12-2010

Identifying Mechanisms Associated with Innate Immunity in Cows Genetically Susceptible to Mastitis

Alexandra Alida Elliott
aelliot8@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Alexandra Alida Elliott entitled "Identifying Mechanisms Associated with Innate Immunity in Cows Genetically Susceptible to Mastitis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Gina M Pighetti, Major Professor

We have read this dissertation and recommend its acceptance:

Jun Lin, Steve Oliver, Tim Sparer, Chunlei Su

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a dissertation written by Alexandra Alida Elliott entitled "Identifying Mechanisms Associated with Innate Immunity in Cows Genetically Susceptible to Mastitis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Gina Pighetti
Major Professor

We have read this dissertation
and recommend its acceptance:

Steve Oliver

Tim Sparer

Jun Lin

Chunlei Su

Accepted for the Council:

Carolyn R. Hodges
Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records)

Identifying Mechanisms Associated with Innate Immunity in Cows Genetically Susceptible to Mastitis.

A Dissertation Presented for
the Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Alexandra Alida Elliott
December 2010

Dedication

I would like to dedicate the dissertation to my parents John and Pam Unguris and my husband Dan Elliott, for their support. Dan, thank you for cheering me up when I've had a bad day and always supporting me. Mom, Dad, thank you for always believing in me.

Acknowledgements

I would like to thank my major professor, Dr. Gina Pighetti for giving me the opportunity to continue my degree at the University of Tennessee. I would like to thank her for her guidance, patience and encouragement throughout my graduate studies and also her friendship. I would also like to thank Dr. Jun Lin, Dr. Steve Oliver, Dr. Tim Sparer and Dr. Chunlei Su for their invaluable knowledge, challenging questions and for critical review of this dissertation.

I would like to thank Dr. John Biggerstaff and Dr. Steve Minkin for their invaluable expertise in microscopy. A big thank you goes out to undergraduate workers Elena Sanchez and Megan Riggle not only for their assistance in the lab, but for their great companionship. I would like to thank Rose Clift not only for her unrivaled expertise in the lab, but also for her continued friendship over the years and Leszek W. for the incredible amount of help he has given me in the lab. Another thanks goes out to Charlie Young and Dean Jenkins for countless blood collections from cows and heifers and to Susan Headrick, Barbra Gillespie, and Mark Lewis for their invaluable help with the mastitis challenges.

Last but not least, I would like to thank my family and husband, Dan Elliott. Dan has always been there to cheer me up when I've had a bad day and has always supported me. My parents have always believed in me and I would like to thank them for that.

Abstract

Mastitis, or mammary gland inflammation, causes the greatest loss in profit for dairy producers. Mastitis susceptibility differs among cows due to environmental, physiological, and genetic factors. Prior research identified a genetic marker in a chemokine receptor, CXCR1, associated with mastitis susceptibility and decreased neutrophil migration. Current research seeks to identify reasons behind mastitis susceptibility by validating this model through in vivo challenge with *Streptococcus uberis* and studying specific mechanisms causing impaired neutrophil migration. Holstein cows with GG (n=19), GC (n=28), and CC (n=20) genotypes at CXCR1+777 were challenged intramammarily with *S. uberis* strain UT888. After challenge 68% of quarters from GG genotype, 74% from CC genotype and only 47% from GC genotype cows had ≥10 colony forming units/ml *S. uberis* for at least two sampling time points ($P<0.05$). However, among infected cows, number of *S. uberis,* somatic cell count, rectal temperature, milk scores and mammary scores were comparable among genotypes throughout infection. These findings suggest that cows with GC genotypes may be more resistant to *S. uberis* mastitis, but have similar responses if infected. To better understand the mechanisms associated with disease resistance, migration patterns in neutrophils from cows with different CXCR1+777 genotypes were evaluated. Neutrophils from cows with GG (n=11) and CC (n=11) genotypes were isolated and stimulated with zymosan activated sera (ZAS). Cells were fixed and stained for F-actin and evaluated for F-actin content, distribution, and cell morphology. Neutrophils from CC cows had significantly lower average F-actin polymerization than GG cows

(P=0.05). Directed migration of neutrophils from GG (n=10) and CC (n=10) genotypes was imaged and tracking data was analyzed for individual cells. Cells from GG genotype traveled further on an X axis and had higher X/Y movement towards IL8 compared to CC genotype, meaning they moved more directly towards IL8. Our findings suggest lower F-actin polymerization in combination with lower ability to directly move towards IL8 could impair neutrophil response to infection in cows with a CC genotype and may contribute to increased mastitis susceptibility. Finding what makes certain cows more susceptible to mastitis could lead to strategies aimed at improved prevention and treatment of mastitis.

Table of Contents

List of figures

List of tables

TABLE PAGE

CHAPTER 1

INTRODUCTION

Mastitis continues to be one of the most significant problems facing dairy producers, causing an estimated loss of up to $2 billion annually. Money is lost due to treatments, discarded milk, lower milk output, decreased quality, and increased labor. Defined as an inflammation of the mammary gland, mastitis is a serious condition which affects every dairy herd. There are approximately 9 million dairy cows in the United States and roughly one third of them will get mastitis at some time during their lactation (NMC 1996). Besides the producer, mastitis affects the consumer as well. An increase in somatic cell count (SCC), common in mammary infections, causes an increase in enzymes within the milk which break down high quality proteins. Shelf-life and taste of milk decrease with increasing enzymes and SCC (Barbano, Ma et al. 2006).

Previous advances regarding milking management and cleanliness have decreased the prevalence of contagious mastitis pathogens, which are spread primarily from cow to cow. However, environmental mastitis pathogens continue to be a challenge for dairy producers (Schukken 2004). *Streptococcus uberis* is one of the most prevalent environmental pathogens causing clinical and subclinical intramammary infections during dry and lactating periods (Oliver 1988; Jayarao, Gillespie et al. 1999). Since there is no way to keep cows in a completely sterile environment in a commercial setting, current research is directed towards improving the immune response against bacteria.

An effective immune response begins with bacteria interacting with cells within the mammary gland, including epithelial cells and leukocytes. These cells release soluble factors including cytokines such as interleukin 1 (IL1), IL6, IL8 and tumor

necrosis factor alpha (TNFα), which induce inflammatory responses such as increased vascular permeability and an influx of leukocytes into the mammary gland (Bannerman 2009). The first leukocytes to migrate into the mammary gland in large numbers are neutrophils, which follow increasing gradients of chemoattractant (Heit, Robbins et al. 2008) given off by bacteria or host cells interacting with bacteria. Directed migration towards the site of infection begins with intracellular signaling, leading to redistribution of the structural protein F-actin to the neutrophil edge where IL8 is in the highest concentration. Increased F-actin polymerization at this edge allows neutrophils to move towards high levels of chemoattractant at the site of infection (Chung and Firtel 2002). One of the most potent neutrophil chemoattractants is interleukin 8 (IL8), which is produced by epithelial cells and leukocytes within the mammary gland. Neutrophils express two surface receptors for IL8, CXCR1 and CXCR2 (Nasser, Raghuwanshi et al. 2007).

Previous findings have identified a genetic marker in the CXCR1 gene associated with increased susceptibility to subclinical mastitis (Youngerman, Saxton et al. 2004). The single nucleotide polymorphism (SNP) is located at position +777 in the CXCR1 gene and results in an amino acid switch from glutamine to histidine (Youngerman, Saxton et al. 2004). This marker has also been found to be associated with decreased neutrophil migration, intracellular calcium release, reactive oxygen species production and increased survival from spontaneous apoptosis (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Rambeaud and Pighetti 2007).

Because neutrophil migration and other key factors in the immune response differ between CXCR1 genotypes and an effective immune response is critical for preventing infection, the hypothesis for this series of studies is that different neutrophil migration patterns in cows with specific CXCR1 genotypes contribute to increased intramammary infection when challenged with *S. uberis*. In order to test this hypothesis, the following objectives were tested:

1. Evaluate directed migration patterns in neutrophils from different CXCR1 genotypes.
2. Evaluate in vivo inflammatory responses after challenge with *S. uberis* among CXCR1 genotypes.

Results from this research will provide a better understanding of migration patterns in cows genetically more susceptible to mastitis and if differences in inflammatory response lead to different infection rates with *S. uberis* among cows with different genotypes. Finding differences in the inflammatory response, including neutrophil migration patterns among cows more susceptible to mastitis may lead to the development of a preventative or treatment which would eliminate inflammatory deficiencies, ultimately decreasing the incidence and severity of mastitis.

CHAPTER 2

LITERATURE REVIEW

I. MASTITIS

Description

Mastitis is the most common and costly disease in dairy cattle, and with an estimated loss of two billion dollars per year, it takes a heavy toll on dairy producers (NMC, 1996). Decreased milk production, treatment, replacement of cows, and discarded milk account for most of this loss. Mastitis occurs when the mammary gland becomes inflamed, due to mechanical injury or most commonly, bacteria entering the mammary gland. Bacteria interact with macrophages and epithelial cells within the mammary gland and these cells release a wide variety of inflammatory mediators which initiate local blood vessel vasodilatation, permeability, and signal white blood cells in the circulation to migrate towards the site of infection (Bannerman 2009). This immune response is initiated in order to destroy the bacteria, repair damaged tissue and return the udder to normal.

Mastitis can be classified as clinical or sub-clinical. There are varying degrees of clinical mastitis and symptoms may include swelling, redness, heat and pain of the udder, fever, depression, and change in appearance and composition of the milk (Merck). For every case of clinical mastitis, there will be 15-40 cases of sub-clinical mastitis (NMC, 1996). Cows with sub-clinical mastitis often go unnoticed because of a lack of visible symptoms. Measuring the number of somatic cells in the milk is the most common practice to identify cows with sub-clinical mastitis (Lukas, Hawkins et al. 2005), however isolating the same bacteria from milk multiple times during a lactation is the best indicator of subclinical mastitis, as increased somatic cell counts are not always observed. Somatic cell count (SCC) measures the number of leukocytes and other

cells per milliliter of milk, and milk from uninfected mammary glands should contain less than 200,000 cells/ml (Rainard and Riollet 2006).

During mastitis, increased permeability and breakdown of tight junctions between epithelial cells leads to an increase of serum proteins such as immunoglobulins and acute phase proteins in milk (Stelwagen, Farr et al. 1997). These proteins are low quality compared to casein, the major protein in non-mastitic milk. The ratio of low quality proteins to high quality proteins increases with infection as casein is able to escape into the bloodstream and serum proteins enter the milk, decreasing milk quality (Urech, Puhan et al. 1999; Boehmer, Ward et al. 2010). An increase in SCC is also associated with decreased milk quality, due to an influx of phagocytic cells, especially neutrophils. At the site of infection, neutrophils release bactericidal components which break down host tissue and proteins as well as kill bacteria (Capuco, Paape et al. 1986). One other reason for decreased milk quality in mastitic cows is bacterial release of toxins and proteases which change milk composition and damage mammary tissue (Sordillo, Nickerson et al. 1989).

Mastitis Pathogens

Bacteria which cause mastitis can be classified as contagious or environmental pathogens. Contagious bacteria colonize skin surrounding the teat duct and usually enter the teat during the milking process. If there is any bacteria present in the machine from the previous cow, or around the cow's teat opening, it can be forced into the teat duct or cistern by the machine (Middleton, Fox et al. 2001). Because contagious

bacteria usually do not survive well in the environment, decreasing cow to cow spread of contaminated milk through good milking practices such as pre- and post- milking teat disinfection and keeping milking equipment clean and functional will decrease the prevalence of these bacteria in the herd (Olde Riekerink, Barkema et al. 2010). One of the most common and problematic contagious bacteria is *Staphylococcus aureus,* a Gram- positive bacteria which does not commonly cause severe clinical mastitis, but mainly sub-clinical infections which can last the entire life of the cow. Cows with subclinical mastitis can have sporadic increases in SCC and decreased milk production. Studies have found that *S. aureus* is able to attach and internalize into mammary epithelial cells (MECs) in order to evade the immune system (Almeida, Matthews et al. 1996).

Over the years, increased milking and sanitation procedures have decreased the amount of mastitis due to contagious pathogens, but environmental pathogens continue to be a challenge (Schukken 2004). Environmental bacteria live in manure, bedding, or soil and enter the teat immediately following milking (Zehner, Farnsworth et al. 1986). The teat orifice stays dilated for 1-2 hours after milking, making it more susceptible to the invasion of bacteria present in the environment (Klaas, Enevoldsen et al. 2005). Common environmental pathogens include *E. coli* and *S. uberis* (Todhunter, Smith et al. 1995). *E. coli* is a Gram- negative bacteria which can cause severe clinical mastitis however many infections are often short lived and eliminated by the cow without antibiotic treatment (Burvenich, Van Merris et al. 2003).

Streptococcus uberis is a Gram- positive bacteria which is one of the most prevalent bacteria present in clinical and subclinical intramammary infections during dry and lactating periods and in heifers (Oliver 1988). Previous in vitro studies have found that *S. uberis* binds to collagen and forms a molecular bridge with the host cell receptor, which increases adhesion to and internalization into mammary epithelial cells (MECs) (Almeida and Oliver 2001). Recent in vitro studies have identified *S. uberis* adhesion molecule (SUAM) expressed on the bacterial surface which binds lactoferrin and uses it as a molecular bridge to bind lactoferrin receptors on MECs (Almeida, Luther et al. 2006; Patel, Almeida et al. 2009). This increases adhesion and internalization of *S. uberis* into MECs in vitro, and could increase *S. uberis* survival in vivo. However, the role of SUAM in evading host defenses has not been as well studied in vivo.

MAMMARY GLAND IMMUNITY

Immunity of the mammary gland involves anatomical, cellular and soluble components which work together to prevent invading pathogens from causing infection.

Anatomical

Innate immunity is considered the first line of defense for the host against bacteria. The first barriers bacteria encounter include epidermis and mucous membranes. Located at the end of the teat, a teat sphincter keeps the entrance to the mammary gland closed tightly, except during milking and up to 2 hours following milking (Klaas, Enevoldsen et al. 2005). If bacteria are able to enter the teat canal during these

times, a lining of keratin serves as an additional barrier by immobilizing most strains of non-encapsulated bacteria (Capuco, Bright et al. 1992). Esterified and non-esterified fatty acids and cationic proteins associated with keratin act as bacteriostatics and bactericidals (Hogan, Smith et al. 1988).

Cellular

If bacteria are able to breach the primary barriers and penetrate deeper into the mammary gland, bacteria will first encounter phagocytic cells, the most common being macrophages in a healthy mammary gland. SCC is a measurement of the number of cells in the milk and should be low in a healthy mammary gland, because the mammary gland is a sterile environment, unlike other body systems such as the intestinal tract. SCC is lowest during peak lactation and highest prior to parturition and following dry off (Burvenich, Van Merris et al. 2003). Somatic cells in a healthy mammary gland consist mainly of macrophages, and a few neutrophils, natural killer cells, lymphocytes, and epithelial cells (Sarikaya, Werner-Misof et al. 2005). Macrophages are phagocytic cells which engulf bacteria entering the gland and help initiate an inflammatory response by producing cytokines as well as unidentified chemotactic mediators. Receptors for opsonins IgG1 and IgG2 have been observed on the surface of macrophages, which leads to increased phagocytosis when bacteria are tagged with those antibodies (Ashfaq and Campbell 1986). However, they are not as bactericidal as neutrophils and their main role is likely to attract neutrophils to the site of infection rather than act as killers themselves. Antigens engulfed by macrophages are presented to T and B cells

which produce antibodies and cytokines to neutralize bacteria (Paape, Shafer-Weaver et al. 2000). Lymphocytes recognize bacteria through specific receptors and represent the adaptive immune response, which allows a faster and more effective immune response the second time the same bacteria is detected. T cells can be subdivided into CD4+ T cells (helper T cells) and CD8+ T cells (cytotoxic T cells) (Kuby Immunology). CD4+ T cells infiltrate the mammary gland during mastitis and release cytokines upon binding to major histocompatibility complex class II (MHC II) receptors on antigen presenting cells, such as macrophages and B cells. The predominant T lymphocyte in healthy bovine mammary glands are CD8+ T cells which are involved in killing host cells infected with intracellular pathogens (Mehrzad, Janssen et al. 2008). The main function of B cells is production of antibodies which can help phagocytic cells recognize and engulf bacteria. Natural killer cells are involved in killing host cells infected with intracellular pathogens through a MHC independent mechanism. Upon binding to an infected cell, granules containing perforin are released and cause a disruption in the bacterial membrane (Shafer-Weaver and Sordillo 1996).

Mammary epithelial cells themselves play a large role in the immune response by producing pro-inflammatory cytokines such as IL6 and IL8 during bacterial infection (Lahouassa, Moussay et al. 2007). Bovine MECs express mRNA for TLR2 and TLR4 as well as IgG1 receptors, and can therefore recognize invading bacteria and alert the immune system (Barrington, Besser et al. 1997; Yang, Zerbe et al. 2008). Specific Fc receptors on MECs are involved in transporting IgG into the mammary gland (Mayer, Doleschall et al. 2005). All of the aforementioned cell types work together to clear

bacteria from the mammary gland, but the most influential cell in innate immunity is the neutrophil.

Neutrophils are commonly called the “first responders” to an infection because they are the first white blood cells to accumulate at the site of infection in large numbers and begin phagocytosis. Neutrophils make up around 20% of somatic cells in the milk of healthy mammary glands and within hours following bacterial infection they increase to around 90% of the population (Paape, Shafer-Weaver et al. 2000). Neutrophils have a multilobed nucleus which allows them to move rapidly through endothelial and epithelial cells on their way to the site of infection. Neutrophils and the cells they interact with are activated by many chemoattractants on their journey to the site of infection. The first step is IL8 induction of slow rolling caused by expression of adhesion molecules P and L selectin on the surface of both neutrophils and endothelial cells lining blood vessels (Kuwano, Spelten et al. 2010). Next, selectins are shed and the expression of B_2 integrins LFA-1 and Mac-1 on neutrophils and ICAM-1 on endothelial cells allows neutrophils to stop rolling and firmly adhere to endothelial cells (Ley 2002). Finally, neutrophils migrate through tissue, following chemoattractants released by macrophages and epithelial cells at the site of infection (Heit, Robbins et al. 2008).

Once neutrophils reach the site of infection, they engulf bacteria and release intracellular granules, containing bactericidal components including defensins, lactoferrin, and molecules which produce reactive oxygen species. There are 4 different types of intracellular granules, including azurophilic, specific, gelatinase, and secretory

granules. Secretory granules contain adhesion molecules necessary for migration and are therefore released first following activation (Borregaard, Miller et al. 1987). Gelatinase granules are secreted next and contain enzymes such as matrix metalloproteinase 9 (MMP9) involved in degrading basement membranes to allow neutrophils to move through the tissue (Dewald, Bretz et al. 1982). Specific granules are released at the site of infection and contain bactericidal proteins such as lactoferrin and adhesion molecules possibly used to attach to bacteria or local cell populations (Baggiolini, Horisberger et al. 1985). Azurophilic granules contain strong bactericidal proteins and myeloperoxidase (MPO), used in the production of reactive oxygen species (Bainton, Ullyot et al. 1971). Because the production of ROS can harm host tissue, these granules are the most regulated and released last (Lacy and Eitzen 2008). MPO catalyzes the reaction of H_2O_2 with a chloride ion to form hypochlorous acid (HOCl) which is a highly bactericidal product. HOCl results in chlorination and inactivation of membrane proteins and replication sites for DNA synthesis (Urban, Lourido et al. 2006). Reactive oxygen intermediates such as H_2O_2 and O_3 are bactericidal also, and kill by damaging DNA, oxidizing fatty acids and proteins, and inactivating enzymes (Weiss 1989). Neutrophils also have extracellular nets, which structurally consist of DNA, histones, and bactericidal proteins from granules which trap and kill bacteria (Medina 2009).

Once neutrophils engulf bacteria, they undergo apoptosis, which is a programmed self-death. Apoptosis can occur extrinsically by activation of Fas receptor (Liles, Kiener et al. 1996) or through intrinsic activation of BAX interacting with the

mitochondria (Santos-Beneit and Mollinedo 2000). Both of these pathways lead to activation of the caspase cascade involving caspase 3, 8, and 9 (Hirata, Takahashi et al. 1998) which leads to DNA fragmentation within the nucleus and the movement of phosphatidyl serine (PS) from the inner cell membrane to the outer cell membrane. PS receptors on macrophages recognize apoptotic neutrophils through PS expression and engulf them (Mikolajczyk, Skrzeczynska-Moncznik et al. 2009). Through apoptosis, the contents of neutrophils are contained within vesicles, which keep reactive oxygen species and other enzymes from destroying local tissue. In contrast, necrosis occurs when the cell breaks apart and the contents are free to destroy local tissue.

Neutrophils isolated from the milk have decreased phagocytic ability and ROS generation compared to blood neutrophils (Jain and Lasmanis 1978). Numerous reasons for this have been described, including reduced energy and increased phagocytosis of milk fat and protein. Neutrophils contain very few mitochondria and receive the majority of their energy through glycolysis (Karnovsky 1968). A previous study found that the amount of glycogen was significantly lower in neutrophils isolated from milk versus blood and that milk neutrophils did not contain glycogen granules which were observed in blood neutrophils (Naidu and Newbould 1973). Another study found that in vitro phagocytosis and ROS production was decreased after neutrophil chemotaxis through epithelial layers (Smits, Burvenich et al. 1999). Neutrophil phagocytosis of fat globules and casein in the milk has been associated with decreased phagocytosis and killing ability (Paape and Guidry 1977). The hypothesis is that neutrophils have less room to ingest bacteria when their phagosomes are full of fat and

protein and if bacteria are ingested, less bactericidal proteins may be present because these compounds already have been used to break down fat and protein.

Humoral Immunity

Antibodies are one of the main soluble factors involved in defense of the mammary gland and consist of IgG_1, IgG_2, IgM, and IgA. IgG_1 is the predominant Ig in a healthy mammary gland, but IgG_2 increases during inflammation (Barrington, Besser et al. 1997). IgA is mainly involved in agglutination of bacteria, which clumps bacteria together preventing it from adhering to host cells (Barrio, Rainard et al. 2003). IgG antibodies mainly act as opsonins by attaching to bacteria and binding to Fc receptors on macrophages and neutrophils increasing the rate of phagocytosis (Fleit, Wright et al. 1982; Perussia, Dayton et al. 1983). Opsonic antibodies are able to provide broad protection against pathogens in the mammary gland before specific adaptive antibodies can be produced.

A healthy mammary gland contains low amounts of small proteins called complement fractions. Normally, complement proteins are inactive, but certain triggers, such as the presence of bacteria and antibodies causes proteases to cleave complement proteins, transitioning them into an active form (Shuster, Kehrli et al. 1997). Activated complement proteins cleave other complement proteins, leading to the complement cascade which ultimately ends with a pore formed in the bacterial membrane causing cell lysis (Rainard, Poutrel et al. 1984). Besides direct bacterial killing, activation of the complement cascade also results in production of c3b which

acts as an opsonin and c5a which acts as a chemoattractant and enhances phagocytosis in neutrophils (Rainard and Poutrel 1995; Rainard, Sarradin et al. 1998). The two main types of complement cascades are alternative and classical. The classical cascade is activated by C1q binding to IgM or IgG associated with antigens or the antigen itself. The alternative pathway begins with spontaneous hydrolysis of C3 and if bacteria is nearby, C3b binds to the surface of bacteria, activating the complement cascade (Rainard 2003). Due to a lack of C1q, the alternative complement cascade predominates in the mammary gland, unless vascular permeability during inflammation allows C1q to enter and produce the classical cascade (Rainard, Poutrel et al. 1984).

