate to the facility two weeks prior to the initiation of research protocols. Cows were divided into sets of ten based on expected calving date for ease of sampling and randomly assigned to the test vaccination (SUAM) or control vaccination (PBS) groups. Cows were housed separately from the core herd and cared for in accordance with ETREC Little River Dairy standard protocols. All animal based work was done in accordance with Institutional Care and Use Committee (IACUC) guidelines.

Vaccination

The test vaccine contained 200µg recombinant SUAM that was isolated and purified by Dr. Stephen Oliver's lab. The control vaccine contained PBS instead of SUAM. Both test and control vaccines contained the same adjuvant, Montanide ISA70VG with an adjuvant/antigen ratio of 70/30. Each cow received a series of three vaccinations where the total volume of each dose was 2mL and given on alternate sides of the neck. Vaccinations were administered in four week intervals at -84 days, -56 days, and -28 days prior to expected calving date. Following each administration,

injection sites and temperatures were closely monitored for adverse reactions to the vaccination.

Challenge

Approximately 1 week prior to calving, secretion samples were collected from each quarter to screen for bacterial presence and aid in selecting the challenge quarter. A frozen stock of *Streptococcus uberis* (UT888) was thawed and allowed to grow at 37°C in Todd Hewitt Broth until turbid. The culture then was serially diluted in PBS to approximately 2000 CFU/mL for infusion into the selected challenge quarter. Each cow was challenged within three days after calving with a total volume of 5mL of the dilute *S. uberis* culture just after milking was complete. The challenge inoculum was diluted serially and plated to determine the actual concentration of *S. uberis* delivered.

Sampling and Basis for Antibiotic Treatment

Time points for collection included: immediately before challenge (0 days) and then 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 14, 21, and 28 days post challenge. Milk samples were used to identify the concentration of *S. uberis* present. Milk and mammary quarter scores were evaluated on a 0-3 scale. Milk scores were defined as follows: 0=normal, 1=small flakes/clots, 2=large clots, 3=stringy, off color, watery, or bloody. Mammary scores were defined as follows: 0=normal, 1=slight swelling, 2=moderate swelling, 3=edema. Scores were evaluated by Charlie Young or Mark Lewis of the ETREC Little River Staff. Three consecutive sets of severe scores (a 2 in both milk and mammary scores, or a 3 in either) was considered grounds for antibiotic treatment. Alternatively, if cows had not cleared *S. uberis* ≥ 14 days post

challenge of their own accord, antibiotic treatment was administered without clinical signs being present. Somatic cell counts were collected and determined by the Tennessee Dairy Herd Information Association (DHIA) lab (Knoxville, TN). Bacteria counts were determined by growth on blood agar plates and were evaluated by Barbara Gillespie or Susan Headrick of the Tennessee Quality Milk Lab (Knoxville, TN) using guidelines established in the NMC lab handbook (NMC 1999). Milk yields were recorded daily by the milking system at the ETREC Little River Dairy.

Haplotyping

A blood sample from each cow was collected via jugular vein prior to the administration of the third vaccination and DNA was isolated from whole blood using UltraClean BloodSpin DNA Isolation Kit (Mo Bio, Carlsbad, CA). Part of the CXCR1 gene was amplified as follows: 1-2 ng of bovine genomic DNA was used as template in a 25 µl reaction containing specific primers and Eppendorf HotMaster Mix (Eppendorf North America; Westbury, NY) according to manufacturer's guidelines. The conditions for amplification were as follows: an initial hot-start denaturation occurred at 94^{o}C for 2min, followed by 50 cycles of 94^{o}C denaturation for 15 sec, 62^{o}C annealing for 20sec, and 72^{o}C extension for 60 sec. After the last cycle, a 10 minute final extension step at 72^{o}C was added before reactions were chilled to 4^{o}C. Amplified products were purified to remove primer and excess nucleotides. Haplotypes were generated using PHASE (Stephens and Scheet 2005; Stephens et al. 2001). The resulting DNA was sequenced using bovine CXCR1 specific primers (Youngerman *et al.* 2004a) and the CXCR1 haplotype was determined based on nucleotide expression at tagSNP positions +621,

+735, +816, and +980 in the CXCR1 gene. The haplotypes of two cows could not be resolved and were not included in analyses.

Statistical Analysis

The experiment was a randomized block design with repeated measures tied to time. Significance was declared at $p<0.05$, and a trend was declared at $p=0.05$ to $p=0.1$. A mixed model using glimmix tested the fixed effects of CXCR1 haplotype, vaccination, time and the two way interactions of each, with time declared as repeated measures. The random effect of cow nested within CXCR1 haplotype also was included. Many covariates were tested for each response variable and include: lactation number, challenge group, rectal temperature, days between calving and challenge (challenge interval), necessity of antibiotic treatment and time between vaccinations and challenge. Of these covariates, necessity of antibiotic treatment was used for milk scores, mammary scores, somatic cell counts, *S. uberis* counts, and milk yield. Analyses were performed in SAS (Cary, NC) using a mixed model analysis of variation with Fisher's LSD for means separation of numeric relationships and chi-square for frequencies. Three cows, 218 (AWQRR x AWQRR), 221 (AWQRR x VWHRR), and 230 (AWQRR x AWQRR) were excluded from all response variable analyses because they were sacrificed early in the trial for experimental reasons and it is unknown if they would require antibiotic treatment. A significant influence of vaccine was not observed for any response variables and will not be discussed.

Results

There were 40 Holstein dairy cows in this study. The dominant haplotype combination was homozygous for AWQRR and represented 28.95% (11/38) of the cows. The second most frequent haplotype combination was AWQRR x VHWKH representing 23.68% (9/38) of the cows. The remaining three haplotype combinations represented smaller percentages of the population and are outlined in Table 13.

The relationship between necessity of antibiotics and time to administration of antibiotics are summarized in Figure 10. The majority of cows (74.29%, 26/35) in the study required treatment with antibiotics. All cows with VWHRR x VWHRR or AWQRR x VWHRR haplotypes required antibiotic treatment and tended to be different from cows with VWHKH x VWHRR haplotype where only 33.33% needed antibiotic treatment ($p=0.0838$). The remaining two haplotypes, AWQRR x VWHKH and AWQRR x AWQRR, had results between both extremes with 75% and 70% of cows needing antibiotic treatment respectively. Cows with the AWQRR x VWHRR haplotype took significantly longer to require treatment, with the administration of the first treatment 13.59 ± 1.73 days post challenge ($p=0.0003$). The remaining four haplotypes required treatment at or before 4.02 ± 1.47 days post challenge.

