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Differential Neutrophil Function and Intracellular Signaling in Dairy Cows with Specific CXCR1 Genotypes

Magdalena Rambeaud
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To the Graduate Council:

I am submitting herewith a dissertation written by Magdalena Rambeaud entitled "Differential Neutrophil Function and Intracellular Signaling in Dairy Cows with Specific CXCR1 Genotypes." 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:

Stephen P. Oliver, Tim Sparer, Arnold Saxton, Alan G. Mathew

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Anne Mayhew

Vice Chancellor and Dean of
Graduate Studies

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DIFFERENTIAL NEUTROPHIL FUNCTION AND INTRACELLULAR SIGNALING IN DAIRY COWS WITH SPECIFIC CXCR1 GENOTYPES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Magdalena Rambeaud

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DEDICATION

This dissertation is dedicated to my husband Fernando, my daughter Julia and my parents Oscar and Cielo Rambeaud. Fer, I draw strength from your love, encouragement and patience. Julia, you are the light of my life. Mami y Papi, thanks for your unwavering support no matter what I endeavors I embark into.

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Last, but not least, I want to thank my husband Fernando for always believing in me and for his unconditional support. What a ride this has been... I draw strength from your love, encouragement and patience. I definitely could not have done this without you.

ABSTRACT

Mastitis continues to be the most economically devastating disease affecting dairy cows worldwide. Neutrophil recruitment to the mammary gland and neutrophil functional ability once there often determine whether a bacterial infection is eliminated or becomes chronic. A slow or ineffective neutrophil attack allows bacteria to continue their assault and generate a longer lasting and potentially more damaging infection. Since neutrophils are key players in the resolution of mastitis, and a polymorphism in CXCR1 +777 (G→C) has been associated with susceptibility to mastitis, the hypothesis of this research was that different CXCR1 +777 genotypes were associated with efficiency of in vitro neutrophil activity and/or IL-8 receptor expression and intracellular signaling. Neutrophil function (migration, adhesion molecule upregulation, survival from spontaneous apoptosis, glutathione levels, reactive oxygen generation and bactericidal activity) as well as CXCR1 and CXCR2 expression and signaling were evaluated in cows with different CXCR1 +777 genotypes. Cows with a CC genotype, which have previously shown increased susceptibility to mastitis, had decreased adhesion molecule upregulation and neutrophil migration towards IL-8 compared to cows with a GG genotype. Furthermore, cows with a CC genotype displayed decreased ROS generation and, paradoxically, increased survival from spontaneous apoptosis. In addition, initial observations revealed that overall IL-8 receptor numbers tended to be lower in cows with a CC genotype compared to

cows with a GG genotype. However, in the presence of a CXCR2 inhibitor (SB225002), CXCR1 affinity was about 5-fold lower in cows with a CC genotype and this may have resulted in an underestimation of receptor numbers in cows with this genotype. Additionally, intracellular calcium ($[Ca^{++}]_i$) release was lower in cows with a CC genotype when cells were stimulated with IL-8 but not epithelial derived neutrophil attractant-78. When neutrophils were stimulated with an optimal dose of IL-8 in the presence of SB225002, allowing only signaling through CXCR1, $[Ca^{++}]_i$ release was lower in cows with a CC genotype, suggesting decreased CXCR1 signaling in these animals, potentially due to lower CXCR1 affinity for IL-8. This research provides evidence for neutrophil functional differences and differential CXC receptor activity and signaling in cows with specific CXCR1 genotypes. Future research aimed at better characterizing CXCR1 and CXCR2 in the bovine as well as corroboration of these findings in an in vivo model may provide prospective ways to enhance or regulate neutrophil function in dairy cows and potentially increase their resistance to mastitis and other inflammatory diseases.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

Chapter 1

INTRODUCTION

Mastitis continues to be the single most limiting factor to profitable dairying. The National Mastitis Council estimates that nearly $200 per cow is lost each year due to lower milk production, increased replacement costs, discarded milk, treatment regimens and labor costs (NMC, 1996). However, the price of mastitis does not stop there. Permanent damage to milk-secreting tissue by chronic infections may reduce milk production in subsequent lactations. Additionally, consumers are demanding a safe, high quality, nutritious food supply. Mastitis is a significant deterrent to these characteristics because it alters milk components and increases cellular content, not to mention the public concern over contributing to bacterial antimicrobial resistance through use of antibiotics to treat infection. Therefore, the ability to identify and cull susceptible animals or to select for resistant animals would be of great benefit, as producers could eliminate costly treatment regimens as well as provide a healthier and safer milk supply for consumers. Historically, this selection process has been based upon the immune response, which is logical as the effectiveness of the immune response often determines whether an infection is eliminated or not (Shuster et al., 1997; Sordillo et al., 1997). However, with the complexity of

innate and acquired immunity, it is highly probable that specific genes or combinations of specific genes would be indicative of host defense ability.

Genes associated with neutrophil function are potential genetic markers for mastitis as neutrophil migration from blood to the site of infection and functional activity once there is essential for the resolution of mastitis (Paape et al., 2000; Kehrli and Harp, 2001). Influx of neutrophils into the mammary gland occurs at a low level for immune surveillance but increases rapidly in response to bacterial infection, where potent cytokines and other inflammatory mediators guide neutrophils towards the site of infection. One of these cytokines, interleukin-8 (IL-8), is a critical mediator of neutrophil function, inducing chemotaxis, adhesion molecule upregulation, phagocytosis, reactive oxygen species generation, cytokine production and cell survival (Kettritz et al., 1998; Chertov et al., 2000). Neutrophils recognize IL-8 through receptors on their surfaces. In humans and other species, two related receptors that recognize IL-8, CXCR1 and CXCR2, have been described. While these receptors share a high degree of structure homology, their ligand specificity differs, with CXCR1 binding only to IL-8 (Devalaraja and Richmond, 1999) and CXCR2 binding to multiple CXC chemokines (Ahuja and Murphy, 1996).

Recent research in our lab has demonstrated a significant association between the incidence of subclinical mastitis and polymorphisms in the CXCR1 receptor gene in Holstein dairy cows (Youngerman et al., 2004b). Cattle with a CC genotype at nucleotide position +777 (Genbank accession number

NM_174360) had a greater incidence of subclinical mastitis than those with a GG genotype at this position. The polymorphism at this position, +777, causes an amino acid change from histidine to glutamine within the third intracellular loop of the receptor where G-protein binding occurs (Damaj et al., 1996a; Youngerman et al., 2004a). Thus, this amino acid change has the potential to influence neutrophil function and subsequent disease resistance. Therefore, the central hypothesis of the present study is that *neutrophil IL-8 receptor expression and/or neutrophil function differ among dairy cows of different CXCR1 SNP +777 genotypes*. Results from this research will provide a better understanding of factors and pathways that contribute to genetic variation in neutrophil activity and mastitis susceptibility. With this knowledge, it may be possible to specifically target these particular factors and pathways for development of pharmacological strategies aimed at either enhancing or restricting neutrophil function depending upon the desired outcome, therefore reducing the incidence, severity and/or duration of mastitis.

Chapter 2

LITERATURE REVIEW

I- NEUTROPHILS

Neutrophils are the primary effector cells against bacterial infections. They play a critical role in protecting the animal from many diseases, such as bacterial pneumonia, retained placenta and mastitis (Paape et al., 1979; Mosier, 1997; Kimura et al., 2002). An efficient protective response requires neutrophils to migrate quickly to the inflammatory site and exert their phagocytic and bactericidal activity. This process is driven by many pro-inflammatory mediators, which regulate neutrophil vascular adhesion, migration and activation.

Description

Mature neutrophils contain a polymorphic segmented nucleus, which allows the cell to line up its nuclear lobes in a straight line to allow rapid migration between endothelial cells to the site of infection (Paape et al., 1979). Neutrophils take their name from the staining properties of their cytoplasmic granules. These granules are neutrophilic, meaning they show no special affinity for either acidic or basic stains but are stained mildly by both (Geneser, 1986). The numerous bactericidal granules present in the cytoplasm are the main weapon used by

neutrophils to combat invading pathogens and can be classified into three categories: primary, secondary and tertiary granules.

a- Primary granules

Primary granules, also called azurophilic granules, are the first to appear during granulopoiesis (Bainton et al., 1971). They are large, electron-dense granules positive for peroxidase activity (Klebanoff, 1970). Primary granules contain bactericidal proteins such as defensins, elastase, bactericidal permeability-increasing protein, acid phosphatase and other lysosomal enzymes; however, the main antibacterial compound derived from these granules is myeloperoxidase (MPO) (Bainton and Farquhar, 1968; Bainton et al., 1971; Welsh and Spitznagel, 1971). Lysozyme, an important bactericidal component found in primary granules of other species which cleaves peptidoglycans from the cell wall of Gram-positive bacteria and from the outer membrane of Gram-negative bacteria (Vakil et al., 1969), is present in very low concentrations in bovine primary granules (Rausch and Moore, 1975).

b- Secondary granules

Secondary granules are peroxidase-negative and not as electron dense as azurophilic granules. The formation of azurophilic granules stops once synthesis of secondary granules begins; therefore, secondary granules outnumber azurophilic granules in mature neutrophils. Secondary granules are characterized by the presence of lactoferrin (Dewald et al., 1982); however, they additionally contain a variety of proteins including, collagenase, gelatinase, small

amounts of lysozyme, complement receptor 3, and neutrophil gelatinase associated lipocalin (NGAL), which may modulate the inflammatory response by binding small lipophilic inflammatory mediators such as platelet activating factor, leukotriene B_4 and lipopolysaccharide (Bainton et al., 1971; Borregaard et al., 1993; Kjeldsen et al., 1993). Secondary granules are mobilized more rapidly than primary granules (Dewald et al., 1982).

c- Tertiary granules

A third "novel" large peroxidase-negative granule has been identified in cows, goats and sheep (Gennaro et al., 1983b). These granules are larger than azurophilic and secondary granules and are the predominant granules in mature bovine neutrophils (Baggiolini et al., 1985). Tertiary granules contain the majority of antimicrobial protein activity in bovine neutrophils (Gennaro et al., 1983a). They contain a group of highly cationic proteins named bactenecins. Two bactenecins with approximate molecular weights of 5000 and 7000 Daltons called Bac5 and Bac7 have been identified (Frank et al., 1990). Both are highly cationic polypeptides with antibacterial properties towards *Escherichia coli*, *Salmonella enterica* Typhimurium and *Klebsiella pneumoniae* (Frank et al., 1990). Tertiary granules are also the storage site for β-defensins, a powerful group of oxygen-independent bactericidal compounds. Defensins are cysteine and arginine rich peptides, containing 38-42 residues, that display bactericidal activity against Gram-positive and Gram-negative bacteria, fungi and viruses (Savioni et al., 1984; Yount et al., 1999).

In addition to the bactericidal granules, neutrophils contain a distinct, highly mobilizable intracellular compartment, the secretory vesicles. Secretory vesicles were discovered due to the observation that the β_2 integrin Mac-1 (CD11b/CD18; described below) was incorporated into the plasma membrane without concomitant exocytosis of granule content (Borregaard et al., 1987). Secretory vesicles are important because of their membrane, particularly rich in receptors, as well as in alkaline phosphatase (which serves as a marker for these vesicles), and cytochrome b_{558}, a component of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (described below) (Borregaard et al., 1987; Burg and Pillinger, 2001). The only known intravesicular content of secretory vesicles is plasma. Secretory vesicles are mobilized when the neutrophil establishes the primary rolling contact with activated endothelium. Mobilization of secretory vesicles may be mediated by signaling through selectins (Crockett-Torabi and Fantone, 1995) or by inflammatory mediators released from endothelial cells (Patel et al., 1994). Therefore, secretory vesicle mobilization due to selectin-mediated rolling on activated endothelium initiates the cascade of events that results in neutrophil adhesion and migration into the inflammatory site.

These regulated storage organelles (granules and secretory vesicles) are not just simple bags of proteolytic or bactericidal proteins that are kept in store until liberated either to the outside of the cell or to the inside of the phagocytic vacuole, but are also important reservoirs of membrane proteins that become

incorporated into the surface membrane of the cell when these organelles fuse with the plasma membrane and exocytose their content (Borregaard et al., 1983). In this way, granules and secretory vesicles may fundamentally change the ability of the neutrophil to interact with its environment. Furthermore, granules and secretory vesicles are mobilized in a hierarchy, which seems adjusted to the different roles these organelles play during the journey of the neutrophil from the circulation to the inflammatory focus. First, secretory vesicles fuse with the plasma membrane, enhancing the potential of the neutrophil for firm adhesion. Second, exocytosis of specific granules, which contain elastase and gelatinase may help with degradation of collagen and elastin fibers present in the basement membrane and extracellular matrix, as well as enhance the phagocytic potential by providing additional CD11b/CD18 molecules that can act as C3b receptors. Lastly, fusion of azurophilic and specific granules within the phagosome creates conditions for oxygen dependent and independent bactericidal activity (Borregaard, 1996).

Neutrophils recognize conserved microbial structures, named pathogen associated molecular patterns, that are relatively invariant within a pathogen class. The receptors that recognize them are called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). Toll-like receptors (TLRs) are an important group of PRRs involved in recognition of lipopolysaccharide, peptidoglycan, lipoteichoic acid, bacterial lipoproteins and zymosan (Beutler, 2000; Medzhitov and Janeway, 2000). This family of receptors has the capacity

to recognize a wide spectrum of stimuli, providing some “specificity” to the innate immune response. The intracellular domains of TLR are similar to the intracellular domain of the interleukin-1 (IL-1) receptor, and these regions are referred to as Toll/IL-1R domain. Activation of TLRs triggers a multi-step signaling pathway highly similar to the one activated by IL-1, which results in activation of particular target genes, mostly through activation of nuclear factor κB (O'Neil and Greene, 1998) and MAPK pathways (Medzhitov, 2001), At least 11 TLRs have been identified (Medzhitov, 2001; Zhang et al., 2004). Expression of all TLRs except TLR3 and TLR11 has been reported in human neutrophils (Hayashi et al., 2003). Specifically for bovine, Menzies and Ingham (2006) described homologues of human TLRs 1-10 in lymphoid tissue, but not specifically in neutrophils, although it can be assumed that bovine neutrophils also express at least some of the known TLRs. TLR activation results in the production of pro-inflammatory cytokines, chemokines, major histocompatibility molecules and co-stimulatory molecules, and TLRs 2, 4 and 6 also play a role in regulating neutrophil apoptosis (Medzhitov, 2001; Jablonska et al., 2006).

An interesting characteristic of bovine neutrophils is that they lack Fc receptors for IgG_1 (Howard et al., 1980; Tao et al., 1995). Consequently, IgG_1 cannot function as an opsonin for bovine neutrophils, which is problematic because IgG_1 is the predominant immunoglobulin present in bovine milk (Lascelles, 1979). In addition, neutrophils express membrane receptors for the

Fc component of IgM and IgG_2 and for complement fraction C3b, which are necessary for mediating phagocytosis of invading bacteria.

In addition, unlike most mammalian species, bovine neutrophils are not chemotactically attracted to N-formylated bacterial peptides (Craven, 1986), which are fairly unique to prokaryotic organisms. Reasons for this are unknown, but might be related to the important function that microbial flora play in ruminant digestion and the fact that it would not be beneficial for animals to develop an immune response towards beneficial microbes involved in digestion (Kehrli and Harp, 2001). It is not known how ruminants efficiently recognize the presence of bacterial peptides in undesirable locations; possibly, N-formylated bacterial peptides are recognized by the ruminant immune system in a more modest manner that results in an indirect initiation of an inflammatory response (Kehrli and Harp, 2001).

Neutrophil recruitment to the inflammatory site

The first step involved in neutrophil recruitment to the site of infection is the initial contact and loose interaction between neutrophils in the bloodstream and endothelial cells from post-capillary venules. Rolling of leukocytes against the blood vessel wall is mediated by members of the selectin family (Zimmerman, 1992). Molecules of the selectin family are heavily glycosylated, single chain integral membrane proteins that include CD62P, CD62E (both present in endothelial cells) and CD62L (present in leukocytes). Rolling of leukocytes is

mediated in part by CD62L which binds to mucin-like glycoproteins (GlyCAM-1 and CD34) expressed on postcapillary endothelial cells (Robbins, 1994). CD62L is crucial for recruitment of neutrophils into inflamed tissues; it acts by slowing down leukocytes upon contact with its specific ligands (Butcher, 1991). Additionally, during an inflammatory response, locally produced cytokines stimulate increased expression of CD62P and CD62E on endothelial cells, which interact with ligands on leukocytes (P-selectin glycoprotein ligand-1 for CD62P, and sialyl Lewis X glycoprotein for CD62E) and further facilitate margination of leukocytes (von Andrian et al., 1993; Moore et al., 1995). Selectins mediate leukocyte transient adhesive contacts with high tensile strength to allow rolling under shear stress (McEver et al., 1995). Neutrophils activated by cytokines or chemoattractants shed CD62L (Kishimoto et al., 1989), which is a prerequisite step for β_2- integrin mediated tight adhesion, possibly permitting neutrophils to "let go" during subsequent stages of transmigration.

Integrins are the major family of cell surface receptors that mediate adhesive cell to cell interactions and attachment to extracellular matrix (Hynes, 1992; Sonnenberg, 1993). Structurally, integrins are heterodimeric glycoproteins made up of alpha and beta chains. The β_2-integrins are mainly involved in leukocyte-endothelial cell contact. The β_2-integrin family consists of distinct α chains, CD11a, CD11b and CD11c that share a common β chain (CD18), all of which have cytosolic domains that interact with the cytoskeleton, allowing stabilization of cell adhesion and providing a framework for signaling proteins.

Distribution of β_2-integrins on leukocyte surfaces varies with cell type and state of activation. The most important β_2-integrin involved in neutrophil recruitment into inflamed tissue is Mac-1 (CD11b/CD18) (Arnaout, 1990). Mac-1 is mainly found on granulocytes with some expression on macrophages and natural killer cells (Arnaout, 1990). Mac-1 is predominantly stored in secretory vesicles; upon cellular activation these secretory vesicles translocate to the cell surface and expression of Mac-1 increases markedly (Miller et al., 1987; Arnaout, 1990). However, expression of integrins on the cell membrane does not guarantee their ability to function as adhesion receptors. Integrins must undergo conversion from inactive to active ligand-binding status, which occurs through a process of clustering and/or altered conformation (Stewart and Hogg, 1996). The stimulus for this activation is initiated by the triggering of other membrane receptors, a route of signal transduction that has been termed “inside out” signaling. In addition to their critical role in neutrophil adhesion, β_2-integrins act as membrane receptors for bacterial pathogens as well as for complement fraction C3b and fibrinogen, and play a role in modulating neutrophil apoptosis (Brown, 1991; Mayadas and Cullere, 2005).

β_2-integrins expressed on leukocytes interact with molecules of the immunoglobulin family. Intracellular adhesion molecule 1 (ICAM-1) is an integral membrane protein that binds to Mac-1 on leukocytes (Diamond et al., 1991). ICAM-1 is expressed in low levels on normal endothelium; however, its expression increases markedly on cytokine- stimulated endothelial cells (Dustin

et al., 1986). Increased ICAM expression is important for high affinity binding of leukocytes to postcapillary venules and subsequent migration into inflamed tissues (Dustin et al., 1986).

In summary, the currently accepted model for neutrophil adhesion and transmigration in acute inflammation, outlined in Figure 1, postulates the following steps (Robbins, 1994):

1) Initial rapid and relatively loose adhesion that accounts for rolling, involving mainly constitutively expressed CD62L and CD62P, and, in cytokine induced endothelium, CD62E.

2) Activation of leukocytes by agents made by endothelium or other cells or emanating from the site of injury, to increase avidity of integrins.

3) Strong binding of leukocytes and endothelial cells, largely through β_2-integrin-ICAM pathway, and consequent transmigration.