Lactoferrin is a protein within the milk produced by MECs and neutrophils which has bacteriostatic and bactericidal functions. The main bacteriostatic property is the ability to bind free ferric ions, which inhibits the growth of bacteria which require iron, such as *E. coli* (Reiter, Brock et al. 1975). *Streptococcus* species are not affected by the chelating property of lactoferrin and have receptors for lactoferrin on their surface, which may help the bacteria attach to MECs (Patel, Almeida et al. 2009). The bactericidal activity of lactoferrin also occurs through the lactoferricin peptide obtained following enzymatic cleavage and involves increased permeability of the bacteria outer membrane (Bellamy, Takase et al. 1992). Lactoferrin increases in the mammary gland during periods of inflammation, including the time of dry off or cessation of milking approximately 60 days prior to calving (Harmon, Schanbacher et al. 1976; Welty, Smith et al. 1976). Lactoferrin can also act as an anti-inflammatory agent by binding to

lipopolysachharide (LPS) and preventing activation of an inflammatory response against it (Wang, Pabst et al. 1995).

Multiple bactericidal proteins within the mammary gland have small roles in resistance individually, but the combination of them works effectively at preventing infection. Lysozyme is a bactericidal protein which cleaves the peptidoglygan layer of the cell wall on bacteria (Persson, Carlsson et al. 1992). Xanthine oxidase is an enzyme present in milk fat globules which catalyzes the production of nitric oxide, leading to the production of peroxynitrate, a strong bactericidal compound (Collins, Parsons et al. 1988). Inducible nitric oxide synthase (iNOS) is an enzyme found in the mammary gland which also catalyses the production of NO, a highly reactive compound. NO reacts with oxygen to form the highly bactericidal peroxynitrite in addition to its metabolites NO_2 and NO_3 and is produced mainly by macrophages and in small amounts by neutrophils and possibly epithelial cells (Boulanger, Bouchard et al. 2001). Beta-defensins are cationic bactericidal proteins produced by neutrophils and MECs which create voltage-dependent channels in the cell membrane causing cell lysis (Selsted, Tang et al. 1996).

Multiple proteins not present in the healthy mammary gland are present during inflammation due to vascular permeability. One protein which enters from the blood is transferrin, which is a chelating agent similar to lactoferrin (Rainard 1986). During a systemic inflammatory response, acute phase proteins (APPs) are produced in the liver and enter the mammary gland through vascular permeability. Important APPs related to mastitis include lactoferrin mentioned earlier and serum amyloid A (SAA). In addition to

entering the gland from blood, MECs also have increased expression of these proteins during mastitis (Molenaar, Harris et al. 2009). Although SAA increases dramatically in plasma and the mammary gland during clinical and subclinical mastitis, its exact role is not yet known (Molenaar, Harris et al. 2009). The large combination of bactericidal proteins observed within the mammary gland help control bacterial infections.

Although not directly bactericidal, cytokines are one of the major soluble inducers of inflammation as well as the resolution of inflammation. Cytokines are small proteins secreted by many cells within the mammary gland (Bannerman 2009). They bind to specific membrane receptors on the cells which produced them, or on other cells and proceed to regulate immunity, inflammation, and hematopoiesis. Interleukin-8 is a chemokine involved with recruitment of neutrophils to the site of infection and is produced by epithelial cells, macrophages and neutrophils (McClenahan, Krueger et al. 2006). Neutrophils and macrophages are also activated by interferon gamma, whose production is stimulated by interleukin-12 (Ellis and Beaman 2004). Tumor necrosis factor and interleukin-1B are involved in the local and systemic immune response . Locally, they promote leukocyte movement towards the site of infection by inducing endothelial adhesion molecule expression (Morzycki, Sadowska et al. 1990) and enhancing phagocytosis and bactericidal activity in neutrophils (Ogle, Noel et al. 1990). Systemically, these two cytokines induce fever and an acute-phase response, resulting in the liver increasing synthesis of proteins needed for the immune response (Brouckaert and Fiers 1996). When the infection has been contained, an increase in interleukin-10 down regulates the production of proinflammatory cytokines, such as

TNFα thereby slowing down the inflammatory process and preventing excessive tissue damage (Cassatella, Meda et al. 1993).

Bacterial Recognition

The invading bacteria contain certain patterns or motifs which immune cells can use to identify them as pathogens and not host cells. These motifs are called pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharide (LPS) found on only Gram- negative bacteria, peptidoglycan (PGN), and certain components of the bacterial cell wall (Oviedo-Boyso, Valdez-Alarcon et al. 2007). These motifs are often essential for bacteria to function properly, so the mutation rate is very low. The ability of the innate immune system to recognize these PAMPs gives some specificity to the innate immune system. PAMPs are recognized by specific receptors located on the surface and inside many different cells throughout the body, including leukocytes, epithelial cells and endothelial cells. These receptors are called Pattern Recognition Receptors (PRRs). One common group of PRRs is the Toll-Like Receptors (TLRs), which are receptors found on or in many different cell types (Takeda and Akira 2001). All of the Toll-like receptors can identify a specific PAMP. For example TLR-2 binds to the PGN in Gram- positive bacteria (Werts, Tapping et al. 2001), TLR-4 binds to the LPS in Gram- negative bacteria (Poltorak, He et al. 1998), and TLR-5 binds to the flagellin in motile bacteria (Hayashi, Smith et al. 2001). TLRs can bind PAMP alone or in combination with other molecules. Lipopolysaccharide Binding Protein (LBP) opsonizes LPS and that combination is recognized by CD14, an opsonic receptor and

this complex then activates TLR-4 (Muta and Takeshige 2001). Although mammary epithelial cells do not have CD14 on the surface of their membrane with which to bind the LPS-LBP complex, these cells can use soluble CD14 (Labeta, Vidal et al. 2000). Activated TLRs initiate a signaling pathway leading to activation of the NF-kB and subsequent production of cytokines such as Tumor Necrosis Factor (TNF), and many different interleukins (Takeda and Akira 2001).

Specific bacteria and even different strains of the same bacteria can cause diverse immune responses in the mammary gland and influence subsequent resistance. Following infection with *E. coli,* cytokines IL1β, IL8, IL6 and TNFα increase within a few hours, neutrophil influx occurs after 3-12 hours and clinical signs occur within 8 hours (Bannerman, Paape et al. 2004). IL1β and TNFα are potent inducers of fever and their peak level in milk corresponds with increased body temperature and IL8 increase corresponds with SCC increase. *S. uberis* infection can take 66 hours to elicit an increase in pro-inflammatory cytokines (TNF α, IL1β, and IL8) and 84 hours to exhibit clinical signs (Rambeaud, Almeida et al. 2003). Following infection with *S. aureus*, IL8 and TNFα are not produced at all and neutrophil influx occurs after 36 hours and is lower in number (Bannerman, Paape et al. 2004).

The collective effort of anatomical, cellular, and humoral immunity in conjunction with bacterial recognition are needed for an effective immune response and subsequent infection resistance. One process in particular, neutrophil migration into the mammary gland, is essential for effective resolution of mastitis (Hill 1981). Quick neutrophil

migration prevents bacteria from multiplying and decreases the number of bacteria within the milk. Because neutrophil migration plays such a key role in the prevention and resolution of mastitis, the next few sections will focus on migration in detail.

NEUTROPHIL MIGRATION

When a neutrophil encounters a chemotactic gradient, it must sense where the chemoattractant is highest and move in that direction. The first step in this process is chemoattractant binding to receptors on the cell surface, resulting in receptor activation and subsequent initiation of intracellular signaling pathways. These pathways involve polarization of the cell, F-actin polymerization and directed migration towards the chemoattractant.

CXCR1 and CXCR2

Two receptors involved in neutrophil migration are G protein coupled receptors (GPCRs) CXCR1 and CXCR2. Highly specific, CXCR1 exclusively binds IL-8, while CXCR2 is less specific and binds to IL-8, epithelial-derived neutrophil attractant (ENA-78), and growth-related oncogenes (GRO) α, β, and γ (LaRosa, Thomas et al. 1992). The most divergent regions between the receptors are the N-terminus, C-terminus and second extracellular loop (Ahuja, Lee et al. 1996).

GPCRs have 7 transmembrane helices with an N-terminal extracellular domain to bind ligands and an intracellular C-terminal domain to direct signaling following receptor activation (Murphy 1994). Trimeric G-proteins bind along intracellular helices

and are involved in signaling pathways leading to migration, phagocytosis, and reactive oxygen species production. G_α, G_β, and G_γ are three G proteins involved in the trimeric complex. G_α consists of two domains, the helical domain and the GTPase or RAS-like domain (Johnston and Siderovski 2007). The helical domain consists of six α helical bundles and forms a lid over the nucleotide binding pocket. The GTPase domain hydrolyzes GTP, provides a binding surface for $G_{\beta\gamma}$, and contains three switch regions. The G_β subunit has a seven bladed propeller conformation with an N-terminal alpha helix that binds to an N-terminal alpha helix on G_γ. The C-terminus of $G_{\beta\gamma}$ has 60 amino acids which correspond with the third intracellular loop of CXCR1, allowing it to bind (Taylor, Jacob-Mosier et al. 1994).

Glycoproteins present in the CXCR1 ligand, IL8 bind to a leucine-rich domain in the CXCR-1 N-terminus positions IL8 to interact with the correct extracellular loops of CXCR1. This binding creates outward movement of helix 6, which opens a pocket that binds the C-terminus of G_α. This causes the release of GDP from G_α and the binding of GTP which causes conformational changes in the 3 switch regions, eliminating the $G_{\beta\gamma}$ binding surface (Oldham and Hamm 2008). Understanding the coupling specificity between G proteins and the receptor has proven difficult due to poor sequence homology of intracellular loops. Closely related receptors activating the same G protein can have dissimilar intracellular loops (Rosenbaum, Rasmussen et al. 2009). The exchange of GDP for GTP on Gα, frees it and $G_{\beta\gamma}$ from the receptor and from each other. G_α activates phospholipase C (PLC) and cleaves PIP2 into IP3 and diacylglycerol (DAG), which are involved in activated cell functions including release of

intracellular calcium (Cicchetti, Allen et al. 2002). PLC is also involved in increasing reactive oxygen intermediates. The $G_{\beta\gamma}$ subunit activates PI3K, leading to functions such as F actin polymerization which will be discussed in the next section.

After CXCR1 and CXCR2 are activated, clusters of serine and threonine residues within the C-terminus are phosphorylated by serine and threonine kinases (Premont, Inglese et al. 1995). GPCR kinases can recognize activated GPCR conformation and drive this process. Arrestins bind the phosphorylated receptor and sterically prevent further G-protein activation. Arrestins then interact with intracellular machinery including clathrin coated pits and mediate receptor endocytosis, where it is degraded or recycled back to the membrane (Marchese, Paing et al. 2008). CXCR2 internalizes much more rapidly than CXCR1 and recovers more slowly than CXCR1. Five minutes following activation, 90% of CXCR2 and only 10% of CXCR1 are internalized (Nasser, Raghuwanshi et al. 2007). Both receptors internalize through an arrestin/dynamin dependent mechanism, however CXCR2 can also internalize through a phosphorylation-independent mechanism.

Polarization

When neutrophils are in the presence of a chemotactic gradient, more GPCRs are activated on the side where chemoattractant is highest. This edge of the cell becomes the “leading edge” through accumulation of actin polymerizing proteins and subsequent increased F-actin polymerization (Janetopoulos and Firtel 2008). The increased F-actin polymerization at the leading edge takes the shape of broad

lamellapodia, involved in propulsion forwards and narrow filopodia, involved in sensing the environment (Pollard and Borisy 2003).

In the first step of polarization, the G proteins discussed earlier activate PI3K at the leading edge, which phosphorylates the 3 position hydroxyl group on the inositol ring of phosphatidylinositol (4,5) P_2 (PIP_2) forming phosphatidylinositol (3,4,5) P_3 (PIP_3) (Wang, Herzmark et al. 2002). PIP_3 sequesters PH domain containing proteins (CRAC, PhdA) to the leading edge by binding their lipid binding domains. The PH domain containing proteins then sequester and activate small Rho family GTPases, including Rho A, Rac1, and Cdc42 at the leading edge (Srinivasan, Wang et al. 2003; Wong, Pertz et al. 2006). Rho A is involved in overall F-actin formation by activating the formin Dia 1 and inactivating cofilin through LIM kinase (Otomo, Otomo et al. 2005). Rac1 is involved in formation of lamellapodia through activation of WAVE, which is necessary for the F-actin branching activity of ARP 2/3 complex (Eden, Rohatgi et al. 2002). Cdc42 is involved in filopodia formation by activating N-WASP and VASP in the filopodia and inactivates actin assembly through PAK kinase (Torres and Rosen 2006). ARP 2/3 complex, cofilin, N-WASP, VASP, and formin Dia1are involved in actin polymerization, so subsequently F-actin polymerization is localized at the leading edge. PTEN localizes along the edges and rear of the cell and prevents localization of PI3K and subsequent activation of PIP_3 at these locations (Heit, Robbins et al. 2008). Without PTEN, the cell could have more than one leading edge and would not migrate as efficiently.

Actin Polymerization

The machinery behind the change of cell shape in polarization and the driving force behind protrusion of the cell forward is F-actin polymerization. In this step, single actin molecules called globular actin (G-actin) are rapidly added to barbed end of filamentous actin (F-actin) and removed from the pointed end, a process called actin treadmilling (Carlier, Laurent et al. 1997). Actin hydrolyzes ATP upon polymerization, creating a difference in the critical concentration of the barbed (cc = 0.06μM) and pointed (cc = 0.6μM) end bound to ADP. To try and achieve steady state concentration, the rate of barbed end elongation equals the pointed end depolymerization and the filament moves forwards and stays the same length (Carlsson 2005). Certain proteins are involved in accelerating actin treadmilling, including cofilin, profilin, and barbed end capping proteins.

Cofilin (also called actin deploymerizing factor (ADF)) is a protein which binds to the sides of ADP-actin filaments and changes their structure, increasing the rate of pointed end depolymerization. By increasing the rate of depolymerization, more singular G-actin molecules are available, speeding up polymerization at the barbed end (Carlier, Ressad et al. 1999). Cofilin is active when non-phosphorylated and LIM kinase inactivates cofilin through phosphorylation. Profilin binds monomeric actin at the barbed end of an actin filament and enhances the exchange of ADP to ATP, necessary for the monomer to be recycled back to the barbed end (Perelroizen, Didry et al. 1996). The presence of cofilin and profilin has been found to increase actin treadmilling 125-fold (Didry, Carlier et al. 1998). Capping proteins include heterotrimeric capping protein,

gelosin and capG. They all function by blocking certain barbed ends, which leads to an increase in G-actin, increasing polymerization of filaments without capping protein, a process called “funneling” (Pantaloni, Le Clainche et al. 2001). PIP2 binds to capping proteins and may assist with severing of capping protein from F-actin as well as inhibit the re-capping of actin filaments. It is still unclear whether PIP2 actually severs the actin filament and whether this uncapping process leads to initiation of actin assembly. The severing of capping proteins from F-actin exposes a broader region of F-actin barbed ends, leading to formation of lamellapodia. Figure 1 summarizes the coordination of F-actin assembly at the cell membrane.

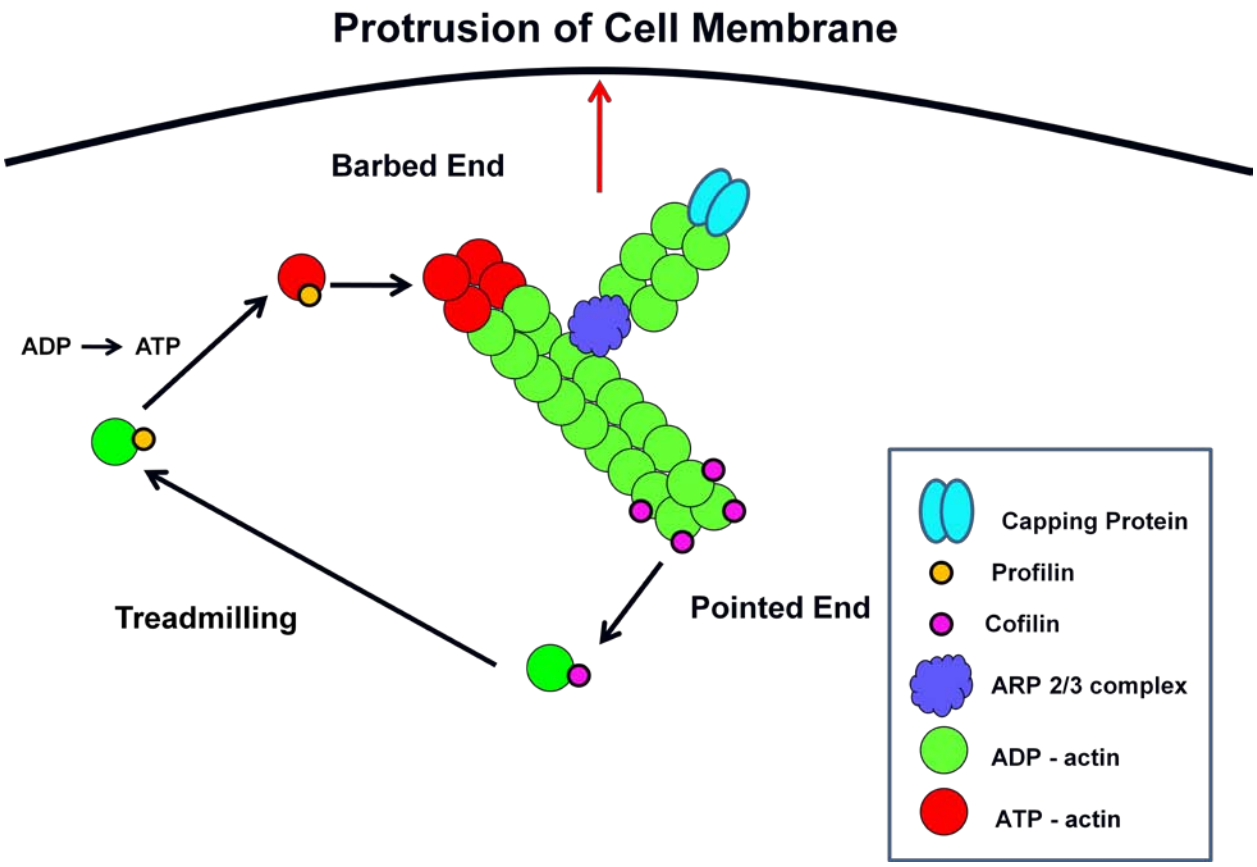


Figure 1. Representation of the interaction of actin and proteins involved in F-actin polymerization. Cofilin assists in severing actin monomers at the pointed end and profilin converts ADP to ATP on the actin monomer, allowing the monomer to bind to the barbed end. ARP 2/3 complex leads to F-actin branching. F-actin polymerization at the cell edge leads to protrusion. Adapted from (Saarikangas, Zhao et al. 2010).

F-actin nucleating proteins assist the formation of new actin filaments. Two very important nucleating proteins associated with actin polymerization are the actin related protein (ARP) 2/3 complex and the formins (mDia1 and mDia2) (Pollard 2007). ARP 2/3 contains two main components (ARP2 and ARP3) and 5 subunits (ARPC1-5). ARPC2 and 4 form the structural core of the complex, ARPC1, 3, and 5 contribute to activation through WASP, and ARP2 and 3 are involved in the nucleation process (Gournier, Goley et al. 2001). Arp 2/3 attaches to a pre-existing actin filament and promotes a new actin molecule to attach at a 70° angle, which promotes branched actin growth (Goley and Welch 2006). Branching is associated with the lamellipodial protrusion, which allows the cell to have a strong foundation to move forward onto. There are two different models of branching involving ARP2/3. In the side branching model, ARP2/3 binds to the side of a pre-existing filament and begins nucleation from there (Amann and Pollard 2001). In the barbed end branching model, ARP2/3 binds to the barbed end of the pre-existing filament and begins nucleation from there (Pantaloni, Boujemaa et al. 2000). Whichever model is correct, activation of the complex is the same and occurs through a VCA domain in WASP protein. The veprolin-homology (V) region of WASP binds monomeric actin, the connecting region (C) interacts with both monomeric actin and the APR 2/3 complex, while the acidic region (A) binds the ARP 2/3 complex (Hufner, Higgs et al. 2001).

Formins, on the other hand, bind to the barbed ends of actin filaments and promote actin binding to create linear actin growth. This type of growth produces filopodial protrusion, which can sense and explore the environment surrounding the cell

(Pollard 2007). Both lamellipodia and filopodia extend in the direction of the chemoattractant. This step occurs very rapidly. Resting neutrophils contain 30% F-actin compared to 60% present in activated neutrophils which have been in contact with stimulants for only 30 sec (Cassimeris, McNeill et al. 1990).

Adhesion

In order for a neutrophil to use the protrusion constructed by F actin polymerization, it must have some form of traction to move forward. The first step in neutrophil adhesion is neutrophil rolling, involving E or P selectins on endothelial cells binding to O-glycans presented as sulfated tyrosine residues on P selectin glycoprotein ligand (PSGL1) (Ley 2002). PSGL1 is located on lipid rafts in the outer membrane of neutrophils and the binding of this molecule to E-selectin on endothelial cells results in beta-integrin activation through a p38 MAPK-dependent pathway (Yago, Shao et al. 2010). L-selectin on neutrophils is also involved in rolling, however neutrophil activation through L-selectin remains unclear (Ley, Laudanna et al. 2007). The next step is expression of β-2 integrins such as leukocyte function-associated antigen 1 (LFA-1) and Mac-1 which bring the neutrophil to a stop by binding to intracellular adhesion molecule 1 (ICAM-1) on endothelial cells . Activation of neutrophil arrest through integrins and ICAM-1 occurs through “inside-out signaling” and “outside-in signaling”. Inside-out signaling occurs when chemokines bind to endothelial cells and neutrophils, leading to increased expression and binding of LFA-1 and ICAM-1. LFA-1 is found in low, intermediate, and high affinity conformations on the neutrophil surface, with high affinity

being the most common (Kuwano, Spelten et al. 2010). Outside-in signaling occurs when the binding of E-selectin to neutrophils induces intermediate and high affinity conformations of LFA-1 on the neutrophil surface, opening the binding pockets and allowing LFA-1 to bind ICAM-1 on endothelial cells (Lefort, Hyun et al. 2009). Why neutrophils have multiple conformations of LFA-1 and how they are used in neutrophil arrest remains unclear. Next, the neutrophil must move through endothelial and epithelial cells to reach the site of infection. Increased vascular permeability and decreased tight junctions between MECs help neutrophils move in between the cells, in addition to proteases released by neutrophils to break remaining tight junctions. Neutrophils are able to migrate through a transcellular route, however only 5-20% of neutrophils use this method (Ley, Laudanna et al. 2007).

Directed Migration

Direct movement of neutrophils into the mammary gland is essential for effective resolution of infection. Neutrophils must sense a specific chemoattractant and ignore other soluble molecules in blood and tissue so they do not get distracted and start moving away from the site of infection (Heit, Robbins et al. 2008). Intermediate chemoattractants such as IL8 are the first to stimulate neutrophils and guide them towards the area of infection through phosphatidylinositol 3 kinase (PI3K) which is located at the leading edge. PI3K localization to the leading edge occurs because phosphatase and tensin homolog (PTEN) which is positioned around the sides and tail of the neutrophil prevents PI3K localization in these areas (Li, Dong et al. 2005). Closer

to the site of infection, neutrophils follow end target chemoattractants such as complement fraction C5a to reach the exact site of infection. Migration towards end-target chemoattractants is mediated through the p38 mitogen activated protein kinase (MAPK) pathway, which is responsible for PTEN localization. When both intermediate and end-target chemoattractants are present, neutrophils will migrate preferentially towards the end target chemoattractant where the foci of infection is located. A study in 2008 by Heit et al. found that this prioritization is mediated by PTEN. PTEN $^{-/-}$ mice not only had neutrophils which moved equally towards end and intermediate target chemoattractants, but were less able to clear an in vivo bacterial infection.