Milk scores or the degree if inflammation, observed at each time point demonstrated AWQRR x VWHRR cows had significantly lower scores then cows of other haplotypes, except for VWHKH x VWHRR cows, which were more comparable. Cows with the AWQRR x VWHRR haplotype combination never reached average milk scores ≥1 for an individual time point and averaged 0.1735 ± 0.1835 overall ($p=0.0263$).

In contrast, VWHRR homozygous cows reached high milk scores (≥1) within three days post challenge and remained high until nine days post challenge and had an average milk score of 1.048 ± 0.2094. The remaining three haplotype combinations shared similarities with both extremes (Figure 11).

Mammary scores for VWHRR homozygous cows were significantly greater than all other genetic backgrounds. Average mammary scores ≥1 were reached within two days post challenge, remained elevated until 11 days post challenge, averaging 1.23 ± 0.1668. The AWQRR x VWHRR cows which had the lowest milk scores compared to most other genetic backgrounds also had significantly lower mammary scores than the VWHRR homozygous and AWQRR homozygous haplotype combinations averaging 0.4123 ± 0.1442 (p=0.0161) (Figure 12).

Somatic cell counts and *S. uberis* counts also were observed at each time point. Haplotype had no effect on somatic cell counts with all ranging between 10^6 and 10^7 c/mL regardless of time point (p=0.1399) (Figure 13). *S. uberis* counts were influenced by haplotype. Cows with the VWHRR x VWHRR haplotype had significantly higher numbers of *S. uberis* isolated from their infected quarter in comparison to cows from the other four haplotypes (p=0.0348). VWHRR x VWHRR cows averaged 981.996 ± 1947.05 CFU/mL whereas the other four haplotypes were 4.108 ± 4.42 CFU/mL or lower (Table 14). Milk yields were recorded on each day of sampling and were not influenced by haplotype (p=0.6126). All haplotypes had average daily weights ranging between 23.21 and 27.34 ± 2.62 kilograms per cow and each haplotype group demonstrated a steady increase over time (Figure 15).

Table 13: Frequency of haplotypes observed in sample population of Holstein dairy cows.

Haplotype	Count	Percentage
VWHRR x VWHRR	5	13.16
VWHKH x VWHRR	7	18.42
AWQRR x VWHRR	6	15.79
AWQRR x VWHKH	9	23.68
AWQRR x AWQRR	11	28.95
Total	38	100.00

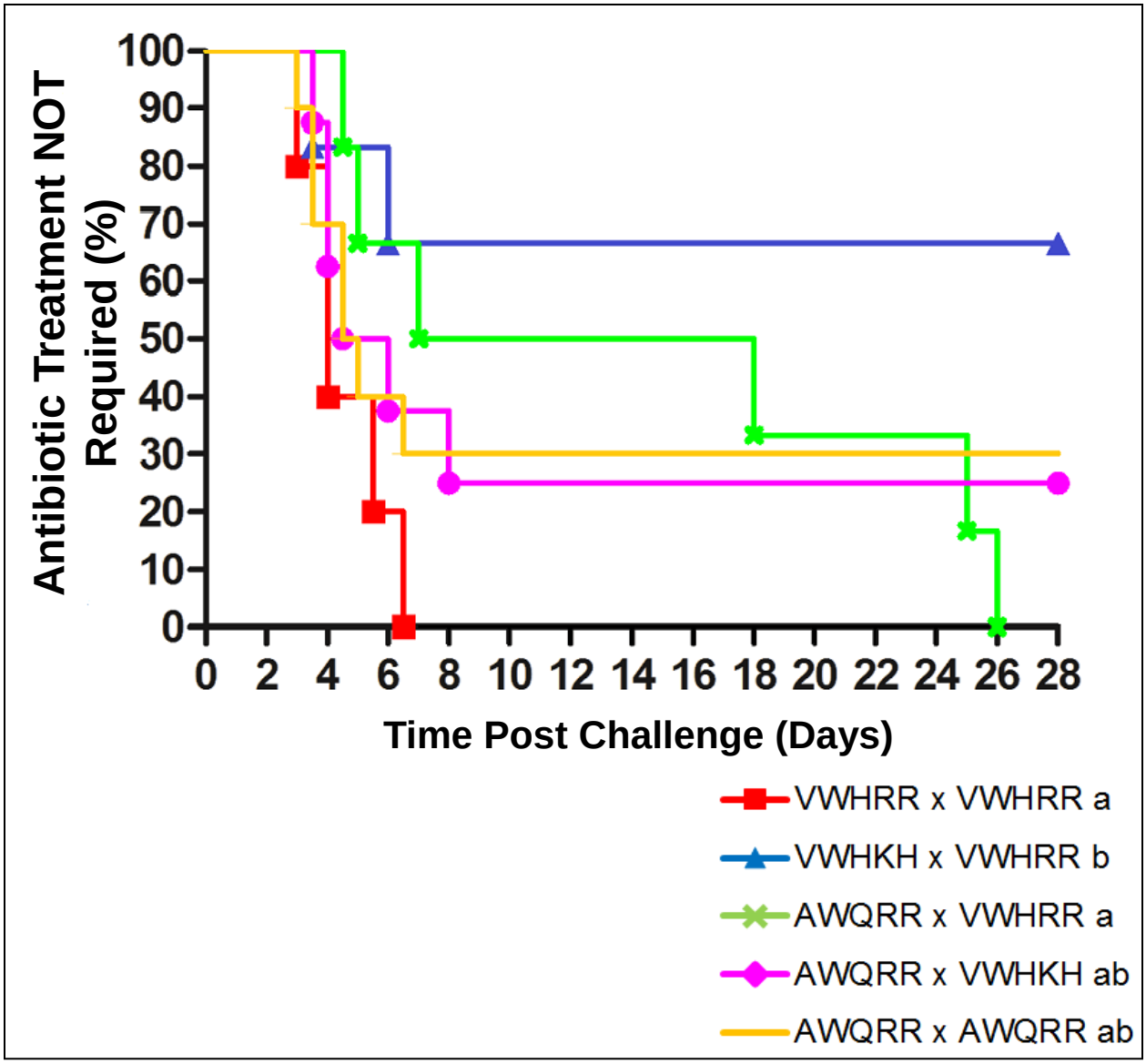

Figure 10: Percentage of and time to antibiotic treatment for each haplotype following *S. uberis* intramammary challenge. Letters (a, b) represent significant differences between haplotypes (p=0.0838).