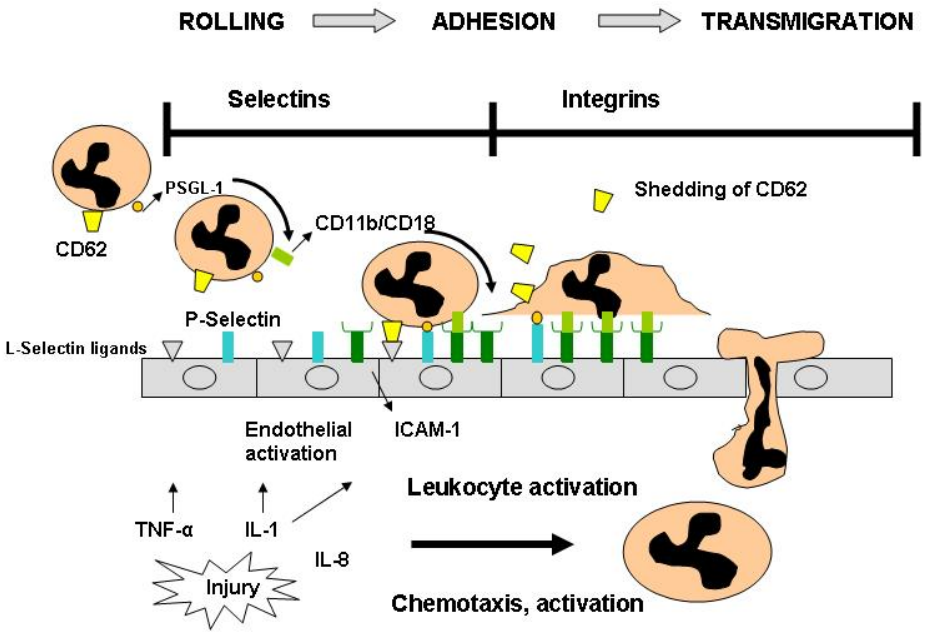

Figure 1. Schematic representation of neutrophil recruitment from the bloodstream into the inflammatory site. Primary contact between circulating neutrophils and endothelium is mediated via selectins and their ligands, which cause the neutrophils to roll along the activated endothelium. Integrins are responsible for tight adhesion to the activated endothelium and consequent transmigration of cells into tissues (adapted from Robbins, 1994).

Neutrophil phagocytosis

The first event to occur in the process of phagocytosis is contact and recognition of the pathogen. Opsonization of bacteria by specific antibodies and/or complement fractions greatly enhances neutrophil phagocytosis (Ohman et al., 1988). Specific antibodies bound to bacteria through their Fab region bind neutrophil Fc receptors through their exposed Fc fraction. The main opsonizing antibodies for bovine neutrophils are IgM and IgG_2 (Howard et al., 1980; Miller et al., 1988). Contrary to human neutrophils, bovine neutrophils do not express $Fc_\gamma RI$ and $Fc_\gamma RII$ receptors (which bind IgG_1 and IgG_2, respectively, in humans), and have a special receptor for IgG_2, the $Fc_\gamma 2$ receptor, which is distinct from all other Fc_γ receptors (Zhang et al., 1995). Complement fraction C3b and C3bi can also act as opsonins, and are respectively recognized by complement receptor 1 and Mac-1 on the neutrophil membrane (Howard et al., 1980).

Non-opsonic phagocytosis may play a particularly important role in the control of bovine intramammary infections by Gram-negative bacteria (Paape et al., 1996). Non-opsonic phagocytosis involves recognition of lectins and hydrophobic motifs on bacterial cell surfaces. Carbohydrate receptors on the cell surface of neutrophils interact with carbohydrate-rich pili of certain Gram-negative bacteria, which results in phagocytosis in the absence of specific opsonins. An interesting possibility is that this neutrophil recognition and adherence of bacteria, even without phagocytosis, may allow immobilization and flushing out of bacteria adhered to neutrophils during milking.

Neutrophil generation of reactive oxygen species (ROS)

In addition to the wide array of bactericidal proteins stored in their cytoplasmic granules, neutrophils produce powerful metabolites from O_2 to defend against microorganisms. These metabolites, called reactive oxygen species (ROS), constitute one of the most potent bactericidal mechanisms of neutrophils because of their ability to oxidize amino acids, nucleotides, and heme proteins, (Weiss, 1989; Densell and Mandell, 1990). The generation of ROS begins with the formation of NADPH oxidase, a multi-subunit enzyme formed only after neutrophil activation (Densell and Mandell, 1990; Burg and Pillinger, 2001). There are at least six components of the oxidase: $p47^{phox}$ (for phagocyte oxidase), $p67^{phox}$, and $p40^{phox}$, which are found in the resting neutrophil as a cytosolic complex; rac-2, a cytosolic, ras-related protein; and $p22^{phox}$ and $gp91^{phox}$, membrane components which comprise cytochrome b_{558} (cyt b_{558}) (Babior et al., 1981; Clark et al., 1990). Translocation of the cytoplasmic components to the membrane and their association with cyt b_{558} render the complex functional. An additional critical step for oxidase function is the phosphorylation of $p47^{phox}$ via protein kinase C (PKC) activation (Clark et al., 1990). Upon activation, $p47^{phox}$ interacts with the cytoskeleton and then moves to the plasma membrane where it associates with cyt b_{558} while also chaperoning $p67^{phox}$ to the membrane (Nauseef et al., 1991). Once assembled, NADPH oxidase generates the highly reactive superoxide radical (O_2^-), which is rapidly converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase.

Myeloperoxidase, released from primary granules, catalyzes a reaction between H_2O_2 and halide ions, especially chloride, to give hypochlorous acid (HOCl), which is extremely reactive and the most bactericidal of oxidants produced by the neutrophil (Densell and Mandell, 1990).

Recently, ROS have been implicated in the activation of proteases and it has been proposed that this activation may represent the primary mechanism of killing. Reeves et al. (2002) have described a novel mechanism by which accumulation of an anionic charge due to ROS generation within the phagocytic vacuole is compensated by a surge of K^+ ions, and the consequent rise in ionic strength facilitates the release of cationic ion proteins from the anionic sulphated proteoglycan matrix of the granules and provides an optimal pH for protease activity. Furthermore, ROS and protease deficiencies lead to comparable reductions in killing efficiency, implying that they act together on the internalized microbe. These findings suggest that it is the proteases, activated through an ROS- dependent mechanism, that are primarily responsible for the destruction of bacteria (Reeves et al., 2002).

Neutrophil apoptosis

Neutrophils can be considered a double-edged sword. On one hand, they provide a beneficial effect by eliminating invading microorganisms; on the other, prolonged exposure of tissues to neutrophil enzymes and metabolites results in tissue damage and sometimes severe disease. Therefore, rapid elimination of

neutrophils by macrophages following bacterial neutralization is essential to minimize injury to the host.

Under physiological conditions, neutrophils have a relatively short half-life (8-10 hours) (Carlson and Kaneko, 1975) and, in the absence of cytokines or other proinflammatory activating agents, aged neutrophils undergo spontaneous apoptosis and are phagocytosed by macrophages (Savill et al., 1989). This process prevents neutrophils from releasing their cytotoxic content into the extracellular environment which would occur if the cells died by necrosis. The neutrophils' constitutively high apoptosis rate can be affected greatly once the cells reach infected tissues, where they are exposed to inflammatory mediators such as interleukin-8 (IL-8), granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-1β (IL-1β), which may prolong their lifespan (Kettritz et al., 1998; Moulding et al., 1998; Dunican et al., 2000; Glynn et al., 2002). Neutrophil rescue from apoptosis by these mediators is considered critical for efficient bacterial clearance, as apoptotic neutrophils have reduced pro-inflammatory functions, including migration and reactive oxygen species generation (Van Oostveldt et al., 2002; Kobayashi et al., 2003).

In cows, neutrophil apoptosis is influenced by stage of lactation. Neutrophils from cows in early lactation had a higher rate of spontaneous apoptosis than neutrophils from cows in mid-lactation (Van Oostveldt et al., 2001). Interestingly, neutrophils from early lactating cows also display impaired neutrophil function (Kehrli et al., 1989). Therefore, it has been speculated that

increased susceptibility of neutrophils to apoptosis during early lactation contributes to impaired neutrophil function and increased severity of mastitis observed during this period (Burvenich et al., 1994).

The ability of a cell to commit to apoptosis is regulated by pro- and anti-apoptotic signaling pathways. The Bcl-2 family of proteins plays a central role in mediating these opposite processes (Adams and Cory, 1998). Bcl-2 proteins can be divided into pro- and anti- apoptotic, and are characterized by the presence of one or more of four distinct Bcl-2 homology (BH) domains that facilitate protein-protein interactions. The pro-apoptotic members or the Bcl-2 family promote cytochrome c release by destabilizing the mitochondrial membrane. Cytochrome c in turn activates downstream effector caspases, including caspase-3 (described below). Anti- apoptotic Bcl-2 proteins exert their effect by forming heterodimers with pro- apoptotic Bcl-2 proteins via BH domains, therefore “neutralizing” the latter and restricting their ability to facilitate cytochrome c release. Neutrophils express a number of Bcl-2 family of proteins, including pro- (Bad, Bak, Bax) and anti- (A1, Bcl-x_L, Bcl-w, Mcl-1) apoptotic members (Chuang et al., 1998; Moulding et al., 1998; Orlofsky et al., 1999; Santos-Beneit and Mollinedo, 2000).

Execution of the apoptotic pathways in neutrophils is largely mediated by caspases, a family of cysteine proteases which cleave their substrates at aspartic acid residues. Caspases exist as inactive zymogens that are activated by proteolytic cleavage (Thornberry and Lazebnick, 1998). Neutrophils have been reported to express at least caspases- 1, 3, 4, 8 and 9 (Santos-Beneit and

Mollinedo, 2000). Of these, caspase-3 and 8 play a central role in mediating neutrophil apoptosis, as studies by Pongracz et al. (1999) and Khwaja and Tatton (1999) reported attenuated neutrophil apoptosis in the presence of caspase-3 and caspase-8 inhibitors. Activation of caspase-8 initiates a proteolytic cascade that results in activation of downstream effector caspases, including caspase-3, with consequent cleavage of intracellular substrates, chromatin fragmentation, and eventual cell death (Hirata et al., 1998). In addition, caspase-3 cleaves and activates protein kinase C-δ, which directly induces apoptosis in neutrophils by a yet unidentified mechanism (Akgul et al., 2001; Pongracz et al., 1999).

Neutrophils express relatively high levels of Fas (CD95), another key regulator of apoptosis. Fas is a prototypic death receptor from the TNF receptor super family of molecules. Ligand-activated Fas induces apoptosis in neutrophils and other cells by recruiting adaptor proteins to its cytoplasmic death domain to form potent signaling complexes known as DISC (death induced signaling complex) (Scaffidi et al., 1998). DISC recruits and activates caspase-8, which then cleaves and activates downstream caspases resulting in protein and DNA fragmentation and cell death. Since circulating neutrophils express higher levels of Fas than other leukocytes, it has been postulated that this would explain their sensitivity to apoptosis. However, the role of Fas in mediating neutrophil apoptosis remains controversial. Several studies have reported neutrophil sensitivity to Fas-induced apoptosis (Liles et al., 1996; Brown and Savill, 1999).

But, neutrophils derived from Fas- or Fas ligand- deficient mice undergo similar rates of apoptosis in vitro as those isolated from control animals (Fecho and Cohen, 1998), suggesting the existence of redundant pathways that mediate neutrophil apoptosis in the absence of Fas signaling.

Neutrophil defense of the bovine mammary gland

Neutrophils play a key role in defense of the bovine mammary gland against bacterial infection. In fact, it has been shown that severity and duration of mastitis are related critically to the promptness of neutrophil migration and to the functional activity of neutrophils once they arrive to the site of infection. Without quick and efficient neutrophil migration and function, infections can persist and lead to acute clinical mastitis, chronic infections, severe tissue damage and potentially death of the cow (Hill, 1981; Kehrli et al., 1989). Interestingly, neutrophils present in milk are less phagocytic than blood neutrophils (Jain and Lasmanis, 1978). This could be due to the fact that milk neutrophils have lower energy reserves in the form of stored glycogen (Newbould, 1973). This theory is supported by the observation that addition of glucose increased in vitro phagocytosis in these cells. The low efficiency of phagocytosis by milk neutrophils has also been attributed to ingestion of milk components such as fat and casein by the cells (Russel et al., 1976; Paape and Guidry, 1977), and more recently, to a direct effect of diapedesis across mammary epithelium, which reduced phagocytosis and oxidative burst of bovine neutrophils (Smits et al.,

1999). Nevertheless, for many mastitis pathogens, if neutrophil recruitment is rapid enough and phagocytic and bactericidal activity is efficient at the beginning of infection, they can prevent establishment of mastitis, or at least result in a controlled, mild and quick-resolving inflammatory reaction.

II- CHEMOKINES AND CHEMOKINE RECEPTORS

Of the many inflammatory mediators that dictate neutrophil migration and activation at the site of infection, the role of chemokines has become increasingly studied in recent years. Chemokines are a group of low-molecular mass proteins with considerable homology at the amino acid level (Oppenheim et al., 1991). Chemokines have been classified into four subfamilies based upon the nature of a cysteine containing signature motif at the amino terminus. While α subfamily members have two cysteines at the amino terminus of the protein separated by an amino acid residue (hence called the CXC subfamily), the β or CC subfamily has two juxtaposed cysteine residues. Members of the γ (or C) subfamily have only one cysteine at the amino terminus and those of the δ (or CX_3C) subfamily exhibit two cysteines separated by three amino acids (Rossi and Zlotnik, 2000). Chemokines were recognized originally for their ability to dictate the migration and activation of selected leukocyte populations (Kunkel et al., 1995). More recently, their role has been recognized in many biological processes, such as angiogenesis, hematopoiesis, cell proliferation, apoptosis, and host defense (Gerard and Rollins, 2001; Esche et al., 2005).

Chemokines mediate their biological effects by binding to G-protein coupled seven-transmembrane receptors that can activate an array of signaling pathways. Chemokine receptors have been classified into subsets based on the family of chemokines with which they interact. Thus, the CXC receptors are used by CXC chemokines, and CC receptors are used by CC chemokines. However, there are fewer chemokine receptors than ligands. As a result, a given chemokine receptor is usually bound by many ligands and vice versa. The redundancy in ligand-receptor interactions might provide a robust mechanism to ensure multiple backups for the defense system (Mantovani, 1999).

CXC chemokines

CXC chemokines can be further subdivided based on the presence or absence of a Glu-Leu-Arg (ELR) amino acid motif immediately preceding the first cysteine residue. Those chemokines containing the ELR sequence bind receptors CXCR1 and CXCR2, whereas chemokines that do not contain the ELR motif bind CXCR3 to CXCR6 (Rossi and Zlotnik, 2000; Hay and Sarau, 2001) (Table 1).

Broadly speaking, CXC chemokines tend to exert their chemotactic and activating effects primarily on neutrophils. Nonetheless, the CXC subfamily also has members targeting T cells, natural killer cells, fibroblasts and endothelial

Table 1. CXC chemokine ligands and receptors (adapted from Gangur et al., 2002 and Esche et al., 2005).

	Chemokine	***Receptor agonist***
ELR +	GRO-α*	CXCR2
	GRO-β*	CXCR2
	GRO-γ*	CXCR2
	ENA-78*	CXCR2
	GCP-2*	CXCR1, CXCR2
	NAP-2	CXCR2
	IL-8*	CXCR1, CXCR2
ELR -	PF4*	Unknown
	ECIP-1*	Unknown
	BRAK/bolekine	Unknown
	MIG	CXCR3
	IP-10	CXCR3
	I-TAC	CXCR3
	SDF-1α/β	CXCR4
	BCA-1	CXCR5
	SR-PSOX	CXCR6

GRO, growth related oncogene; ENA-78, epithelial derived neutrophil activating peptide 78; GCP-2, granulocyte chemotactic protein 2; NAP-2, neutrophil activating peptide 2; IL-8, interleukin-8; PF4, platelet factor 4; ECIP-1, epithelial cell inflammatory protein 1; BRAK, breast- and kidney-expressed chemokine; MIG, monokine-induced by interferon-γ; IP-10, interferon-γ inducible protein 10; I-TAC, interferon-γ inducible T-cell chemoattractant; SDF-1, stromal cell derived factor 1; BCA-1, B-cell attracting chemokine; SR-PSOX, scavenger receptor for phosphatidylserine and oxidized lipoprotein.

Chemokines with an asterisk (*) have been described in bovine (gene and/or protein).

cells. Because neutrophils are the primary cell type expressing CXCR1 and CXCR2, this portion of the review will focus on CXCR1 and CXCR2 expression, function and signaling pathways pertaining to neutrophils.

CXC receptors: CXCR1 and CXCR2

a- Structure

CXCR1 and CXCR2 are members of the large family of serpentine receptors with seven transmembrane domains that couple to *Bordetella pertussis* toxin-sensitive heterotrimeric G-proteins for signal transduction (Murphy, 1994). The amino terminus is located on the extracellular region, while the carboxyl terminus is located intracellularly in the cytoplasm and confers the ability of the receptor-ligand complex to internalize and initiate signal transduction (Ben Baruch et al., 1995). Structurally similar, CXCR1 and CXCR2 amino acid sequences express 77% homology, but exhibit different binding characteristics. CXCR1 displays greater ligand specificity than CXCR2 and binds with high affinity to IL-8 and granulocyte chemotactic protein-2 (K_d=1-2 nM) (LaRosa et al., 1992; Lee et al., 1992). CXCR2 binds multiple CXC chemokines in addition to IL-8, including epithelial derived neutrophil attractant-78 (ENA-78; K_d=2.2 nM), neutrophil activating peptide-2 (NAP-2; K_d=0.7 nM), and growth related oncogene (GRO)-α, -β, and -γ (K_d=1-2 nM) (Lee et al., 1992; Schumacher et al., 1992; Ahuja and Murphy, 1996; Bozic et al., 1996). Most of the divergent residues between the two receptors are clustered in three regions: 1) the N-terminal

segment, 2) the region from transmembrane domain (TMD) 4 to the end of the second extracellular loop, and 3) the C-terminal cytoplasmic tail (Ahuja and Murphy, 1996). The first two of these regions determine receptor-binding specificity (LaRosa et al., 1992; Suzuki et al., 1994; Ahuja et al., 1996). A recent study by Weathington et al. (2006) reported a novel peptide ligand for CXCR1 and CXCR2 derived from extracellular matrix degradation with structural homology to CXC chemokines, providing an interesting link between degradation of extracellular matrix with neutrophil recruitment.

On unstimulated neutrophils, the two receptors are expressed in approximately equal numbers (Moser et al., 1991; Lee et al., 1992) and in the presence of stimulating ligand both receptors are rapidly internalized. The internalization mechanism appears to be similar to that used by many other G-protein coupled receptors (GPCR). In brief, agonist binding to the receptor induces a change in conformation necessary for interaction of the receptor with GPCR kinases (GRKs), which specifically phosphorylate GPCRs at serine and threonine residues found in their carboxyl terminal tails and/or third intracellular loops (Premont et al., 1995). GRK-mediated phosphorylation serves to promote β-arrestin binding, which uncouples the receptor from G-protein activation (Ferguson et al., 1998). Interaction of β-arrestin with clathrin and the AP-2 adapter complex results in the formation of clathrin coated pits and subsequent endocytosis of agonist-bound receptors. Following agonist removal, internalized CXCR1 and CXCR2 may be recycled to the cell surface, thereby enabling a

subsequent round of signaling (Chuntharapai and Kim, 1995; Rose et al., 2004). Under experimental conditions, CXCR1 is rapidly re-expressed (within minutes). The rapid re-expression of CXCR1 after down modulation with maximal agonist levels suggests that CXCR1 may play a more active role at the site of inflammation, where agonist concentration is higher (Chuntharapai and Kim, 1995). Re-expression of CXCR2 is considerably slower and occurs at lower agonist inputs, which would suggest that CXCR2 may play a more important role in the initiation of neutrophil migration distant from the site of inflammation where agonist concentration is at the picomolar level (Chuntharapai and Kim, 1995; Rose et al., 2004). Interestingly, it has been reported recently that the threshold levels of agonist required for endocytosis of CXCR1 and CXCR2 were ~10-fold or higher (EC_{50}≥50 nM) than those needed for maximal calcium flux and chemotactic responses (5 and 10 nM respectively) (Rose et al., 2004). This finding suggests that rather than being integral to the process of cell migration, receptor endocytosis may be a terminal stop signal when cells reach the focus of inflammation where chemoattractant concentrations are the highest.

b- Signal transduction pathways

CXCR1 and CXCR2 exert their effects by activating heterotrimeric G proteins. These signaling molecules are composed of α, β and γ subunits and are themselves guanine nucleotide binding proteins with intrinsic GTPase activity (Exton, 1997). Upon G-protein activation by ligated cell surface receptors, α subunits dissociate from the β-γ complex. Dissociation results in conformational

changes within the guanine nucleotide binding site of the α subunit, the release of GDP and its replacement by GTP, which is prevalent in the cytosol of resting cells. Free α subunits (complexed with GTP) and β-γ subunits then interact with different cellular targets.