MASTITIS SUSCEPTIBILITY

Causes of susceptibility

A number of factors influence the susceptibility of a dairy cow to mastitis, including diet, age, stage of lactation, and genetics. Negative energy balance at the time of parturition has been shown to increase mastitis susceptibility, while an increase in dietary selenium and vitamin E has been shown to decrease mastitis susceptibility (Smith, Harrison et al. 1984). Cows in negative energy balance compensate by releasing energy stores in the form of non-esterified fatty acids (NEFA) leading to β-hydroxybutyric acid (BHBA) production and have high levels of both products circulating in the blood and milk. NEFA and BHBA decrease neutrophil phagocytosis, ROS production, and production of NETs (Hoeben, Heyneman et al. 1997; Grinberg, Elazar

et al. 2008). A recent study found a decrease in IL8 signaling, glucocorticoid receptor signaling, and oxidative stress response in mammary tissue in negative energy balance cows following challenge with *S. uberis* (Moyes, Drackley et al. 2009). Older cows are generally more susceptible to infection than younger cows (Burvenich, Van Merris et al. 2003). Cows are especially susceptible to mastitis at parturition and at dry off (Shuster, Lee et al. 1996; Jayarao, Gillespie et al. 1999). Increased levels of glucocorticoids, especially cortisol at the time of parturition has been associated with altered neutrophil function, such as decreased ROS production and CD62L expression (Mehrzad, Dosogne et al. 2001; Madsen, Weber et al. 2002). Surprisingly, neutrophils at parturition have higher survival regulated by increases in glucocorticoids which decrease expression of pro-apoptotic genes (FADD, BAK) and increase expression of anti-apoptotic genes (IL8, BAFF) (Burton, Madsen et al. 2005). Alteration in neutrophil function is likely a main cause of susceptibility around parturition. At the time of dry off, cessation of milking leads to an engorged mammary gland, which results in milk leaking from teats and breaking down of intracellular junctions between MECs (Stelwagen, Farr et al. 1997). Milk leaking indicates the teat end is open, which increases the risk of bacteria entering the gland. Once bacteria are inside the gland, milk is not being removed by daily milking, so bacteria are able to stay in the gland. Macrophages and neutrophils within the gland also have decreased phagocytic ability following dry off, possibly due to increased ingestion of apoptotic MECs (Paape, Miller et al. 1992).

Genetic selection for mastitis resistance

The idea of selective breeding is to manipulate genetics of a population to produce desired phenotypes, or traits. Heritability measures the degree by which genetic variation reflects phenotypic variation. Since mastitis is the most costly disease in the dairy industry, producers want to select for cows with mastitis resistance. Genetic selection against mastitis can happen indirectly, by the producer culling cows which are more susceptible, or directly by selecting for traits genetically correlated to decreased mastitis. Direct genetic selection can focus on specific genes associated with favorable traits (oligenic selection) or use a large number of genes, each with small additive effects to select for favorable traits (polygenic selection) (Detilleux 2009). Recent advances that will advance both types of genetic selection include sequencing of the bovine genome, discovery of thousands of DNA markers in the form of single nucleotide polymorphisms (SNPs) and decreased costs of genotyping. A SNP is a change in one base pair between paired chromosomes in an animal or within a species.

One polygenic selection process called genomic selection involves selecting cows based on genomic breeding values (GEBV) (Hayes, Bowman et al. 2009). To calculate GEBV, the entire genome is divided into small segments and effects of each segment are estimated in a reference population of animals which have been genotyped and phenotyped. Cows in following generations can be genotyped for specific markers to determine which chromosome segments they carry. GEBV is then predicted by summing the estimated effects of the segments the animal carries across the whole genome.

One of the more obvious traits to select for in polygenic selection is lower occurrence of clinical mastitis. However, clinical mastitis has on average low heritability (3%-10%), likely due to different units of observation (cow vs quarter), dependence on veterinarian or producer diagnosis, and record keeping (Zwald, Weigel et al. 2006). Heritability estimates for clinical mastitis does increase in Norway, Denmark and Sweden, where records of all veterinary treatments since the early 80's are required and lead to more accurate estimates (Heringstad, Klemetsdal et al. 2007). Clinical mastitis can be identified by bacteriological analysis, but it is not available on a large scale due to labor and price. SCC is a common trait for indicating mastitis and many producers are now genetically selecting for sires which produce cows with lower SCCs. The genetic correlation between SCC and clinical mastitis is between 50 and 80% averaging around 78% (Heringstad, Gianola et al. 2006). More records are available on SCC, due to monthly collection and reports and heritability is 10-15%. However there are some limitations to using SCC for reducing mastitis susceptibility, including if SCC are only collected once monthly, a cow could have been infected with a bacteria such as *E. coli* and eliminated the infection in between sampling days. In a few studies, SCC was higher in cows which resisted challenge with *S. aureus* than those which did not (Piccinini, Bronzo et al. 1999; Beaudeau, Fourichon et al. 2002). Other studies found that neutrophils from cows with lower EBV for SCC had greater functional ability around calving, and higher numbers of circulating mononuclear cells (Kelm, Detilleux et al. 1997). A recent study suggests that selecting for low average SCC alone is not as accurate as selecting for multiple traits describing SCC and mammary gland health (de

Haas, Ouweltjes et al. 2008). Three groups of alternative SCC traits include: SCC traits defined on the basis of lactation stage, occurrence of excessive SCC, and SCC traits on the basis of patterns in peak SCC. Since clinical and subclinical infections are commonly correlated with different SCC traits, having a combination of different traits may be more effective at selecting against both clinical and subclinical infections rather than grouping infections together. A recent study used several alternative SCC traits to examine which ones would be the best to genetically reduce clinical and subclinical mastitis (Windig, Ouweltjes et al. 2010). SCC factors which were strongly correlated with clinical and subclinical mastitis, including SCC in early and late lactation, suspicion of infection based on increased SCC, extent of increased SCC, and presence of a peak pattern in SCC.

Aside from SCC and clinical mastitis, there are other traits which may be useful in selecting against mastitis. Moderate heritability for neutrophil phagocytosis (30-70%), migration (20-50%), and serum complement activity (40-50%) were observed (Detilleux, Koehler et al. 1994). Fore udder attachment, udder depth, teat length, and milking speed have also been correlated with mastitis and are currently used in selection. Higher, more tightly attached udders as well as slower milking times are associated with lower SCC and less clinical mastitis (Boettcher, Dekkers et al. 1998).

One advantage of genetically selecting for cows that are more resistant to mastitis is that since their immune response is likely better, they may be more resistant to other common infections, such as metritis. One major limitation of genomic selection is having good phenotypic information from a large number of animals. Another

limitation is that a separate system must be set up for each population, including different breeds of cattle due to different SNPs.

Genetic markers for mastitis susceptibility

In addition to genomic selection, which selects for individual traits over a large number of genes, identifying markers or SNPs in specific genes associated with the immune response is another way to genetically select for mastitis resistance. Markers in genes for cytokines and their receptors in addition to proteins involved in immune cell function have been investigated. A recent study identified SNPs in IL10, the IL10 receptor, TGF-β and the TGF-β receptor and found that SNPs in the IL10 receptor were correlated to estimated breeding values (EBV) for SCC in bulls (Verschoor, Pant et al. 2009). Another study found SNPs in TLR4 to be correlated with EBV for SCC in bulls (Sharma, Leyva et al. 2006). Because the binding of bacteria to TLR4 is one of the first steps in initiating an immune response and IL10 is associated with down-regulating the immune response at the end of an infection to prevent excess tissue damage, these SNPs may be suitable markers to include in genetic selection.

Aside from testing genotypes of bulls for correlations with EBV values, another way to study SNPs is through the cows themselves. One such study genotyped each cow in the population directly and identified a polymorphism in the CXCR1 gene, a receptor for the chemoattractant IL8, associated with increased susceptibility to subclinical mastitis (Youngerman, Saxton et al. 2004). In Youngerman's study, cows with a CC genotype at position +777 on CXCR1 had a higher incidence of bacterial

intramammary infection and a lower SCC compared to cows with a GG or GC genotype at this position.

A series of studies performed by Rambeaud compared neutrophil functions among +777 CXCR1 genotypes. Cows with a CC genotype had lower migration, adhesion molecule expression, intracellular calcium release, and reactive oxygen species production (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Rambeaud and Pighetti 2007). Surprisingly, cows with a CC genotype had increased survivability compared to the other genotypes. This pattern is similar to neutrophils around parturition which have decreased functional abilities and increased survivability. Because cows are more susceptible to mastitis around parturition and cows with a CC genotype are more susceptible to mastitis, there could be a common intracellular signaling pathway in both populations. Future studies of the CXCR1SNP will increase understanding of why mastitis susceptibility differs among genotypes and will be aimed at specific migration patterns and inflammatory response following in vivo challenge.

CONCLUSIONS

Mastitis is one of the largest challenges facing dairy producers and increased knowledge of why certain cows are more susceptible to mastitis is needed to decrease the prevalence and severity of this costly disease. Anatomical, cellular, and humoral immunity combined with bacterial recognition are necessary for effective disease resistance and any impairment in these factors could increase disease susceptibility.

One aspect in particular, neutrophil migration into the mammary gland is necessary for resolution of mastitis (Hill 1981). Deficiencies in certain factors associated with neutrophil migration such as actin polymerization and polarization cause impaired directed migration. Because differences in subclinical mastitis and neutrophil migration have been observed in cows with different CXCR1+777 genotypes, the current study is aimed at identifying the cause of decreased migration and validating this model by comparing the immune response following intramammary challenge with *Streptococcus uberis* among genotypes. The hypothesis for the current studies is that neutrophil migration patterns differ between genotypes and may contribute to increased intramammary infection when challenged with *S. uberis*.

CHAPTER 3

COWS GENETICALLY MORE SUSCEPTIBLE TO MASTITIS HAVE ALTERED NEUTROPHIL MIGRATION PATTERNS

This chapter is a paper by the same name that will be submitted for publication.

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper include: i) design of experiment, ii) animal selection and sample collection, iii) Cell isolation and most of the labwork, iv) most of the data analysis, v) most of the gathering and interpreting of the literature, vi) most of the writing of this paper.

Abstract

The largest loss in profit for dairy farmers occurs with mastitis, an inflammation of the mammary gland. Our prior research identified a marker in the CXCR1 gene associated with mastitis and decreased neutrophil migration in vitro. Because neutrophil migration is critical for eliminating most infections, our ongoing research seeks to identify the specific mechanisms causing impaired migration. The first study evaluated actin polymerization, one of the first steps in neutrophil migration, in cows with different CXCR1+777 genotypes. Neutrophils from cows with GG (n=11) and CC (n=11) genotypes were isolated and stimulated with zymosan activated sera (ZAS). Cells were fixed and stained for F-actin and subsequently evaluated for F-actin content, distribution, and cell morphology. Neutrophils from cows with the CC genotype had significantly lower F-actin polymerization than cows with the GG genotype (P=0.05). Because F-actin polymerization drives neutrophil movement, lower amounts could partially explain reduced migration. In contrast, cell morphology and F-actin distribution was similar between genotypes. Our second study focused on directed migration of

neutrophils towards interleukin-8 (IL8). Migration of neutrophils from GG (n=5) and CC(n=5) genotypes was captured by microscopy and velocity, acceleration, distance of the path, distance from origin, largest X distance and largest Y distance were analyzed for individual cells. Neutrophils from cows with the GG genotype traveled further on an X axis and had higher X vs Y movement compared to cells from cows with the CC genotype, meaning they moved more directly towards IL8. Our findings suggest lower F-actin polymerization in combination with a lower ability to directly and efficiently move towards the site of infection could impair neutrophil response to infection in cows with the CC genetic background and may contribute to increased mastitis susceptibility.

Keywords: F-actin, immunity, chemotaxis, neutrophil, CXCR1

Introduction

Mastitis, an inflammation of the mammary gland, accounts for the largest loss in profit for dairy farmers (NMC 1996). Inflammation occurs when bacteria enter the mammary gland and interact with host cells resulting in release of inflammatory mediators which guide immune cells towards the site of infection. The first cells to show up in great number are neutrophils, attracted by chemoattractants such as interleukin 8 (IL8) and complement C5a (Bannerman 2009). The ability of neutrophils to migrate into the mammary gland is required for resolution of mastitis (Hill 1981). In order to clear the infection, neutrophils need to move directly and efficiently from blood into mammary tissue.

Neutrophils know which direction to travel by following chemotactic gradients of increasing concentration (Zhelev, Alteraifi et al. 2004; Heit, Robbins et al. 2008). As they move towards the site of infection, neutrophils first encounter early-target chemoattractants such as IL8, which guides them towards the general infected area and then encounter end-target chemoattractants closer to the site of infection such as C5a which guide them to the specific infection site (Heit, Robbins et al. 2008). Chemoattractants bind to specific receptors on neutrophils and initiate intracellular signaling which leads to reorganization of organelles and structural components of the cell necessary for cell movement. One main class of receptors for chemoattractants are G-protein coupled receptors (GPCR), which consist of Gα and Gβ/Gγ subunits. Once activated, the Gβ/Gγ subunit activates phosphatidyl inositol 3 kinase (PI3K) and phospholipase C (Zarbock and Ley 2008). PI3K activation causes pH-domain containing proteins (PKB, CRAC, phdA) to migrate to the leading edge, where the chemoattractant concentration is the greatest. These proteins have many different functions, including sensing direction and activating nucleation factors leading to polymerization of F-actin at the leading edge of the cell (Chung and Firtel 2002). F-actin polymerization is required for formation of lamellipodia and filopodia which move the cell forward (Watts, Crispens et al. 1991; Koestler, Auinger et al. 2008). One nucleation factor involved in the branching of F-actin filaments is Actin related protein (ARP 2/3) complex, which consists of two main proteins (ARP2 and ARP3) and five subunit proteins. ARP 2/3 attaches to the side of pre-existing F-actin filaments at a 70° angle

and forms new filaments, leading to branched F-actin (Welch 1999; Pollard and Borisy 2003).

A cow could be more susceptible to mastitis if neutrophil migration into the mammary gland is impaired. Our prior research identified a polymorphism at position +777 in the CXCR1 gene, a receptor for the chemoattractant IL8. This polymorphism has been associated with an increased susceptibility to mastitis and decreased neutrophil migration in vitro (Youngerman, Saxton et al. 2004; Rambeaud and Pighetti 2005). Because neutrophil migration is critical for eliminating most infections, our ongoing research seeks to identify specific mechanisms causing impaired migration in cows with different CXCR1 genotypes. Chemoattractant sensing followed by activation of intracellular signaling pathways leading to F-actin polymerization and cell migration are all necessary for the neutrophil to reach the site of infection. If any step in this process is not functioning correctly, neutrophils will not be able to migrate effectively, possibly leading to increased susceptibility to infection. This study tested this hypothesis by evaluating actin polymerization, cell morphology, protein expression, and directed migration in cows with different CXCR1+777 genotypes.

Materials and methods

Animal selection

Ten Holstein heifers and 22 cows located at the East Tennessee Research and Education Center were used in this study. Heifers and cows were paired based on CXCR1 genotypes, determined by PCR amplification and sequencing at The University of Tennessee molecular biology core facility (Youngerman, Saxton et al. 2004). Cows were additionally paired on age and stage of lactation. All cows were free from clinical mastitis and milk samples were collected immediately prior to blood collection to determine the SCC, which was performed with a Somacount 300 cell counter (Bentley Instruments, Chaska, MN) by the Dairy Herd Improvement Association (DHIA) laboratory at The University of Tennessee, Knoxville, TN. All animal experimentation was approved by The University of Tennessee Institutional Animal Care and Use Committee. Prior to neutrophil isolation, an aliquot of blood was removed for determination of red and white blood cell counts using an automated cell counter (Vet Count IIIB; Mallinckrodt, Phillipsburg, NJ, USA). Cows with SCCs >200,000 cells/ml and white blood cell counts >12,000 were not used. In addition, smears were obtained to determine leukocyte differential counts. Neutrophil purity after isolation was greater than 97%.

F-actin quantitiation

Neutrophils from cows with CXCR1+777 GG (n=11) and CC (n=11) genotypes were isolated from whole blood as described previously (Rambeaud and Pighetti 2005).

Neutrophils were stimulated with 5% zymosan activated sera (ZAS) or Hank's Balanced Salt Solution (HBSS; pH 7.2; Cellgro, Herndon, VA) for 0, 15, 30, 60, 90, 120, and 180 sec and fixed with 3.7% paraformaldehyde (Sigma, St. Louis, MO, USA). Cells then were permeabilized with 2% saponin. F-actin in the cells was stained using 1.5 U of alexa fluor 488-phaolloidin conjugate (Invitrogen, Carlsbad, CA, USA) and cells were subsequently evaluated for F-actin content, distribution, and cell morphology. F-actin content was quantified by mean fluorescent intensity of F-actin stain using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). A total of 10,000 cells were counted for each cow and time point.

Cell morphology

Neutrophils at time points 0, 30, and 90 sec from the F-actin assay were given a score from 1 to 4 based on F-actin distribution and cell morphology. Cells receiving a score of 1 were perfectly round, with even F-actin distribution throughout the cell; those receiving a score of 2 had F-actin clumping and a slightly rough plasma membrane; cells with a score of 3 had F-actin clumping around the edges of the cell and moderate roughness of the plasma membrane; cells receiving a score of 4 had all F-actin located along edges of the cell and marked ruffled outer edges (Fig 2). A total of 100 neutrophils were scored for each cow and time point.

Figure 2

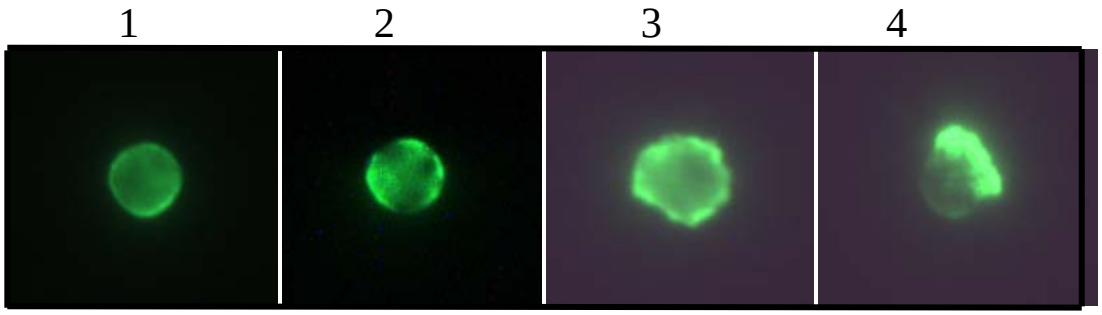


Figure 2. Cell morphology and F-actin distribution after stimulation with 5% Zymosan Activated Serum. Neutrophils were fixed with 3.7% paraformaldehyde and F-actin was stained with alexa fluor 488 phalloidin. A cell score of 1 represents a non-activated cell, 2 = slightly polarized, 3 = moderately polarized, 4 = fully polarized.

Western blotting

A total of $1.5x10^7$ neutrophils were pelleted and lysed with Mammalian Protein Extraction Reagent containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). Stimulated cells were incubated with 5% ZAS for 30 min before lysis. Proteins (30 µg/well) were separated on a 12% SDS page gel. Each gel contained samples from stimulated and unstimulated cells from one pair of GG and CC genotype cows (n=10GG, 10CC). The page gel was transferred to a Polyvinylidene fluoride (PVDF) membrane and stained for 5 min with Ponceau stain (Roche, Indianapolis, IN, USA) to ensure even protein loading. The membrane was blocked for 1 hr in 10% blocking buffer (Roche, Indianapolis, IN, USA) followed by a 2 hour incubation with mouse anti–human ARP2 primary antibody (Santa Cruz, Santa Cruz, CA, USA) diluted 1:300 in Tris-buffered saline tween-20 (TBST) with 5% blocking buffer. Goat anti-mouse secondary antibody horseradish peroxidase conjugate (Pierce Biotechnology, Rockford, IL, USA) was diluted 1:5,000 in TBST with 5% blocking buffer and incubated with the membrane for 1 hr. Membranes were then incubated in West Dura chemilluminescent substrate for 5 min at room temperature. Chemilluminescence was detected using x-ray film and band intensity was determined by spot densitometry.

Directed cell migration under agarose

Neutrophils from heifers with GG (n=5) and CC (n=5) genotypes were isolated, resuspended at 1 x 10^7 cells/ml in Hanks Balanced Salt Solution (HBSS) and stained

with either SYTO 64 red fluorescent stain, or SYTO16 green fluorescent stain (Invitrogen, Carlsbad, CA, USA)), with one color assigned to each genotype. Delta T dishes (Bioptechs, Butler, PA, USA) were coated with 0.5mg/ml collagen (Roche, Basel, Switzerland) and filled with 2ml of 1% agarose (Cambrex, Rutherford, NJ, USA) dissolved in RPMI media and 10% Fetal Bovine Serum (FBS). Three wells of 3mm diameter were cut into the 1% agarose gel 2mm apart (Figure 3) (Heit and Kubes 2003). Ten µl of bovine IL8 with a concentration of 100 µg/ml (Kingfisher, St. Paul, MN, USA) were placed in the far right well and 10 µl of HBSS + 2mM Ca + 2mM Mg + 0.5% BSA were placed in the far left well. Ten µl of solution containing $1x10^5$ total neutrophils from each genotype were added to the center well and a glass cover slip was placed over all three wells. Plates were incubated for 45 min in a 37ºC incubator and then moved to a Nikon microscope with a delta T heated microscope stage adaptor set at 37ºC. Cells were imaged on a 40x objective and pictures were taken every 15 sec for 30 min. Fluorescent images of cells were taken at 0 and at 30 min (Figure 4). Red fluorescence was imaged with a TRITC filter and green fluorescence was imaged using a FITC filter. After 30 min of imaging, a wide fluorescent image was captured showing all cells which migrated away from the center well. Concentric rings were drawn around the well at distances of 0-600 µm and 600-1,200 µm away from the well. A vertical line was drawn down the center of the well to separate the IL8 side from the HBSS side. The number of cells from each genotype which traveled from the edge of the well 0-600 µm and 600-1,200 µm were counted.

Figure 3.

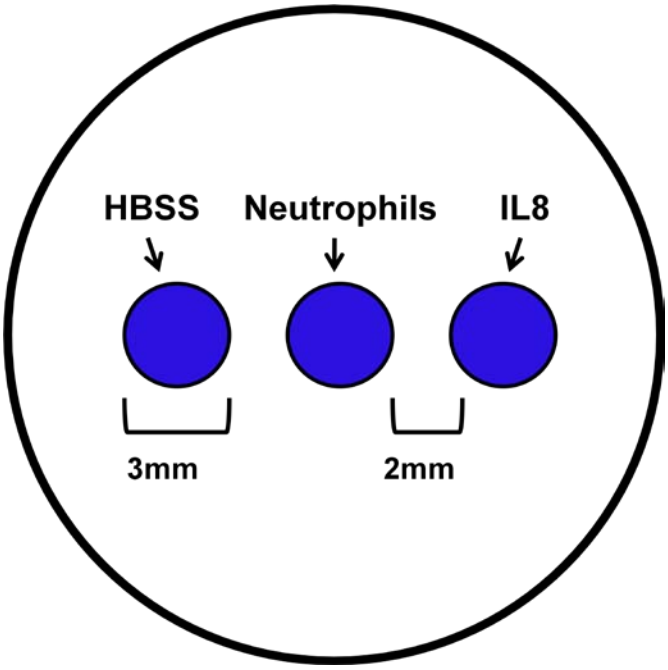


Figure 3. Plate setup. Three wells 3mm in diameter were cut into 1% agarose 2mm apart. Ten µl of HBSS was placed in one outer well and 10 µl of IL8 was placed in the other outer well. Ten µl containing $1x10^5$ total neutrophils from each genotype was placed in the center well.