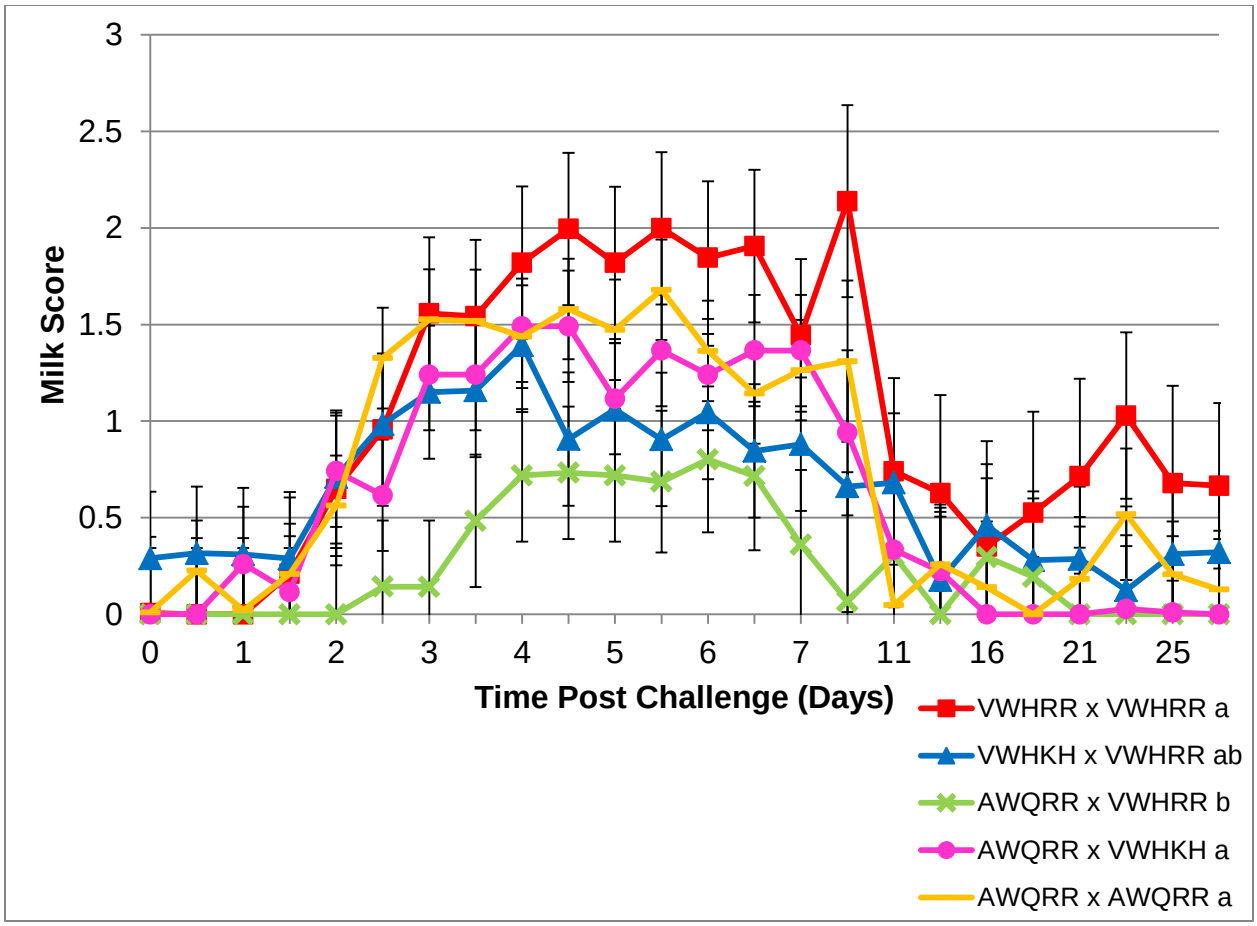

Figure 11: The effect of CXCR1 haplotype combination on milk scores following *S. uberis* intramammary challenge. A covariate of necessity of antibiotic treatment was included (p=<0.0001). Letters (a, b) represent significant differences between haplotypes (p=0.0263).