Activated α subunits hydrolyze the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) by activating phospholipase C (PLC). PIP_2 hydrolysis results in the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) and release of arachidonic acid. IP_3 induces Ca^{++} release from intracellular stores; DAG, in conjunction with Ca^{++}, allows for activation of protein kinase C (PKC) (Wu et al., 1993). PKC phosphorylates $p47^{phox}$, a required step for $p47^{phox}$ translocation to the plasma membrane and subsequent NADPH oxidase assembly and ROS generation (Clark et al., 1990). However, it is now accepted that CXCR1 and CXCR2 do not initiate ROS generation per se, but rather prime cells for enhanced ROS production by other stimuli (Green et al., 1996; Galligan and Coomber, 2000; Mitchell et al., 2003). Moreover, PKC activation mediates neutrophil adhesion by upregulating and activating CD11b/CD18 (Takami et al., 2002). Additionally, Ca^{++} increases are required for the detachment of pseudopods during migration (Hendey et al., 1992).

Activated β-γ subunits remain near the receptor-ligand complexes, recruiting and activating phosphatidylinositol 3-kinase (PI3K), which in turn generates phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 aids in directing neutrophil chemotaxis as it concentrates in the front of migrating cells and

confers polarity (Weiner et al., 2002). In addition, PIP_3 activates protein kinase B (Akt) and small GTPases, resulting in increased cell adherence by adhesion molecule upregulation (Takami et al., 2002) and directed migration along the chemotactic gradient (Mukaida, 2000). Furthermore, Akt activation rescues neutrophils from spontaneous apoptosis by three mechanisms: 1) phosphorylation of BAD protein. BAD, when not phosphorylated, promotes apoptosis by forming an inhibiting heterodimer with anti-apoptotic members of the Bcl-2 family. Phosphorylated BAD dissociates from Bcl-2, thereby increasing the anti-apoptotic effects of the Bcl-2 family (Klein et al., 2000); 2) phosphorylation of extracellular signal-regulated kinase (ERK), which in turn activates transcription of survival proteins such as A1 and Mcl-1 via NFkB (Akgul et al., 2001); and 3) phosphorylation and consequent inactivation of procaspase-9, an important member of the cell death pathway (Cardone et al., 1998).

There is ongoing debate regarding the differential roles of CXCR1 and CXCR2 in neutrophils. Conflicting information exists as to whether neutrophil chemotaxis is mediated by one or both receptors. A study reported that both receptors mediate chemotaxis (Loetscher et al., 1994); however, this study was performed in transfected Jurkat cells and findings were not corroborated in neutrophils. In contrast, White et. al. (1998) reported that SB225002, a CXCR2 antagonist, inhibited neutrophil chemotaxis and margination both in vitro and in vivo (in human neutrophils and in rabbits, respectively), suggesting that CXCR2 is responsible for these functions. To add to the controversy, antibodies to

CXCR1 inhibited neutrophil migration across infected human uroepithelial cells, but antibodies to CXCR2 had no effect, suggesting a primary role of CXCR1 in the migration process (Godaly et al., 2000). It seems evident that both CXCR1 and CXCR2 are capable of signaling for chemotaxis; however the relative importance of each receptor regarding this process remains to be determined. Regarding other neutrophil functions, it has been well documented that IL-8 activation of NADPH oxidase and phospholipase D is mediated solely by CXCR1 (L'Heureux et al., 1995; Jones et al., 1996).

c- Receptor expression and disease

Expression levels of CXCR1 and CXCR2 have been associated with disease processes in humans and mice. For instance, neutrophil CXCR1 expression was reduced in children prone to acute pyelonephritis compared to healthy control patients, but CXCR2 expression did not vary (Frendeus et al., 2000). Furthermore, IL-8 receptor knock-out mice exhibited dysfunctional neutrophil migration and severe disease in a urinary tract infection model, with neutrophils unable to traverse the mucosal barrier and accumulating under the epithelium, which resulted in neutrophil abscesses, high numbers of bacteria and symptoms of systemic disease (Hang et al., 2000). Since the mammary gland can be considered a similar mucosal system, it is tempting to speculate that a comparable pattern could occur in cows more susceptible to mastitis, where neutrophils of susceptible cows are slow or unable to reach the mammary lumen and therefore bacteria are able to replicate freely. Regarding CXCR2 expression,

patients with severe sepsis exhibited a 50% reduction in expression compared with normal donors. Moreover, in vitro neutrophil migration to CXCR2 specific chemokines ENA-78 and GRO-α was significantly reduced in septic patients (Cummings et al., 1999). In addition, CXCR2 knockout (KO) mice showed impaired neutrophil migration and increased susceptibility to *Toxoplasma gondii* infection (Del Rio et al., 2001). Interestingly, spleen cells from CXCR2 KO mice also exhibited defective production of TNF-α and IFN-γ (both type 1 cytokines, important in intracellular pathogen infections) after *Toxoplasma gondii* infection, and serum levels of IFN-γ were lower in CXCR2 KO mice compared to wild type mice (Del Rio et al., 2001). These findings suggest that neutrophils play an immunoregulatory role in initiating type 1 cytokine responses, and impaired neutrophil function due to a CXCR2 defect may affect this response.

There is indirect evidence that bovine neutrophils express CXCR1 and CXCR2, as they display functional activity towards IL-8, ENA-78 and GRO-α (Li et al., 2002b). Li et. al. (2002a) reported CXCR1 and CXCR2 receptor expression in bovine neutrophils through labeling with human CXCR1 and CXCR2-specific antibodies. In addition, a mRNA sequence is publicly available for one of the bovine IL-8 receptors and is thought to be CXCR2 (Genbank accession No. NM 17_4360). However, the possibility that bovine neutrophil CXCR1 and/or CXCR2 expression varies among cows and may influence neutrophil function and/or susceptibility to disease is a distinct one and requires further investigation.

III- SINGLE NUCLEOTIDE POLYMORPHISMS AND DISEASE RESISTANCE

The term “single nucleotide polymorphism” (SNP) is applied to variable nucleotide bases within a DNA molecule for which the rarer base is present within the population at >1% frequency (Kirk et al., 2002). A novel approach in disease research is to correlate SNP patterns with phenotypes (e.g. normal versus disease) to directly associate SNPs that act either as markers and/or are causative for disease. While a few Mendelian diseases result from defects in one gene, it is suspected that variation in the presence/absence of most common diseases is attributable, at least in part, to polymorphisms in multiple interacting genes.

Humans and mice

SNPs in chemokine receptors have been associated with decreased receptor function or expression, as well as with disease prevalence or progression. For instance, polymorphisms in the fractalkine receptor CX_3CR1 have been associated with HIV disease progression and decreased chemokine binding (Faure et al., 2000). HIV-infected patients homozygous for CX_3CR1 I-249 M-280, a variant haplotype affecting two amino acids (isoleucine-249 and methionine-280), progressed to AIDS more rapidly than those with other haplotypes. Functional CX_3CR1 analysis showed that fractalkine binding was reduced among patients homozygous for this particular haplotype (Faure et al., 2000). The haplotype I-249 M-280 was also evaluated as a risk factor for

coronary artery disease by Moatti et. al. (2001). I-249 heterozygosity was associated with a markedly reduced risk of acute coronary events, and I-249 heterozygote individuals showed decreased number of fractalkine binding sites per cell (Moatti et al., 2001). Additionally, SNPs in the CCR2, CCR5, and CXCR4 genes have shown association with HIV disease progression (Kageyama et al., 2001).

Polymorphisms within the CXCR2 gene have been identified in humans (Kato et al., 2000; Renzoni et al., 2000). Of these, homozygosity at position +785 CC and at position +1208 TT had higher frequency in individuals with systemic sclerosis compared to controls (Renzoni et al., 2000). It is possible that polymorphisms in bovine CXCR2 may also be associated with disease susceptibility or progression.

Bovine

Grosse et al. (1999) identified four SNPs within the third exon of the bovine interleukin-8 receptor locus. When combined together, these four SNPs generated five different haplotypes that were transmitted with Mendelian inheritance patterns (Grosse et al., 1999). Research conducted in our laboratory on dairy cattle identified five SNPs within the same 311 nucleotide base pair region (Youngerman et al., 2004a). These polymorphisms were determined to occur at positions +612 (G→A), +684 (G→A), +777 (G→C), +858 (C→A) and +861 (A→G) when compared to the available bovine IL8 receptor sequence

(Genbank accession No. NM_174360). The current annotation by Genbank suggests the identity of this sequence is CXCR2. However, more recent evidence from our laboratory proposes that this sequence in fact corresponds to CXCR1 (Pighetti and Rambeaud, 2006); therefore, the latter nomenclature will be used. Four of these SNPs (+612, +777, +858, +861) were previously reported in beef cattle (Grosse et al., 1999). A nonsynonymous substitution at position +777 (G→C) results in a histidine replacement of glutamine at amino acid residue 245. This may have direct implication in the functionality of the receptor since amino acid 245 is located in the receptor's third intracellular loop, a critical site for G-protein coupling and activation (Damaj et al., 1996a). The four other SNPs result in synonymous nucleotide changes.

Youngerman et al. (2004b) also examined the association between different CXCR1 genotypes and haplotypes (a combination of genotypes) as well as differences within each of the 5 SNPs with mastitis incidence. A total of 42 Jersey cows and 37 Holstein cows were sequenced for SNP genotype and analyzed for incidence of subclinical and clinical mastitis. Holstein cows expressing the CC genotype at position +777 had greater incidence of subclinical mastitis (37%) than those with a GC or GG genotype at this position (21 and 22% respectively) (Youngerman et al., 2004b). No differences were observed among Jersey cows, where the CC genotype is present in very low frequency, making this comparison difficult. Since cows exhibit higher somatic cell scores (SCS) when a bacterial infection is present, it was expected that cows with a CC

genotype exhibited increased SCS compared to GG cows. Surprisingly, no differences were observed among cows of different genotypes. The absence of increased SCS despite bacterial presence suggests a potential defect in one or more of the mechanisms necessary for neutrophil migration to the mammary gland.

IV- SUMMARY AND STATEMENT OF THE PROBLEM

Neutrophils play a critical role in the resolution of intramammary infections and other bacterial diseases. Furthermore, the severity and duration of mastitis is related critically to the promptness of leukocyte migration and the bactericidal activity of leukocytes at the site of infection (Hill, 1981; Kehrli et al., 1989). Polymorphisms in different chemokine receptors including CXCR1 have been correlated with receptor expression and function, as well as with disease susceptibility and progression in humans and mice. Polymorphisms for CXCR1 also have been identified in dairy cattle and associated with mastitis susceptibility. In addition, the polymorphism located at position +777 causes an amino acid substitution at residue 245 which may affect CXCR1 signaling and consequent neutrophil function in dairy cattle. Alternatively, the CXCR1 +777 polymorphism may serve as a marker for SNPs located within other segments of the CXCR1 coding sequence that more directly affect receptor binding and function, or for other genes located in the vicinity of CXCR1 which may impact immune function. Therefore, *the hypothesis of the present study is that different*

CXCR1 SNP +777 genotypes are associated with differential interleukin-8 receptor expression and/or neutrophil function in dairy cows. In order to test this hypothesis, the following *research objectives* were conducted*:*

1. Evaluate various neutrophil functions in cows with different CXCR1 SNP +777 genotypes

2. Characterize CXCR1 and CXCR2 expression and mechanisms associated with altered neutrophil function in cows with different CXCR1 SNP +777 genotypes

Note:

During the course of the research described in this dissertation a Genbank misannotation in the available sequence for the bovine IL-8R was found. It is now known that the available mRNA sequence (previously annotated as CXCR2) is in fact CXCR1 (Pighetti and Rambeaud, 2006). As a result, chapters 3 and 4 of this dissertation refer to the gene as CXCR2 because that was the identity of the sequence at the time the experiments were conducted, and this is how the data was reported and published. However, chapters 1, 2, 5 and 6 reflect the new knowledge gained and uses the correct nomenclature.

CHAPTER 3

IMPAIRED NEUTROPHIL MIGRATION ASSOCIATED WITH SPECIFIC BOVINE CXCR2 GENOTYPES

This chapter is a paper by the same name published in the journal *Infection and Immunity* in 2005 by Magdalena Rambeaud and Gina M. Pighetti:

Rambeaud, M., Pighetti, G.M. Impaired neutrophil migration associated with specific bovine CXCR2 genotypes. *Infection and Immunity* 73 (8), 4955-4959.

My use of “we” in this chapter refers to my co-author and myself. My primary contributions to this paper include (1) Design of the experiment and animal selection, (2) sample collection, (3) most cell isolations and the majority of the labwork, (3) all of the data analysis (4) most of the gathering and interpreting of the literature, (5) most of the writing of this paper.

I- ABSTRACT

Bovine mastitis continues to be the most detrimental factor for profitable dairying. Recent research conducted within our laboratory has identified a genetic marker in the CXCR2 gene associated with mastitis susceptibility. The objective of the present study was to evaluate the migratory ability of neutrophils

from cows with different CXCR2 +777 genotypes. Neutrophils isolated from peripheral blood of 30 Holstein cows were tested for in vitro migration and adhesion molecule expression. Cows with the CC or GC genotype at CXCR2 +777 showed significantly lower neutrophil migration to recombinant human interleukin-8 (rhIL-8) than cows with the GG genotype ($P<0.05$). Cows with the CC genotype at CXCR2 +777 also showed decreased neutrophil migration to zymosan activated serum when compared to these same cows ($P<0.05$). Decreased upregulation of CD18 expression was observed after stimulation with rhIL-8 in cows expressing the CXCR2 +777 CC genotype when compared to cows expressing the GG genotype ($P<0.05$). A similar trend was observed for CD11b ($P<0.10$). However, no difference in CD62 downregulation was observed with respect to genotype. These results provide initial evidence for association between a single nucleotide polymorphism and neutrophil function in dairy cows, and offer potential insight into specific mechanisms affected in cows more susceptible to mastitis.

II- INTRODUCTION

Infectious diseases caused by bacteria negatively impact every animal industry. Effective elimination of bacterial infections such as mastitis in dairy cattle requires four basic steps: bacterial recognition, inflammatory mediator release, leukocyte recruitment from the bloodstream and bacteria removal. When infection occurs, resident macrophages and mammary epithelial cells release

inflammatory mediators that signal the body for help. These chemical messengers include chemotactic complement fractions, prostaglandins, leukotrienes and acute phase cytokines (interleukin-1, tumor necrosis factor and interleukin-8 [IL-8]), and are responsible for massive neutrophil migration from the circulation to the site of infection (Paape et al., 1979; Craven, 1983; Rose et al., 1989; Riollet et al., 2000).

The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by cytokine, chemokine and complement receptors. Two chemokine receptors present on neutrophil surfaces, CXCR1 and CXCR2, are required for maximum neutrophil function during infection (Holmes et al., 1991; Del Rio et al., 2001). Interleukin-8 is a high affinity ligand for both receptors, but shares CXCR1 with the lower affinity ligand granulocyte chemotactic protein-2. Besides IL-8, CXCR2 also can bind growth related oncogene (GRO)-α, GRO-β, GRO-γ, neutrophil activating peptide-2, and epithelial-derived neutrophil attractant-78. (Ahuja and Murphy, 1996; Wuyts et al., 1998). In neutrophils, recognition of chemokines by CXCR1 and CXCR2 induces β_2 integrin upregulation and chemotaxis, as well as enhancement of reactive oxygen species generation and phagocytosis of pathogens (Ahuja and Murphy, 1996; Caswell et al., 1999; McClenahan et al., 2000; Li et al., 2002b; Mitchell et al., 2003). The CXC receptors are members of the large family of serpentine receptors with seven transmembrane domains (7TMD) that couple to *Bordetella pertussis* toxin-sensitive heterotrimeric G-proteins for signal transduction. G-

protein coupling to the receptor results in activation of phospholipase C, formation of the second messenger inositol 1,4,5-trisphosphate and the subsequent increase in cytosolic free calcium concentration (Wu et al., 1993; Baggiolini et al., 1994), as well as activation of phospyatidylinositol 3'kinase and consequent generation of phospyatidylinositol 3-phosphate (Mukaida, 2000). All these mechanisms mediate many of the events necessary for proper neutrophil activation and migration to eliminate invading pathogens.

Effective neutrophil recruitment to the site of infection also requires adhesion molecules from the selectin and β_2 integrin families. Neutrophil selectin CD62L slows down cells upon contact with its specific ligands (Butcher, 1991) and allows neutrophils to roll along vascular endothelial cells. Neutrophils activated by cytokines or chemoattractants subsequently shed CD62L (Kishimoto et al., 1989) as a prerequisite for β_2 integrin mediated tight adhesion. The most important β_2 integrin involved in neutrophil recruitment into inflamed tissue is CD11b/CD18 (Arnaout, 1990), which is predominantly stored in cytoplasmic granules. Upon cellular activation by inflammatory mediators, these cytoplasmic granules translocate to the cell surface and CD11b/CD18 expression increases (Miller et al., 1987), causing neutrophils to bind firmly to endothelial cells through interaction with its specific ligand, intercellular adhesion molecule 1.

Influx of neutrophils into the mammary gland is critical for the resolution of mastitis; without quick and efficient neutrophil migration, infections can persist and lead to acute clinical mastitis, chronic infections, severe tissue damage and

potentially death of the cow (Hill, 1981; Kehrli et al., 1989). Interleukin-8 is one of the ligands that binds CXCR2 with high affinity (Ahuja and Murphy, 1996), and is at least partly responsible for the massive influx of neutrophils to the mammary gland during bacterial infection (Shuster et al., 1997; Riollet et al., 2000; Rambeaud et al., 2003). Upon arrival at the site of infection, neutrophils phagocytose and kill intramammary bacteria through reactive oxygen species generation and antibacterial granule proteins (Roth, 1994).Once an infection is cleared, neutrophils undergo apoptosis and are phagocytosed by macrophages (Savill et al., 1989). This rapid clearance is important because neutrophil lysosomal enzymes and respiratory burst products can damage mammary gland tissue (Harmon and Heald, 1982; Capuco and Akers, 1986; Paape et al., 2000).

Single nucleotide polymorphisms (SNP) within the CXCR2 gene have been identified and evaluated for their potential association with disease in humans (Kato et al., 2000; Renzoni et al., 2000). Of these, homozygosity at position +785 CC and at position +1208 TT was found in high frequency in individuals with systemic sclerosis compared to healthy controls (Renzoni et al., 2000). It is possible that polymorphisms in bovine CXCR2 are also associated with disease susceptibility or progression, and may potentially be used to select cows that are more resistant to disease. Recent research conducted in our laboratory has identified 5 SNPs in a 311 base pair segment of the CXCR2 gene of dairy cows (Youngerman et al., 2004a). Polymorphisms at position +612, +777 and +861 showed a significant association with subclinical mastitis (Youngerman

et al., 2004b). One of these, the SNP located at position +777 (G→C), results in amino acid 245 glutamine→histidine replacement. This may have direct implication in the functionality of the receptor since amino acid 245 is located in the receptor's third intracellular loop, a critical site for G-protein coupling and activation (Damaj et al., 1996a). Holstein cows expressing the CC genotype at position +777 had increased incidence of subclinical mastitis (37%) when compared to Holstein cows that expressed the CG (21%) or GG genotype (22%) (Youngerman et al., 2004b). Based on this phenotypic observation, and since neutrophil recruitment to the site of infection is critical for the resolution of mastitis, we hypothesized that neutrophil migration and adhesion molecule expression would differ among cows of different CXCR2 +777 genotypes.