Figure 4

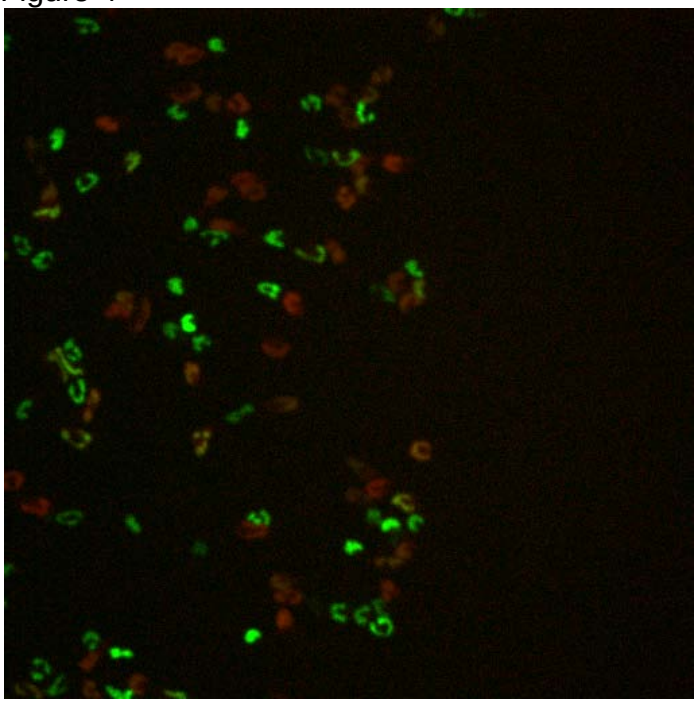


Figure 4. Isolated neutrophils were stained with either green or red SYTO live-cell stain, based on their CXCR1 +777 genotypes (GG or CC). Before time lapse photography began, one fluorescent image was captured to differentiate between genotypes for cell tracking.

Cell tracking was performed using Nikon viewer software and included 5 to 10 active cells from each genotype. Tracking data included individual cell velocity, acceleration, distance of the cell path, displacement, largest X (horizontal) distance the cell moved, and largest Y (vertical) distance the cell moved. Only cells with continuous movement, without running into other cells, were used in tracking measurements. Tracking was performed for 16 consecutive time points/images. One pair of heifers was used per day (GG=1, CC=1). Each pair of heifers was used on two separate days and was assigned a different dye color each day to test dye effect on migration ability.

Statistical Analysis

Analysis of variance was completed with mixed models using SAS software (SAS 9.1; SAS Institute Inc., Cary, NC, USA). To determine the effect of CXCR1 +777 genotype on F-actin content over time, a repeated measures design was used, with each day separated into a block. For cell morphology, genotypes were compared by the number of cells in each rating. To determine the effect of CXCR1 +777 genotype on average cell morphology, F-actin distribution, and ARP2 expression at 0 and 30 min, a randomized block design, blocked on day was used. To determine the effect of CXCR1 +777 genotype on neutrophil velocity, acceleration, pathlength, displacement, largest x distance, largest y distance, x displacement, and y displacement, a randomized block design blocked on day with a split plot of dye and sampling was used. Statistical

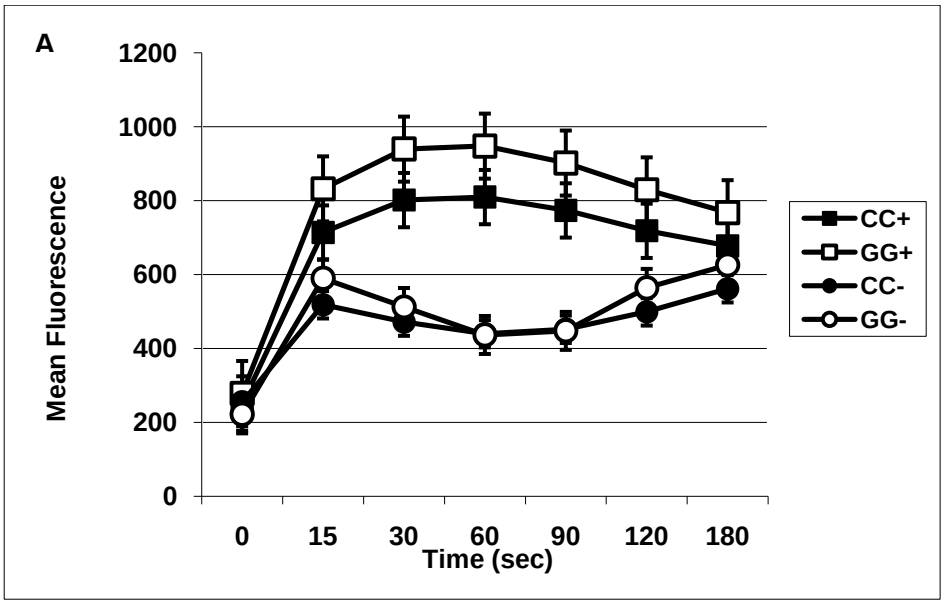
significance of genotype was declared at a *P* level of ≤ 0.05 and a trend towards significance was declared at a P level of ≤ 0.1.

Results

Because F-actin polymerization at the leading edge of the neutrophil is one of the first steps in migration, we compared F-actin polymerization between genotypes. Neutrophils from cows with a CC genotype had lower F-actin polymerization over time (Figure 5a) and significantly lower average F-actin polymerization than neutrophils from cows with the GG ($P \leq 0.05$) after stimulation with ZAS (GG=786 ± 65, CC=678 ± 65) (Figure 5b). F-actin content was similar between CC and GG genotypes after treatment with the control.

Once stimulated, F-actin polymerizes at the neutrophil leading edge and causes plasma membrane ruffling. To assess F-actin distribution and cell morphology among GG and CC genotypes, cells were scored 1 (unactivated) through 4 (highly activated) (Table 1). Cell morphology and F-actin distribution scores were similar among GG and CC genotypes at all time points, suggesting that resting cells had similar F-actin patterns and following stimulation the ability of F-actin to accumulate at cell edges was not impaired.

Figure 5.



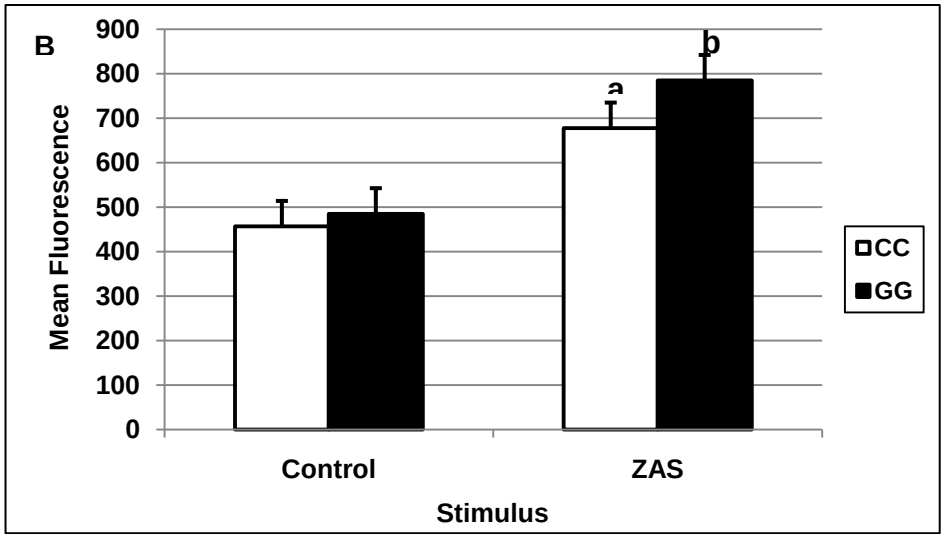


Figure 5. Amount of F-actin in cows with different CXCR1 genotypes (GG=solid symbols, CC=open symbols) a) from 0 to 180 sec after addition of 5% Zymosan Activated Serum (ZAS) (squares)(+) or Hanks Balanced Salt Solution (HBSS) (circles) (-) (genotype P≤0.05; ti me P≤0.05; genotype*time P≥0.05). b) Average amount of F-actin content among cells stimulated with HBSS or 5% ZAS (P<0.05). Results are expressed as mean fluorescent intensity with data reported as least square means ± standard errors of means.

Table 1. Percent of neutrophils with scores based on neutrophil morphology and F-actin distribution after stimulation with ZAS

Time	Genotype	Resting cell Score: 1 % ± SE		2 % ± SE		3 % ± SE		Completely Polarized 4 % ± SE	
0 sec	GG	**39**	3	**59**	3	**2**	2	**0**	
	CC	**36**	3	**62**	3	**2**	2	**0**	
30 sec	GG	**23**	3	**51**	3	**24**	2	**2**	0.7
	CC	**22**	3	**49**	3	**27**	2	**1**	0.7
90 sec	GG	**6**	3	**31**	3	**40**	2	**22**	2
	CC	**7**	3	**30**	3	**43**	2	**19**	2
P value	Genotype	0.63		0.97		0.21		0.61	
	Time	<0.0001		<0.0001		<0.0001		<0.0001	
	Genotype*Time	0.73		0.67		0.78		0.83	

Numerous proteins including the ARP2/3 complex are involved in F-actin polymerization at the leading edge, resulting in membrane ruffling and the formation of lamellipodia. Western blots were used to assess the amount of ARP2 protein present in un-stimulated neutrophils and neutrophils stimulated for 30 min with ZAS (Figure 6). The amount of ARP2 appeared to be similar among genotypes and among stimulated versus unstimulated cells. Measurement of band density was used to confirm this observation. A ratio comparing band density of unstimulated cells and stimulated cells within a genotype confirmed that the level of ARP2 was similar between genotypes (ratio ± SE; GG=0.94 ± 0.05, CC=0.88 ± 0.05; $P > 0.05$).

Because the ability of neutrophils to directly follow a chemoattractant gradient is crucial for quick infiltration into the mammary gland, the number of neutrophils migrating towards IL8 and HBSS was assessed (Table 2). A similar number of neutrophils from GG and CC genotypes moved towards IL8 within 600 µm from the starting well (GG=266 ± 39 cells, CC=228 ± 49 cells; $P>0.05$) and 600-1,200 µm from the starting well (GG=120 ± 22 cells, CC=76 ± 22 cells; $P>0.05$). On the control side of the well, there were also equal numbers of GG and CC genotype neutrophils within 600 µm (GG=164 ± 46 cells, CC=131 ± 46 cells; $P>0.05$) and 600-1,200 µm away from the starting well (GG=51 ± 25 cells, CC=53 ± 25 cells; $P>0.05$). Additionally, the ratio of the number of cells migrating towards IL8 to the number migrating towards HBSS 600-1,200µm away from the well was similar between GG and CC genotypes (GG=4.4 ± 1.4, CC=5.0 ± 1.4) ($P>0.05$).

Fig. 6

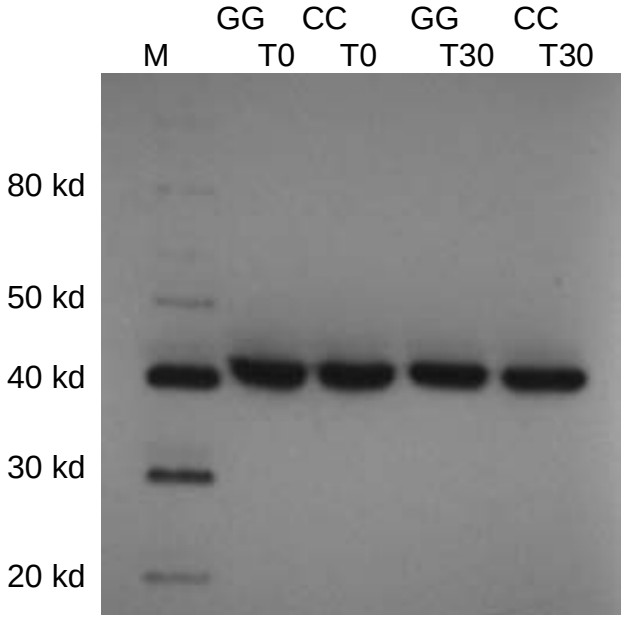


Figure 6. Western blot of ARP2 protein. Neutrophils from each genotype (GG=10, CC=10) were either unstimulated (T0) or stimulated for 30 min with 5% Zymosan Activated Serum (T30). Proteins from whole cell lysates (30 µg/well) were separated on a 12% SDS PAGE gel. Mouse anti-human ARP2 1:300 primary antibody, and goat anti mouse HP conjugate 1:5,000 secondary antibody were used.

Table 2. Number of neutrophils traveling away from the origin

Distance from center well	IL8			Control		
	GG	*CC*	*P value=*	*GG*	*CC*	*P value=*
0-600 µm	**266** ± 49	**228** ± 49	0.44	**164** ± 46	**131** ± 46	0.24
600-1200 µm	**120** ± 22	**76** ± 22	0.16	**51** ± 25	**53** ± 25	0.94

To assess the movement of individual neutrophils, a tracking program was used on 5 - 10 active cells from each genotype and migration data were collected (Table 3). Average neutrophil velocity and acceleration were used to assess neutrophil speed and were similar between CC and GG genotypes. Pathlength and displacement were used to measure the amount of cell movement regardless of direction. Total pathlength measured the entire path the cell traveled, while displacement measured from the starting position to the ending position, regardless of where the cell traveled in between these two points. Cells from cows with the CC genotype had a similar pathlength and displacement compared to cells from cows with the GG genotype when migrating towards IL8 or HBSS. A straight-line ratio (displacement / pathlength) was used to assess the ability of neutrophils to move linearly, with a ratio of one representing a perfectly straight line. Interestingly, CC genotype neutrophils had a lower straight-line ratio than GG genotype neutrophils when moving towards HBSS (GG=0.76 ± 0.03 µm, CC=0.67 ± 0.04 µm; P=0.06). When moving towards IL8, straight line ratios of GG genotype neutrophils remained the same as when moving towards HBSS and CC ratios increased to similar levels as GG genotype cells (GG=0.76 ± 0.03 µm, CC=0.73 ± 0.03 µm; P>0.1). One limitation of the straight-line ratio is that it does not describe the directionality of the cell's path - only how straight the path is.

Table 3. Neutrophil tracking between genotypes

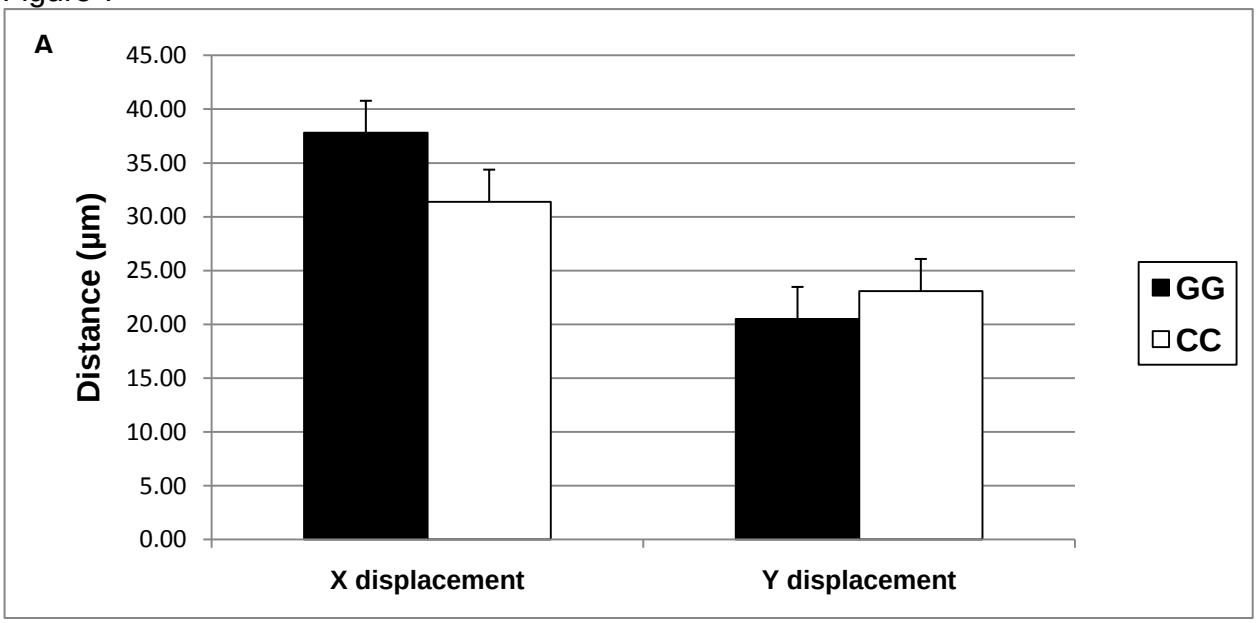
Treatment	Genotype	Velocity (nm/s) (±SE)	Acceleration (nm/s^2)	Pathlength (µm)	Displacement (µm)	Displacement / pathlength
IL8	GG	**270** ± 10	**1.0** ± 0.1	**60.8** ± 5	**45.6** ± 4	**0.76** ± 0.03
	CC	**260** ± 10	**1.5** ± 0.1	**57.4** ± 5	**40.9** ± 4	**0.73** ± 0.03
P value		**.53**	**0.61**	**0.31**	**0.18**	**0.40**
HBSS	GG	**240** ± 20	**1.0** ± 0.5	**51.5** ± 5	**36.4** ± 5	**0.76** ± 0.03
	CC	**240** ± 20	**1.0** ± 1.7	**51.1** ± 5	**34.6** ± 5	**0.67** ± 0.04
P value		**0.98**	**0.97**	**0.93**	**0.40**	**0.06**

Because directed movement is essential for neutrophils to move towards the site of infection, movement of cells on an X-Y axis was analyzed, with the X axis being the linear path from the center well to the IL8 or HBSS well, and Y being the vertical line perpendicular to X. The maximum amount of X and Y movement throughout all time points and the X-Y displacement from starting to ending position were measured. Neutrophils from cows with the CC genotype did not move as well towards IL8 as neutrophils from cows with the GG genotype, having a lower total X distance (GG=39.1 ± 3 µm, CC = 33.5 ± 2.6 µm; P= 0.08) and a lower X/Y ratio (GG=2.13 ± .24, CC=1.66 ± .21; P ≤ 0.05). Similarly, displacement on the X axis (GG= 37.8 ± 3 µm, CC=31.4 ± 3 µm; Figure 7a) and ratio of X/Y displacement (GG=2.38 ± 0.27, CC=1.64 ± 0.22; P≤0.1; Figure 7b) was also lower in the CC genotype In contrast, the vertical distance or total distance and displacement on the Y axis was similar between genotypes.

Discussion

Previous studies identified a genetic marker in the CXCR1 gene associated with increased susceptibility to mastitis and decreased neutrophil migration. Because efficient neutrophil migration into the mammary gland is essential for resolution of mastitis, we hypothesized that certain mechanisms associated with neutrophil migration would be altered in cows with genotypes more susceptible to mastitis.

Figure 7



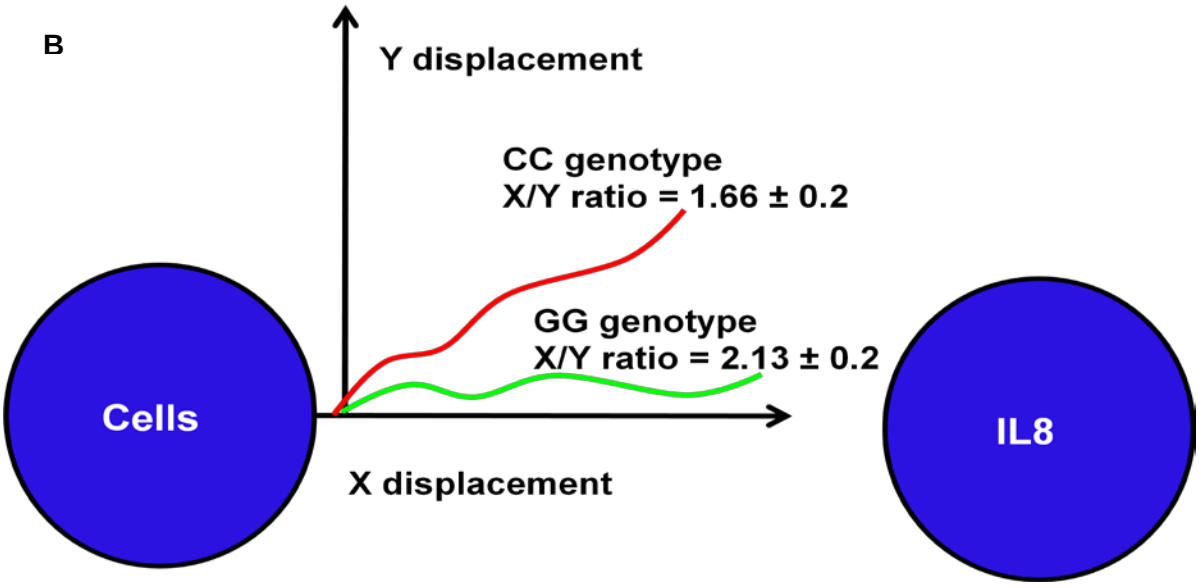


Figure 7. Neutrophil directional movement towards IL8 was measured through X and Y displacement values obtained through a cell tracking program. a) Displacement in a straight line from the starting well to the IL8 well was measured on the X axis and perpendicular movement was measured on the Y axis (left graph axis) for GG genotype (black bars) and CC genotype (white bars) (P≤ 0.1). b) The ratio between X and Y movement (right graph axis) was calculated for GG and CC genotypes (P≤ 0.1).

Neutrophil migration begins with a chemoattractant binding to its specific receptor followed by intracellular signaling. This intracellular signaling is responsible for activities such as expression of adhesion molecules and initiation of F-actin polymerization (Chung, Funamoto et al. 2001; Le Clainche and Carlier 2008). F-actin polymerization is the driving force at the leading edge of llamelipodia and is necessary for neutrophil migration (Pollard and Borisy 2003; Stricker, Falzone et al. 2010). In this study the overall amount of F- actin polymerized was lower in neutrophils from cows with the CC genotype compared to cows with the GG genotype when stimulated with ZAS. Within the non-stimulated control cells, similar levels were observed between genotypes. This suggests that resting neutrophils have similar amounts of F-actin between genotypes, but after stimulation, neutrophils from cows with the CC genotype are not able to polymerize as much F-actin as those from the GG genetic background.

F-actin polymerization is stimulated and regulated by intracellular signaling involving a wide range of proteins, including the ARP 2/3 complex. Polymerizing proteins such as the ARP 2/3 complex help actin monomers attach to F-actin filaments at the leading edge of an activated cell (Pollard and Borisy 2003). Previous studies have found that the absence of ARP2/3 can decrease the amount of F-actin polymerized and subsequently alter morphology and migration (Akin and Mullins 2008). However, in this study, similar amounts of ARP2 were observed in stimulated and unstimulated neutrophils from CC and GG genotypes, suggesting ARP2 is not altered in the CC genotype and decreased F-actin polymerization is due to a different protein or

mechanism. The ARP2/3 complex contains 6 subunits other than ARP2 which are needed for the complex to be fully functional (Balcer, Daugherty-Clarke et al. 2010). One of the other components may be limited in the CC genotype and influence complex formation. Additionally, certain proteins such as Wiscott Aldrich Syndrome Protein are directly involved in attaching ARP2/3 complex to the side of an F-actin filament and represent another possibility for altered actin polymerization (Millard, Sharp et al. 2004). This defect occurs in patients with Wiscott Aldrich Syndrome and decreases the ability of neutrophils and other immune cells to polarize and migrate (Snapper and Rosen 1999).