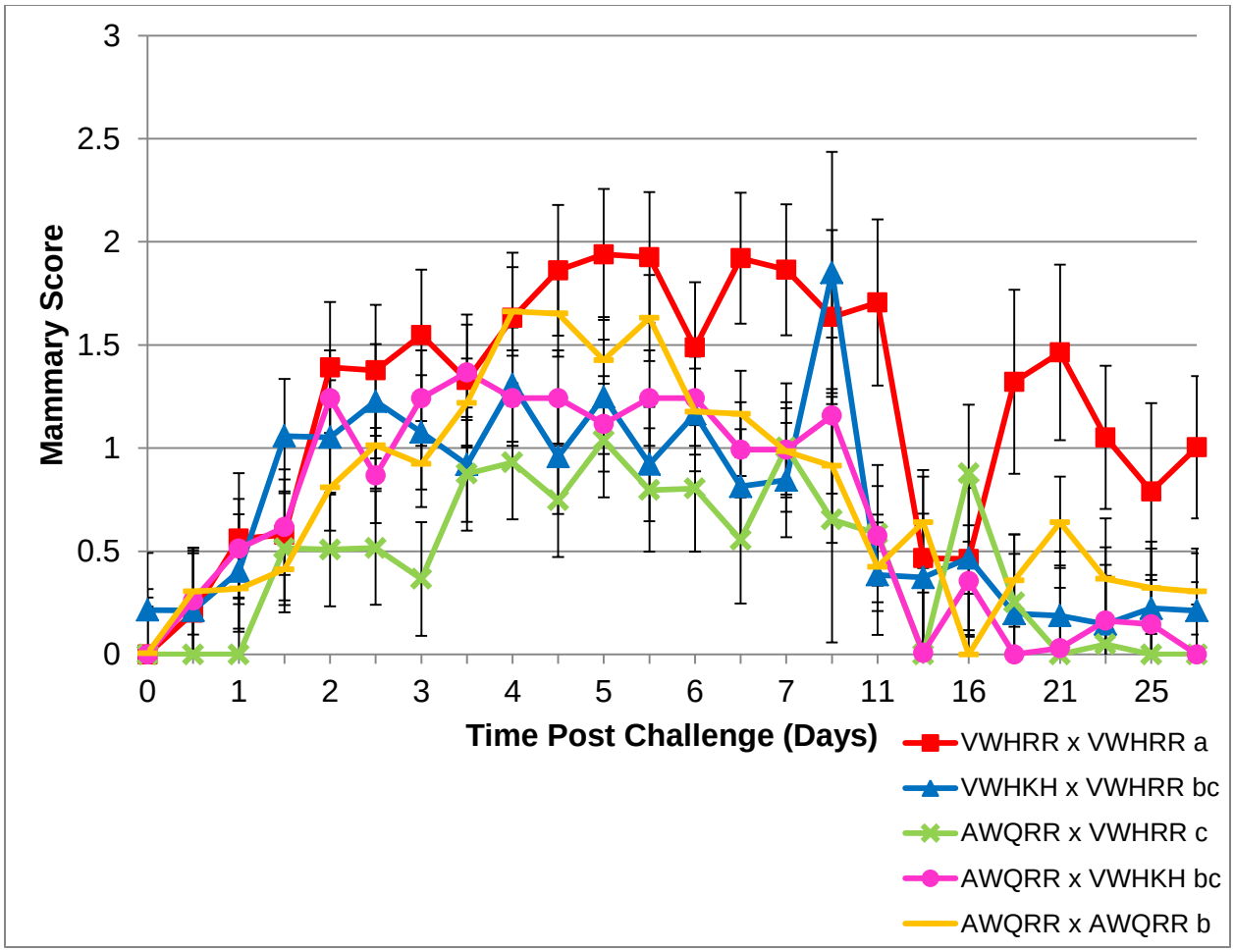

Figure 12: The effect of haplotype on mammary scores following *S. uberis* intramammary challenge. A covariate of necessity of antibiotic treatment was included (p=0.0002). Letters (a, b, c) represent significant differences between haplotypes (p=0.0161).

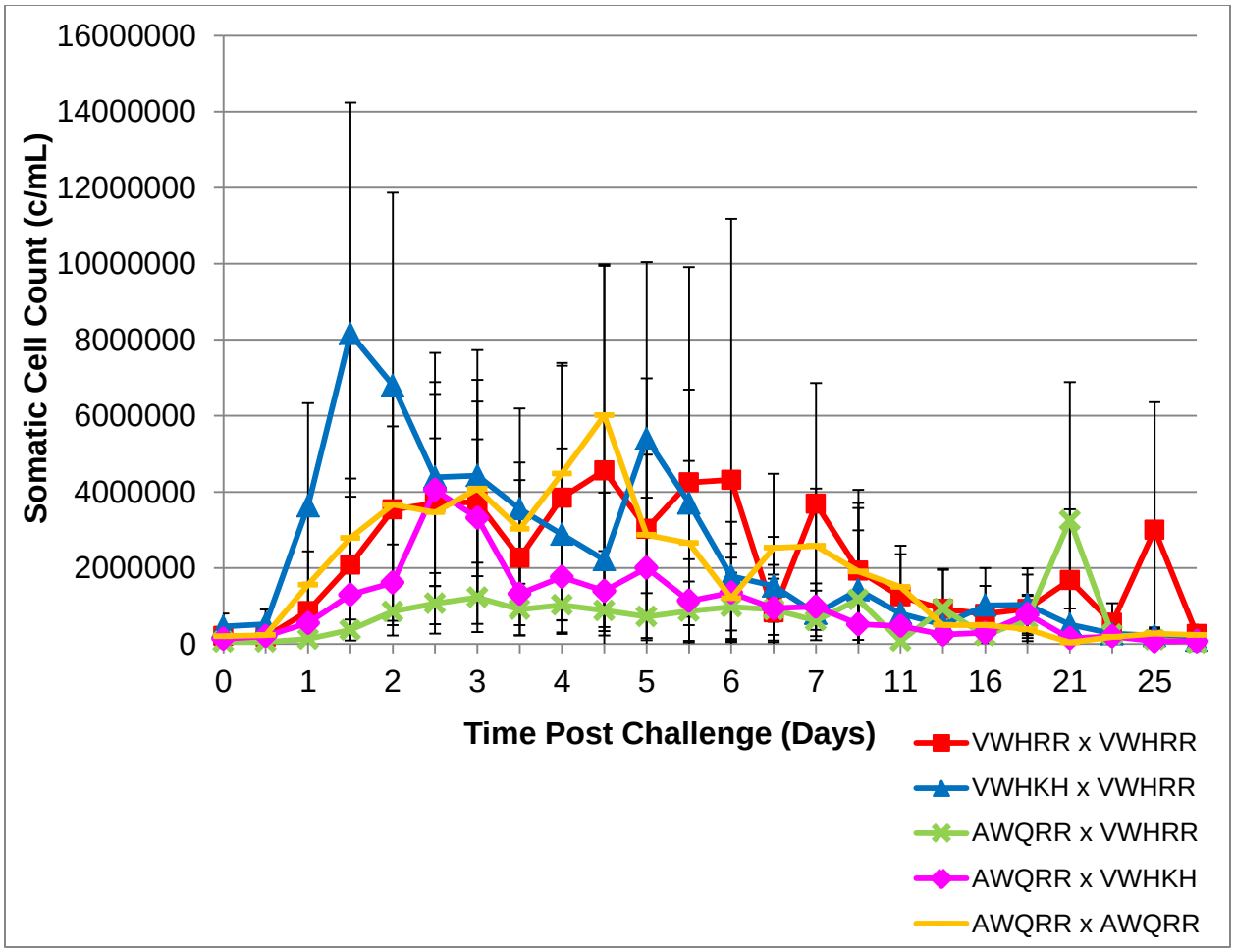

Figure 13: The effect of haplotype on somatic cell counts following *S. uberis* intramammary challenge. A covariate of necessity of antibiotic treatment was included (p=<0.0001). No significant differences were observed in somatic cell counts across haplotypes (p=0.1399).