II- MATERIALS AND METHODS

Animal selection and sample collection

Thirty Holstein cows (6 cows/day; 2 cows from each genotype) from the Knoxville Experiment Station in mid-to late lactation (at least 100 days in milk) were used in this study. Cows were selected based on their CXCR2 +777 genotype (GG, GC, CC, n=10 each), which was determined by MALDI-TOF mass spectroscopy at a commercial facility (Geneseek, Lincoln, NE). All cows were free from clinical mastitis, and milk samples were collected aseptically immediately prior to blood collection to determine the presence or absence of subclinical intramammary infection. Blood (60 ml) was collected by jugular

venipuncture in syringes containing 2X acid citrate dextrose anticoagulant (10%vol/vol) and processed immediately for neutrophil isolation. Prior to isolation an aliquot was removed for determination of red and white blood cell counts using an automated cell counter (VetCount IIIB; Mallinckrodt, Phillipsburg, NJ). In addition, smears were obtained to determine leukocyte differential counts.

Neutrophil isolation

Neutrophils were isolated from blood as described previously (Aarestrup et al., 1994) with some modifications. Blood was centrifuged at 860 $\times$ *g* for 30 minutes at 4ºC, and the plasma and buffy coat were discarded. After repeating this step, neutrophils were isolated from the remaining erythrocytes by adding double-distilled water for 30 seconds and then a 3X concentration of RPMI 1640 medium (Sigma, St. Louis, MO, USA) was added to regain isotonicity of the solution. After a second lysis of red blood cells, remaining neutrophils (96% average purity) were washed with Hank's balanced salt solution (HBSS, pH 7.2; Cellgro, Herndon, VA) and resuspended in the appropriate media and cell concentration for the different functional assays as described below. Viability was assessed by trypan blue dye exclusion and always exceeded 97%. Average white blood cell count among cows was 7.5×10^3 cells/ml.

Zymosan activated serum preparation

Zymosan activated serum (ZAS) was obtained by incubating bovine serum with yeast cell wall particles (Zymosan A; Sigma, St. Louis, MO) at a concentration of 10 mg/ml for 30 minutes at 37ºC in a shaking water bath. After incubation, zymosan particles were pelleted by centrifugation, and serum was collected and stored at –20ºC until use.

Adhesion molecule expression

Indirect immunofluorescent analysis of CD11b, CD18 and CD62L expression on isolated neutrophils was performed using murine monoclonal antibodies and fluorescein-conjugated antibody to mouse IgG. Neutrophils (4×10^6 cells/ml in HBSS) were preincubated with HBSS (unstimulated control), 400 ng/ml recombinant human IL-8 (rhIL-8; R&D systems, Minneapolis, MN), 10% ZAS or 10 nM phorbol myristate acetate (PMA; Sigma, St. Louis, MO) at 39ºC for 30 minutes while mixing horizontally. After preincubation, 50 µl of neutrophil suspension were added to 96-well round bottom microtitre plates containing 50 µl IgG as a negative control (10 µg/ml; Caltag Laboratories, Burlingame, CA), monoclonal antibodies to bovine CD11b (MM10A, 5 µg/ml; VMRD Inc., Pullman, WA, USA), bovine CD18 (BAQ30A, 10 µg/ml; VMRD Inc.) or bovine CD62 (BAQ92A, 5 µg/ml; VMRD Inc.). Plates were incubated at 4ºC for 30 minutes, washed twice with HBSS, and 100 µl of fluorescently labeled goat anti-mouse IgG (Calbiochem, La Jolla, CA, USA) were added to each well. Samples were

incubated at 4ºC for 30 minutes, washed as before, and resuspended in 2% formaldehyde in 0.15M phosphate buffered saline (PBS). Samples were stored at 4ºC in the dark until analyzed by flow cytometry. Data are expressed as percent of median fluorescence intensity increase over the HBSS-stimulated (negative) control.

Flow cytometry

For adhesion molecule expression, neutrophils were analyzed using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Dot plots were gated for neutrophils based on forward and side scatter characteristics, and the median fluorescence intensity was calculated after plotting fluorescence of histograms. Fluorescence associated with neutrophils incubated with nonspecific mouse IgG instead of anti CD11b, CD18 or CD62L was considered the control for nonspecific fluorescence.

Neutrophil migration

The ability of neutrophils to migrate towards rhIL-8 and ZAS was evaluated using a 48-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD) as described previously (Bizarri et al., 2001). Briefly, PBS-0.1% bovine serum albumin (BSA) as negative control, 100 ng/ml rhIL-8 or 5% ZAS were seeded in the lower compartment of the chemotaxis chamber, whereas neutrophils in PBS-

0.1% BSA (4×10^5 total cells/well) were added to the upper chamber. The chamber was incubated at 39ºC, 5%CO_2 for 30 minutes, the polycarbonate filter separating the compartments removed, washed with PBS and scraped three times, fixed with methanol and stained with DiffQuik for counting. A total of 10 fields at 40X amplification were counted per well. Samples were assayed in triplicate; results were expressed as mean total number of neutrophils migrated per well.

Statistical analysis

Analysis of variance was done with mixed models using SAS software (SAS 8.2, SAS Institute Inc, Cary, NC). A randomized block design with replication was used to determine the effect of CXCR2+777 genotype on different neutrophil functions. The statistical model was $y_{ijk}=\mu + D_i + G_j + D*G_{ij} + C(D*G)_{ijk}$, where μ=overall mean, D=day, G=genotype, and C=cow. Data are presented as least square means with associated standard error. Statistical significance was declared at $P<0.05$ and a trend toward significance was declared at $P<0.10$.

III- RESULTS AND DISCUSSION

During mastitis and other inflammatory diseases, potent cytokines (interleukin-1, tumor necrosis factor, IL-8, etc) and other inflammatory mediators (complement fractions such as C3a and C5a, prostaglandins, leukotrienes, etc.)

guide neutrophils towards the site of infection (Giri et al., 1984; Rose et al., 1989; Shuster et al., 1993; Shuster et al., 1997; Riollet et al., 2000; Rambeaud et al., 2003). This recruitment and the functional ability of neutrophils once there often determine whether a bacterial infection is cleared or becomes chronic (Hill, 1981; Kehrli et al., 1989). A slow or ineffective neutrophil attack allows bacteria to continue their assault and generate a longer lasting and potentially more damaging infection. Since neutrophils are key players in the resolution of mastitis, and a polymorphism in CXCR2 +777 (G→C) had been associated with mastitis susceptibility, we hypothesized that different CXCR2 +777 genotypes would be associated with efficiency of neutrophil migratory ability in vitro.

As a prerequisite for neutrophil migration from blood into tissues, CD62L must be shed and β_2 integrin expression and/or affinity must be increased by neutrophils (Borregaard et al., 1994). Therefore, we evaluated adhesion molecule expression following stimulation with IL-8, complement fractions C3a and C5a through stimulation with ZAS, and PMA. No differences in CD62L downregulation in response to rhIL-8, ZAS or PMA were observed among CXCR2 genotypes ($P>0.05$; data not shown). However, following incubation with rhIL-8, but not PMA or ZAS, the increase in CD18 expression by neutrophils from cattle with a CC genotype was approximately half that of neutrophils from cattle with a GG genotype and resulted in a significant difference between the two ($P<0.05$; Figure 2; Stimulation with PMA or ZAS not shown). Similarly, upregulation of CD11b after stimulation with IL-8 was reduced by approximately

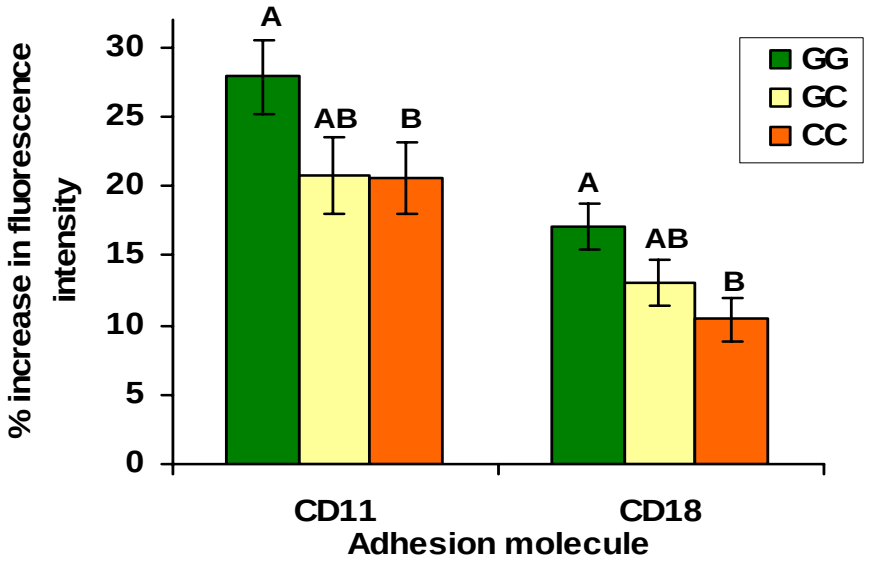

Figure 2. Upregulation of CD11b and CD18 in neutrophils of cows with different CXCR2 +777 genotypes after stimulation with rhIL-8. Results are expressed as percent increase in median fluorescence intensity after stimulation compared to unstimulated control neutrophils. Data are reported as least square means ± SEM. CD11b bars with no common letter differ, $P<0.10$. CD18 bars with different letter above differ, $P<0.05$.

a third in neutrophils from cattle with a CC genotype compared to those with a GG genotype ($P<0.10$; Figure 2; Stimulation with PMA or ZAS not shown). As both CD11b and CD18 are stored predominately in cytoplasmic granules (Arnaout, 1990), the decreased expression in neutrophils from cattle with the CC genotype may be due to lower baseline expression of these proteins in the cytoplasmic granules, or reduced signaling from CXCR1 and/or CXCR2 which impairs granule translocation to the cell surface (Miller et al., 1987; Arnaout, 1990). However, no differences were observed in CD11b or CD18 expression increase after stimulation with PMA or ZAS, which act independently of CXCR activation; this finding would suggest that overall CD11b or CD18 protein levels do not differ among genotypes. Regardless of the mechanism, altered upregulation of CD11b/CD18 may interfere with firm adherence of the neutrophil to the endothelium and the promptness of migration to the inflammatory site. Studies are currently underway to determine if firm adhesion is impacted.

Once firm adhesion occurs by binding of the β_2 integrins to the endothelial surface, transmigration through the blood vessel to the site of infection takes place, and potent chemoattractants such as IL-8, C5a and bacterial products direct neutrophils towards the site of infection. The effectiveness of this response upon bacterial invasion plays a critical role in the resolution of mastitis (Hill, 1981; Kehrli et al., 1989). As such, we evaluated the ability of neutrophils from cattle with different CXCR2 genotypes to migrate in vitro (Figure 3). Cows expressing the CC genotype for CXCR2 +777 had significantly fewer neutrophils migrate in

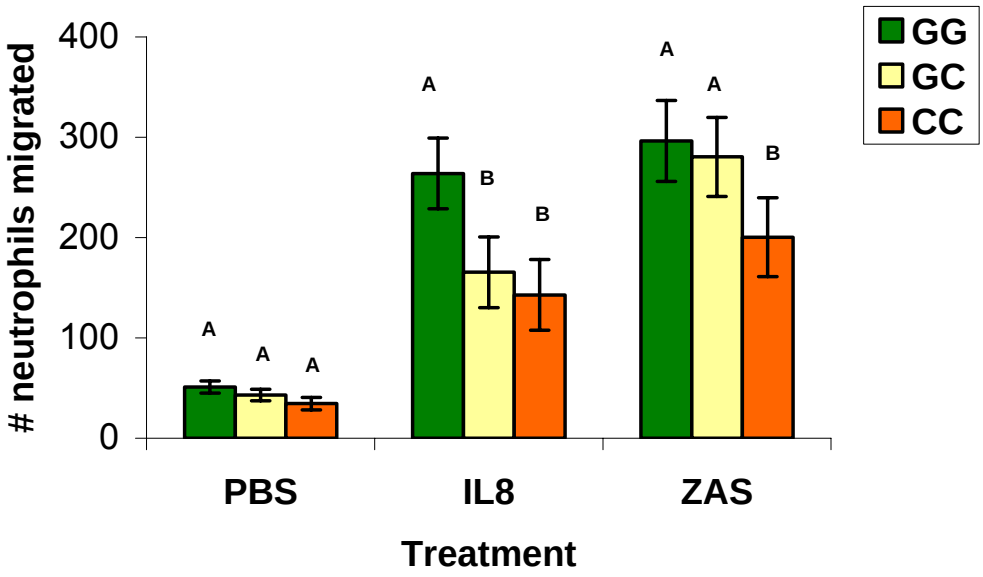

Figure 3. Neutrophil migration in cows of different CXCR2 +777 genotype after stimulation with PBS-0.1% BSA (PBS), recombinant human IL-8 (IL-8) or 5% zymosan activated serum (ZAS). Results are reported as number of neutrophils migrated/well. Data are expressed as least square means ± SEM. Within each treatment, bars with different letters above differ ($P<0.05$).

response to both rhIL-8 and ZAS when compared to neutrophils from cattle with the GG genotype ($P<0.05$). Interestingly, neutrophils from cattle with a heterozygous genotype responded differently depending upon the stimulus. When neutrophils were stimulated with rhIL-8, those from heterozygous cows responded similarly to neutrophils from cattle with the CC genotype. In contrast, when stimulated with ZAS, neutrophils from heterozygous cows migrated at levels comparable to those cells from cows with the GG genotype.

The reduction in neutrophil migration as well as CD11b/CD18 upregulation in cattle with a CC genotype suggests that neutrophil migration also may be impaired in vivo in cattle with the same genotype and may partially explain the greater incidence of subclinical intramammary infections, determined by bacteriological culture, observed in these cows (Youngerman et al., 2004b). With presence of subclinical intramammary infections, it would be expected that higher numbers of neutrophils would be present in the mammary gland in order to fight off infection. However, this expected increase in neutrophil migration, as measured by the average lifetime somatic cell counts in milk, was not observed in cows with a CC genotype (Youngerman et al., 2004b) and supports the hypothesis of impaired neutrophil migration in vivo in these animals. The combination of the in vitro and in vivo data also indicates that the defect/s does not result in an all or none response, but is a subtle reduction in the number and/or timing of neutrophils migrating. A ‘slight’ delay in either of these factors

may be sufficient to allow initial proliferation and invasion of bacteria, and subsequent establishment of chronic subclinical infections.

Both neutrophil adhesion molecule (CD11b/CD18) upregulation and migration in response to rhIL-8 were lower in cattle with a CC genotype. One possible explanation for this response may be directly related to the identified polymorphism in CXCR2 (G→C) that a causes glutamine→histidine replacement. This amino acid lies within the third intracellular loop of the receptor and has the potential to affect neutrophil activity during infection. This hypothesis is supported by work reported by Damaj et al. (1996a) which identified amino acid residues within the third intracellular loop of both human IL-8 receptors, CXCR1 and CXCR2, that were involved in mediating neutrophil calcium signaling and mobilization during IL-8 stimulation. Thus, the amino acid substitution at residue 245 may affect CXCR2 signaling and consequent neutrophil function in dairy cattle. Alternatively, the CXCR2 +777 polymorphism may serve as a marker for SNPs located within other segments of the CXCR2 coding sequence that more directly affect receptor binding and function.

Neutrophil migration was not only impaired in response to IL-8, but ZAS as well in cattle with the CC genotype in comparison to the GG genotype. As ZAS does not activate CXCR2, this would suggest that polymorphisms within that gene may not be the cause of reduced neutrophil migration and altered adhesion molecule expression in cattle with a CC genotype. Zymosan activated serum contains the complement fraction C5a. Similar to IL-8, C5a acts on neutrophils

through a specific 7TMD receptor (Hopken et al., 1997). Since the C5a receptor is also a G-protein coupled receptor, it is possible that intracellular pathways that lead to neutrophil migration and are common to both receptors may be altered in cows expressing the CC genotype. Further supporting this idea, CXCR2 and C5a both activate phospholipase C in a pertussis toxin-sensitive manner (Oppenheim et al., 1991; Wu et al., 1993).

V- CONCLUSION

In conclusion, this study provides a potential mechanism to explain the genetic differences in mastitis susceptibility observed in cattle with different CXCR2 +777 genotypes. Cows expressing the CXCR2 +777 CC genotype, which have previously shown higher prevalence of intramammary infections, exhibited impaired neutrophil migration and adhesion molecule upregulation compared to cows of the GG genotype. This information may offer insight into specific mechanisms that are affected in cows more susceptible to mastitis, and provide possible targets for generating new strategies to either prevent or treat mastitis in dairy cattle.

VI- ACKNOWLEDGEMENTS

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Chapter 4

ASSOCIATION OF A BOVINE CXCR2 GENE POLYMORPHISM WITH NEUTROPHIL SURVIVAL AND KILLING ABILITY

This chapter is a paper by the same name published in the journal *Veterinary Immunology and Immunopathology* in 2006 by Magdalena Rambeaud, Rose Clift and Gina M. Pighetti:

Rambeaud, M., Clift, R. Pighetti, G.M. Association of a bovine CXCR2 gene polymorphism with neutrophil survival and killing ability. *Veterinary Immunology and Immunopathology* 111 (3-4), 231-238.

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) Design of the experiment and animal selection, (2) sample collection, (3) most cell isolations, (4) all of the reactive oxygen species generation and apoptosis assays (3) all of the data analysis (4) most of the gathering and interpreting of the literature, (5) most of the writing of this paper.

I- ABSTRACT

Recent research in our lab has demonstrated a significant association between the incidence of subclinical mastitis and specific polymorphisms of the

CXCR2 gene in Holstein dairy cows. This gene encodes a receptor for interleukin-8 (IL-8), a key regulator of neutrophil migration, killing and survival. Because of the importance of this gene in neutrophil function, we hypothesized that differences in neutrophil killing and survival may exist among the CXCR2 genotypes and potentially contribute to the observed variation in intramammary infections. To test this hypothesis, neutrophils were isolated from cows representing each CXCR2 +777 genotype (GG, GC or CC) and tested for suppression of apoptosis, reactive oxygen species (ROS) generation, glutathione levels, and bactericidal activity. A significant increase in survival was observed in neutrophils from cows with a CC genotype when compared to those with a GG genotype in response to IL-8, but not dexamethasone. In contrast, a significant reduction in neutrophil ROS generation in response to phorbol-13-myristate-12 acetate (PMA) was observed in cows with a CC genotype when compared to those with a GG genotype. However, no differences in bactericidal activity or glutathione levels were observed among genotypes. The functional activity of neutrophils from cows heterozygous for this polymorphism was intermediate between those with homozygous genotypes for those assays where differences were observed among homozygous genotypes. In summary, our results suggest that neutrophils from Holstein cows with different CXCR2 genotypes vary in their ability to suppress apoptosis and produce ROS. These differences have the potential to influence overall neutrophil function and may partially explain the variation observed with respect to mastitis in vivo. These results provide a foundation for future research aimed at better understanding the basic

differences between dairy cows genetically more or less susceptible to mastitis and has the potential to provide novel preventive and therapeutic measures against inflammatory diseases such as mastitis.

II- INTRODUCTION

Bacterial infections constitute a major source of economic loss in dairy and beef cattle. Mastitis is the most prevalent disease of dairy cows (NMC, 1996), whereas bacterial pneumonia is one of the most frequent diseases and causes of mortality in feedlot cattle (Mosier, 1997). A critical component of host defenses against bacterial infection are neutrophils, due to their ability to eliminate invading bacteria by phagocytosis, discharge of granule contents (Jain and Lasmanis, 1978; Baggiolini et al., 1985; Watson et al., 1995), reactive oxygen species (ROS) generation (Densell and Mandell, 1990), and subsequent protease activation (Reeves et al., 2002). The generation of ROS begins with the formation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, a multi-subunit enzyme formed only after neutrophil activation (Densell and Mandell, 1990; Burg and Pillinger, 2001). NADPH-oxidase generates the highly reactive superoxide radical (O_2^-), which can be converted by myeloperoxidase to extremely reactive hypochlorous acid, which can destroy invading bacteria. Reactive oxygen species constitute one of the most potent bactericidal mechanisms of neutrophils because of their ability to oxidize amino acids, nucleotides, and heme proteins, (Densell and Mandell, 1990; Burg and Pillinger,

2001). Myeloperoxidase, as well as several components of NADPH oxidase are released from neutrophilic granules following activation. Neutrophil specific and azurophilic granules also release bactericidal proteins such as bacterial permeability increasing protein, defensins, elastase, and cathelicidins that aid in killing phagocytosed bacteria (Gennaro et al., 1983a; Roth, 1994; Risso, 2000). Recently, ROS have been implicated in the activation of proteases (Reeves et al., 2002). These findings suggest that it is the proteases, activated through an ROS-dependent mechanism, that are primarily responsible for the destruction of the bacteria.