Even though the overall amount of F-actin differed between genotypes in ZAS stimulated neutrophils, morphology and F-actin distribution was similar between genotypes in stimulated and unstimulated cells. This suggests that after activation, neutrophils from cows with the CC genotype were equally able to direct F-actin to the cell edge and cause an activated morphology, despite not having as much F-actin as GG genotype neutrophils. Similar polarization also supports the similar number of cells which moved outside the center well observed between genotypes because polarity is necessary for efficient chemotaxis (Weiner 2002). In an adherent neutrophil moving towards a chemoattractant, F-actin localizes to one side of the cell where chemoattractant is highest (the leading edge) and myosin localizes at the sides and tail of the cell to prevent formation of more than one leading edge (Sanchez-Madrid and del Pozo 1999). One limitation of the morphology assay used in this study is the

neutrophils were suspended in a solution of uniform chemoattractant instead of being adherent and able to polarize towards a chemoattractant gradient. Therefore, it is unknown whether cells would have formed a single leading edge and adherent neutrophils which lack a leading edge and have F-actin around the entire edge of the cell do not migrate effectively towards an increasing gradient of chemoattractant (Wang, Herzmark et al. 2002). Future studies involving F-actin distribution and morphology in adherent neutrophils randomly migrating and moving towards a chemoattractant would be useful to explain migration differences between CXCR1 genotypes.

Because F-actin is the driving mechanical force behind cell movement, lower F-actin in cells from cows with the CC genotype could result in cells not moving as fast or as far as the GG genotype. However when individual neutrophils were tracked, velocity, acceleration, and the overall distance which neutrophils traveled, measured by pathlength and displacement were similar between genotypes. This suggests that the decrease in F-actin did not have a significant impact on the ability of neutrophils to migrate non-directionally. Decreased F-actin in CC genotype neutrophils was observed up to 3 min after stimulation while directed migration was studied after 30 min of stimulation, suggesting that decreased F-actin polymerization in CC genotype neutrophils could be an initial phenomenon and the F-actin in these cells could increase to an equal amount as GG genotype neutrophils over time. One other possibility is that even though CC genotype neutrophils had lower F-actin, the level was high enough to sustain cellular functions.

While assembly and disassembly of actin filaments is involved in cell motility, directed migration is dependent on gradient sensing and cell polarity (Janetopoulos and Firtel 2008). The ability of neutrophils to travel directly towards an increasing chemoattractant gradient without wasted movement is essential for their quick infiltration to the site of infection. Neutrophils from cows with the CC genotype had a lower total X distance and displacement directly towards an increasing concentration of IL8. Since path-length and overall displacement were similar between genotypes, this suggests that CC genotype neutrophils had more wasted movement on the Y axis. This is supported by the lower X/Y ratio observed in CC genotype cells, meaning they had less X axis movement and more Y axis movement towards IL8. The X/Y ratio and X axis movement was similar between genotypes on the HBSS side of the well (data not reported), suggesting it is a pattern observed in CC genotype neutrophils undergoing chemotaxis. A deficiency in migration towards chemoattractant in patients with rheumatoid arthritis has been associated with increased bacterial infections in these patients (Aglas, Hermann et al. 1998). This suggests that abnormal directed migration in CC genotype neutrophils could affect increased mastitis observed in this genotype.

Directed migration begins with the chemoattractant binding to a receptor on the cell surface. If a SNP in the CXCR1 receptor causes a change in conformation, receptor affinity for IL8 could decrease, decreasing the ability of neutrophils to sense the chemoattractant. A previous study found that neutrophils from cows with a CC genotype most likely had CXCR1 receptors with lower affinity for IL8, however detection

was not CXCR1 specific, so they were unable to rule out a decrease in the actual number of receptors (Rambeaud and Pighetti 2007). Following binding of chemoattractant, the receptor undergoes a conformational change which releases Gα and Gβγ proteins bound to the receptor. These proteins activate PI3K at the leading edge of the cell where the chemoattractant is highest in concentration (Hannigan, Huang et al. 2004). PI3K locally phosphorylates PIP2 into PIP3, which causes small GTPases such as Rac and PH domain containing proteins to localize at the leading edge of the cell (Wang, Herzmark et al. 2002). PH domain containing proteins activate and cause F-actin polymerizing proteins such as formins and ARP2/3 to localize at the leading edge. A change in receptor structure or function due to a SNP such as the CXCR1 +777 SNP could affect G protein binding to and release from the receptor, which could lead to defective activation in any of the proteins involved in downstream signaling. Previous studies have found a decrease in polarization and directed migration in cells lacking $G_{\beta\gamma,}$ PI3K, PIP3, and the small GTPase cdc42 (Hannigan, Zhan et al. 2002; Wang, Herzmark et al. 2002; Li, Hannigan et al. 2003). Furthermore, the same pathways which activate F-actin polymerization and directed migration are involved in other activities in activated cells, such as reactive oxygen species production, intracellular calcium increase, and expression of adhesion molecules (Paape, Bannerman et al. 2003). Previous studies on these activities found that neutrophils from cows with the CC genotype also had decreased ROS production, intracellular calcium and expression of adhesion molecules, suggesting a common

deficiency in intracellular signaling (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Rambeaud and Pighetti 2007).

There were similar numbers of neutrophils from both genotypes outside the well following the migration assay. This suggests that even though the tracked CC genotype neutrophils were less able to directly migrate towards IL8, overall, neutrophils from cows with the CC genotype were still able to migrate outside the center well. Cells outside the well were counted 1 hr after stimulation with IL8 and had only traveled 1,200 µm away from the center well. In vivo neutrophils must travel further distances for longer periods of time, and the lower x/y ratio may have a larger impact over time in the CC genotype, leading to neutrophils not reaching the infection site as efficiently. Neutrophils contain very few mitochondria and receive the majority of their energy through glycolysis (Karnovsky 1968). A previous study found that the amount of glycogen was significantly lower in neutrophils isolated from the milk versus the blood and that milk neutrophils did not contain glycogen granules which were observed in blood neutrophils (Naidu and Newbould 1973). If neutrophils with a CC genotype use more energy by taking a less direct path to the site of infection, they may have less energy to kill the bacteria once inside the mammary gland. Adhesion molecules involved in migration are contained within secretory granules which are the first to be released during adhesion and specific granules which contain proteins involved in phagocytosis and bacterial killing (Lacy and Eitzen 2008). It is not clear whether migrating neutrophils release granules other than secretory granules, but if neutrophils

from CC cows are taking a longer path to get to the site of infection, there could be an increased chance of release of specific granules if more adhesion molecules are needed during migration. Furthermore, F-actin regulates the translocation of granules to the surface and prevents granules containing bactericidal proteins from being released before reaching the site of infection (Jog, Rane et al. 2007) and CC cows had lower amounts of F-actin. Release of intracellular granules before reaching the site of infection will decrease the amount of bactericidal proteins available to kill bacteria, since granular proteins are produced when neutrophils are still in the bone marrow and packaged into granules to be used later (DiStasi and Ley 2009). To support this, one study found decreased bacterial phagocytosis and ROS production in neutrophils following chemotaxis through mammary epithelial cells (Smits, Burvenich et al. 1999). A previous in vitro study did not find any differences in killing ability among CXCR1 genotypes; however neutrophils did not undergo chemotaxis prior to bactericidal assays (Rambeaud, Clift et al. 2006).

The current study found altered F-actin polymerization in neutrophils stimulated with ZAS, which does not signal through CXCR1. ZAS contains the complement fraction C5a, and the C5a receptor is a GPCR with similar structure and downstream signaling as CXCR1 (Ward 2009). Altered GPCR structure or downstream signaling common to both receptors would affect neutrophils stimulated with ZAS or IL8, as seen in the current study. The CXCR1 SNP could be a marker for other SNPs within the CXCR1

gene or within adjacent genes on the chromosome, which regulates neutrophil chemotaxis more closely.

In conclusion, these data suggest that genetic differences in neutrophil migration could be due to differences in the amount of F-actin formation, less efficient migration towards IL8, or a combination of both. If it takes longer for neutrophils to get to the site of infection, because they are not able to migrate directly and efficiently through the tissue, this could lead to increased mastitis, as observed in cows with the CC genotype. Finding the reasons behind what makes some cows more genetically vulnerable to infection will provide an understanding which will help develop targeted strategies to prevent and treat mastitis infections.

CHAPTER 4

RESPONSES OF DIFFERENT CXCR1 GENOTYPES AFTER EXPERIMENTAL CHALLENGE WITH *STREPTOCOCCUS UBERIS*

This chapter is a paper by the same name that will be submitted for publication.

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper include: i) some of the sample collection, iii) most of the genotyping, iv) most of the data analysis, v) most of the gathering and interpreting of the literature, vi) most of the writing of this paper.

Abstract

Mastitis, an inflammation of the mammary gland, accounts for the largest loss in profit for dairy farmers. Our prior research identified a polymorphism in the CXCR1 gene associated with mastitis and research is ongoing to identify what makes some cows genetically more vulnerable to infection. This study evaluated the susceptibility of cows with different genotypes at position +777 in the CXCR1 gene to experimental infection by *Streptococcus uberis*. Holstein dairy cows with GG (n=19), GC (n=28), and CC (n=20) genotypes were challenged intramammarily with *S. uberis* UT888 and samples were collected either once or twice daily for 7 or 14 d. After the challenge, 68% of quarters from the GG genotype, 74% of quarters from the CC genotype and only 47% of quarters from the GC genotype had at least 100 colony forming units (cfu)/ml *S. uberis* for at least two sampling time points (P=0.03) and were considered to be infected. Among all cows, the number of *S. uberis* isolated in milk was similar among genotypes at d 0 through 2. However, GC genotypes had fewer *S. uberis* than CC genotypes on d 3 and 4, and only GG genotypes on day 7 (P≤0.05). Similarly, GC cows had a lower mean SCC over time compared to GG and CC genotypes (GG=349 ± 7, GC=162 ± 4,

CC= 369 ± 7 x1000 cells/ml; P=0.04). Among cows which developed infection, the number of *S. uberis* in the milk, somatic cell count, rectal temperature, milk scores and mammary scores were all comparable among genotypes throughout infection. The ability of heterozygous cows to resist infection is most likely due to variation in early immune responses based on how quickly differences between infected and non infected animals occurred. Finding the reasons behind what makes some cows more genetically vulnerable to infection will provide an understanding which will help develop targeted strategies to prevent and control mastitis.

Introduction

Mastitis, an inflammation of the mammary gland, continues to account for the largest loss in profit for dairy farmers (NMC 1996). Inflammation occurs when bacteria enter the mammary gland and interact with mammary epithelial cells and leukocytes which release inflammatory mediators such as interleukin (IL) 1β, IL6, IL8, and tumor necrosis factor alpha (TNFα) (Uthaisangsook, Day et al. 2002; Rambeaud, Almeida et al. 2003; Lee, Bannerman et al. 2006). These mediators induce fever, vasodilatation, vascular permeability, release of acute phase proteins, and act as messengers recruiting circulatory leukocytes to the site of infection (Gouwy, Struyf et al. 2005; Bannerman 2009). This host immune response, in addition to toxins secreted by bacteria, can lead to tissue damage and altered milk composition observed in clinical mastitis (Zhao and Lacasse 2008). Mastitis can also become subclinical and/or chronic, with cows often showing no symptoms and the only detection being bacteria isolated

from the milk. Subclinical mastitis can lead to decreased milk production, and sporadic periods of high somatic cell counts (SCC) (Lukas, Hawkins et al. 2005). Somatic cell count, a measure of cell concentration within the milk, consists mainly of leukocytes, and can lead to decreased milk quality (Barbano, Ma et al. 2006).

The inflammatory response is directly involved in whether the infection is cleared quickly or becomes chronic (Hill 1981; Burvenich, Van Merris et al. 2003). Because the immune response is variable between individual cows, multiple studies have focused on genetic resistance to mastitis. Markers associated with mastitis and SCC have been found in genes involved in the immune system including lactoferrin and IL10 receptors (Verschoor, Pant et al. 2009; Huang, Wang et al. 2010). One such study identified a polymorphism in the CXCR1 gene, a receptor for the chemoattractant IL8, associated with increased susceptibility to subclinical mastitis and decreased neutrophil migration in vitro (Youngerman, Saxton et al. 2004; Rambeaud and Pighetti 2005). Cows with a CC genotype at position +777 on CXCR1 had a greater incidence of intramammary infection and a lower SCC compared to cows with a GG or GC genotype at this position (Youngerman, Saxton et al. 2004). However, later studies performed on Canadian and German Holsteins observed similar SCCs among +777 genotypes but did not investigate infection status (Leyva-Baca, Schenkel et al. 2008; Goertz, Baes et al. 2009).

Over time, mastitis control programs have decreased the prevalence of contagious pathogens. However, environmental pathogens continue to cause many cases of clinical and subclinical mastitis (Schukken 2004). *Streptococcus uberis* is one of the most prevalent environmental bacteria and is associated with clinical and subclinical intramammary infections during dry and lactating periods (Oliver 1988; Jayarao, Gillespie et al. 1999). Differences in subclinical mastitis susceptibility have been found among CXCR1 genotypes during field-based studies (Youngerman, Saxton et al. 2004); however, direct evaluation of the immune response and susceptibility to an experimental bacterial challenge among CXCR1 genotypes has not been studied. Because differences among genotypes have been found in invitro studies on neutrophil function and in field studies on subclinical mastitis incidence, we hypothesize that cows with different +777 genotypes will differ in the rate of intramammary infection and inflammatory response when challenged intramammarily with *S. uberis*.

Materials and methods

Animal selection

Challenges were performed on 4 separate dates: twice in November/December 2004 and twice in January 2008. Holstein cows in their second or third lactation used as control cows for other studies were used for all current challenges. Cows from the 2004 study were in mid-lactation and in the 2008 study cows were in late-lactation. Milk samples were collected three consecutive d prior to challenge and all cows had no symptoms of clinical mastitis and initial SCC <200,000 cells/ml. SCCs were performed

at The University of Tennessee DHIA lab using a Somacount 300 cell counter (Bentley Instruments, Chaska, MN, USA). All animal protocols were approved by UT Institutional Animal Care and Use Committee (IACUC).

In the 2004 challenge, 19 cows were assigned randomly to one of two challenge dates (challenge date 1 = 9 cows; challenge date 2 = 10 cows). In the 2004 challenge, one uninfected mammary quarter was selected randomly and infused with *S. uberis*. In the 2008 challenge, 48 cows were assigned randomly to one of the two challenge dates (challenge date 3 = 24 cows; challenge date 4 = 24 cows). In this study, two uninfected mammary quarters were chosen randomly and infused with *S. uberis* one week prior to dry off. Cows from all challenges were genotyped on the CXCR1 gene at position +777. Genotypes were determined by PCR amplification and sequencing was done at The University of Tennessee molecular biology core facility (Youngerman, Saxton et al. 2004).

Bacterial suspension

Cows were challenged with *S. uberis* UT888, which was isolated previously from a cow with clinical mastitis. Stock bacterial cultures were stored at -80 ºC until thawed. Once thawed, cultures were plated on a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Beckton Dickinson Microbiology Systems, Cockeysville, MD, USA), followed by overnight incubation at 37 ºC in a 5% CO_2 incubator. Three colonies were then subcultured at 37 °C in Todd Hewitt broth (Difco, Detroit, MI, USA) for 7 h to

a concentration of approximately 5 x 10^8 CFU/ml. Bacterial cultures were then diluted in sterile PBS to various concentrations stated in the inoculation procedure below.

Inoculation Procedure

Within 30 min following the morning milking, teats were cleaned using individual disposable paper towels and the teat end was sanitized thoroughly using gauze containing isopropyl alcohol. *S. uberis* was inoculated into the teat using a sterile syringe and teat cannula. In the 2004 study, 3ml of inoculum containing a total of 10,200 colony forming units were inoculated into the teat cistern on the first challenge day, and 10,500 cfu were inoculated on the second challenge date. In the 2008 study, 3.6ml of inoculum containing 5,399 cfu were inoculated per teat. The inoculum was massaged into the mammary gland and teats were dipped with a post milking teat disinfectant following challenge.

Sampling

In the 2004 study, milk and blood samples were collected immediately prior to challenge and daily for 14 d after challenge. In the 2008 study, milk samples were collected prior to challenge, twice daily for the first 3 d and once on d 4 and 7 after challenge. Blood samples were collected on the day of challenge for both studies. Milk samples for SCC were collected in plastic snap cap vials (Capitol Vial Co.) containing Bromopropyl-B as a preservative. Somatic cell counts were performed using a Somacount 300 cell counter (Bentley Instruments, Chaska, MN) by the Dairy Herd Improvement Association (DHIA) laboratory at The University of Tennessee, Knoxville, TN. Milk samples for

microbiological examination were collected aseptically as described previously (Oliver, King et al. 1990), and stored at -20°C. Bacterial examination of milk samples were performed according to the National Mastitis Council recommendations as previously described (Oliver, King et al. 1990). The detection limit was 1000 cfu/ml in the 2004 study and 4000 cfu/ml in the 2008 study. Milk samples were diluted in 100 µl PBS and four 10µl drops of each dilution were plated on trypticase soy agar to determine the number of bacteria per ml of milk.

Animal Evaluation

Rectal temperature, milk scores and mammary scores were collected at the same time points as milk samples stated previously. Appearance of milk and clinical status of the mammary gland was determined using the following scale:

Milk: 1 = normal, 2 = a few flakes, 3 = small slugs, 4 = large slugs/clots, and 5 = stringy/watery.

Mammary gland:

1 = normal; the udder is pliable when totally milked out. Heat, pain, redness, and/or swelling is not detectable; cows exhibits no signs of discomfort.

2 = slight swelling; the udder is less pliable with some firmness as if not totally milked out. Additional milking or stripping does not return the gland to normal. Redness, heat and pain are generally not detectable and cows generally do not exhibit signs of discomfort.

3 = moderate swelling; the udder is definitely firm, reddened and warm to the touch. The udder does not return to normal size when milked out. The cow generally exhibits signs of discomfort (irritable, performs a stepping motion with feet and/or kicks) during prepping and milking procedures.

4 = severe swelling; the udder is very hard, red, hot and noticeably larger than other mammary quarters before milking with little or no change in size following milking. The cow is extremely uncomfortable and very irritable.

Cows which developed clinical mastitis were closely monitored and received antibiotic treatment, if necessary.

Statistical Analysis

Analysis of variance was completed with mixed models using SAS software (SAS 9.1; SAS Institute Inc., Cary, NC). Because the number of bacteria inoculated varied, maximum *S. uberis* counted and stage of lactation varied between the 2004 and 2008 studies, data were blocked on challenge date when performing analysis so the two studies could be combined. To show the degree of variation among studies, descriptive statistics of bacteria, SCC, milk and mammary scores, and rectal temperature can be found in Table 4.

To determine the effect of CXCR1 +777 genotype on number of *S uberis*, SCC, rectal temperature, milk and mammary scores, a repeated measures design was used, with each challenge separated by block. SCC and second time point of *S uberis* isolation was log transformed by a value of 0.1 due to variability within the data. For single

Table 4. Challenge statistics from 2004 and 2008 studies

	2004 Study			**2008 Study**		
	Mean	Median	Range	Mean	Median	Range
S. uberis (cfu/ml)	22	0	0 - 100	84	0	0 - 400
SCC (cells/ml)	6,899	1,802	6 - 99,950	1,004	131	0 - 64,160
Milk Score	1.99	1.00	1 – 5	1.16	1.00	1 - 5
Mammary Score	2.37	2.00	1 – 5	1.18	1.00	1 - 4
Rectal Temperature (°C)	38.4	38.4	37.1 - 40.6	39	38.9	37.9 - 41.4

measurement data, such as starting SCC, and peak temperature, a randomized block design was used, with each challenge separated into a different block. A contingency table was used to determine the effect of CXCR1 +777 genotype on infection status. Because we were testing the effect of genotype on various factors, genotype was the treatment for all ANOVA analyses. ANOVA level of significance was set at $P < 0.05$.

Results

Infection status

Multiple definitions of infection based on increasing concentrations of bacteria were used to compare infection rate among CXCR1 +777 genotypes. For the 2004 study, only one quarter was challenged and infection definitions included 10 colony forming unit per ml (cfu/ml), 100 cfu/ml, or 1,000 cfu/ml isolated from the challenged mammary quarter on two separate time points within the 14 d study (Table 5). Among all definitions, cows with a GG genotype had the lowest percent infected, cows with the GC genotype were intermediate and cows with the CC genotype had the highest percent infected. Infection rates stayed the same from day 1 through 7, at which point certain cows were treated with antibiotics due to discomfort. The greatest difference among genotypes occurred when at least 100 cfu/ml and 1,000 cfu/ml were isolated twice, with 4 out of the 7 GG (57%), 5 out of 7 GC (71%), and 5 out of the 5 CC (100%) genotype cows becoming infected ($P=0.25$). Although not significant, the considerably lower percentage of infected cows with GG and GC genotypes was promising as it showed similar trends to the field-based study by Youngerman et al. (2004). A second study in

Table 5. 2004 Challenge - Percent of cows infected within 14 days after challenge among genotypes with various infection definitions.

	Study 1 +777 SNP			
	GG	GC	CC	P value
10 cfu/ml seen 2x	71%	86%	100%	0.40
100 cfu/ml seen 2x	57%	71%	100%	0.25
1,000cfu/ml seen 2x	57%	71%	100%	0.25
N=	7	7	5	

2008 had an increased sample size and allowed greater focus on the early stages of infection.

In the second study, two quarters were challenged per cow, so the infection rate could be studied in cows having no quarters infected, at least one quarter infected, or both quarters infected. When infection rate within individual quarters was evaluated, cows with a GC genotype had significantly fewer infections than GG and CC genotype cows regardless of definition (Table 6). When infection was based on individual cows (both quarters), a significant difference ($P<0.05$) was observed among genotypes with infection of 10 cfu/ml *S. uberis* with 10 out of 12 GG (83%), 9 out of 21 GC (43%), and 12 out of 15 CC (80%) becoming infected. A similar trend ($P<0.10$) was observed when less strict infection criteria (≥100 cfu/ml) were used. The percent of infected quarters remained constant from 12 h through 36 h for GC and CC genotypes and 48 h for GG genotype cows at which point infection rate decreased through day 4 (Figure 8). Infection rates were not evaluated past d 4, because on d 5, cows exhibiting discomfort were provided antibiotics to alleviate infection and would have skewed the data. By collecting samples every 12 hr during the first three d, we were able to determine that the second isolation of *S. uberis* occurred 6-7 h later in cows with a CC genotype compared to heterozygous cows (^{ab}GG=27±0.4 hr, ^{a}GC=26±0.4, ^{b}CC=33±0.4; $P=0.06$).

Table 6. 2008 Challenge - Percent of cows infected with *S. uberis* within 7 days post challenge in both quarters (infection per cow) and percent of quarters infected among genotypes with various infection definitions.

	Infection per quarter				**Infection per cow (both quarters)**			
	GG	GC	CC	P value	GG	GC	CC	P value
10 cfu/ml seen 2x	83%	52%	80%	**0.01**	83%	43%	80%	**0.02**
100 cfu/ml seen 2x	71%	43%	70%	**0.03**	58%	29%	60%	0.06
1,000 cfu/ml seen 2x	67%	26%	57%	**0.001**	42%	14%	47%	0.08
N =	24	42	30		12	21	15	

Figure 8.