Table 14: The effect of haplotype on S. uberis counts following S. uberis intramammary challenge.

	Haplotype[i]				
Day	**VWHRR x VWHRR[a]**	**VWHRR x VWHKH[b]**	**VWHRR x AWQRR[b]**	**VWHKH x AWQRR[b]**	**AWQRR x AWQRR[b]**
0	0	0.01	0	0	0
0.5	619.72	12424.73	20.41	25.22	18.04
1	9714.68	162951.56	2296.61	72.32	67.04
1.5	18257.3	40792.42	623.59	590.14	364.43
2	70083.38	298.56	48.6	295.49	7969.93
2.5	337238.32	169.47	0.71	1101.13	53153.56
3	400230.59	6314.32	38.74	602.46	127893.25
3.5	201136.67	269.71	430.84	892.44	188686.6
4	791880.14	71.99	777.07	1315.69	66615.61
4.5	302270.55	110.63	318.79	609.51	54650.87
5	237818.62	33.05	2167.32	7754.51	27477.93
5.5	613894.22	32.95	234.22	408.95	7742.82
6	58667279	10.43	94.14	79.4	732.87
6.5	1.456E+09	25.55	5.79	8.55	3.54
7	2.16	0.18	0.65	0.22	0
9	0.01	0	0	0.09	0
11	0	0	0	0	0
14	0.02	0.02	0.11	0	0
16	1.69	0.03	0	0	0
18	0	0.08	0	0	0.01
21	1302.37	0.03	0.01	0	0.01
23	47.84	0.02	0	0	0
25	107505.49	0	0	0	1.64
28	47.22	0	0	0	0
SEM[ii]	413183348.8	29692.88	993.72	2004.05	57759.93

[i] Haplotype combinations with different letters differ (p=0.0348) with a covariate of necessity of antibiotic treatment (p=0.0002).

[ii] SEM: standard error of the mean.

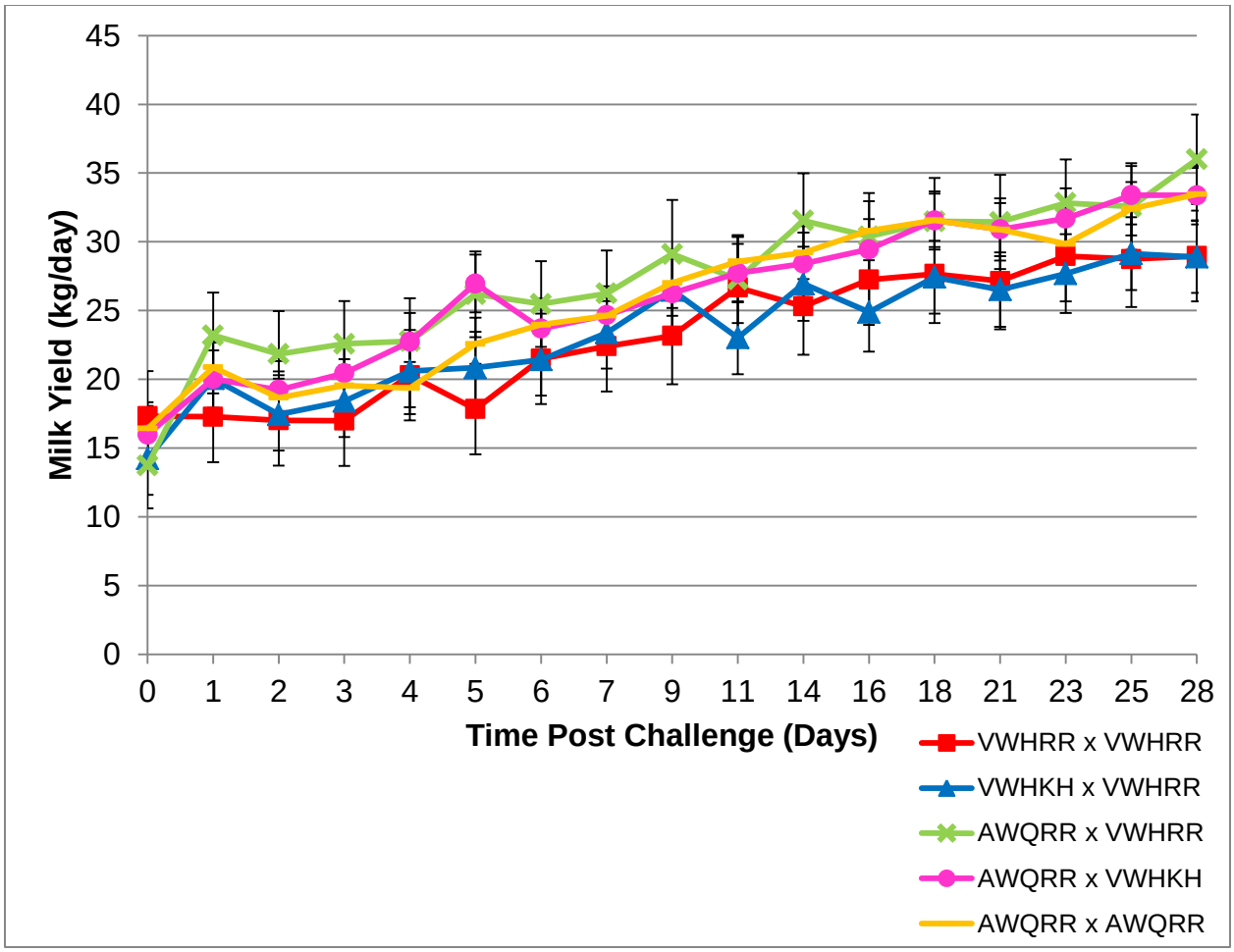

Figure 14: The effect of haplotype on milk yield following *S. uberis* intramammary challenge. A covariate of necessity of antibiotic treatment was included (p=0.2211). No significant differences were observed in somatic cell counts across haplotypes (p=0.6126).