In the absence of cytokines or other proinflammatory activating agents, aged neutrophils undergo spontaneous apoptosis and are phagocytosed by macrophages (Savill et al., 1989). This process prevents neutrophils from releasing their cytotoxic content into the extracellular environment which would occur if the cells died by necrosis. The neutrophils' constitutively high apoptosis rate can be affected greatly once the cells reach infected tissues, where they are exposed to inflammatory mediators such as interleukin-8 (IL-8), granulocyte-monocyte colony stimulating factor and interleukin-1β, which may prolong their lifespan (Kettritz et al., 1998; Moulding et al., 1998; Dunican et al., 2000; Glynn et al., 2002). Neutrophil rescue from apoptosis by these mediators is considered critical for efficient bacterial clearance, as apoptotic neutrophils have reduced pro-inflammatory functions, including migration and reactive oxygen species generation (Van Oostveldt et al., 2002; Kobayashi et al., 2003).

Since neutrophil migration and functionality are essential for resolution of bacterial infections (Paape et al., 2000; Kehrli and Harp, 2001), genes associated with neutrophil function are potential genetic markers for disease resistance. The chemokine receptors CXCR1 and CXCR2 are expressed on the surface of neutrophils and are required for maximum neutrophil function during infection (Holmes et al., 1991; Del Rio et al., 2001). In neutrophils, recognition of chemokines by CXCR1 and CXCR2 induces β_2 integrin upregulation and chemotaxis, as well as enhancement of reactive oxygen species generation and phagocytosis of pathogens (Ahuja and Murphy, 1996; Caswell et al., 1999; McClenahan et al., 2000; Li et al., 2002b; Mitchell et al., 2003). Additionally, CXCR1 and CXCR2 activation protects neutrophils from spontaneous apoptosis (Kettritz et al., 1998; Glynn et al., 2002). This may be advantageous during the early stages of infection, since it may allow normally short-lived neutrophils to survive longer, and exert their bactericidal activity in a localized area of infection. Recent research in our lab has demonstrated an association between the incidence of subclinical intramammary infections and polymorphisms in the CXCR2 gene in Holstein dairy cows (Youngerman et al., 2004b). More specifically, cattle with a CC genotype at nucleotide position +777 (Genbank accession number U19947) had a greater incidence (37%) of subclinical mastitis than those with a GC or GG genotype at this position (21 and 22% respectively). Furthermore, we have also reported that neutrophils from cows with a CC genotype exhibited impaired neutrophil migration and CD11b/CD18 upregulation in vitro (Rambeaud and Pighetti, 2005). The CXCR2 polymorphism at position

+777 causes an amino acid change from histidine to glutamine within the third intracellular loop of the receptor where G-protein binding occurs (Damaj et al., 1996a; Youngerman et al., 2004a). Thus this amino acid change has the potential to influence neutrophil function and subsequent disease resistance because the product of the CXCR2 gene is a receptor for IL-8, a key regulator of neutrophil migration, killing, and survival (Chertov et al., 2000; Glynn et al., 2002). Because of the importance of neutrophil function in the resolution of bacterial infections, we hypothesized that differences in neutrophil killing and survival may exist among the CXCR2 +777 genotypes and provide a potential mechanism for the observed variation in intramammary infections.

III- MATERIALS AND METHODS

Animal selection and sample collection

Holstein cows from the Knoxville Experiment Station in mid-to late lactation (at least 100 days in lactation) were used in this study (ROS and apoptosis assays, n=20 cows/genotype; glutathione and bactericidal assay, n=10 cows/genotype). Cows were selected based on their CXCR2 +777 genotype (GG, GC or CC), which was determined by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry at a commercial facility (Geneseek, Lincoln, NE). All cows were free from clinical mastitis, and milk samples were collected aseptically immediately prior to blood collection to determine the presence or absence of subclinical intramammary infection. Blood

(60 ml) was collected by jugular venipuncture in syringes containing acid citrate dextrose anticoagulant (10%vol/vol) and processed immediately for neutrophil isolation. Prior to isolation an aliquot was removed for determination of red and white blood cell counts using an automated cell counter (VetCount IIIB; Mallinckrodt, Phillipsburg, NJ). In addition, smears were obtained to determine leukocyte differential counts.

Neutrophil isolation

Neutrophils were isolated from blood as described previously (Rambeaud and Pighetti, 2005). Neutrophils (96% average purity) were washed with Hanks' balanced salt solution (HBSS, pH 7.2; Cellgro, Herndon, VA) and resuspended in the appropriate media and cell concentration for the different functional assays as described below. Viability was assessed by trypan blue dye exclusion and always exceeded 95%. Average white blood cell count among cows was 7.2×10^3 cells/ml.

Neutrophil survival from spontaneous apoptosis

To determine neutrophil suppression of apoptosis dependent and independent of CXCR2 activation, 10^6 cells in RPMI (Sigma, St. Louis, MO) containing 1% FBS (Hyclone, Logan, UT) were incubated with media alone, 50 nM recombinant human IL-8 (rhIL-8, R&D Systems, Minneapolis, MN), or 100 nM

dexamethasone (Acros Organics, Geel, Belgium) for 22-24 hours. Neutrophils were then collected and evaluated for apoptosis by Annexin V staining (BD Biosciences Pharmingen, San Diego, CA) following manufacturer's instructions. Data are reported as percent of Annexin V positive cells. In addition, the rate of decrease in apoptotic cells (expressed as percent decrease in apoptosis) was calculated by comparing each treatment (IL-8 and dexamethasone) to control (untreated) cells within each genotype.

Neutrophil ROS generation

The neutrophils' respiratory burst was quantitated by flow cytometry as described by Lehmann et. al. (2000). Neutrophils were incubated with 1 μM dihydrorhodamine 123 (Molecular Probes, Eugene, OR) at 39ºC for 5 minutes and then with 10 and 100 nM phorbol-12-myristate-13 acetate (PMA; Sigma) at 39ºC for 15 minutes to induce ROS generation. Neutrophils incubated under the same conditions with no stimulus served as a negative control. Cells were then placed on ice and analyzed by flow cytometry within 1 hour.

Glutathione assay

Glutathione (GSH) levels before and after 24 hour incubation with 0 and 50 nM rhIL-8 were monitored as described by Allen et. al. (2001) with some modifications. Briefly, 10^6 cells were added to 96 well plates in triplicate, lysed

with Triton X-100 (Fisher Scientific, Pittsburg, PA) and mixed with 5% sulfosalicylic acid (Sigma). Glutathione reaction buffer (221.3 μM NADPH, 462.6 μM 5,5' dithiobis-2-nitrobenzoic acid, 10.6-12.3 U/ml GSH reductase, 92.7 mM sodium phosphate, 4.1 mM EDTA [all from Sigma]) was then added to each well, and absorbance at 405 nm was read on a microplate reader at 30 second intervals for 5 minutes. Glutathione levels were calculated by extrapolation from a standard curve with 0-10 μM GSH.

Bactericidal assay

Neutrophil bactericidal activity was evaluated by colorimetric determination of formazan production as described previously by Stevens et al. (1991). Briefly, opsonized and non-opsonized *Staphylococcus aureus* and *Streptococcus uberis* (both isolated from cows with clinical mastitis) were incubated in 96-well flat bottom plates with 10^6 neutrophils/well at a ratio of 10 bacteria per neutrophil. Following 1 hour of incubation, neutrophils were lysed with 2% saponin and the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT, 2%; Calbiochem, San Diego, CA) was added to each well. Plates were incubated with extraction buffer overnight to lyse all bacterial cells and debris. The ability of live bacteria to reduce MTT to purple formazan was quantitated by measuring optical density at 562 nm. The percentage of bacteria killed by neutrophils was determined by extrapolation from a standard formazan

curve derived by incubating MTT with a known number of bacteria. Samples were assayed in quadruplicate.

Flow cytometry

For ROS generation and Annexin V staining, neutrophils were analyzed using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). For ROS generation, dot plots were gated for neutrophils based on forward and side scatter characteristics and the median fluorescence intensity was calculated after plotting fluorescence of histograms. Unstimulated cells were considered the negative control. For apoptosis determination cells were not gated in order to account for all apoptotic cells present. Unstained cells served to set Annexin V negative and positive gates in order to determine percent of apoptotic cells.

Statistical analysis

Analysis of variance was done with mixed models using SAS software (SAS 8.2, SAS Institute Inc, Cary, NC). A randomized block design with replication was used to determine the effect of CXCR2 +777 genotype on different neutrophil functions. The statistical model was $y_{ijk}=\mu + D_i + G_j + D*G_{ij} + C(D*G)_{ijk}$, where μ=overall mean, D=day, G=genotype, and C=cow. Neutrophil ROS data were converted to $\log_{10}$ to maintain a normal distribution for statistical

analysis. Data are presented as least square means with associated standard error. Statistical significance was declared at $p<0.05$, and a trend towards significance was declared at $p<0.10$.

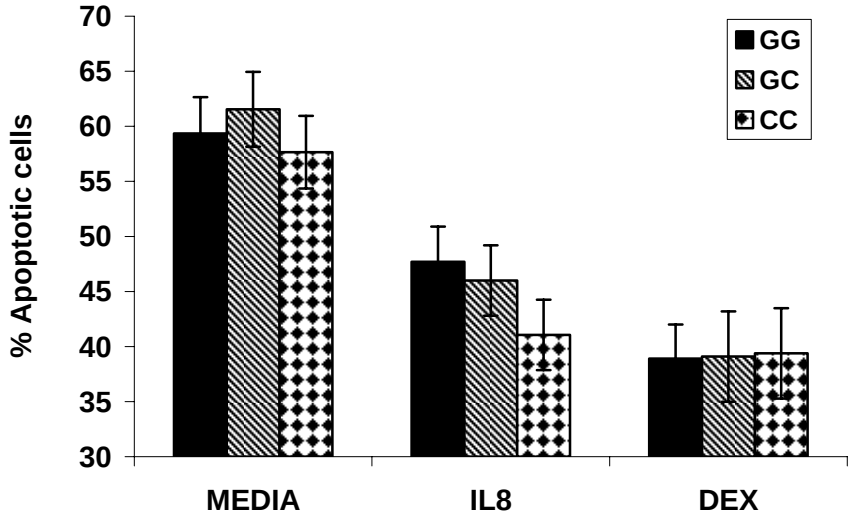
IV- RESULTS

Neutrophil survival from spontaneous apoptosis

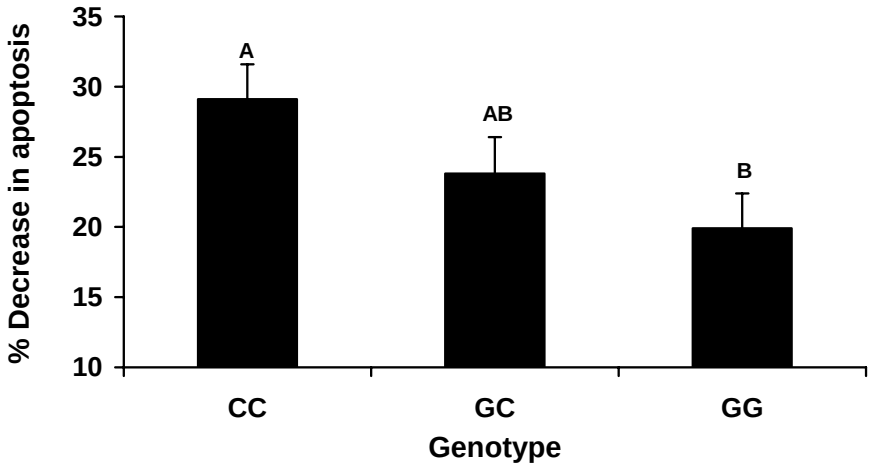
After incubation for 22-24 hours, ~60% of untreated neutrophils were apoptotic regardless of cow genotype. Treatment with either 50 nM rhIL-8 or 100 nM dexamethasone caused significant suppression of spontaneous apoptosis in neutrophils of cows from all genotypes (Figure 4A). However, neutrophils from cows with a CC genotype tended to respond better to the anti-apoptotic effect of rhIL-8, evidenced by a lower percentage of apoptotic cells compared to neutrophils of cows with a GG genotype ($p=0.08$; Figure 4A). No difference in apoptosis levels was observed among genotypes when neutrophils were treated with dexamethasone ($p>0.05$; Figure 4A).

Apoptosis levels in untreated neutrophils showed some variation (although not significant). In order to account for this slight variation in “baseline” apoptosis (i.e., untreated cells), the rate of decrease in apoptosis was calculated for each genotype by studying the percent decrease of apoptosis in each treatment compared to the untreated control. When cells were incubated with rhIL-8, the reduction in apoptotic cells was more pronounced in neutrophils from cows expressing the CC genotype compared to neutrophils from cows with a GG

Figure 4. Neutrophil survival from spontaneous apoptosis. Neutrophils from cattle with different CXCR2 +777 genotypes (GG, GC and CC, n=20 each) were incubated in RPMI-1% FBS alone (MEDIA), or RPMI-1% FBS with 50 nM rhIL-8 (IL8) or 100 nM dexamethasone (DEX) for 22-24 hours at 39°C. Apoptosis was determined by fluorescent detection of Annexin V staining. A) Data are reported as percent apoptosis positive cells (i.e., Annexin V positive). B). Percent reduction in apoptosis after incubation with IL-8 relative to control samples that did not receive IL-8. Data are presented as the mean reduction in apoptosis ± SEM for 20 cows within each genotype. Bars with different letters above differ (p=0.03).


70
65
60
55
50
45
40
35
30
% Apoptotic cells
GG
GC
CC
MEDIA
IL8
DEX

(A)


35
30
25
20
15
10
% Decrease in apoptosis
A
AB
B
CC
GC
GG
Genotype

(B)

genotype (p=0.03; Figure 4B). This difference was not observed when cells were treated with dexamethasone (data not shown).

Neutrophil ROS generation

Approximately 100% of the cells stimulated with 10 nM and 100 nM PMA were capable of generating ROS, regardless of the cows' genotype. However, comparison of the median level of fluorescent intensity (as an indicator of overall ROS generation) following stimulation with 10 nM PMA revealed that neutrophils from cattle with a CC genotype produced significantly less ROS than neutrophils from cows with a GG genotype, with heterozygous cows intermediate between the two (p=0.04; Figure 5). A similar decrease in ROS, although not significant, was observed following stimulation with 100 nM PMA (p=0.16; Figure 5).

GSH levels

Neutrophil GSH levels were evaluated to address the possibility that oxidant stress may contribute to the observed differences in neutrophil survival and ROS generation among genotypes. As expected, GSH levels decreased from 5.78 ± 0.16 μM to 1.7 ±0.16 μM after incubation for 24 hours regardless of genotype. In addition, incubation with 50 nM rhIL-8 resulted in slightly higher neutrophil GSH levels in all genotypes compared to incubation without rhIL-8 (2.07 ± 0.16 μM vs. 1.7 ±0.16 μM, $p<0.05$; data not shown). However, GSH

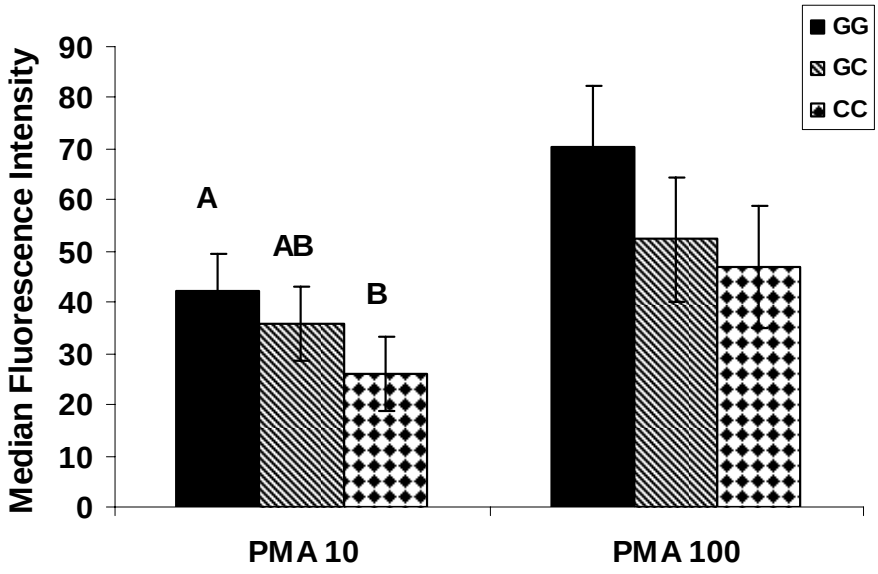


Figure 5. Neutrophil generation of reactive oxygen species following stimulation with PMA. Neutrophils from cattle with different CXCR2 +777 genotypes were activated with 10 nM and 100 nM PMA. Data are presented as the mean of the median fluorescence intensity ± SEM for 20 cows within each genotype. Bars with different letters above differ (p<0.05).

levels did not differ among genotypes either before or after a 24 hour incubation with or without 50 nM rhIL-8 (p>0.05; data not shown).

Bactericidal activity

Neutrophil bactericidal activity was evaluated on *S. aureus* and *S. uberis*, two common mastitis pathogens. Overall killing ability of neutrophils was higher when *S. aureus* and S*. uberis* were opsonized with specific antiserum compared to non-opsonized bacteria in cows of all CXCR2 genotypes (Table 2). However, in spite of the observed difference in ROS generation among cows of different genotypes, bactericidal activity was similar among genotypes for opsonized and non-opsonized *S. aureus* and *S. uberis* (p>0.05; Table 2).

V- DISCUSSION

Once neutrophils have reached the site of infection, they exert their bactericidal action through generation of ROS and release of cytoplasmic granules within the phagosome (Gennaro et al., 1983a; Leino and Paape, 1993). A common means of assessing the ability of neutrophils to generate ROS is through stimulation of the protein kinase C (PKC) signal transduction pathway by treatment of neutrophils with PMA (van Eeden et al., 1999). Our findings suggest that neutrophils from all genotypes are capable of generating ROS in response to PMA; however, the overall effectiveness differs with respect to a cow's genotype

Table 2. Bactericidal activity of neutrophils isolated from Holstein cows with different CXCR2 genotypes.

Genotype	*S. uberis* Non-opsonized	*S. uberis* Opsonized	*S. aureus* Non-opsonized	*S. aureus* Opsonized
GG	23.7 ± 2.6	53.7 ± 3.5	5.8 ± 1.9	29.9 ± 5.8
GC	21.5 ± 2.6	54.4 ± 3.6	4.5 ± 1.9	31.2 ± 5.8
CC	19.3 ± 2.6	51.1 ± 3.4	6.6 ± 1.9	32.4 ± 5.8

Neutrophils from cattle with different CXCR2 +777 genotypes (GG, GC and CC, n=10 each) were incubated with opsonized and non-opsonized *S. aureus* and *S. uberis* (10:1 bacteria to neutrophil ratio) and bactericidal activity was determined by colorimetric determination of formazan production. Results are expressed as percent of bacteria killed ± SEM for 10 cows within each genotype.

(Figure 5) and agrees with our prior findings regarding neutrophil migration (Rambeaud and Pighetti, 2005). The lower ROS generation observed in cows with a CC genotype could result in inefficient bacterial clearance and potential establishment of chronic infections. However, no differences were observed in the ability of neutrophils to kill either *S. uberis* or *S. aureus*, two common mastitis pathogens. This was somewhat unexpected in light of the variation observed in ROS generation. A variety of factors could contribute to the lack of differences observed with respect to bactericidal activity: ROS levels were sufficient to activate proteases, bacteria activated alternative pathways via Toll-like receptors, bacterial numbers (10 bacteria per neutrophil) were low enough that the ROS generated and/or proteases activated were sufficient for killing, or neutrophils were not 'primed' for killing. This last possibility may have considerable impact as neutrophils in vivo are primed for killing through pre-activation by chemokines such as IL-8 (Mitchell et al., 2003). Further studies are being conducted to evaluate these possibilities.