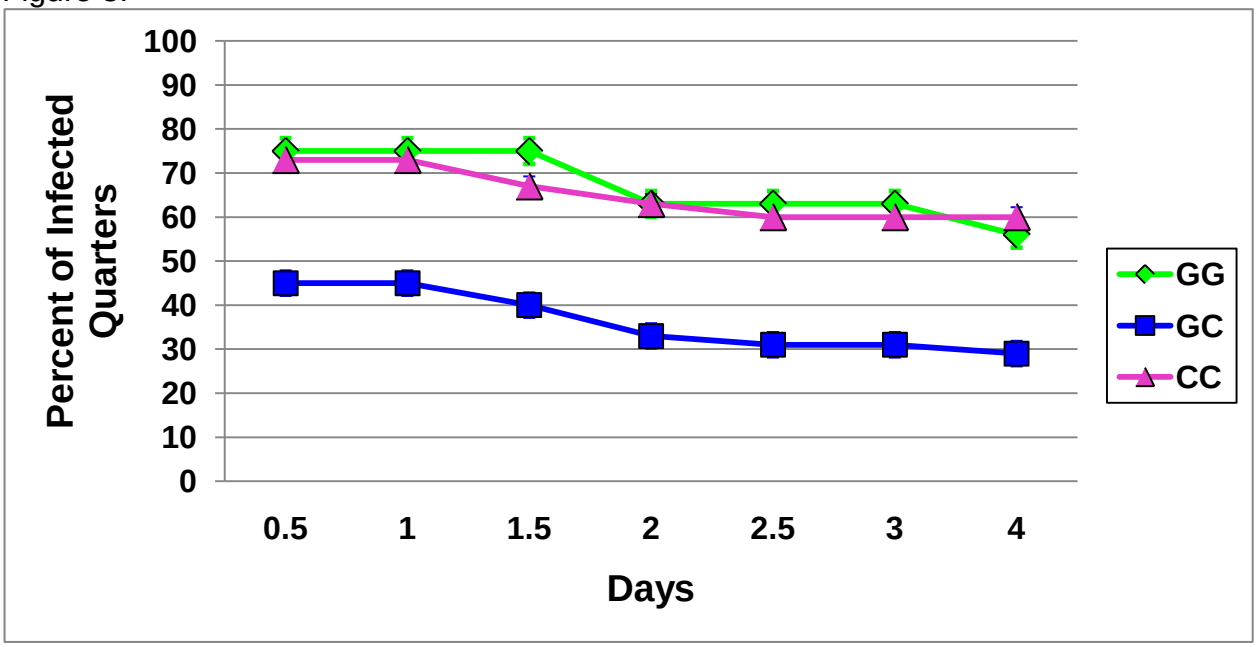


Figure 8. Percent of infected quarters among genotypes up through day 4 in the 2008 study. Infection is defined as 100cfu/ml isolated at two separate time points. Day 4 was the cutoff, because some cows began receiving antibiotics on day 5.

S. uberis, somatic cell concentrations and circulating WBC count in all cows

To increase the ability to detect different responses among genotypes, the two challenge studies were combined based on an infection with at least 10 cfu/ml isolated at least twice in the same quarter. Common time points were used to analyze and report the data (days 0, 1, 2, 3, 4, 7). Among challenged cows, the GC genotype had the lowest mean *S. uberis* concentration (cfu/ml), while CC and GG genotypes had the highest mean *S. uberis* concentration over time (GG=74 ± 0.8 cfu/ml, GC=40 ± 0.4 cfu/ml, CC=77 ± 0.8 cfu/ml; P = 0.07), which corresponds with lower infection rates observed in the heterozygous group. When examining individual time points, *S. uberis* concentration was similar among genotypes at days 0 through 2. However, GC genotypes had fewer *S. uberis* than CC genotypes on d 3 and 4, and only GG genotypes on d 7 (P≤0.05; Figure 9a).

Following experimental challenge, SCC among genotypes was similar to number of *S. uberis* observed among genotypes. Cows with the GC genotype had a lower mean SCC over time compared to both homozygous (GG and CC) genotypes (GG=349 ± 7, GC=162 ± 4, CC= 369 ± 7 x1000 cells/ml; P=0.04). However unlike *S. uberis* concentrations, SCC were similar among genotypes within a given day, (Figure 9b). Interestingly, cows with a heterozygous genotype had significantly higher circulating white blood cell counts at the start of infection than CC genotype cows (^{ab}GG=8.7 ± 0.2, ^{a}GC=9.4 ± 0.2, ^{b}CC=7.5 ± 0.2; P = 0.04) when both studies were combined (Table 7).

Figure 9a

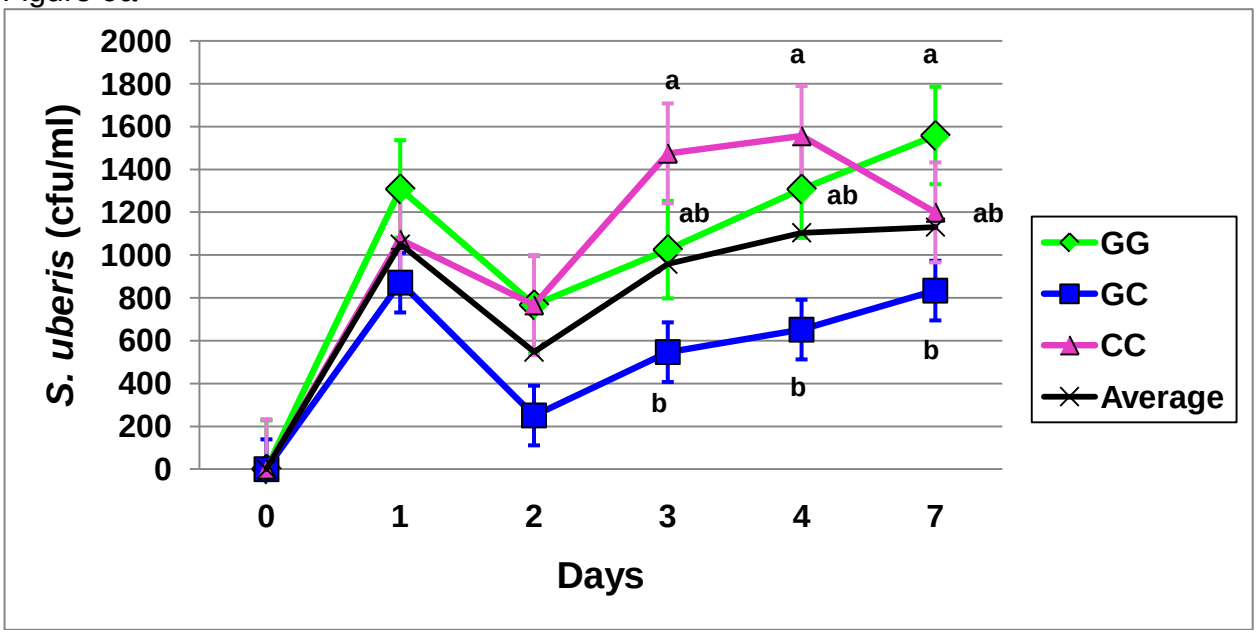


Figure 9a. The number of *S. uberis* isolated from milk of cows with different CXCR1 +777 genotypes. Includes infected and uninfected cows. Different letters represent significant difference between genotypes calculated by least squared means (genotype P =0.07; time P < 0.001; genotype*time P = 0.03).

Figure 9b

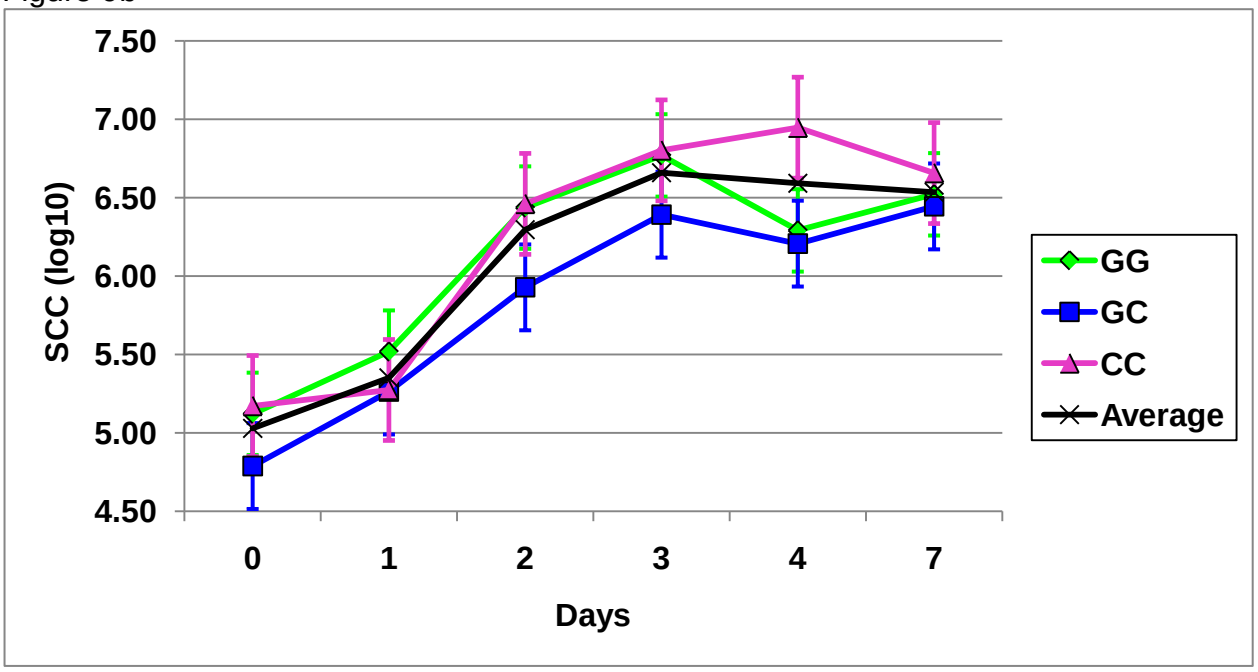


Figure 9b. SCC (log10) from cows with different CXCR1 genotypes. Includes infected and uninfected cows. (genotype P =0.04; time P < 0.001; genotype*time P = 0.36).

Table 7. Circulating white blood cell (WBC) and neutrophil concentrations of cows prior to challenge.

	GG	**GC**	**CC**	P value
Circulating WBC count x 1000 cells/ml	8.7 ± 0.2 ab	9.4 ± 0.2 a	7.5 ± 0.2 b	**0.04**
Neutrophil %	42 ± 3	42 ±3	41 ± 3	**0.98**
Neutrophils x 1000 cells/ml	3.6 ± 0.5	4.0 ± 0.4	3.1 ± 0.4	**0.17**
N=	19	28	20	

However, the number and percent of circulating neutrophils was similar among genotypes.

To identify potential differences in the immune response among genotypes, only cows which became infected with at least 10 cfu/ml *S. uberis* were included in the following analysis. *S. uberis* concentration in all genotypes increased the day after challenge, dropped slightly day 2, increased again on day 3, and became steady for days 4 and 7 (Figure 10). Once infected, the initial, peak, final, and overall mean *S. uberis* concentration were similar among CXCR1 +777 genotypes.

SCC increased in all genotypes on days 2 and 3 and dropped in GG and GC genotype cows on day 4 at which time the CC genotype cows continued to increase and peaked at 14 x 10^6 cells/ml (Figure 11). By day 7, SCC of CC genotype cows had decreased to around 6 million cells/ml and was similar to GC and GG genotypes. However, despite the SCC influx, the number of *S. uberis* in the milk remained high. SCC over the course of infection, peak SCC, and SCC on day of challenge were similar among genotypes.

Animal evaluation

In order to further compare the inflammatory response among genotypes, rectal temperature as well as milk and mammary scores were obtained. As in SCC and *S. uberis* analyses, only infected cows were included in order to assess the response to infection. Rectal temperature in all genotypes increased on day 3 and remained elevated through day 7 (Table 8). CC genotype cows had an elevated temperature

Figure 10.

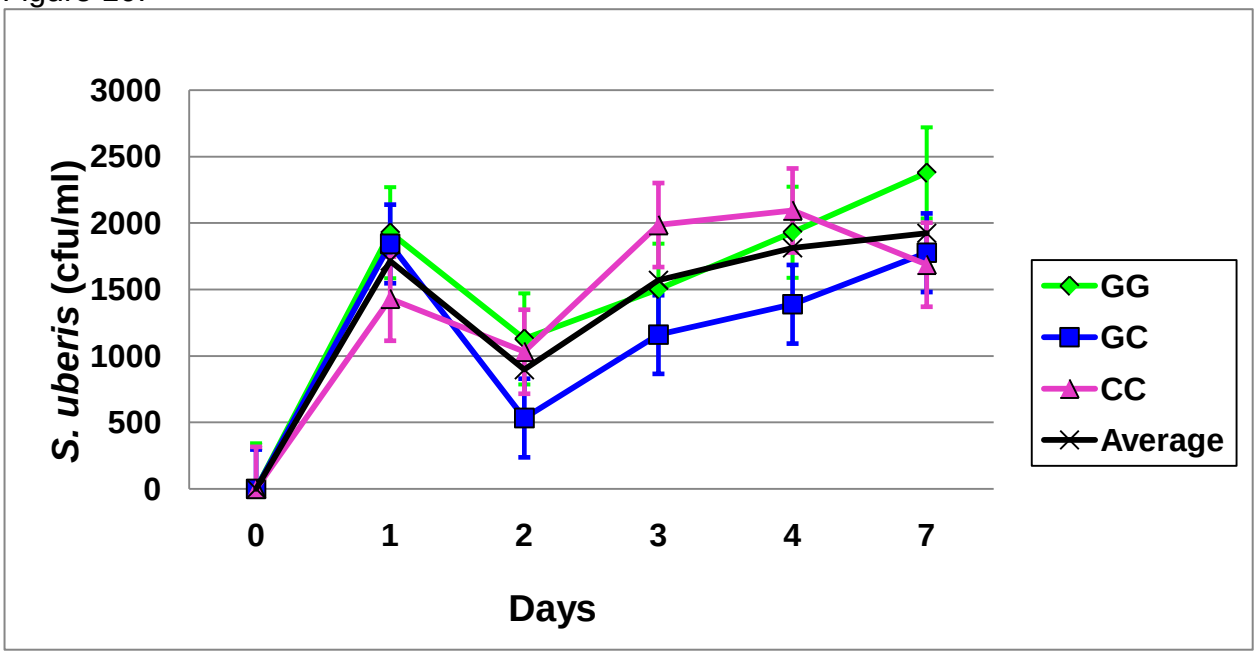


Figure 10. *S. uberis* concentration isolated from milk among +777 genotypes. Includes only cows which were infected (100cfu/ml seen 2x) (genotype P =0.44; time P ≤ 0.001; genotype*time P = 0.35).

Figure 11.

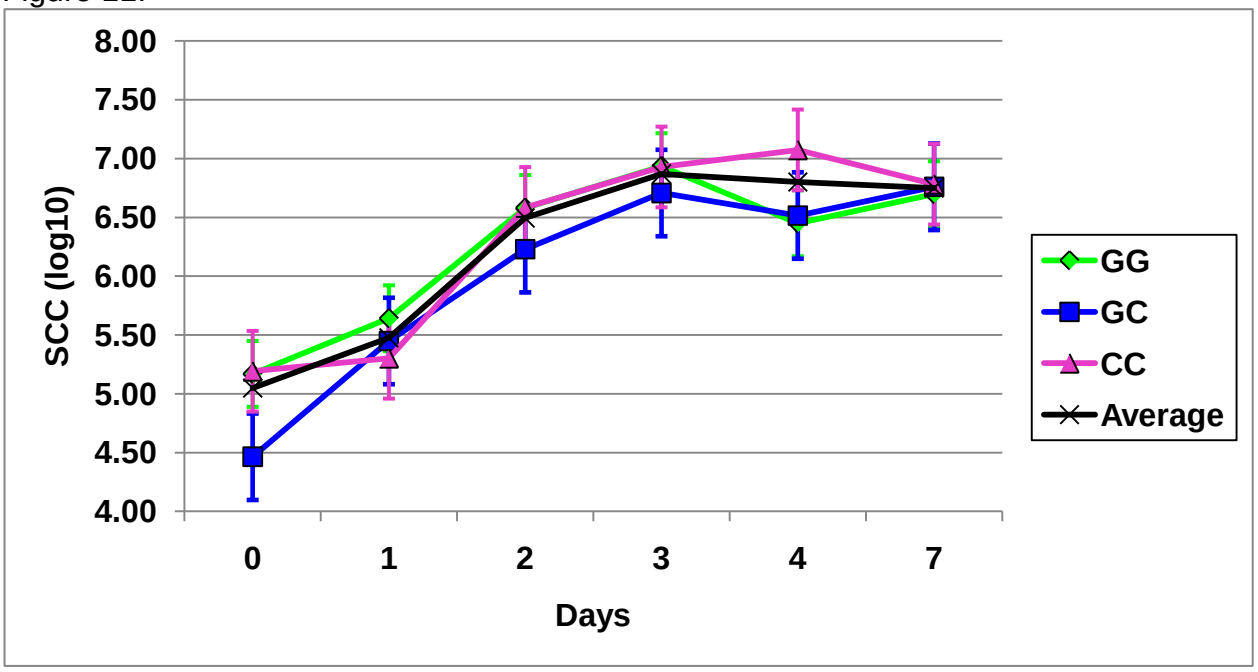


Figure 11. Somatic cell count (log10) among +777 CXCR1 genotypes. Includes only cows which were infected (100cfu/ml seen 2x). (genotype P =0.12; time P < 0.001; genotype*time P value = 0.72).

Table 8. Rectal temperature, milk score, and mammary score among genotypes in infected cows on days 0-7.

		Day 0	± SE	Day 1	± SE	Day 2	± SE	Day3	± SE	Day 4	± SE	Day 7	± SE	P value
Rectal	**GG**	38.6	0.4	38.7	0.4	38.7	0.4	39.1	0.4	38.9	0.4	38.9	0.4	0.25
Temp °C	**GC**	38.7	0.4	38.6	0.4	38.7	0.4	39.0	0.4	38.8	0.4	39.3	0.4	
	CC	38.6	0.4	38.7	0.4	38.7	0.4	39.2	0.4	39.1	0.4	39.2	0.4	
Milk	**GG**	1.00	0.2	1.00	0.2	1.05	0.2	1.72	0.2	2.14	0.2	2.93	0.2	0.19
score	**GC**	1.00	0.2	1.00	0.2	1.13	0.2	1.26	0.2	1.70	0.2	2.50	0.2	
	CC	1.00	0.2	1.00	0.2	1.12	0.2	1.54	0.2	2.19	0.2	2.54	0.2	
Mammary	**GG**	1.00	0.3	1.00	0.3	1.24	0.3	1.90	0.3	2.33	0.3	2.93	0.3	0.40
score	**GC**	1.00	0.3	1.00	0.3	1.13	0.3	1.39	0.3	1.70	0.3	2.40	0.3	
	CC	1.00	0.3	1.00	0.3	1.27	0.3	1.77	0.3	2.27	0.3	2.38	0.3	

compared to GG and GC genotype cows on days 3 and 4 but decreased to similar levels by day 7. Rectal temperature throughout infection, starting temperature, and peak temperature were comparable among genotypes.

Abnormal milk and mammary scores indicate inflammation inside the mammary gland due to bactericidal products released by immune cells or toxins released by the bacteria. Milk scores steadily increased and were similar among genotypes from d 2 to d 7 (Table 8). Mammary scores also increased steadily from d 2 to d 7 and were similar among genotypes (Table 8).

Discussion

Cows with the GC genotype were consistently more resistant to bacterial intramammary infection following bacterial challenge with *S. uberis* compared to the CC genotype in prior field data and current data from two separate challenges. Previous studies on neutrophil function may partially explain increased resistance in cows with GC genotypes. Multiple functions in activated cells, such as adhesion molecule expression, migration, and reactive oxygen species (ROS) production are necessary for effective clearance of an infection. Studies on adhesion molecule expression and ROS production found neutrophils from heterozygous cows had adhesion molecule expression and ROS production in between that of the two homozygous genotypes, with CC genotype neutrophils expressing the lowest amount (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006). When neutrophil migration was measured,

heterozygous neutrophils had increased migration similar to GG genotype neutrophils when migrating towards zymosan activated sera, but decreased migration, similar to CC genotype neutrophils when migrating towards IL8 (Rambeaud and Pighetti 2005). Interestingly, neutrophils from CC genotype cows had increased survival when stimulated with IL8 compared to GG genotype cows, with heterozygous cows remaining in the middle (Rambeaud, Clift et al. 2006). Neutrophils from heterozygous cows could benefit from having functional activities on levels in between GG and CC genotypes. Heterozygous cows have lower adhesion molecule expression, migration, and ROS production than GG genotype cows, but not enough to limit their abilities to fight infection, as might be happening in CC genotype cows. Neutrophil survival is higher in GC than GG genotype cells, which may allow them to stay alive in the mammary gland longer than GG genotype cows. This hypothesis is supported by previous studies which have found that at parturition, when cows are more susceptible to mastitis, neutrophils have increased survival and decreased functions such as migration and ROS production (Madsen, Weber et al. 2002). This matches the functional pattern observed in neutrophils from CC cows. Increased survival in combination with efficient migration and ROS production in neutrophils from heterozygous cows could make them more efficient at resisting infection.

Aside from the protection which neutrophils provide against mammary infection, there are numerous other factors inside the mammary gland which are involved in the immune response. Once bacteria enter the mammary gland, one of the first responses

is the production of pro-inflammatory cytokines and antimicrobial proteins by epithelial cells following activation of Toll-Like Receptors within the gland (Griesbeck-Zilch, Meyer et al. 2008). Previous studies have shown that IL8 and C5a levels in milk significantly increase within 24 to 36 h following challenge with *S. uberis* (Rambeaud, Almeida et al. 2003; Bannerman, Paape et al. 2004). While the initial response of epithelial cells to bacteria may not differ among genotypes, IL8 produced in response to TLR activation may produce different effects among genotypes if CXCR1 is expressed by mammary epithelial cells. Although it is not known whether bovine mammary epithelial cells typically express CXCR1, human mammary stem cells and human bronchial epithelial cells both express CXCR1 (Farkas, Hahn et al. 2005; Ginestier, Liu et al. 2010). This suggests it is possible that mammary epithelial cells express CXCR1 and may respond to IL8 levels within the gland. If the CXCR1 SNP results in altered receptor function and/or altered intracellular pathways, epithelial cells may produce different amounts of pro-inflammatory and bactericidal molecules among genotypes in response to IL8, leading to different infection rates.

Other leukocytes within the mammary gland which could be involved in early resistance are CD8+ T cells. CD8+ T cells, which comprise the largest population of lymphocytes in the mammary gland, are involved in killing host cells infected with bacteria (Mehrzad, Janssen et al. 2008). Because *S. uberis* has been found to be internalized by mammary epithelial cells in vitro (Patel, Almeida et al. 2009), this suggests CD8+ T cells could play a key role in clearing infected epithelial cells during infection with this

organism. Human CD8+ T cells express CXCR1 (Takata, Tomiyama et al. 2004) and we expect the same to be true in the bovine. If the CXCR1 SNP results in altered receptor function, intracellular signaling could be altered and the ability of CD8+ T cells to migrate into the mammary gland and kill bacteria could differ between genotypes, leading to the different infection rates observed.

Regardless of whether neutrophils or CD8+ T cells play a larger role in resistance, an increased number of WBCs in general may impact infection rate. Cows with a GC genotype had higher circulating WBC counts compared to CC genotype cows at the time of challenge. However, circulating WBCs have the greatest impact on immune response when they migrate into the mammary gland and SCC was found to be similar among genotypes in the first two days following challenge. This suggests that increased WBC in GC genotype cows has little impact on their increased resistance, or an increased SCC occurred in GC genotype cows in between sampling time points and was therefore not detected. An increased circulating WBC count in cows which did not become infected compared to those which did become infected regardless of genotype supports the idea that increased WBC count played some sort of role in resistance.

In addition to potential insight regarding the influence of CXCR1 genotypes on resistance to infection, this study also indicated the potential need for extended sampling times when evaluating *S. uberis* infection. In 2004, samples were collected every 24 h for 14 d with an overall infection rate of 74%. In contrast, 2008 samples

were collected every 12 h for the first 3 d and then d 4 and 7 with an overall infection rate of 58%. Closer examination of the data from 2004 revealed that 20-30% of cows with GC and CC genotypes did not have a second isolation of *S. uberis* or an increase in SCC until after d 7. This finding suggests that the overall infection rate may have been underestimated in 2008. While in 2004, the early stages of infection may have been underestimated as the second isolation of *S. uberis* occurred on a 12 h interval versus a 24 h interval in 10-12% of cows in 2008.