Discussion

The objective of this study was to determine the association between CXCR1 haplotype and resistance or susceptibility to *S. uberis* based mastitis. The common observed infection rate for *S. uberis* following experimental challenge is about 70% (Rambeaud *et al.* 2003). However, we observed a 100% infection rate based on *S. uberis* presence. This difference in number of cows developing infection is most likely attributed to differences in days in milk (DIM). Cows in our study were three or less DIM and cows in the Rambeaud *et al.* (2003) study were between 11 and 38 DIM. Cows are more susceptible to developing mastitis within the periparturient period due mainly to decreased neutrophil function (Cai *et al.* 1994; Oliver and Sordillo 1988; Sordillo and Streicher 2002). The 100% infection rate caused us to reevaluate our definition of resistance in this study. As a result, we focused on the need for antibiotic treatment, which resulted in three groups. The first group contained cows with haplotype combinations VWHRR x VWHRR and AWQRR x VWHRR where 100% of cows required antibiotic treatment. Cows with the VWHRR x VWHRR haplotype combination were highly inflammatory. They developed average milk and mammary scores ≥1 within 2.5 days post challenge, reached *S. uberis* counts statistically higher than other haplotype combinations, and all required antibiotic treatment within seven days post challenge. Contrastingly, the AWQRR x VWHRR haplotype combination still required 100% antibiotic treatment but were much less inflammatory in response. AWQRR x VWHRR cows did not reach milk or mammary scores ≥1 and had *S. uberis* counts statistically similar to all haplotype combinations besides VWHRR x VWHRR. Half of the AWQRR x VWHRR cows received treatment within seven days post challenge as a

consequence of inflammatory milk and mammary scores. The other half did not receive treatment until 18-26 days post challenge and were received antibiotic treatment because cows were still shedding *S. uberis.* The second group contained AWQRR x VWHKH and AQWRR x AWQRR cows with only 70 or 75% requiring treatment. The last group contained cows with the VWHKH x VWHRR haplotype combination where only 33.33% required antibiotic treatment. Within the groups requiring less than 100% antibiotic treatment, cows developed elevated milk and mammary scores ≥1 within two days post challenge and had statistically similar *S. uberis* counts to all haplotypes except VWHRR homozygous cows. Cows that required antibiotic treatment in the groups with less than 100% antibiotic treatment were all treated by 7 days post challenge for severe clinical signs.

The variation observed amongst the different groups can potentially be explained by the theoretical influence of amino acid changing SNPs in the CXCR1 gene. VWHRR homozygous cows required 100% antibiotic treatment within seven days post challenge because of severe clinical signs. These cows are homozygous for the +365 SNP that corresponds to amino acid position 122 in the CXCR1 amino acid sequence. The valine at position 122 has been associated with ligand binding and strength due to its location in the first transmembrane domain, which is essential for any downstream effects to be carried out (Leong *et al.* 1994; Monteclaro and Charo 1996). These cows are also homozygous for the tryptophan amino acid at position 206 in the CXCR1 amino acid sequence. This is a conserved amino acid across all of the observed haplotypes because the alternative ghis a stop codon that results in no receptor expression or a

truncated receptor which both result in an inability for CXCR1 to respond to ligand. Cows homozygous for the VWHRR haplotype are homozygous for the C allele at the +735 SNP and histidine at position 245 in the amino acid sequence. The 245 position has been witnessed to associate with neutrophil migration, survival, and ROS generation as well as intracellular calcium signaling. Expression of histidine at position 245 instead of the conserved glutamine residue has been tied to decreases in these functions in homozygous cows (Rambeaud *et al.* 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005). This suggests that VWHRR homozygous cows may have decreased ability to induce internal calcium signaling and subsequent neutrophil migration, survival, and ROS generation. Furthermore, the +980 and +995 SNPs corresponding to arginine at positions 327 and 332 respectively in the amino acid sequence have been linked to the ability to bind internal mediators and initialize internal signaling, internalize the receptor and induce migration, although more importance is given to the 327 position (Fan et al. 2002; Fan et al. 2000; Raman et al. 2010). Expression of arginine at the 327 position in CXCR2 alters the **K**ILAIHGLI motif and reduces the ability of the C-terminus to bind internal mediators and cause downstream effects such as receptor internalization and subsequent migration (Fan et al. 2002; Fan et al. 2000; Raman et al. 2010; Yang et al. 1999). The potential decrease in internal signaling and receptor internalization that could be associated with VWHRR homozygous cows could allow *S. uberis* to establish infection easier and proliferate more within the gland. The greater *S. uberis* load may lead to the highly inflammatory responses seen and the need for all to receive antibiotic treatment within seven days post challenge.

VWHRR homozygous and VWHKH x VWHRR cows displayed dramatic differences in their need for antibiotic treatment. Only 33.33% of VWHKH x VWHRR cows required treatment as opposed to 100% of VWHRR homozygous cows. Cows with the VWHKH x VWHRR haplotype combination share several similarities with VWHRR homozygous cows having one identical allele and the other allele differing in only the two terminal amino acids. This suggests that they too may have decreased ability to induce neutrophil migration, survival, and ROS generation as well as internal calcium signaling (Rambeaud *et al.* 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005). The terminal lysine and histidine residues are involved in the **K**ILAIHGLI motif of the C-terminus of the CXCR1 receptor. The **K**ILAIHGLI motif is linked to adaptin-2/LASP-1 and HSP-70 interacting protein (Hip) binding to the C-terminus of CXCR1 (Fan *et al.* 2002; Fan *et al.* 2000; Raman *et al.* 2010). Mutations within this motif have demonstrated decreased adaptin-2 or Hip binding resulting in decreased receptor internalization and subsequent migration (Raman et al. 2010). Cows with the VWHKH x VWHRR haplotype combination possess one mutated and one wild type haplotype for this motif suggesting they may have intermediate ability to bind adaptin-2 or Hip and cause subsequent receptor internalization and migration. However, because VWHKH x VWHRR cows displayed a decreased need for antibiotic treatment compared to VWHRR homozygous cows, this suggests that the C-terminal amino acid positions 327 and 332 play a greater role in the ability of CXCR1 to properly respond to ligand stimulation in comparison to the 245 amino acid. Furthermore, these terminal amino acids are most likely responsible in providing some VWHKH x VWHRR cows with the ability to clear *S. uberis* mastitis without the aid of antibiotic treatment.