The reduced ROS generation observed in cows with a CC genotype compared to cows with a GG genotype, together with prior findings in which cows with a CC genotype exhibited impaired neutrophil migration and adhesion molecule upregulation upon stimulation with IL-8 (Rambeaud and Pighetti, 2005), seem to contrast with the increased neutrophil survival displayed by these cows. One potential explanation may be tied to differences in ROS generation as recent research by Scheel-Toellner et. al. (2004) has demonstrated that ROS

accumulation plays a central role in triggering neutrophil spontaneous apoptosis through generation of ceramide and consequent clustering of death receptors. In support of this theory, neutrophils deficient in NADPH oxidase undergo apoptosis at a reduced rate (Kasahara et al., 1997), and incubation of neutrophils under hypoxic conditions significantly inhibits apoptosis (Mecklenburgh et al., 2002). It is possible that neutrophils of cows with a CC genotype survived better upon incubation with IL-8 due to a constitutively lower capacity to generate ROS in these cells. Since GSH acts as a powerful scavenger of ROS, and GSH depletion induces neutrophil apoptosis (Watson et al., 1997), GSH levels were evaluated in cows of different CXCR2 genotypes before and after incubation for 24 hours with or without IL-8. However, no differences in GSH levels were observed among cows of different genotypes regardless of time or stimulation, suggesting that GSH does not play a critical role in elucidating the differences observed in induced ROS production and decrease in apoptosis among genotypes.

Another possible explanation for the apparent contradiction of the observed response in spontaneous apoptosis relative to other functions in neutrophils from CC cattle compared to GG cattle may be tied to differences in receptor activation and signal transduction. As mentioned previously, IL-8 activates two receptors – CXCR1 and CXCR2. Because these two receptors are not completely redundant, a defect in either receptor has the potential to influence the degree of subsequent neutrophil activity. For instance, CXCR1 has

been demonstrated to preferentially activate the respiratory burst (Jones et al., 1996), whereas CXCR2 primarily induces survival from spontaneous apoptosis (Glynn et al., 2002). The presence of the polymorphism within the CXCR2 gene also does not preclude the defect from being within the CXCR1 gene, as the two genes are in close proximity on the human chromosome and most likely are in linkage disequilibrium. The exact position of the CXCR1 gene in the bovine is unknown, but bioinformatic analysis of AC151135.5, a working draft of the bovine genome, indicates that CXCR1 and CXCR2 are separated by approximately 25,000 bp (unpublished observations). The causative mutation also may be in a cellular process downstream of IL-8 and both its receptors. This hypothesis is supported by the observed variation in ROS generation among the CXCR2 +777 genotypes following activation with PMA, an analogue of diacylglycerol that activates PKC (Gschwendt et al., 1991). Ongoing studies within the laboratory are in the process of testing each of these hypotheses.

VI- CONCLUSION

In summary, our results indicate that neutrophils from Holstein cows with different CXCR2 genotypes vary in their ability to generate ROS and suppress apoptosis. When taken alone these alterations in function may seem minor. However these findings, in conjunction with differences reported previously in neutrophil migration and adhesion molecule upregulation, represent the first time within the bovine system where it has been possible to identify genetic

differences in neutrophil function. Furthermore, these changes offer a possible functional mechanism for differences in subclinical mastitis observed among cattle with different genotypes at CXCR2 +777. Future research aimed at better understanding these basic differences as well as confirming these results in vivo has the potential to provide novel preventive and therapeutic measures against inflammatory diseases such as mastitis.

VII- ACKNOWLEDGEMENTS

The aid of John Hodges, Mark Campbell, Charlie Young and Dean Jenkins for each of their roles with respect to this project is appreciated greatly. The research reported within this manuscript was supported by The University of Tennessee Food Safety Center of Excellence, The College of Veterinary Medicine Center of Excellence and The University of Tennessee Agricultural Experiment Station.

Chapter 5

DIFFERENTIAL CALCIUM SIGNALING IN DAIRY COWS WITH SPECIFIC CXCR1 GENOTYPES POTENTIALLY RELATED TO INTERLEUKIN-8 RECEPTOR FUNCTIONALITY

This chapter is a paper by the same name which will be submitted for publication in the *Journal of Leukocyte Biology* in 2006 by Magdalena Rambeaud and Gina M. Pighetti.

My use of “we” in this chapter refers to my co-author and myself. My primary contributions to this paper include (1) design of the experiment and animal selection, (2) sample collection, (3) most cell isolations, (4) iodination of interleukin-8, (3) all of the binding assays, (4) neutrophil RNA isolation and real time PCR analysis (5) all of the calcium release assays (6) all of the data analysis (7) most of the gathering and interpreting of the literature, (8) most of the writing of this paper.

I- ABSTRACT

Neutrophil migration and activation are critical components of innate immunity and are mediated by a variety of inflammatory mediators which include interleukin-8 (IL-8) and epithelial derived neutrophil activating peptide-78 (ENA-78). Limited knowledge on the expression of receptors for these inflammatory

mediators (CXCR1 and CXCR2) in bovine, in addition to the association of different CXCR1 +777 genotypes with impaired neutrophil function, prompted evaluation of CXCR1 and CXCR2 mRNA and protein expression, ligand binding affinity, and intracellular receptor signaling in neutrophils from cows with different CXCR1 +777 genotypes. Initial observations revealed that overall IL-8 receptor numbers tended to be lower in cows with a CC genotype compared to cows with a GG genotype. However, in the presence of SB225002, a CXCR2 inhibitor, CXCR1 affinity was about 5-fold lower in cows with a CC genotype and may have resulted in an underestimation of receptor numbers in cows with this genotype. In addition, intracellular calcium ($[Ca^{++}]_i$) release was lower in cows with a CC genotype when cells were stimulated with IL-8 but not ENA-78. Furthermore, when neutrophils were stimulated with an optimal dose of IL-8 in the presence of SB225002, $[Ca^{++}]_i$ release was lower in cows with a CC genotype, suggesting differential CXCR1 signaling among genotypes. These findings offer knowledge of the role that each of these receptors plays in the inflammatory response in the bovine and provide insight into the potential mechanisms that may be affected in neutrophils of cows with different CXCR1 genotypes. A greater understanding of these mechanisms will enhance our ability to develop preventive and therapeutic strategies against inflammatory based diseases.

II- INTRODUCTION

Neutrophils are the first line of defense against most bacterial infections. An effective protective response requires neutrophils to migrate quickly to the inflammatory site and exert their phagocytic and bactericidal activity. This process is driven by many pro-inflammatory mediators, which regulate neutrophil vascular adhesion, migration and activation. Chemokines of the CXC family are an important group of neutrophil chemoattractants and activators, which include interleukin-8 (IL-8), growth-related oncogene α (GRO- α), and epithelial neutrophil activating peptide-78 (ENA-78). CXC chemokines bind to G-coupled 7-transmembrane domain receptors, named CXCR1 and CXCR2 (Lee et al., 1992; Ahuja and Murphy, 1996). G-protein coupling to these receptors results in activation of phospholipase C, formation of the second messenger inositol 1,4,5-trisphosphate and the subsequent increase in cytosolic free calcium concentration ($[Ca^{++}]_i$) (Wu et al., 1993; Baggiolini et al., 1994; Damaj et al., 1996b), as well as activation of phosphatidylinositol 3- kinase and consequent generation of phosphatidylinositol 3-phosphate (Mukaida, 2000). All these mechanisms mediate many of the events necessary for proper neutrophil activation and migration to eliminate invading pathogens.

Structurally similar, human CXCR1 and CXCR2 amino acid sequences have 77% homology, but exhibit different binding characteristics. CXCR1 displays greater ligand specificity than CXCR2 and binds with high affinity to IL-8 and granulocyte chemotactic protein-2 (K_d=1-2 nM) (LaRosa et al., 1992; Lee et

al., 1992). CXCR2 binds multiple CXC chemokines in addition to IL-8, including ENA-78 (K_d=2.2 nM), neutrophil activating peptide-2 (K_d=0.7 nM), and GRO-α, -β, and -γ (K_d=1-2 nM) (Lee et al., 1992; Schumacher et al., 1992; Ahuja and Murphy, 1996; Bozic et al., 1996). On unstimulated neutrophils the two receptors are expressed in approximately equal numbers (Moser et al., 1991; Lee et al., 1992) and in the presence of stimulating ligand both receptors are rapidly internalized. There is indirect evidence that bovine neutrophils express CXCR1 and CXCR2, as they display functional activity towards IL-8, ENA-78 and GRO-α (Li et al., 2002a; Rambeaud and Pighetti, 2005; Rambeaud et al., 2006). In addition, Li et. al. (2002a) reported CXCR1 and CXCR2 receptor expression in bovine neutrophils through labeling with human CXCR1 and CXCR2-specific antibodies. However, to the best of our knowledge, bovine CXCR1 and/or CXCR2 protein and/or mRNA expression has not been described.

The critical role of CXCR1 and CXCR2 is evidenced in IL-8 receptor knock-out mice, which exhibited dysfunctional neutrophil migration and severe disease in a urinary tract infection model (Hang et al., 2000) as well as impaired neutrophil migration and increased susceptibility to *Toxoplasma gondii* infection (Del Rio et al., 2001). Furthermore, neutrophil CXCR1 expression was reduced in children prone to acute pyelonephritis compared to healthy control patients (Frendeus et al., 2000), and CXCR2 expression in septic patients was reduced compared to control patients (Cummings et al., 1999). CXCR2 expression was also reduced in women with recurrent urinary tract infections (Smithson et al.,

2005). These findings point towards a central role of CXCR1 and/or CXCR2 in influencing the outcome of certain inflammatory processes.

Research conducted in our laboratory has identified a polymorphism in the bovine CXCR1 gene that has shown association with mastitis susceptibility. In prior publications, this polymorphism was reported to be in CXCR2. However, subsequent comparison of recently available bovine genome sequence to the human CXCR1 and CXCR2 sequences has revealed this gene to be annotated incorrectly and to be CXCR1 (Pighetti and Rambeaud, 2006). Cows with a CC genotype at position +777 showed increased incidence of subclinical mastitis compared to cows with a GG genotype at the same position (Youngerman et al., 2004a; Youngerman et al., 2004b). Furthermore, cows with a CC genotype exhibited impaired neutrophil migration, adhesion molecule upregulation and reactive oxygen species generation, as well as increased survival by suppression of apoptosis (Rambeaud and Pighetti, 2005; Rambeaud et al., 2006). The polymorphism at position +777 (G→C) results in a histidine replacement of glutamine at amino acid residue 245, which occurs in the receptor's third intracellular loop. This may have direct implication in the functionality of the receptor since amino acid 245 is located in the receptor's third intracellular loop, a critical site for G-protein coupling and activation (Damaj et al., 1996a). Based on these observations, we hypothesized that cows of different CXCR1 +777 genotypes would exhibit different CXCR1 and/or CXCR2 expression, ligand binding affinity, and/or intracellular receptor signaling.

III- MATERIALS AND METHODS

Animal selection and sample collection

Holstein cows from the Knoxville Experiment Station in mid-to late lactation (at least 100 days in lactation) were used in this study (receptor binding assays, n=5 cows/genotype; real time PCR, n=6 cows/genotype; intracellular calcium release assay, n=18 cows/genotype). Cows were selected based on their CXCR2 +777 genotype (GG or CC), which was determined by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry at a commercial facility (Geneseek, Lincoln, NE) or by PCR amplification and sequencing at the University of Tennessee molecular biology core facility (Youngerman et al., 2004a). All cows were free from clinical mastitis, and milk samples were collected immediately prior to blood collection to determine the number of somatic cells present to account for subclinical intramammary infections. Blood was collected by jugular venipuncture in syringes containing acid citrate dextrose anticoagulant (10%vol/vol) and processed immediately for neutrophil isolation. Prior to isolation an aliquot was removed for determination of red and white blood cell counts using an automated cell counter (VetCount IIIB; Mallinckrodt, Phillipsburg, NJ). In addition, smears were obtained to determine leukocyte differential counts.

Neutrophil isolation

Neutrophils were isolated from blood as described previously (Rambeaud and Pighetti, 2005). Neutrophils (98% average purity) were washed with Hanks' balanced salt solution (HBSS, pH 7.2; Cellgro, Herndon, VA) and resuspended in the appropriate media and cell concentration for the different functional assays as described below. Viability was assessed by trypan blue dye exclusion and always exceeded 95%. Average white blood cell count among cows was 8.1×10^3 cells/ml.

Iodination of IL-8

Iodination of IL-8 was performed by using the chloramine T method as described by Petersen et. al. (1994). Briefly, each reaction mixture contained 0.5 nmol of IL-8 (Peprotech, Rocky Hill, NJ) dissolved in 20 µl of 0.1 M phosphate buffer (pH 7.4), 0.5 mCi $Na^{125}I$ (Amersham Biosciences, Piscataway, NJ), and 10 µl chloramine T (1 mg/ml in 0.1 phosphate buffer; Sigma, St. Louis, MO). After 30 s of incubation at room temperature, the reaction was terminated by the addition of 200 µl sodium metabisulfate (25 µg/ml in phosphate buffer). The mixture was then loaded onto a Sephadex G-25 column equilibrated with PBS-0.1%BSA. The iodinated chemokine was eluted in 500 µl fractions. Those fractions containing the iodinated chemokine were pooled and stored in aliquots at -20ºC until use. Specific activity for IL-8 was 588 Ci/mmol.

Binding assays with radiolabeled IL-8

Binding assays were performed essentially as described by Moser et. al. (Moser et al., 1991) with a few modifications. Briefly, neutrophils were resuspended at $1x10^7$ cells/ml in binding media (RPMI 1640 [Sigma] + 20mM HEPES [Roche Diagnostics, Indianapolis, IN] + 1% BSA). When necessary, the CXCR2 inhibitor SB225002 (Calbiochem, San Diego, CA) was added to a subset of cells immediately prior to conducting the binding assay to yield a final inhibitor concentration of 1 μM. 100 μl of cells ($1x10^6$ neutrophils) were added to duplicate sets of tubes with 100 μl of binding media containing ^{125}I IL-8 (0.5 and 1 nM) and increasing concentrations of unlabeled chemokine (0.008-125 nM). After incubating the cells for ~2 h at 4ºC, cells were washed with binding media to remove unbound chemokines, the supernatant was aspirated, the cell pellets were sliced off and radioactivity was measured in a γ-counter. Global curve fitting of results obtained with the two doses of iodinated chemokine was performed using Prism 4.0 (Graphpad Software Inc., San Diego, CA) to calculate K_d and number of binding sites/cell.

Neutrophil RNA isolation

Total RNA was isolated from neutrophils using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Neutrophils were lysed in TRIzol at a concentration of $7x10^6$ cells/ml by vigorous vortexing and pipetting for 10 minutes. RNA concentration and purity was determined using a

BioPhotometer (Eppendorf Scientific, Westbury, NY). RNA quality was checked in all samples by 28S and 18S rRNA band visualization on a 1.2% agarose denaturing gel. RNA was then treated with DNAse (Promega, Madison, WI) at 1U/ug of RNA for 30 minutes at 37ºC. Samples were immediately purified and concentrated with RNeasy MinElute columns (Qiagen, Valencia, CA) as described by the manufacturer.

Real time RT-PCR

CXCR1 and CXCR2 mRNA expression was assessed by quantitative real time RT-PCR (n=6 cows/genotype). Neutrophil RNA (2 µg) was reverse transcribed into cDNA in a 25 µl reaction containing 1 µg oligo $(dT)_{15}$ primer and 1 µg random hexamers (both from Promega), RT buffer (provided by the RT manufacturer; final concentrations of 50 mM Tris-HCl, ph 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM spermidine), 1 mM dNTPs, 40 U of RNAsin (Promega) and 30 U of AMV reverse transcriptase (Promega). The reaction was incubated at 25ºC for 10 min, continued at 42ºC for 45 min and finished at 65ºC for 5 min. For every sample, an additional reaction was prepared as described above but without AMV-RT to control for genomic DNA contamination. Quantitative real time RT-PCR was performed on an IQ iCycler real time PCR detection system (BioRad, Hercules, CA) using the IQ SYBR green PCR supermix (BioRad) and CXCR1, CXCR2 and β-actin specific primer pairs designed with Primer3 software (http://frodo.wi.mit.edu/cgi-

bin/primer3/primer3_www.cgi). Primers used were: CXCR1 forward (5'-ggaggggtttgaggatgagt), CXCR1 reverse (5'- gccaggttcagcaggtagac), CXCR2 forward (5'- gcgatgaagattttggcaat), CXCR2 reverse (5'- gccaggttcagcaggtagac), β-actin forward (5'- cggcattcacgaaactacct), and β-actin reverse (5'-gggcagtgatctctttctg). Reactions were performed in triplicate for 30 cycles at an annealing temperature of 60ºC. β-actin was included in all Q-RT-PCR analyses as the normalizing gene. Results were computed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and expressed as fold change in gene expression relative to GG genotype after normalizing for β-actin gene expression.

$[Ca^{++}]_i$ measurement

Changes in intracellular Ca^{++} were measured using the fluorescent calcium indicator Indo-1AM (Molecular Probes, Eugene, OR) as described by Sparer et. al. (Sparer et al., 2004). Isolated neutrophils were resuspended to 1-5X10^6 cells/ml in HBSS. Cells were labeled with Indo-1AM (1μg/ml in DMSO) at 3μl/ml. Cells were incubated at room temperature for 45 minutes on a nutator mixer, washed with HBSS and resuspended in HBSS + 1.3 mM $CaCl_2$ + 8 mM $MgSO_4$ + 1% FBS. Two ml of cell suspension were added to a heated stirring cuvette on a PTI fluorometer (Photon Technologies Inc., Lawrenceville, NJ). To induce intracellular Ca^{++} accumulation via CXCR-mediated pathway, IL-8 was added to a final concentration of 12.5 nM (optimal dose) and 1.56 nM (suboptimal dose), and ENA-78 was added to a final concentration of 100 nM

(optimal dose) and 12.5 nM (suboptimal dose). In a subset of cows, $[Ca^{++}]_i$ measurements were performed in the presence of the CXCR2 inhibitor SB225002 at a concentration of 1 μM. The ratio of 400nm/490nm was plotted over the acquisition period, and the magnitude of the Ca^{++} peak was recorded. Data are presented as least square means ± SEM of peak fluorescence ratio corrected for background fluorescence in each cow.

Data analysis

Analysis of variance was performed using the mixed procedure of SAS (SAS 9.1, SAS Institute Inc, Cary, NC). A randomized block design was used to determine the effect of genotype on different parameters. The statistical model was $y_{ij}=\mu + D_i + G_j + D*G_{ij}$, where μ=overall mean, D=day, and G=genotype. Receptor number data were log transformed to maintain a normal distribution. Data are presented as least square means with associated standard error. Statistical significance was declared at $p<0.05$, and a trend towards significance was declared at $p<0.10$.

IV- RESULTS

^{125}I IL-8 binding to neutrophils of cows with different CXCR1 genotypes

In order to calculate ^{125}I IL-8 binding parameters (K_d and B_{max}) for cows of different genotypes, a homologous competition binding assay was performed. By

performing the assay with 2 concentrations of radiolabeled chemokine, data from both curves can be analyzed using global curve fitting (parameter sharing) which minimizes errors that may arise from fitting a single curve (Motulsky and Christopoulos, 2003). Representative results from one cow are shown in Figure 6. ^{125}I IL-8 binding assays revealed that ^{125}I IL-8 binds to bovine neutrophils with high affinity regardless of genotype (K_d values 1.26 nM ± 0.28 and 1.13 nM ± 0.28 for CC and GG genotypes, respectively; P>0.05, Figure 7A). However, cows with a CC genotype tended to express a lower number of receptors/cell (P=0.089; figure 7B). When the CXCR2 inhibitor SB225002 was added (therefore only allowing ^{125}I IL-8 binding to CXCR1), K_d values were slightly higher for both genotypes, although not significantly different from each other (P=0.12, Figure 7C), and numbers of receptors also did not differ (P=0.47, Figure 7D). Interestingly, the number of calculated receptor sites/cell stayed constant for cows with a CC genotype whether the SB225002 inhibitor was used or not (13290 ± 3090 and 13248 ± 3618 sites/cell respectively), but receptor numbers dropped (as expected) in cows with a GG genotype when the SB225002 inhibitor was added (8885 ± 4046 and 22574 ± 3090 with and without inhibitor, respectively). However, these results should be interpreted with caution due to the fact that the variability in the data was very large.