Additionally, the two studies challenged cows at different stages of lactation. Cows from the 2004 study were challenged at mid lactation, the stage of lactation at which cows are least susceptible to *S. uberis* mastitis (Jayarao, Gillespie et al. 1999). Whereas cows from the 2008 study were challenged 7 days prior to dry off, a stage of lactation at which cows are more susceptible to *S. uberis* mastitis (Oliver 1988). During late lactation, altered concentrations of sex steroids such as estrogen and progesterone, as well as regulatory proteins such as insulin-like growth factor (IGF-I) could influence immune responses during this time. High estrogen levels typically observed during late gestation have been found to decrease neutrophil viability following diapedesis (Lamote, Meyer et al. 2004). In contrast, IGF-1 concentrations decrease over lactation. Because IGF-I is involved in priming cells for production of pro-inflammatory cytokines such as IL6, IL8, and TNF-α, decreased levels of IGF-I observed in late lactation are thought to decrease production of these cytokines and could dampen the strength of the immune response (Kooijman, Coppens et al. 2002; Kooijman, Coppens et al. 2003). Additionally

during late lactation, the level of lactoferrin increases within milk. Recent in vitro studies have identified *S. uberis* adhesion molecule (SUAM) expressed on the bacterial surface which binds lactoferrin and uses it as a molecular bridge to bind lactoferrin receptors on MECs (Almeida, Luther et al. 2006; Patel, Almeida et al. 2009). This increases adhesion and internalization of *S. uberis* into MECs in vitro, and could affect infection rate in late lactation. Thus differing levels of sex steroids and other proteins in the milk during different stages of lactation could have affected the infection rates observed among genotypes between the two studies.

Cows with a GG genotype had variable responses to bacterial intramammary infection in field and challenge studies. In the field study and the 2004 challenge, GG genotype cows were less susceptible to mastitis compared to those with a CC genotype, and heterozygous cows in the middle. However, in the 2008 challenge study, the infection rate was similar among GG and CC genotype cows. Different infection rates in GG genotype cows among field data and two challenges could be caused by a number of factors including type and number of bacteria, duration of infection, and environmental factors. In the field study, multiple bacterial strains (*S. aureus, C. bovid*, and coagulase negative *Staph*) were isolated from the mammary gland of cows. Specific bacteria and even different strains of the same bacteria can cause diverse immune responses in the mammary gland and influence subsequent resistance. *E. coli* can cause clinical signs within 8 h of infection and cytokines IL1, IL8, and TNF to increase within 16 h (Bannerman, Paape et al. 2004). This is compared to *S. uberis* which can take 32 h to

elicit an increase in pro-inflammatory cytokines and 84 h to exhibit signs of clinical mastitis (Bannerman, Paape et al. 2004). Even within the *S. uberis*, different strains can elicit different timing of immune responses (Rambeaud, Almeida et al. 2003; Bannerman, Paape et al. 2004). If cows with a GG genotype are less susceptible to bacteria other than *S. uberis* or a strain of *S. uberis* other than UT888, this could partially explain the variation in results of the field study versus challenge data. For example, bacteria such as *S. aureus* do not cause upregulation of IL8 within the mammary gland; therefore, polymorphisms in the CXCR1 gene might not have as great an impact on infection.

Duration of infection can also influence the relationship with CXCR1 genotypes and could be another factor in the differences between challenge and field studies. Challenge data was collected over several days for one or two weeks compared to field data which was collected once every few months. Cows with mastitis in the field study have more chronic infections, but samples from challenges were not collected past day 7 or 14, limiting infection rate to these days. GG genotype cows in the second challenge study had higher infection rates than heterozygous genotype cows, but it is not known if the chronic infection rate in the GG genotype would have decreased to similar levels as heterozygous. Numerous other factors must be assessed, including nutrition status and season when comparing field data and challenge data.

In conclusion, cows with a GC +777 genotype were more resistant to infection with *S. uberis.* However once infected, responses were similar among genotypes and reinforces that early immune responses are critical for mastitis resistance. Because the GC genotype was shown to resist infection better than the CC genotype in one field study and two challenge studies, the +777 SNP offers an excellent model for studying mastitis resistance and potentially a marker for genetic selection. Future studies aimed at finding why the GC genotype is more resistant to infection, including challenges with different types of bacteria and studying soluble and cellular components within the mammary gland will help explain overall infection susceptibility. Understanding why certain cows are more susceptible to mastitis will help lead to improved prevention and control strategies.

Chapter 5
SUMMARY AND CONCLUSIONS

Mastitis continues to be one of the most costly problems challenging dairy producers. Because of this, numerous studies have been directed towards finding reasons for differences in the immune response of cows less susceptible to mastitis. Previous research has found a SNP in the CXCR1 gene at position +777 which is associated with mastitis susceptibility and deficient neutrophil functions such as migration and ROS production (Youngerman, Saxton et al. 2004; Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006). Because efficient neutrophil migration into the mammary gland is necessary for quick resolution of infection and deficiencies were found in neutrophil migration among +777 CXCR1 genotypes, the hypothesis of the current study was that neutrophil migration patterns differ between genotypes and may contribute to different immune responses in cows with specific CXCR1 genotypes are involved with increased intramammary infection when challenged with *S uberis*.

To test this hypothesis, the first step was to evaluate different migration patterns in vitro. Migration patterns evaluated included F-actin polymerization, F-actin distribution and morphology, and directed migration towards IL8. When neutrophils were incubated with ZAS and stained for F-actin, neutrophils from GG genotypes had an overall increased amount of F-actin polymerization compared to neutrophils from CC genotypes. However increased F-actin polymerization did not affect the ability of F-actin to accumulate along the edges of the cell or outer edge morphology following stimulation with ZAS. Resting neutrophils had similar F-actin amounts and distribution as well as morphology. ARP 2, which is part of the ARP 2/3 complex, a key molecule involved in F-actin polymerization and lamellipodia formation, was found in similar

amounts within CC and GG genotype neutrophils, suggesting that ARP 2 itself does not play a role in decreased F-actin among CC genotype neutrophils. However, the ARP2/3 complex is made up of 2 main molecules and 5 subunit molecules and any alteration to these proteins or how they fit together could impact actin polymerization.

To observe directed migration of neutrophils towards IL8, an under agarose assay was used with time lapse microscopy. Following a direct comparison in migration between neutrophils from cows with different genotypes, CC genotype neutrophils had a lower maximum X distance and displacement directly towards an increasing concentration of IL8. Since path-length and overall displacement were similar between genotypes, this suggests that CC genotype neutrophils had more wasted movement on the Y axis. CC genotype cells also had a lower X/Y ratio, meaning they had less X axis movement and more Y axis movement towards IL8. Lower X and X/Y ratio migration was not observed on the HBSS side of the well, suggesting it is a pattern observed in CC genotype neutrophils undergoing chemotaxis. Since there were similar numbers of neutrophils from both genotypes outside the well following the migration assay, this suggests that even though the tracked CC genotype neutrophils were less able to directly migrate towards IL8, overall, neutrophils with a CC genotype were still able to migrate outside the center well.

The next step in testing the hypothesis was to compare the inflammatory response and infection status of cows challenged with *S uberis* among CXCR1 genotypes. Among previous field studies and current challenge studies, cows with a heterozygous genotype (GC) had a significantly lower infection rate than cows with the

CC genotype, whereas cows with a GG genotype varied in relation to heterozygous and CC genotype cows. Previous in vitro studies have found that heterozygous cows have functional levels in between those of GG and CC genotypes, suggesting that they may have the best combination of neutrophil functions to resist infection (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Rambeaud and Pighetti 2007). Heterozygous cows have lower adhesion molecule expression, migration, and ROS production than GG genotype cows, but not enough to limit their abilities to fight infection, as might be happening in CC genotype cows. Neutrophil survival is higher in heterozygous than GG genotype cells, which may allow them to stay alive in the mammary gland longer than GG genotype cows. Besides the protection of neutrophils, there are numerous factors within the mammary gland involved in the immune response which may differ between genotypes. Human CD8+ T cells and bronchial epithelial cells express CXCR1 (Takata, Tomiyama et al. 2004; Farkas, Hahn et al. 2005) and it is possible these cell types express CXCR1 within the bovine mammary gland. Mammary epithelial cells produce many key pro-inflammatory cytokines, such as IL1β, IL6, and IL8 (Griesbeck-Zilch, Meyer et al. 2008) and CD8+ T cells are involved in killing host cells infected by bacteria (Mehrzad, Janssen et al. 2008). A decreased response in either of these cells could have a negative impact on infection rate.

One of the first steps of mammary infection is neutrophil influx into the mammary gland and the first step of neutrophil migration is chemoattractants produced at the site of infection binding to G-protein coupled receptors (GPCRs) on the neutrophil surface. Downstream signaling from activated GPCRs leads to F-actin polymerization at the

leading edge of the cell where chemoattractant concentration is highest. A change in receptor structure or function due to a SNP such as the CXCR1 +777 SNP could affect G protein binding to and release from the receptor, which could lead to defective activation in any of the proteins involved in downstream signaling. Previous studies have found a decrease in polarization and directed migration in cells lacking $G_{\beta\gamma,}$ PI3K, PIP3, and the small GTPase cdc42 (Hannigan, Zhan et al. 2002; Wang, Herzmark et al. 2002; Li, Hannigan et al. 2003), which are all activated in pathways following GPCR binding to ligand. Furthermore, the same pathways which activate F-actin polymerization and directed migration are involved in other activities in activated cells, such as reactive oxygen species production, intracellular calcium increase, and expression of adhesion molecules (Paape, Bannerman et al. 2003), all of which were altered among CXCR1 genotypes in previous studies, suggesting a common deficiency in intracellular signaling. Alteration in these activities could influence the early immune response to bacteria, such as *S. uberis*.

Aside from intracellular signaling, numerous cellular activities including trafficking of intracellular granules are partially regulated by F-actin polymerization (Jog, Rane et al. 2007). Intracellular granules contain molecules necessary for an effective immune response, including adhesion molecules, reactive oxygen species (ROS) and lactoferrin (Faurschou and Borregaard 2003). Granular contents could be released in different amounts among genotypes due to different regulation of granule trafficking to the cell surface. This is supported by the current finding that CC genotype neutrophils have less F-actin than GG genotype and previous in vitro studies have found decreased ROS

production in CC genotype neutrophils. In the 2004 *S. uberis* challenge and field studies, cows with a GG genotype had a lower infection rate than CC genotype cows, and following infection the number of *S. uberis* and inflammatory responses were similar. This suggests that early factors such as release of bactericidal proteins from granules and production of ROS from the first cells to arrive at the site of infection may play a role in infection rate.

F-actin involvement in the release of intracellular granules also could have affected the under agarose migration assay. Neutrophils isolated from a GG and CC genotype cow were placed together in the center well to allow simultaneous evaluation of responses and minimize experimental variation. However, with the changes in F-actin polymerization neutrophils from GG genotype cows could have greater release of intracellular granules into the solution, which may benefit neutrophils from cows with the CC genotype. This potential for 'cross-action' may explain why in previous migration studies an increased number of neutrophils from GG genotype cows moved towards IL8 and in the current study, similar numbers of neutrophils migrated outside the center well towards IL8. One other reason for the similar number of neutrophils from GG and CC genotype cows migrating outside the center well could be that the IL8 gradient was released from a circular well and did not reach all of the neutrophils at the same time which would have occurred in the previous study (Rambeaud and Pighetti 2005). This could have affected the overall number of cells which migrated outside the well, but not the close image where tracking was performed on individual cells since all cells in the close image were responding to the same gradient.

Direct migration of neutrophils into the mammary gland is critical for effective resolution of mastitis. Neutrophils from cows with a CC genotype had lower x/y movement towards IL8, meaning they had more wasted movement. If they waste more energy getting to the site of infection, they might have less energy to engulf and destroy *S. uberis* leading to increased infection rate observed in CC genotype cows. A previous study found that the amount of glycogen was significantly lower in neutrophils isolated from the milk versus the blood and that milk neutrophils did not contain glycogen granules which were observed in blood neutrophils (Naidu and Newbould 1973). Furthermore, a study found decreased bacterial phagocytosis and ROS production in neutrophils following chemotaxis through mammary epithelial cells (Smits, Burvenich et al. 1999). In previous in vitro studies which did not find any differences in killing ability among CXCR1 genotypes, neutrophils did not undergo chemotaxis prior to bactericidal assays (Rambeaud, Clift et al. 2006).

One limitation of the current study was the choice to evaluate only the GG and CC genotype cows in the in vitro assays evaluating F-actin polymerization and directed migration. This choice was based on prior studies where neutrophil function in GC genotype cows was in between the functional activity of GG and CC genotype cows and would not provide as much information as the homozygous genotypes which represented the two extremes. Additionally, the directed migration assays could only be performed on GG and CC genotype neutrophils due to availability of contrasting dyes. However, decreased infection rate in GC genotype cows following intramammary challenge warrants further study of the immune response in this genotype. Although it

should be noted that since SCC was similar among genotypes throughout all time points following challenge, it is likely that neutrophil migration is not the main reason behind increased resistance.

In conclusion, genetic differences among CXCR1 genotypes in neutrophil migration could be due to differences in the amount of F-actin polymerization, less efficient migration towards IL8, or a combination of both. If it takes longer for neutrophils to get to the site of infection, because they are not able to migrate directly and efficiently through the tissue, this could lead to increased infection rate when challenged with *S. uberis* seen in the CC genotype. Cows with a GC genotype may be less susceptible to infection with *S. uberis*, however after becoming infected responses were similar among genotypes suggesting that early immune response is critical in mastitis susceptibility. Because heterozygous cows have shown to resist infection better than the CC genotype in one field study and two challenge studies, the +777 SNP would likely be a good model for studying mastitis susceptibility. Future studies aimed at finding out if the CXCR1 SNP is receptor mediated and why the GC genotype is more resistant to infection, including soluble and cellular components within the mammary gland during infection and protein expression within the neutrophil itself will help explain overall infection susceptibility. Identifying a specific protein or mechanism leading to increased resistance in GC cows may result in the development of increased prevention or treatment of mastitis. If a specific protein or mechanism leads to increased resistance in GC cows, it could be associated with increased resistance in the overall population, besides the CXCR1 genotype. Modifying the protein or mechanism through

vaccine or treatment could make cows susceptible to mastitis more resistant. Finding the reasons behind what makes some cows more genetically vulnerable to infection will provide an understanding which will help develop targeted strategies to prevent and treat mastitis infections.

REFERENCES

Aglas, F., J. Hermann, et al. (1998). "Abnormal directed migration of blood polymorphonuclear leukocytes in rheumatoid arthritis. Potential role in increased susceptibility to bacterial infections." Mediators Inflamm **7**(1): 19-23.

Ahuja, S. K., J. C. Lee, et al. (1996). "CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct." J Biol Chem **271**(1): 225-232.

Akin, O. and R. D. Mullins (2008). "Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex." Cell **133**(5): 841-851.

Almeida, R. A., D. A. Luther, et al. (2006). "Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM)." Vet Microbiol **115**(1-3): 183-191.

Almeida, R. A., K. R. Matthews, et al. (1996). "Staphylococcus aureus invasion of bovine mammary epithelial cells." J Dairy Sci **79**(6): 1021-1026.

Almeida, R. A. and S. P. Oliver (2001). "Role of collagen in adherence of Streptococcus uberis to bovine mammary epithelial cells." J Vet Med B Infect Dis Vet Public Health **48**(10): 759-763.

Amann, K. J. and T. D. Pollard (2001). "The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments." Nat Cell Biol **3**(3): 306-310.

Ashfaq, M. K. and S. G. Campbell (1986). "The influence of opsonins on the bactericidal effect of bovine alveolar macrophages against Pasteurella multocida." Cornell Vet **76**(2): 213-221.

Baggiolini, M., U. Horisberger, et al. (1985). "Identification of three types of granules in neutrophils of ruminants. Ultrastructure of circulating and maturing cells." Lab Invest **52**(2): 151-158.

Bainton, D. F., J. L. Ullyot, et al. (1971). "The development of neutrophilic polymorphonuclear leukocytes in human bone marrow." J Exp Med **134**(4): 907-934.

Balcer, H. I., K. Daugherty-Clarke, et al. (2010). "The p40/ARPC1 subunit of Arp2/3 complex performs multiple essential roles in WASp-regulated actin nucleation." J Biol Chem **285**(11): 8481-8491.

Bannerman, D. D. (2009). "Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows." J Anim Sci **87**(13 Suppl): 10-25.

Bannerman, D. D., M. J. Paape, et al. (2004). "Innate immune response to intramammary infection with Serratia marcescens and Streptococcus uberis." Vet Res **35**(6): 681-700.

Bannerman, D. D., M. J. Paape, et al. (2004). "Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection." Clin Diagn Lab Immunol **11**(3): 463-472.

Barbano, D. M., Y. Ma, et al. (2006). "Influence of raw milk quality on fluid milk shelf life." J Dairy Sci **89 Suppl 1**: E15-19.
Barrington, G. M., T. E. Besser, et al. (1997). "Expression of immunoglobulin G1 receptors by bovine mammary epithelial cells and mammary leukocytes." J Dairy Sci **80**(1): 86-93.
Barrio, M. B., P. Rainard, et al. (2003). "Assessment of the opsonic activity of purified bovine sIgA following intramammary immunization of cows with Staphylococcus aureus." J Dairy Sci **86**(9): 2884-2894.
Beaudeau, F., C. Fourichon, et al. (2002). "Risk of clinical mastitis in dairy herds with a high proportion of low individual milk somatic-cell counts." Prev Vet Med **53**(1-2): 43-54.
Bellamy, W., M. Takase, et al. (1992). "Identification of the bactericidal domain of lactoferrin." Biochim Biophys Acta **1121**(1-2): 130-136.
Boehmer, J. L., J. L. Ward, et al. (2010). "Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification." J Dairy Sci **93**(2): 593-603.
Boettcher, P. J., J. C. Dekkers, et al. (1998). "Development of an udder health index for sire selection based on somatic cell score, udder conformation, and milking speed." J Dairy Sci **81**(4): 1157-1168.
Borregaard, N., L. J. Miller, et al. (1987). "Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils." Science **237**(4819): 1204-1206.
Boulanger, V., L. Bouchard, et al. (2001). "Induction of nitric oxide production by bovine mammary epithelial cells and blood leukocytes." J Dairy Sci **84**(6): 1430-1437.
Brouckaert, P. and W. Fiers (1996). "Tumor necrosis factor and the systemic inflammatory response syndrome." Curr Top Microbiol Immunol **216**: 167-187.
Burton, J. L., S. A. Madsen, et al. (2005). "Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows." Vet Immunol Immunopathol **105**(3-4): 197-219.
Burvenich, C., V. Van Merris, et al. (2003). "Severity of E. coli mastitis is mainly determined by cow factors." Vet Res **34**(5): 521-564.
Capuco, A. V., S. A. Bright, et al. (1992). "Increased susceptibility to intramammary infection following removal of teat canal keratin." J Dairy Sci **75**(8): 2126-2130.
Capuco, A. V., M. J. Paape, et al. (1986). "In vitro study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows." Am J Vet Res **47**(3): 663-668.
Carlier, M. F., V. Laurent, et al. (1997). "Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility." J Cell Biol **136**(6): 1307-1322.
Carlier, M. F., F. Ressad, et al. (1999). "Control of actin dynamics in cell motility. Role of ADF/cofilin." J Biol Chem **274**(48): 33827-33830.
Carlsson, A. E. (2005). "The effect of branching on the critical concentration and average filament length of actin." Biophys J **89**(1): 130-140.

Cassatella, M. A., L. Meda, et al. (1993). "Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide." J Exp Med **178**(6): 2207-2211.

Cassimeris, L., H. McNeill, et al. (1990). "Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities." J Cell Biol **110**(4): 1067-1075.

Chung, C. Y. and R. A. Firtel (2002). "Signaling pathways at the leading edge of chemotaxing cells." J Muscle Res Cell Motil **23**(7-8): 773-779.

Chung, C. Y., S. Funamoto, et al. (2001). "Signaling pathways controlling cell polarity and chemotaxis." Trends Biochem Sci **26**(9): 557-566.

Cicchetti, G., P. G. Allen, et al. (2002). "Chemotactic signaling pathways in neutrophils: from receptor to actin assembly." Crit Rev Oral Biol Med **13**(3): 220-228.

Collins, R. A., K. R. Parsons, et al. (1988). "Histochemical localization and possible antibacterial role of xanthine oxidase in the bovine mammary gland." J Dairy Res **55**(1): 25-32.

de Haas, Y., W. Ouweltjes, et al. (2008). "Alternative somatic cell count traits as mastitis indicators for genetic selection." J Dairy Sci **91**(6): 2501-2511.

Detilleux, J. C. (2009). "Genetic factors affecting susceptibility to udder pathogens." Vet Microbiol **134**(1-2): 157-164.

Detilleux, J. C., K. J. Koehler, et al. (1994). "Immunological parameters of periparturient Holstein cattle: genetic variation." J Dairy Sci **77**(9): 2640-2650.

Dewald, B., U. Bretz, et al. (1982). "Release of gelatinase from a novel secretory compartment of human neutrophils." J Clin Invest **70**(3): 518-525.

Didry, D., M. F. Carlier, et al. (1998). "Synergy between actin depolymerizing factor/cofilin and profilin in increasing actin filament turnover." J Biol Chem **273**(40): 25602-25611.

DiStasi, M. R. and K. Ley (2009). "Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability." Trends Immunol **30**(11): 547-556.

Eden, S., R. Rohatgi, et al. (2002). "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck." Nature **418**(6899): 790-793.

Ellis, T. N. and B. L. Beaman (2004). "Interferon-gamma activation of polymorphonuclear neutrophil function." Immunology **112**(1): 2-12.

Farkas, L., M. C. Hahn, et al. (2005). "Expression of CXC chemokine receptors 1 and 2 in human bronchial epithelial cells." Chest **128**(5): 3724-3734.

Faurschou, M. and N. Borregaard (2003). "Neutrophil granules and secretory vesicles in inflammation." Microbes Infect **5**(14): 1317-1327.

Fleit, H. B., S. D. Wright, et al. (1982). "Human neutrophil Fc gamma receptor distribution and structure." Proc Natl Acad Sci U S A **79**(10): 3275-3279.

Ginestier, C., S. Liu, et al. (2010). "CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts." J Clin Invest **120**(2): 485-497.

Goertz, I., C. Baes, et al. (2009). "Association between single nucleotide polymorphisms in the CXCR1 gene and somatic cell score in Holstein dairy cattle." J Dairy Sci **92**(8): 4018-4022.
Goley, E. D. and M. D. Welch (2006). "The ARP2/3 complex: an actin nucleator comes of age." Nat Rev Mol Cell Biol **7**(10): 713-726.
Gournier, H., E. D. Goley, et al. (2001). "Reconstitution of human Arp2/3 complex reveals critical roles of individual subunits in complex structure and activity." Mol Cell **8**(5): 1041-1052.
Gouwy, M., S. Struyf, et al. (2005). "Synergy in cytokine and chemokine networks amplifies the inflammatory response." Cytokine Growth Factor Rev **16**(6): 561-580.
Griesbeck-Zilch, B., H. H. Meyer, et al. (2008). "Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells." J Dairy Sci **91**(6): 2215-2224.
Grinberg, N., S. Elazar, et al. (2008). "Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic Escherichia coli." Infect Immun **76**(6): 2802-2807.
Hannigan, M., L. Zhan, et al. (2002). "Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis." Proc Natl Acad Sci U S A **99**(6): 3603-3608.
Hannigan, M. O., C. K. Huang, et al. (2004). "Roles of PI3K in neutrophil function." Curr Top Microbiol Immunol **282**: 165-175.
Harmon, R. J., F. L. Schanbacher, et al. (1976). "Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and alpha-lactalbumin during acute experimental and natural coliform mastitis in cows." Infect Immun **13**(2): 533-542.
Hayashi, F., K. D. Smith, et al. (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature **410**(6832): 1099-1103.
Hayes, B. J., P. J. Bowman, et al. (2009). "Invited review: Genomic selection in dairy cattle: progress and challenges." J Dairy Sci **92**(2): 433-443.
Heit, B. and P. Kubes (2003). "Measuring chemotaxis and chemokinesis: the under-agarose cell migration assay." Sci STKE **2003**(170): PL5.
Heit, B., S. M. Robbins, et al. (2008). "PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils." Nat Immunol **9**(7): 743-752.
Heringstad, B., D. Gianola, et al. (2006). "Genetic associations between clinical mastitis and somatic cell score in early first-lactation cows." J Dairy Sci **89**(6): 2236-2244.
Heringstad, B., G. Klemetsdal, et al. (2007). "Selection responses for disease resistance in two selection experiments with Norwegian red cows." J Dairy Sci **90**(5): 2419-2426.
Hill, A. W. (1981). "Factors influencing the outcome of Escherichia coli mastitis in the dairy cow." Res Vet Sci **31**(1): 107-112.
Hirata, H., A. Takahashi, et al. (1998). "Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis." J Exp Med **187**(4): 587-600.