Cows with the AWQRR x VWHRR haplotype combination also required 100% antibiotic treatment, but were much less inflammatory then VWHRR homozygous cows. Their lower inflammatory response suggests the AWQRR haplotype may impair the ability to kill *S. uberis* efficiently. These cows are heterozygous for the +365 SNP that corresponds to amino acid position 122. The 122 position is conserved across five species and has been linked to ligand binding and strength due to its location in the first transmembrane domain (Leong et al. 1994; Monteclaro and Charo 1996; Pighetti et al. 2012). An amino acid change from valine to alanine could cause a conformational change or alter the hydrophobicity of the transmembrane domain and potentially result in a reduced ability to bind ligand or reduced binding strength (Leong *et al.* 1994; Monteclaro and Charo 1996). These cows are also heterozygous for the 245 amino acid position. In previous studies, cows that are heterozygous at the 245 position display have displayed neutrophil migration, survival, ROS generation, and internal calcium signaling ability similar to both homozygous extremes (Rambeaud et al. 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005). Overall, this suggests the AWQRR haplotype could be impaired in ligand binding as well as adaptin-2 or Hip binding and subsequent receptor internalization and migration. The proposed abilities of each haplotype within the AWQRR x VWHRR haplotype combination suggest they may have decreased ability to bind ligand and carry out subsequent downstream effects of ligand binding. This could explain the low inflammatory response coupled with the inability to completely clear *S. uberis* from the gland and require antibiotic treatment due to *S. uberis*' continued presence witnessed with these cows.

The remaining two haplotype combinations, AWQRR x VWHKH and AWQRR x AWQRR, required intermediate levels (70-75%) of antibiotic treatment. These haplotype combinations share similarities in proposed receptor ability with the other two antibiotic treatment groups previously discussed. The AWQRR x VWHKH cows are heterozygous for amino acid positions 122, 245, 327, and 332. This indicates they will most likely display intermediate abilities in ligand binding tied to position 122; intermediate neutrophil migration, survival, killing ability and internal calcium signaling tied to the 245 position; and intermediate abilities in initiating internal signaling based on the altered **K**ILAIHGLI motif tied to positions 327 and 332 as previously discussed. The AWQRR homozygous cows will most likely display decreased ability to bind ligand due to being homozygous for alanine at position 122 and decreased ability to bind adaptin-2 or Hip to initialize internalization and subsequent migration because of the homozygous arginines at positions 327 and 332 altering binding ability of the KILAIHGLI motif. The proposed abilities of the AWQRR x VWHKH and AWQRR x AWQRR cows can be linked to the responses witnessed for these cows within our study where the majority (70-75%) of cows required antibiotic treatment. Those cows that required antibiotic treatment displayed inflammatory signs and were treated within 7 days post challenge indicating a possible inability to properly control the infection through immune responses.

Increased somatic cell counts are often associated with a state of infection within the mammary gland because immune cells are heavily recruited to the site to fight infection. Somatic cells are present within milk when the mammary gland is healthy at

lower concentrations, generally ≤ 200,000 c/mL, but increase dramatically upon incidence of infection to about 700,000 c/mL or higher, but can vary depending on pathogen (Bannerman *et al.* 2004; Rambeaud *et al.* 2003). The findings of this study followed a similar pattern with somatic cell counts originating at approximately 150,000 c/mL and resulting in over 8,000,000 c/mL. Because of the association of the 245 amino acid position with neutrophil migration (Rambeaud and Pighetti 2005), initial thoughts were that somatic cell counts would differ based on amino acid expression at position 245. However, several studies have provided conflicting results. Youngerman *et al.* (2004a) demonstrated Holsteins heterozygous at the 245 position displayed higher somatic cell scores (SCS) than Holsteins homozygous for histidine at the 245 position and Jerseys showed no such association. Subsequent studies did not confirm an association between the 245 position and SCS (Beecher *et al.* 2010; Goertz *et al.* 2009; Leyva-Baca *et al.* 2008). Our study, looking at the effect of haplotype instead of a single SNP, also did not display differences in somatic cell counts. The IL-8/CXCR1 ligand-receptor complex is not the only complex responsible for neutrophil migration. Other ELR^+ chemokines including, GRO-α, β, γ, ENA-78, IL-8, GCP-2 and NAP-2 are ligands for the functionally similar CXCR2 receptor (Ahuja and Murphy 1996; Lee *et al.* 1992). Binding of these ELR^+ chemokines to the CXCR2 receptor has been demonstrated to induce neutrophil migration (Geiser *et al.* 1993; Lahouassa *et al.* 2008; Walz *et al.* 1991; Walz *et al.* 1989). Thus it is possible that differences in somatic cell counts are not witnessed based on CXCR1 haplotype because the other ligand-receptor complexes aid in recruiting neutrophils. Furthermore, during the infection state, the bulk of somatic cells in milk are neutrophils, but other cell populations are present and

include: macrophages, lymphocytes (CD4+ and CD8+ T-cells) and epithelial cells (Boutinaud and Jammes 2002). Thus, an increase in another subpopulation of somatic cells may contribute to the lack of variation observed.

This research indicates there are differences in responses to *S. uberis* experimental challenge based upon CXCR1 haplotype. All cows developed mastitis indicating resistance could not be attributed to CXCR1 haplotype in an experimental challenge model given under immune compromised conditions. However, differences in susceptibility based on CXCR1 haplotype were observed relative to the need for antibiotic treatment as well as the degree of bacterial growth and inflammation. Of these, cows with a VWHKH x VWHRR haplotype combination required only 33.33% antibiotic treatment and may represent a desirable genetic marker due to their proposed ability to clear infection efficiently without antibiotic treatment during a highly susceptible period.