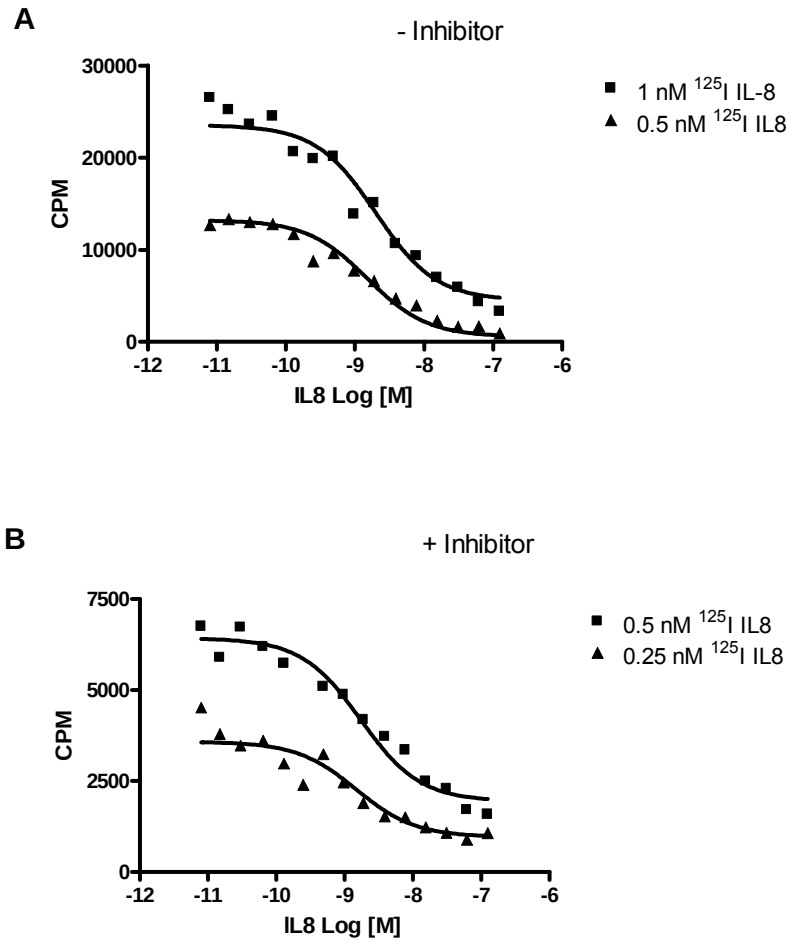
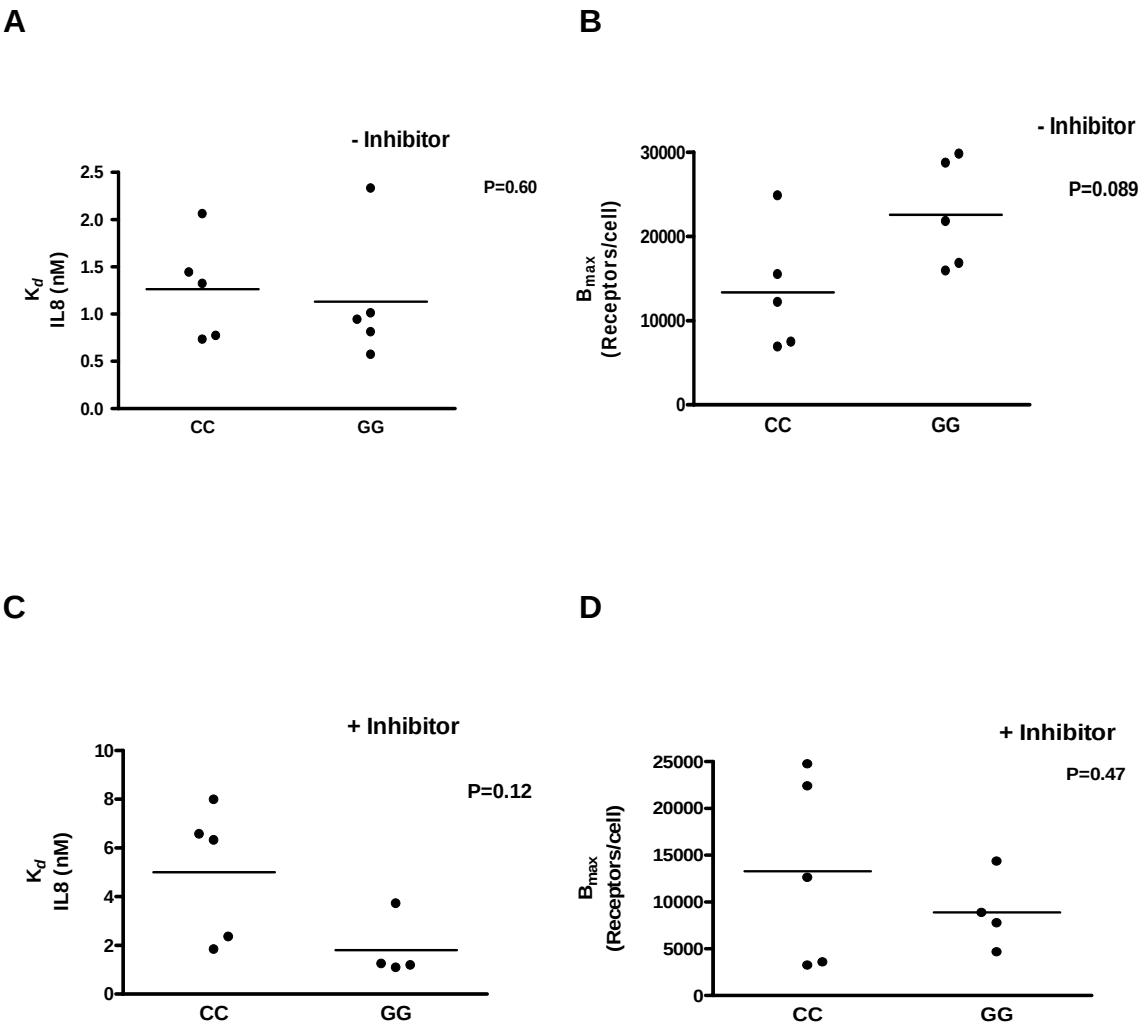


Figure 6. ^{125}I IL-8 homologous competition binding assay results from one representative cow. Neutrophils (10^6 cells/tube in duplicate) were incubated for 2 h with (A) 0.5 and 1 nM ^{125}I IL-8 (tubes with no inhibitor) or (B) 0.25 and 0.5 nM ^{125}I IL-8 (tubes with inhibitor) in the presence of increasing concentrations of unlabeled IL-8 (0.008-125 nM). For each cow, data were analyzed using global curve fitting to calculate K_d and B_{max} values.

Figure 7. Binding parameters of ^{125}I IL-8 to neutrophils of cows with different CXCR1 genotypes. *Top panel.* Binding parameters (K_d in A and B_{max} in B) of ^{125}I IL-8 to neutrophils of cows with different CXCR1 genotypes (GG and CC, n=5 each). Neutrophils were incubated for 2 h with 0.5 and 1 nM ^{125}I IL-8 in the presence of increasing concentrations of unlabeled IL-8 (0.008-125 nM). A) K_d values did not differ among CXCR1 genotypes. B) Receptor sites/cell tended to be lower in cows with a CC genotype than in cows with a GG genotype. *Bottom panel.* Binding parameters (K_d in C and B_{max} in D) of ^{125}I IL-8 to neutrophils of cows with different CXCR1 genotypes (GG and CC, n=5 each) in the presence of the CXCR2 inhibitor SB225002 (1 μM). Neutrophils were incubated for 2 h with 0.25 and 0.5 nM ^{125}I IL-8 in the presence of increasing concentrations of unlabeled IL-8 (0.008-125 nM). No differences were observed among cows of different genotypes for either parameter (p>0.10).


A
- Inhibitor
P=0.60
K_d IL8 (nM)
2.5
2.0
1.5
1.0
0.5
0.0
CC
GG
B
- Inhibitor
P=0.089
B_{max} (Receptors/cell)
30000
20000
10000
0
CC
GG
C
+ Inhibitor
P=0.12
K_d IL8 (nM)
10
8
6
4
2
0
CC
GG
D
+ Inhibitor
P=0.47
B_{max} (Receptors/cell)
25000
20000
15000
10000
5000
0
CC
GG

CXCR1 and CXCR2 mRNA expression

CXCR1 and CXCR2 mRNA expression was confirmed and quantitated by real time-RT PCR. Despite the trend for lower overall receptor numbers observed in cows with a CC genotype with the ^{125}I IL-8 binding assays, CXCR1 and CXCR2 mRNA expression did not differ among cows of both genotypes (least square means ± SEM for CXCR1 were 1.034± 0.148 and 1.053± 0.148 for GG and CC cows, respectively; least square means ± SEM for CXCR2 were 1.026 ± 0.115 and 0.887 ± 0.115 for GG and CC cows, respectively; p>0.3). In addition, within each cow mRNA levels for each receptor was similar (data not shown), suggesting approximately equal levels of CXCR1 and CXCR2 expression.

$[Ca^{++}]_i$ measurement

Stimulation of bovine neutrophils with IL-8 and ENA-78 induced an increase in $[Ca^{++}]_i$ (Figure 8). However, as shown in Figure 8, when neutrophils were stimulated with IL-8 the magnitude of the $[Ca^{++}]_i$ peak differed among cows of different genotypes. Cows with a CC genotype exhibited a smaller $[Ca^{++}]_i$ peak compared to cows with a GG genotype when neutrophils were stimulated with a suboptimal dose of IL-8 (p=0.0001) and a similar trend was observed when cells were stimulated with an optimal dose of IL-8 (p=0.08). This difference was not observed when neutrophils were stimulated with ENA-78 at either the optimal or suboptimal dose (Figure 8, p>0.10).

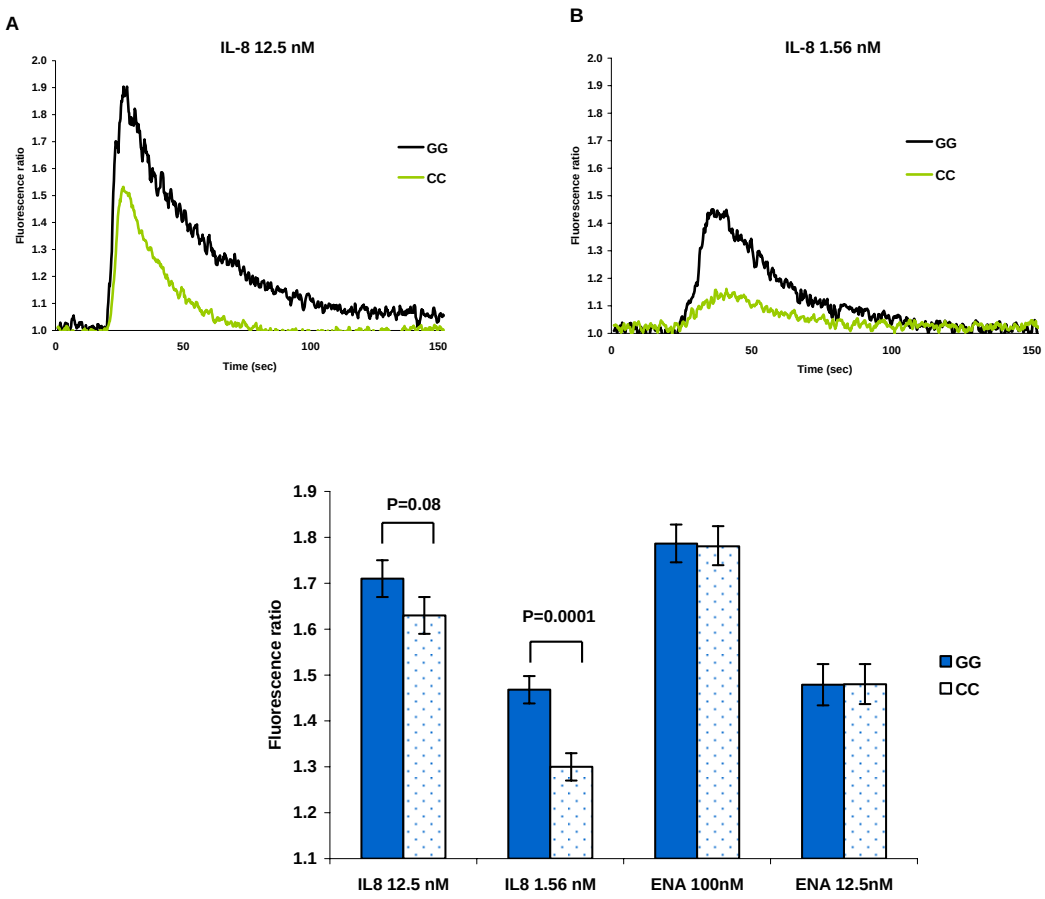


Figure 8. Intensity of $[Ca^{++}]_i$ peak on neutrophils of cows of different CXCR1 genotypes upon stimulation with 12.5 and 1.56 nM IL-8 (optimal and suboptimal dose, respectively), and 100 nM and 12.5 nM ENA-78 (optimal and suboptimal dose, respectively). *Top panel.* Representative traces for one cow of each genotype (GG, black line; CC, grey line) after IL-8 stimulation with 12.5 nM (A) and 1.56 nM (B). *Bottom panel.* Intensity of $[Ca^{++}]_i$ peaks (mean ± SEM) after stimulation with IL-8 and ENA-78. IL-8 stimulation, *n*=18 cows/genotype; ENA-78 stimulation, *n*=10 cows/genotype.

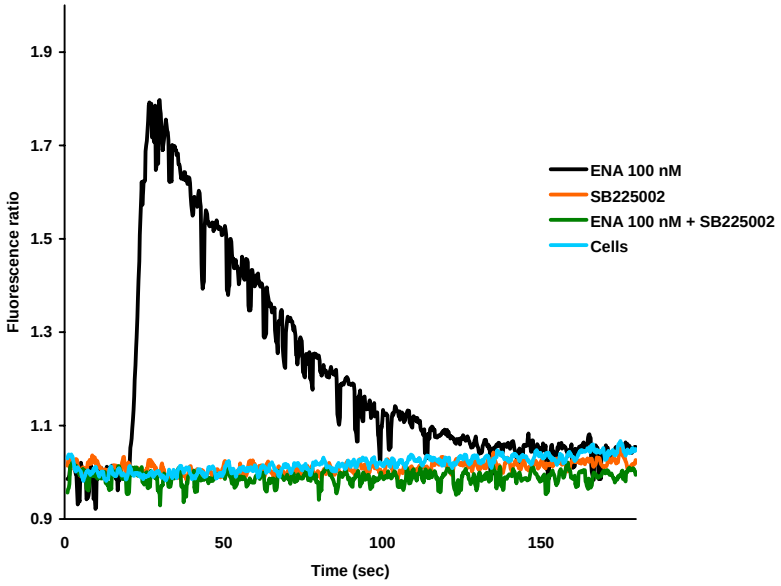
To further characterize the difference in $[Ca^{++}]_i$ release observed among genotypes when neutrophils were stimulated with IL-8, the CXCR2 inhibitor SB225002 was used. The addition of 1 μM SB225002 to bovine neutrophils completely blocked $[Ca^{++}]_i$ release upon ENA-78 stimulation (Figure 9), confirming the specificity of this compound as a CXCR2 inhibitor in our system. Since CXCR2-mediated $[Ca^{++}]_i$ release is blocked at this inhibitor dose, any $[Ca^{++}]_i$ release observed when neutrophils are stimulated with IL-8 under these conditions can be attributed to signaling through CXCR1. When neutrophils were stimulated with IL-8 in the presence of 1 μM SB225002, $[Ca^{++}]_i$ release was decreased, although to different degrees depending on the IL-8 dose (Figure 10). At an optimal dose of IL-8 (12.5 nM), cows with a CC genotype showed lower $[Ca^{++}]_i$ release compared to cows with a GG genotype (p=0.015). On the other hand, when a suboptimal dose of IL-8 was used (1.56 nM), the $[Ca^{++}]_i$ release was abrogated regardless of genotype (p>0.10).

V- DISCUSSION

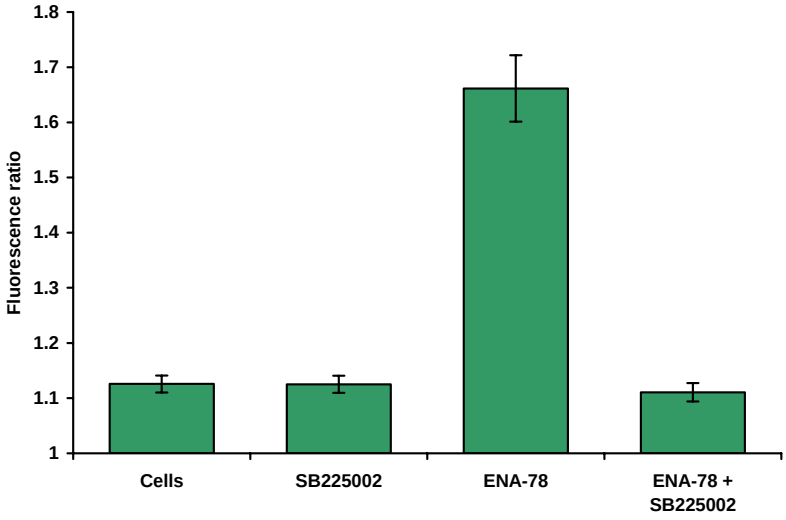
In order to elucidate some of the observed differences in subclinical mastitis incidence as well as neutrophil functionality in cows with different CXCR1 genotypes, (Youngerman et al., 2004b; Rambeaud and Pighetti, 2005; Rambeaud et al., 2006), we evaluated CXCR1 and CXCR2 expression, affinity, and ability to induce $[Ca^{++}]_i$ release in neutrophils of cows with different CXCR1 genotypes. The affinity of IL-8 for its receptors on bovine neutrophils did not differ

Figure 9. $[Ca^{++}]_i$ release in neutrophils of cows with different CXCR1 genotypes stimulated with ENA-78 in the presence of SB225002, a CXCR2 inhibitor. The addition of 1 μM SB225002 immediately before ENA-78 stimulation (100 nM) blocked $[Ca^{++}]_i$ release. A) Representative traces from one cow. B) Intensity of $[Ca^{++}]_i$ peaks (means ± SEM; n=9 cows) in neutrophils without stimulation (Cells), neutrophils with inhibitor only (SB225002), neutrophils stimulated with ENA-78 (ENA-78) and neutrophils stimulated with ENA-78 in the presence of 1 μM SB225002 (ENA-78 + SB225002).