Hoeben, D., R. Heyneman, et al. (1997). "Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils." Vet Immunol Immunopathol **58**(2): 165-170.
Hogan, J. S., K. L. Smith, et al. (1988). "Growth responses of environmental mastitis pathogens to long-chain fatty acids." J Dairy Sci **71**(1): 245-249.
Huang, J., H. Wang, et al. (2010). "Single nucleotide polymorphisms, haplotypes and combined genotypes of lactoferrin gene and their associations with mastitis in Chinese Holstein cattle." Mol Biol Rep **37**(1): 477-483.
Hufner, K., H. N. Higgs, et al. (2001). "The verprolin-like central (vc) region of Wiskott-Aldrich syndrome protein induces Arp2/3 complex-dependent actin nucleation." J Biol Chem **276**(38): 35761-35767.
Jain, N. C. and J. Lasmanis (1978). "Phagocytosis of serum-resistant and serum-sensitive coliform bacteria (Klebsiella) by bovine neutrophils from blood and mastitic milk." Am J Vet Res **39**(3): 425-427.
Janetopoulos, C. and R. A. Firtel (2008). "Directional sensing during chemotaxis." FEBS Lett **582**(14): 2075-2085.
Jayarao, B. M., B. E. Gillespie, et al. (1999). "Epidemiology of Streptococcus uberis intramammary infections in a dairy herd." Zentralbl Veterinarmed B **46**(7): 433-442.
Jog, N. R., M. J. Rane, et al. (2007). "The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets." Am J Physiol Cell Physiol **292**(5): C1690-1700.
Johnston, C. A. and D. P. Siderovski (2007). "Receptor-mediated activation of heterotrimeric G-proteins: current structural insights." Mol Pharmacol **72**(2): 219-230.
Karnovsky, M. L. (1968). "The metabolism of leukocytes." Semin Hematol **5**(2): 156-165.
Kelm, S. C., J. C. Detilleux, et al. (1997). "Genetic association between parameters of inmate immunity and measures of mastitis in periparturient Holstein cattle." J Dairy Sci **80**(8): 1767-1775.
Klaas, I. C., C. Enevoldsen, et al. (2005). "Cow-related risk factors for milk leakage." J Dairy Sci **88**(1): 128-136.
Koestler, S. A., S. Auinger, et al. (2008). "Differentially oriented populations of actin filaments generated in lamellipodia collaborate in pushing and pausing at the cell front." Nat Cell Biol **10**(3): 306-313.
Kooijman, R., A. Coppens, et al. (2002). "Igf-I inhibits spontaneous apoptosis in human granulocytes." Endocrinology **143**(4): 1206-1212.
Kooijman, R., A. Coppens, et al. (2003). "IGF-I stimulates IL-8 production in the promyelocytic cell line HL-60 through activation of extracellular signal-regulated protein kinase." Cell Signal **15**(12): 1091-1098.
Kuwano, Y., O. Spelten, et al. (2010). "Rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils." Blood **116**(4): 617-624.
Labeta, M. O., K. Vidal, et al. (2000). "Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14." J Exp Med **191**(10): 1807-1812.

Lacy, P. and G. Eitzen (2008). "Control of granule exocytosis in neutrophils." Front Biosci **13**: 5559-5570.
Lahouassa, H., E. Moussay, et al. (2007). "Differential cytokine and chemokine responses of bovine mammary epithelial cells to Staphylococcus aureus and Escherichia coli." Cytokine **38**(1): 12-21.
Lamote, I., E. Meyer, et al. (2004). "Influence of 17beta-estradiol, progesterone, and dexamethasone on diapedesis and viability of bovine blood polymorphonuclear leukocytes." J Dairy Sci **87**(10): 3340-3349.
LaRosa, G. J., K. M. Thomas, et al. (1992). "Amino terminus of the interleukin-8 receptor is a major determinant of receptor subtype specificity." J Biol Chem **267**(35): 25402-25406.
Le Clainche, C. and M. F. Carlier (2008). "Regulation of actin assembly associated with protrusion and adhesion in cell migration." Physiol Rev **88**(2): 489-513.
Lee, J. W., D. D. Bannerman, et al. (2006). "Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR." Vet Res **37**(2): 219-229.
Lefort, C. T., Y. M. Hyun, et al. (2009). "Outside-in signal transmission by conformational changes in integrin Mac-1." J Immunol **183**(10): 6460-6468.
Ley, K. (2002). "Integration of inflammatory signals by rolling neutrophils." Immunol Rev **186**: 8-18.
Ley, K., C. Laudanna, et al. (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade updated." Nat Rev Immunol **7**(9): 678-689.
Leyva-Baca, I., F. Schenkel, et al. (2008). "Polymorphisms in the 5' upstream region of the CXCR1 chemokine receptor gene, and their association with somatic cell score in Holstein cattle in Canada." J Dairy Sci **91**(1): 407-417.
Li, Z., X. Dong, et al. (2005). "Regulation of PTEN by Rho small GTPases." Nat Cell Biol **7**(4): 399-404.
Li, Z., M. Hannigan, et al. (2003). "Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42." Cell **114**(2): 215-227.
Liles, W. C., P. A. Kiener, et al. (1996). "Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils." J Exp Med **184**(2): 429-440.
Lukas, J. M., D. M. Hawkins, et al. (2005). "Bulk tank somatic cell counts analyzed by statistical process control tools to identify and monitor subclinical mastitis incidence." J Dairy Sci **88**(11): 3944-3952.
Madsen, S. A., P. S. Weber, et al. (2002). "Altered expression of cellular genes in neutrophils of periparturient dairy cows." Vet Immunol Immunopathol **86**(3-4): 159-175.
Marchese, A., M. M. Paing, et al. (2008). "G protein-coupled receptor sorting to endosomes and lysosomes." Annu Rev Pharmacol Toxicol **48**: 601-629.
Mayer, B., M. Doleschall, et al. (2005). "Expression of the neonatal Fc receptor (FcRn) in the bovine mammary gland." J Dairy Res **72 Spec No**: 107-112.

McClenahan, D., R. Krueger, et al. (2006). "Interleukin-8 expression by mammary gland endothelial and epithelial cells following experimental mastitis infection with E. coli." Comp Immunol Microbiol Infect Dis **29**(2-3): 127-137.
Medina, E. (2009). "Neutrophil extracellular traps: a strategic tactic to defeat pathogens with potential consequences for the host." J Innate Immun **1**(3): 176-180.
Mehrzad, J., H. Dosogne, et al. (2001). "Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation." J Dairy Res **68**(3): 399-415.
Mehrzad, J., D. Janssen, et al. (2008). "Increase in Escherichia coli inoculum dose accelerates CD8+ T-cell trafficking in the primiparous bovine mammary gland." J Dairy Sci **91**(1): 193-201.
Middleton, J. R., L. K. Fox, et al. (2001). "Management strategies to decrease the prevalence of mastitis caused by one strain of Staphylococcus aureus in a dairy herd." J Am Vet Med Assoc **218**(10): 1615-1618, 1581-1612.
Mikolajczyk, T. P., J. E. Skrzeczynska-Moncznik, et al. (2009). "Interaction of human peripheral blood monocytes with apoptotic polymorphonuclear cells." Immunology **128**(1): 103-113.
Millard, T. H., S. J. Sharp, et al. (2004). "Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex." Biochem J **380**(Pt 1): 1-17.
Molenaar, A. J., D. P. Harris, et al. (2009). "The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence." Biomarkers **14**(1): 26-37.
Morzycki, W., J. Sadowska, et al. (1990). "Interleukin-1 and tumour necrosis factor alpha induced polymorphonuclear leukocyte-endothelial cell adhesion and transendothelial migration in vitro: the effect of apical versus basal monolayer stimulation." Immunol Lett **25**(4): 331-340.
Moyes, K. M., J. K. Drackley, et al. (2009). "Gene network and pathway analysis of bovine mammary tissue challenged with Streptococcus uberis reveals induction of cell proliferation and inhibition of PPARgamma signaling as potential mechanism for the negative relationships between immune response and lipid metabolism." BMC Genomics **10**: 542.
Murphy, P. M. (1994). "The molecular biology of leukocyte chemoattractant receptors." Annu Rev Immunol **12**: 593-633.
Muta, T. and K. Takeshige (2001). "Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 Reconstitution of TLR2- and TLR4-activation by distinguishable ligands in LPS preparations." Eur J Biochem **268**(16): 4580-4589.
Naidu, T. G. and F. H. Newbould (1973). "Glycogen in leukocytes from bovine blood and milk." Can J Comp Med **37**(1): 47-55.
Nasser, M. W., S. K. Raghuwanshi, et al. (2007). "CXCR1 and CXCR2 activation and regulation. Role of aspartate 199 of the second extracellular loop of CXCR2 in CXCL8-mediated rapid receptor internalization." J Biol Chem **282**(9): 6906-6915.

NMC (1996). Current concepts of bovine mastitis. National Mastitis Council. Madison, WI.
Ogle, J. D., J. G. Noel, et al. (1990). "The effects of cytokines, platelet activating factor, and arachidonate metabolites on C3b receptor (CR1, CD35) expression and phagocytosis by neutrophils." Cytokine **2**(6): 447-455.
Olde Riekerink, R. G., H. W. Barkema, et al. (2010). "Management practices associated with the bulk-milk prevalence of Staphylococcus aureus in Canadian dairy farms." Prev Vet Med **97**(1): 20-28.
Oldham, W. M. and H. E. Hamm (2008). "Heterotrimeric G protein activation by G-protein-coupled receptors." Nat Rev Mol Cell Biol **9**(1): 60-71.
Oliver, S. P. (1988). "Frequency of isolation of environmental mastitis-causing pathogens and incidence of new intramammary infection during the nonlactating period." Am J Vet Res **49**(11): 1789-1793.
Oliver, S. P., S. H. King, et al. (1990). "Efficacy of chlorhexidine as a postmilking teat disinfectant for the prevention of bovine mastitis during lactation." J Dairy Sci **73**(8): 2230-2235.
Otomo, T., C. Otomo, et al. (2005). "Structural basis of Rho GTPase-mediated activation of the formin mDia1." Mol Cell **18**(3): 273-281.
Oviedo-Boyso, J., J. J. Valdez-Alarcon, et al. (2007). "Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis." J Infect **54**(4): 399-409.
Paape, M. J., D. D. Bannerman, et al. (2003). "The bovine neutrophil: Structure and function in blood and milk." Vet Res **34**(5): 597-627.
Paape, M. J. and A. J. Guidry (1977). "Effect of fat and casein on intracellular killing of Staphylococcus aureus by milk leukocytes." Proc Soc Exp Biol Med **155**(4): 588-593.
Paape, M. J., R. H. Miller, et al. (1992). "Influence of involution on intramammary phagocytic defense mechanisms." J Dairy Sci **75**(7): 1849-1856.
Paape, M. J., K. Shafer-Weaver, et al. (2000). "Immune surveillance of mammary tissue by phagocytic cells." Adv Exp Med Biol **480**: 259-277.
Pantaloni, D., R. Boujemaa, et al. (2000). "The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins." Nat Cell Biol **2**(7): 385-391.
Pantaloni, D., C. Le Clainche, et al. (2001). "Mechanism of actin-based motility." Science **292**(5521): 1502-1506.
Patel, D., R. A. Almeida, et al. (2009). "Bovine lactoferrin serves as a molecular bridge for internalization of Streptococcus uberis into bovine mammary epithelial cells." Vet Microbiol **137**(3-4): 297-301.
Perelroizen, I., D. Didry, et al. (1996). "Role of nucleotide exchange and hydrolysis in the function of profilin in action assembly." J Biol Chem **271**(21): 12302-12309.
Persson, K., A. Carlsson, et al. (1992). "Immunoglobulins, lysozyme and lactoferrin in the teat and udder of the dry cow during endotoxin-induced inflammation." Zentralbl Veterinarmed B **39**(3): 165-174.

Perussia, B., E. T. Dayton, et al. (1983). "Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells." J Exp Med **158**(4): 1092-1113.
Piccinini, R., V. Bronzo, et al. (1999). "Study on the relationship between milk immune factors and Staphylococcus aureus intramammary infections in dairy cows." J Dairy Res **66**(4): 501-510.
Pollard, T. D. (2007). "Regulation of actin filament assembly by Arp2/3 complex and formins." Annu Rev Biophys Biomol Struct **36**: 451-477.
Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." Cell **112**(4): 453-465.
Poltorak, A., X. He, et al. (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-2088.
Premont, R. T., J. Inglese, et al. (1995). "Protein kinases that phosphorylate activated G protein-coupled receptors." FASEB J **9**(2): 175-182.
Rainard, P. (1986). "Bacteriostasis of Escherichia coli by bovine lactoferrin, transferrin and immunoglobulins (IgG1, IgG2, IgM) acting alone or in combination." Vet Microbiol **11**(1-2): 103-115.
Rainard, P. (2003). "The complement in milk and defense of the bovine mammary gland against infections." Vet Res **34**(5): 647-670.
Rainard, P. and B. Poutrel (1995). "Deposition of complement components on Streptococcus agalactiae in bovine milk in the absence of inflammation." Infect Immun **63**(9): 3422-3427.
Rainard, P., B. Poutrel, et al. (1984). "Assessment of hemolytic and bactericidal complement activities in normal and mastitic bovine milk." J Dairy Sci **67**(3): 614-619.
Rainard, P. and C. Riollet (2006). "Innate immunity of the bovine mammary gland." Vet Res **37**(3): 369-400.
Rainard, P., P. Sarradin, et al. (1998). "Quantification of C5a/C5a(desArg) in bovine plasma, serum and milk." Vet Res **29**(1): 73-88.
Rambeaud, M., R. A. Almeida, et al. (2003). "Dynamics of leukocytes and cytokines during experimentally induced Streptococcus uberis mastitis." Vet Immunol Immunopathol **96**(3-4): 193-205.
Rambeaud, M., R. Clift, et al. (2006). "Association of a bovine CXCR2 gene polymorphism with neutrophil survival and killing ability." Vet Immunol Immunopathol **111**(3-4): 231-238.
Rambeaud, M. and G. M. Pighetti (2005). "Impaired neutrophil migration associated with specific bovine CXCR2 genotypes." Infect Immun **73**(8): 4955-4959.
Rambeaud, M. and G. M. Pighetti (2007). "Differential calcium signaling in dairy cows with specific CXCR1 genotypes potentially related to interleukin-8 receptor functionality." Immunogenetics **59**(1): 53-58.
Reiter, B., J. H. Brock, et al. (1975). "Inhibition of Escherichia coli by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of E. coli." Immunology **28**(1): 83-95.

Rosenbaum, D. M., S. G. Rasmussen, et al. (2009). "The structure and function of G-protein-coupled receptors." Nature **459**(7245): 356-363.
Saarikangas, J., H. Zhao, et al. (2010). "Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides." Physiol Rev **90**(1): 259-289.
Sanchez-Madrid, F. and M. A. del Pozo (1999). "Leukocyte polarization in cell migration and immune interactions." EMBO J **18**(3): 501-511.
Santos-Beneit, A. M. and F. Mollinedo (2000). "Expression of genes involved in initiation, regulation, and execution of apoptosis in human neutrophils and during neutrophil differentiation of HL-60 cells." J Leukoc Biol **67**(5): 712-724.
Sarikaya, H., C. Werner-Misof, et al. (2005). "Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows." J Dairy Res **72**(4): 486-492.
Schukken, Y. H. S., H. (2004). Coping with mastitis pathogen shift. Why mastitis culturing improves treatment and control strategies. National Mastitis Council, Bloomington, Minnesota.
Selsted, M. E., Y. Q. Tang, et al. (1996). "Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils." J Biol Chem **271**(27): 16430.
Shafer-Weaver, K. A. and L. M. Sordillo (1996). "Enhancing bactericidal activity of bovine lymphoid cells during the periparturient period." J Dairy Sci **79**(8): 1347-1352.
Sharma, B. S., I. Leyva, et al. (2006). "Association of toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls." J Dairy Sci **89**(9): 3626-3635.
Shuster, D. E., M. E. Kehrli, Jr., et al. (1997). "Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with Escherichia coli." Infect Immun **65**(8): 3286-3292.
Shuster, D. E., E. K. Lee, et al. (1996). "Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation." Am J Vet Res **57**(11): 1569-1575.
Smith, K. L., J. H. Harrison, et al. (1984). "Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms." J Dairy Sci **67**(6): 1293-1300.
Smits, E., C. Burvenich, et al. (1999). "Diapedesis across mammary epithelium reduces phagocytic and oxidative burst of bovine neutrophils." Vet Immunol Immunopathol **68**(2-4): 169-176.
Snapper, S. B. and F. S. Rosen (1999). "The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization." Annu Rev Immunol **17**: 905-929.
Sordillo, L. M., S. C. Nickerson, et al. (1989). "Pathology of Staphylococcus aureus mastitis during lactogenesis: relationships with bovine mammary structure and function." J Dairy Sci **72**(1): 228-240.

Srinivasan, S., F. Wang, et al. (2003). "Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis." J Cell Biol **160**(3): 375-385.
Stelwagen, K., V. C. Farr, et al. (1997). "Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components." Am J Physiol **273**(1 Pt 2): R379-386.
Stricker, J., T. Falzone, et al. (2010). "Mechanics of the F-actin cytoskeleton." J Biomech **43**(1): 9-14.
Takata, H., H. Tomiyama, et al. (2004). "Cutting edge: expression of chemokine receptor CXCR1 on human effector CD8+ T cells." J Immunol **173**(4): 2231-2235.
Takeda, K. and S. Akira (2001). "Roles of Toll-like receptors in innate immune responses." Genes Cells **6**(9): 733-742.
Taylor, J. M., G. G. Jacob-Mosier, et al. (1994). "Binding of an alpha 2 adrenergic receptor third intracellular loop peptide to G beta and the amino terminus of G alpha." J Biol Chem **269**(44): 27618-27624.
Todhunter, D. A., K. L. Smith, et al. (1995). "Environmental streptococcal intramammary infections of the bovine mammary gland." J Dairy Sci **78**(11): 2366-2374.
Torres, E. and M. K. Rosen (2006). "Protein-tyrosine kinase and GTPase signals cooperate to phosphorylate and activate Wiskott-Aldrich syndrome protein (WASP)/neuronal WASP." J Biol Chem **281**(6): 3513-3520.
Urban, C. F., S. Lourido, et al. (2006). "How do microbes evade neutrophil killing?" Cell Microbiol **8**(11): 1687-1696.
Urech, E., Z. Puhan, et al. (1999). "Changes in milk protein fraction as affected by subclinical mastitis." J Dairy Sci **82**(11): 2402-2411.
Uthaisangsook, S., N. K. Day, et al. (2002). "Innate immunity and its role against infections." Ann Allergy Asthma Immunol **88**(3): 253-264; quiz 265-256, 318.
Verschoor, C. P., S. D. Pant, et al. (2009). "SNPs in the bovine IL-10 receptor are associated with somatic cell score in Canadian dairy bulls." Mamm Genome **20**(7): 447-454.
Wang, D., K. M. Pabst, et al. (1995). "Lipopolysaccharide-inactivating activity of neutrophils is due to lactoferrin." J Leukoc Biol **57**(6): 865-874.
Wang, F., P. Herzmark, et al. (2002). "Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils." Nat Cell Biol **4**(7): 513-518.
Ward, P. A. (2009). "Functions of C5a receptors." J Mol Med **87**(4): 375-378.
Watts, R. G., M. A. Crispens, et al. (1991). "A quantitative study of the role of F-actin in producing neutrophil shape." Cell Motil Cytoskeleton **19**(3): 159-168.
Weiner, O. D. (2002). "Regulation of cell polarity during eukaryotic chemotaxis: the chemotactic compass." Curr Opin Cell Biol **14**(2): 196-202.
Weiss, S. J. (1989). "Tissue destruction by neutrophils." N Engl J Med **320**(6): 365-376.
Welch, M. D. (1999). "The world according to Arp: regulation of actin nucleation by the Arp2/3 complex." Trends Cell Biol **9**(11): 423-427.
Welty, F. K., K. L. Smith, et al. (1976). "Lactoferrin concentration during involution of the bovine mammary gland." J Dairy Sci **59**(2): 224-231.

Werts, C., R. I. Tapping, et al. (2001). "Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism." Nat Immunol **2**(4): 346-352.
Windig, J. J., W. Ouweltjes, et al. (2010). "Combining somatic cell count traits for optimal selection against mastitis." J Dairy Sci **93**(4): 1690-1701.
Wong, K., O. Pertz, et al. (2006). "Neutrophil polarization: spatiotemporal dynamics of RhoA activity support a self-organizing mechanism." Proc Natl Acad Sci U S A **103**(10): 3639-3644.
Yago, T., B. Shao, et al. (2010). "E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin alphaLbeta2-mediated slow leukocyte rolling." Blood **116**(3): 485-494.
Yang, W., H. Zerbe, et al. (2008). "Bovine TLR2 and TLR4 properly transduce signals from Staphylococcus aureus and E. coli, but S. aureus fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder." Mol Immunol **45**(5): 1385-1397.
Youngerman, S. M., A. M. Saxton, et al. (2004). "Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle." J Dairy Sci **87**(8): 2442-2448.
Youngerman, S. M., A. M. Saxton, et al. (2004). "Novel single nucleotide polymorphisms and haplotypes within the bovine CXCR2 gene." Immunogenetics **56**(5): 355-359.
Zarbock, A. and K. Ley (2008). "Mechanisms and consequences of neutrophil interaction with the endothelium." Am J Pathol **172**(1): 1-7.
Zehner, M. M., R. J. Farnsworth, et al. (1986). "Growth of environmental mastitis pathogens in various bedding materials." J Dairy Sci **69**(7): 1932-1941.
Zhao, X. and P. Lacasse (2008). "Mammary tissue damage during bovine mastitis: causes and control." J Anim Sci **86**(13 Suppl): 57-65.
Zhelev, D. V., A. M. Alteraifi, et al. (2004). "Controlled pseudopod extension of human neutrophils stimulated with different chemoattractants." Biophys J **87**(1): 688-695.
Zwald, N. R., K. A. Weigel, et al. (2006). "Genetic analysis of clinical mastitis data from on-farm management software using threshold models." J Dairy Sci **89**(1): 330-336.

VITA

Alexandra Elliott was born on February 12, 1982 in Ellicott City, Maryland. She attended primary and secondary school in Damascus, Maryland and graduated from Damascus High School in 2000. From there, she went to Murray State University where she majored in pre-veterinary science and received her Bachelor's of Science degree in May 2004. She went on to graduate in December 2005 from Murray State University with her Master's of Science degree in Agriculture Science. In fall 2010, she graduated from the University of Tennessee with a Doctor of Philosophy degree in Animal Science.