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Chapter V: Conclusion

The first objective of this study was to evaluate types of cells expressing CXCR1 and CXCR2 in the bovine mammary gland. We identified expression of CXCR1 on alveolar epithelial cells, fibroblasts and leukocytes. Expression of CXCR1 has been witnessed on mammary stem cells, cancerous mammary epithelial cells, and epithelial cells from other species and/or tissues, thus CXCR1 expression on alveolar epithelial cells in the mammary gland was not a novel discovery (Ben-Baruch 2003; Miller et al. 1998; Wislez et al. 2006). The expression of CXCR1 on both fibroblasts and leukocytes were not unexpected findings as CXCR1 has been observed on cultured human fibroblasts (Moser *et al.* 1993), neutrophils (Grob *et al.* 1990), dendritic cells (Sallusto *et al.* 1998), natural killer cells (Berahovich *et al.* 2006), CD4+ T-cells (Gasser *et al.* 2006), CD8+ T-cells (Takata *et al.* 2004), and eosinophils (Petering *et al.* 1999). Expression of CXCR2 on alveolar epithelia cells and leukocytes also was demonstrated. Neither expression of CXCR2 on alveolar epithelial cells nor leukocytes were unexpected due to the prior discovery of CXCR2 expression on cultured epithelial cells (Park *et al.* 2010), neutrophils (Sprenger *et al.* 1994), and eosinophils (Petering *et al.* 1999).

The results of the first objective indicate that not only leukocytes, but also alveolar epithelial cells and fibroblasts are capable of responding to released ELR^+ chemokines indicative of an inflammatory state. For neutrophils, binding of ELR^+ chemokine to CXCR1 or CXCR2 induces migration, survival and modifies secreted chemokine/cytokine profiles (Baggiolini and Clark-Lewis 1992; Grob *et al.* 1990; Kettritz *et al.* 1998; Soehnlein *et al.* 2008). Expression of CXCR1 and CXCR2 on alveolar epithelial cells, fibroblasts, and other leukocytes theoretically provides the same benefits

of increasing migration, increasing survival and modifying chemokine/cytokine profiles. Future studies should evaluate the *in vitro* abilities of bovine mammary alveolar epithelial cells, fibroblasts and leukocyte subsets for their abilities to migrate, survive and modify chemokine/cytokine profiles upon addition of ELR^+ chemokines.

Expression of CXCR1 or CXCR2 in human mammary tissue is typically linked to cancer cells (Ben-Baruch 2003; Charafe-Jauffret *et al.* 2009). However, unlike the bovine mammary gland, lactation in the human mammary gland is a temporary state, thus native expression of CXCR1 and CXCR2 in the bovine mammary gland could be attributed to the perpetual state of lactation. To test this, mammary tissue samples from non-lactating heifers, dried off cows, and cows of varying stages of lactation should be assessed for CXCR1 and CXCR2 native expression. Furthermore, expression of the receptor does not demand a correlation to ligand secretion, indicating that the receptor may be present and unbound and only responds when ligand is released. An examination of what transcription factors and promoter sequences allow CXCR1 and CXCR2 expression in the bovine mammary gland could better illuminate this relationship.

The second objective of this study was to determine the association of CXCR1 haplotype on resistance and susceptibility to direct *S. uberis* challenge. We observed a 100% infection rate resulting in the reevaluation our definition of resistance in this study that focused on the need for antibiotic treatment. This resulted in three groups: VWHRR x VWHRR and AWQRR x VWHRR where 100% required antibiotic treatment, AWQRR x VWHKH and AQWRR x AWQRR where 70 or 75% required antibiotic treatment and VWHKH x VWHRR where 33.33% required antibiotic treatment.

The results from the second objective indicate that the combination of individual SNPs within a haplotype as well as the haplotype combination play an intricate role in the phenotype observed. Cows homozygous for the VWHRR or AWQRR haplotype shared the terminal two arginine residues and all required treatment. This indicates that the C-terminus involving the **K**ILAIHGLI motif is essential for combating infection through internal binding of adaptin-2 or Hip and subsequent receptor internalization and chemotaxis (Fan et al. 2002; Fan et al. 2000; Raman et al. 2010). Although focused on by several previous studies, the amino acid residue at position 245 does not appear to have as great an influence on observed phenotype as the terminal 327 and 332 residues. Further evidence of this comes from the VWHKH x VWHRR cows displaying the ability to clear the infection without the aid of antibiotics in the majority of cases. However, VWHRR homozygous and AWQRR homozygous cows did differ greatly in the phenotypic level of inflammation observed. VWHRR cows were highly inflammatory and AWQRR homozygous cows displayed the least amount of overall inflammation of all observed haplotype combinations in this study. This indicates that the 245 position may be greatly linked to the intensity of the generated immune response most likely tied to the neutrophil migration, survival, ROS generation and internal calcium signaling observed with the glutamine residue at this position (Rambeaud et al. 2006; Rambeaud and Pighetti 2007; Rambeaud and Pighetti 2005). These cows also differed at the 122 amino acid position. The 122 position has been previously associated with ligand binding and strength and may also be associated with the differences observed between the two homozygous haplotype groups likely tied to the ability to bind ligand (Leong et al. 1994; Monteclaro and Charo 1996).

To further support these conclusions, transfectants representative of each haplotype group should be generated and evaluated *in vitro* for their ability to migrate, survive, and modify chemokine/cytokine profiles upon stimulation with ELR^+ chemokines. Furthermore, the infection state is associated with cellular damage. Damaged cells are typically cleared by macrophages (Gordon 1998). If cows of a certain haplotype group (VWHRR x VWHRR) are less capable of resisting mastitis, the bacteria will be present within the gland in higher numbers and capable of causing more cell damage. The end result will be more scar tissue and less active parenchyma to produce milk. These cows would then be more likely to be culled from the herd at a younger age then cows of other haplotype groups due to decreased milk yield. An evaluation of how long cows of each haplotype group stay in the active milking herd could divulge if a trend is present and if further studies are of interest.

Coupling together the knowledge from these two objectives could be the beginning of understanding the important role that the CXCR1/IL-8 receptor-ligand complex plays in mastitis. Based on our results, we know that several populations of cells are natively expressing CXCR1 in the bovine mammary gland and that differences are seen based on CXCR1 haplotype in response to experimental *S. uberis* challenge. Therefore, the proposed abilities of each haplotype can be linked not only to immune cells but also epithelial cells and some fibroblasts. Combining the previously proposed *in vitro* studies to test the migration, survival, and chemokine/cytokine profiles of all cell populations expressing CXCR1 and each CXCR1 haplotype combination should provide invaluable information potentially leading to reduces mastitis.

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Vita

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