A


1.9
1.7
1.5
1.3
1.1
0.9
Fluorescence ratio
0
50
100
150
Time (sec)
ENA 100 nM
SB225002
ENA 100 nM + SB225002
Cells

B


1.8
1.7
1.6
1.5
1.4
1.3
1.2
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1
Fluorescence ratio
Cells
SB225002
ENA-78
ENA-78 +
SB225002

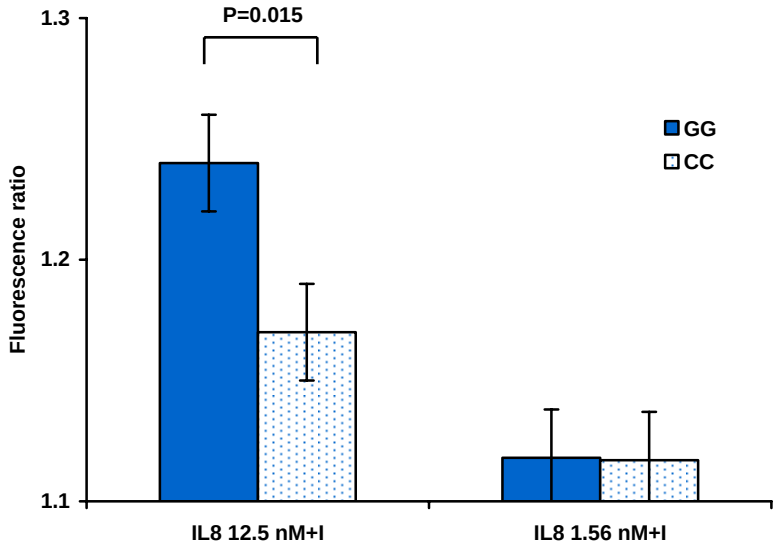

Figure 10. Intensity of $[Ca^{++}]_i$ peaks (means ± SEM) in neutrophils of cows with different CXCR1 genotypes stimulated with IL-8 in the presence of the CXCR2 inhibitor SB225002 (1 μM). When an optimal dose of IL-8 was used, $[Ca^{++}]_i$ release was decreased ~50% in cows with a CC genotype compared to cows with a GG genotype. When an suboptimal dose of IL-8 was used, $[Ca^{++}]_i$ release was abrogated in cows of both genotypes.

among cows of different CXCR1 genotypes (Figure 7A), and is similar to that observed in human neutrophils (K_d =1-2 nM) (LaRosa et al., 1992; Lee et al., 1992). However, calculated IL-8 receptors/neutrophil tended to be about half as many in cows with a CC genotype compared to cows with a GG genotype (Figure 7B). This finding may explain, in part, reduced migration and adhesion molecule upregulation (Rambeaud and Pighetti, 2005), as well as decreased $[Ca^{++}]_i$ release when neutrophils of cattle with a CC genotype were stimulated with IL-8 (Figures 8 and 10, this paper). However, the basis for reduced IL-8 binding are not immediately evident as a decrease in CXCR1 or CXCR2 numbers, affinity, or some combination of the two could lead to this observation. Efforts were made to directly assess neutrophil CXCR2 receptor expression and affinity. Unfortunately, a functional CXCR2 ligand was unattainable because ENA-78 was not able to be labeled with three different iodination methods (chloramine-T, Iodogen and Bolton-Hunter reagent), nor was GRO-α a potential alternative because prior experiments from our laboratory indicated extremely low or non-existent neutrophil migration or $[Ca^{++}]_i$ release (unpublished observations).

Because of the observed differences in neutrophil function (Rambeaud and Pighetti, 2005; Rambeaud et al., 2006) as well as IL-8 receptor binding ability, the intensity of neutrophil $[Ca^{++}]_i$ release was quantitated following stimulation with IL-8 and ENA-78. Two doses of chemoattractant were evaluated: an optimal dose which elicited maximum $[Ca^{++}]_i$ release in a dose titration curve

(data not shown), and a sub-optimal dose which resulted in a lower $[Ca^{++}]_i$ peak, but was more similar to chemoattractant concentrations found in vivo, and therefore more relevant biologically (Allman-Iselin et al., 1994; Caswell et al., 1998; Rambeaud et al., 2003). When stimulated with an optimal dose of IL-8, a CXCR1 and CXCR2 ligand (Figure 8, bottom panel), neutrophils of cows with a CC genotype showed ~10% lower $[Ca^{++}]_i$ release than cows with a GG genotype; this difference became more pronounced when cells were stimulated with a suboptimal dose of IL-8, where ~40% lower $[Ca^{++}]_i$ release was observed. This difference in neutrophil $[Ca^{++}]_i$ release was not observed following stimulation with ENA-78, a CXCR2 specific ligand (Figure 8, bottom panel), suggesting that signaling through CXCR1 differs among cows of the two genotypes, and that CXCR1 may be implicated in decreased IL-8 binding ability. The observation that the difference in $[Ca^{++}]_i$ release was more pronounced when the suboptimal dose of IL-8 was used (Figure 8, bottom panel) may be informative relative to infection in vivo because, as mentioned previously, the suboptimal dose mimics IL-8 concentrations found in vivo. Along those lines, it is tempting to speculate that cows with a CC genotype, which have shown increased susceptibility to mastitis and impaired neutrophil migration in vitro, may not respond as effectively to neutrophil IL-8 stimulation in vivo due to decreased intracellular signaling, which could potentially allow the establishment of bacterial infection.

Based upon the differences observed in $[Ca^{++}]_i$ release and IL-8 binding ability by neutrophils of cows with a CC genotype, it can be logically

hypothesized that CXCR1 numbers or affinity is impaired in cows with this genotype. Ideally, to test this hypothesis it would be necessary to perform binding and $[Ca^{++}]_i$ release assays with each receptor separately. In order to do this, two approaches could be followed: cloning and expression of CXCR1 and CXCR2 from cows of different genotypes (which would allow to test each receptor's affinity towards different ligands), or blocking of each receptor with specific inhibitors or antibodies in neutrophils of cows with different genotypes, (which would allow determination of receptor numbers as well). Unfortunately, a CXCR1 inhibitor or antibodies that cross react with bovine CXCR1 and CXCR2 are unavailable at this time (unpublished observations). For the current experiment, the CXCR2 specific inhibitor SB225002 was used to characterize IL-8 binding and $[Ca^{++}]_i$ release responses mediated through CXCR1.

At the concentration used in our study (1 μM), SB225002 has been demonstrated to completely block IL-8 binding to CXCR2 (White et al., 1998). Our results confirm this finding since SB225002 completely blocked $[Ca^{++}]_i$ release induced by ENA-78 (Figure 9). Consequently, all binding or signaling occurring under these conditions can be attributed to CXCR1. Although not significant, the K_d values for CXCR1 were ~5 fold higher (5 nM) higher than those determined for CXCR1 and CXCR2 combined (1-2 nM), especially in cows with a CC genotype (Figure 7C), suggesting that these cows exhibited lower IL-8 affinity for CXCR1, which could explain the lower $[Ca^{++}]_i$ release observed in these cows when neutrophils were stimulated with IL-8. Even though the variation in the data

was large, it is tempting to speculate that the lower affinity of CXCR1 in cows with a CC genotype may also contribute to the decreased functionality observed in neutrophils of these cows. We were unable to determine IL-8 affinity for CXCR2 due to the lack of an available CXCR1 inhibitor, or the affinity of CXCR2 for any other CXCR2 ligand for reasons described previously.

A surprising finding was that in cows with a CC genotype, the number of calculated receptor sites/cell was similar both in the presence or absence of SB225002 (Figures 7B and 7D). We can assume this is not due to lack of CXCR2 expression in cows with a CC genotype, as neutrophils from cows of both genotypes exhibited strong $[Ca^{++}]_i$ release responses following stimulation with ENA-78, a CXCR2 specific ligand (Figure 8, bottom panel). One possible explanation for this finding is that IL-8 binding to CXCR2 is somehow impaired in cows with a CC genotype, and therefore all or most of the IL-8 binding observed in these cows even in the absence of the inhibitor is to CXCR1. However, when SB225002 was added to bovine neutrophils, ^{125}I IL-8 binding was decreased, as evidenced by a ~50% decrease in overall counts per minute (Figure 6; representative results from one cow), which would indicate that about 50% of the receptors are CXCR2. This occurred consistently in every cow, regardless of genotype. We found similar levels of CXCR1 and CXCR2 mRNA expression in all cows, further supporting the possibility that CXCR1 and CXCR2 are expressed in approximately equal numbers in bovine. This finding offers an apparent contradiction with calculated receptor numbers: if overall counts per

minute decreased by half in presence of the CXCR2 inhibitor, receptor numbers should have also decreased, but in cows with a CC genotype they remained constant. However, if CXCR1 affinity for IL-8 is indeed lower in cows with a CC genotype, estimated overall receptor numbers may be underrepresented in these cows due to lower binding of IL-8 to CXCR1. Low CXCR1 affinity of IL-8 may also explain the decreased $[Ca^{++}]_i$ release observed when cells were stimulated with IL-8, both in the absence and presence of a CXCR2 inhibitor (see below).

When neutrophils were stimulated with an optimal dose of IL-8 in the presence of SB225002, the intensity of $[Ca^{++}]_i$ release decreased by approximately 70% regardless of genotype, indicating that under these in vitro conditions the majority of neutrophil $[Ca^{++}]_i$ release upon IL-8 stimulation is mediated through CXCR2. Interestingly, when a suboptimal dose of IL-8 was used in the presence of SB225002, $[Ca^{++}]_i$ release was blocked regardless of genotype, which would suggest that at lower IL-8 doses, intracellular signaling is achieved primarily through CXCR2. This finding is consistent with those in human neutrophils, where it is thought that CXCR1 may play a more active role at the site of inflammation where agonist concentration is higher (Chuntharapai and Kim, 1995), and that CXCR2 may play a more important role in the initiation of neutrophil migration distant from the site of inflammation where agonist concentration is lower (Chuntharapai and Kim, 1995; Rose et al., 2004).

A closer examination of $[Ca^{++}]_i$ release following stimulation with the optimal dose of IL-8 in the presence of SB225002, revealed that $[Ca^{++}]_i$ release

continued to be ~40-50% lower in cows with a CC genotype compared to cows with a GG genotype, further supporting the possibility that these differences were due to differential CXCR1 signaling. In addition, the lower $[Ca^{++}]_i$ release observed in these cows may be explained by the apparent lower affinity for IL-8 to CXCR1 suggested by the binding assays. Alternatively, it is possible that since the +777 polymorphism causes an amino acid substitution from glutamine to histidine at the receptor's third intracellular loop, G-protein binding, activation and consequent intracellular signaling events may impacted in cows with a CC genotype (Damaj et al., 1996a).

Overall lower receptor numbers or receptor affinity in cows with a CC genotype may be responsible, at least in part, for the lower functionality of neutrophils in these cows upon IL-8 stimulation. This finding is not unprecedented in relation to disease, as an association between chemokine receptor number expression or affinity and disease susceptibility and/or cell functionality has been documented in studies performed in humans. For instance, patients with a heterozygous fractalkine receptor CX_3CR1 genotype at amino acid 249 exhibited lower receptor numbers in peripheral blood mononuclear cells than those individuals with a homozygous genotype (Moatti et al., 2001). Furthermore, individuals with a heterozygous genotype also had a markedly reduced risk of acute coronary events independent of established coronary risk factors (Moatti et al., 2001). The potential explanation for this association is that since fractalkine can be anchored to its receptor and act as a

potent adhesion factor, reduced receptor expression would result in reduced monocyte adhesion to damaged endothelium, therefore reducing the risk of acute cardiovascular events. Another study found that HIV-infected individuals with certain CX_3CR1 haplotypes exhibited reduced fractalkine binding and progressed to AIDS more rapidly than those with other haplotypes (Faure et al., 2000). Specifically for CXC receptors, CXCR1 expression was reduced in children prone to acute pyelonephritis (Frendeus et al., 2000) and CXCR2 expression was lower in women prone to urinary tract infections compared to healthy control patients (Smithson et al., 2005). Since neutrophils are critical players in eliminating bacterial infections in the urinary tract (Hang et al., 2000), lower CXCR1 and/or CXCR2 expression may account for impaired ability of neutrophils to clear an infection in these patients.

VI- CONCLUSION

In conclusion, this study provides insight into potential mechanisms that may be affected in neutrophils of cows with different CXCR1 genotypes. Cows with a CC genotype at position +777 in the CXCR1 gene showed lower IL-8 receptor expression as well as decreased $[Ca^{++}]_i$ release upon IL-8 stimulation compared to cows with a GG genotype at this position. In addition, the finding that $[Ca^{++}]_i$ release was lower in cows with a CC genotype in the presence of a CXCR2 inhibitor suggests that the observed differences may be due to decreased CXCR1 signaling, potentially due to lower CXCR1 affinity for IL-8.

These findings may partially explain some of the observed differences in neutrophil functionality and mastitis incidence in cows with these genotypes.

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Chapter 6

SUMMARY AND CONCLUSIONS

Neutrophil recruitment to the mammary gland and neutrophil functional ability once there often determine whether a bacterial infection is cleared or becomes chronic. A slow or ineffective neutrophil attack allows bacteria to continue their assault and generate a longer lasting and potentially more damaging infection. Since neutrophils are key players in the resolution of mastitis, and a polymorphism in CXCR1 +777 (G→C) has been associated with susceptibility to mastitis, the hypothesis of this research was that different CXCR1 +777 genotypes were associated with efficiency of in vitro neutrophil activity and/or interleukin-8 (IL-8) receptor expression and intracellular signaling.

As a first step, neutrophil adhesion molecule expression and migration were evaluated upon stimulation with IL-8, phorbol-13-myristate-12 acetate (PMA) and zymosan activated serum (ZAS), in order to determine whether differences among genotypes, if observed, were CXCR- mediated (IL-8 stimulation) or not (ZAS stimulation, which contains C3a and C5a complement fractions; and stimulation with PMA, a non-specific protein kinase C [PKC] activator). CD62 was downregulated equally in both genotypes. However, CD11b/CD18 upregulation was lower in cows with a CC genotype compared to cows with a GG genotype, but only when neutrophils were stimulated with IL-8.

The finding that no differences were observed when neutrophils were stimulated with ZAS or PMA suggests that overall CD11b or CD18 protein levels are similar among genotypes, and that the observed differences are due to reduced signaling through CXCR1 and/or CXCR2, which would result in impaired secretory vesicle translocation to the cell surface. In addition, neutrophil migration towards both IL-8 and ZAS was impaired in cows with a CC genotype compared to cows with a GG genotype, which suggests that a downstream signaling mechanism common to both pathways is also impacted in cows with a CC genotype.

We also examined neutrophil reactive oxygen species (ROS) generation and survival from spontaneous apoptosis in cows of different genotypes. Generation of ROS is vital for elimination of pathogens, and neutrophil rescue from spontaneous apoptosis by inflammatory mediators is considered critical for efficient bacterial clearance, as apoptotic neutrophils have reduced pro-inflammatory functions. Neutrophils from cattle with a CC genotype produced significantly less ROS than neutrophils from cows with a GG genotype. The lower ROS generation observed in neutrophils from cows with a CC genotype could result in inefficient bacterial clearance and potential establishment of chronic infections. In addition, neutrophils from cows with a CC genotype exhibited increased survival from spontaneous apoptosis when incubated with IL-8. These two observations may be related, as recent research by Scheel-Toellner et. al. (2004) has demonstrated that ROS accumulation plays a central role in triggering

neutrophil spontaneous apoptosis. It is possible that neutrophils of cows with a CC genotype survived better upon incubation with IL-8 due to a constitutively lower capacity to generate ROS in these cells. Another possible explanation for the apparent contradiction of the observed response in spontaneous apoptosis relative to other functions in neutrophils from CC cattle compared to GG cattle may be tied to differences in signal transduction. For instance, PMA acts as a diacylglycerol analog that activates PKC (Gschwendt et al., 1991), which in turn activates nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and results in subsequent ROS generation (Dekker et al., 2000). Since PMA-stimulated ROS generation is independent of CXCR1 activation, our observations would suggest that PKC activation and/or NADPH oxidase levels or activity differ among genotypes independently of CXCR1 action. Protein kinase C is not a single enzyme but comprises a family of related isotypes, the most important isoforms expressed in neutrophils being PKC-β_I, β_{II} and δ (Kent et al., 1996; Khwaja and Tatton, 1999), which are responsible for NADPH oxidase activation and subsequent ROS generation (Dekker et al., 2000). In addition, PKC-δ plays a role in spontaneous and induced apoptosis, as inhibition of the enzyme reduces neutrophil apoptosis, or alternatively, increases neutrophil survival (Khwaja and Tatton, 1999). Therefore, it is possible that decreased PKC-δ levels or activity may explain why cows with a CC genotype exhibit lower ROS generation as well as increased neutrophil survival. Furthermore, IL-8-mediated CD11b/CD18 upregulation, which differed among cows of different genotypes as well, is also mediated primarily by PKC activation, presumably PKC- δ (Takami et

al., 2002). These findings would argue for a role of PKC- δ in explaining, at least in part, some of the observed differences in neutrophil function among cows of different genotypes.

In order to elucidate some of the observed differences in neutrophil functionality in cows with different CXCR1 genotypes, we evaluated CXCR1 and CXCR2 expression, affinity, and ability to induce intracellular Ca^{++} ($[Ca^{++}]_i$) release in neutrophils of cows with different CXCR1 genotypes. Initial observations revealed that overall IL-8 receptor numbers tended to be lower in cows with a CC genotype compared to cows with a GG genotype. However, in the presence of SB225002, a CXCR2 inhibitor, CXCR1 affinity was about 5-fold lower in cows with a CC genotype and may have resulted in an underestimation of receptor numbers in cows with this genotype. Relative CXCR1 and CXCR2 mRNA expression was similar within each cow regardless of genotype, further supporting approximately equal CXCR1 and CXCR2 expression. Furthermore, intracellular signaling, measured by $[Ca^{++}]_i$ release, was lower in cows with a CC genotype, whether the CXCR2 inhibitor was used or not, also suggesting impaired CXCR1 signaling upon IL-8 activation. Lower IL-8 affinity for CXCR1 and/or lower intracellular signaling in cows with a CC genotype may partly explain the decreased neutrophil migration and adhesion molecule upregulation, and the increased survival from apoptosis observed when neutrophils from these cows were stimulated with IL-8.

Several limitations were found during the course of this research, mostly due to the lack of available reagents to work in the bovine system. Unfortunately, many human reagents (chemokines, antibodies) do not cross react with bovine cells; we found this particularly limiting in performing the receptor binding assays, as we were unable to obtain a viable CXC2 ligand, which limited our findings a good extent. We also tried to determine receptor expression indirectly by using commercially available monoclonal antibodies towards human CXCR1 and CXCR2, but all the reagents tested did not cross react with bovine neutrophils. Therefore, it becomes imperative to develop the necessary reagents to elucidate some of the questions remaining to be answered. Specifically, the development of antibodies for bovine CXCR1 and CXCR2 would be of great benefit not only to definitely characterize receptor numbers and affinity on neutrophils of cows with different genotypes, but also to allow development of additional assays which may involve receptor blocking and/or detection. Furthermore, these antibodies would allow better description of the role of each receptor in other bovine tissues, such as epithelial and endothelial cells, monocytes and lymphocytes.

The significance of the CXCR1 +777 polymorphism may be two-fold: first, the change in base pairs (G→C) which causes an amino acid change from glutamine to histidine in the receptor's third intracellular loop, may have a causative effect on receptor functionality, and may potentially explain some of the observed differences in neutrophil function such as migration, adhesion molecule upregulation and survival from apoptosis, upon IL-8 stimulation. Evidence for this

hypothesis is provided by work by Damaj et. al. (1996a) which identified amino acid residues within the third intracellular loop of both human IL-8 receptors, CXCR1 and CXCR2, that were involved in mediating neutrophil calcium signaling and mobilization during IL-8 stimulation. On the other hand, the polymorphism may be a marker for other SNPs located within other segments of the CXCR1 gene that more directly affect receptor binding or function. Along those lines, research within our laboratory has identified at least three additional nonsynonymous polymorphisms within the CXCR1 gene (unpublished observations). In addition, the polymorphism at position +777 may also act as a marker for other genes located in the vicinity of the IL-8 receptor locus, which may be tied to disease susceptibility. For instance, CXCR1 is in close proximity to CXCR2 and natural resistance associated macrophage protein-1, both of which have shown to be polymorphic and associated with immune responses during infection (Bellamy, 1999; Renzoni et al., 2000). Lastly, the observations of differential migration towards ZAS and overall lower ROS generation following stimulation with PMA in cows with a CC genotype, both processes unrelated to CXCR1 or CXCR2 activation, would suggest that the +777 CXCR1 polymorphism is a marker for other genes and/or traits, possibly related to downstream signaling events or common intracellular signaling pathways.

In conclusion, this research provides evidence for neutrophil functional differences and differential CXC receptor activity and signaling in cows with specific CXCR1 genotypes. The combination of in vitro data reported in this

research with in vivo data from previous studies suggests that the impaired functionality of neutrophils observed in cows with a CC genotype does not result in an all-or-none response, but rather in a subtle reduction in the number, timing, bactericidal activity, survival, or intracellular signaling of neutrophils, or a combination of any of these factors, which may result in increased susceptibility to mastitis. Future research aimed at better characterizing CXCR1 and CXCR2 in the bovine as well as corroboration of these findings in an in vivo model may provide prospective ways to enhance or regulate neutrophil function in dairy cows and potentially increase their resistance to mastitis and other inflammatory diseases.

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