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I am submitting herewith a thesis written by Angela Pollock Loughery entitled "Relationship of Intracellular Signaling Pathways with Migration in Bovine Neutrophils with Different CXCR1+777 Genotypes." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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**RELATIONSHIP OF INTRACELLULAR SIGNALING
PATHWAYS AND MIGRATION IN BOVINE
NEUTROPHILS WITH DIFFERENT CXCR1+777
GENOTYPES**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Angela Pollock Loughery

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DEDICATION

This thesis is dedicated to my family who have always believed in me and supported me in everything I have embarked on throughout my life and to my husband, T.C., for always encouraging me with love and patience.

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supports, encourages, and most of all always reminds what is truly important in life.

ABSTRACT

Prior research in our lab has demonstrated a significant association between the incidence of subclinical mastitis and neutrophil functions with polymorphisms of the CXCR1 gene in Holstein dairy cattle. The objective of this study was to evaluate the specificity of the response relative to CXCR1 and potential involvement of intracellular signaling pathways. Migration was evaluated in cows of both homozygous genotypes (n=7 each) in response to high and low doses of interleukin-8 (IL -8; specific for CXCR1 and CXCR2), epithelial neutrophil activating peptide (ENA-78; specific for CXCR2) or zymosan activated sera (ZAS; complement receptors). Cows with a GG genotype had significantly more migration in response to both 12.5 and 50 nM doses of IL -8 ($p<0.05$) compared to cows with a CC genotype. Although a similar trend was observed for 50 nMENA-78 ($p<0.07$), this was not evident with the 12.5 nM dose. This result was comparable to ZAS, where the 5% dose caused significantly more neutrophil migration in cows with a GG genotype than those with a CC genotype ($p<0.03$), while the response to the 1% dose of ZAS was similar between genotypes. p38 MAPK and PKB phosphorylation were evaluated prior to and following stimulation with the high doses of each ligand. Relative increases in p38 MAPK phosphorylation were greater in neutrophils from cows with a GG genotype when compared to those with a CC genotype regardless of treatment with rhu-IL-8, rhu-ENA-78, and ZAS or not ($p<0.001$), But CC cows had higher baseline levels of p38-MAPK phosphorylation than GG cows. Therefore, the lower increase in phosphorylation in neutrophils from CC cows may have been due to higher

starting levels. Increases in PKB phosphorylation also were greater in neutrophils from cows with a GG genotype when compared to a CC genotype when treated with ZAS, but not other ligands. Because no differences between genotypes were observed at time zero this may be tied to the altered p38 MAPK pathway. Future research will be aimed at further elucidating the signaling pathways causing functional differences in neutrophils from cows with different CXCR1 genotypes.

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Part I
INTRODUCTION

Mastitis, an inflammation of the mammary gland, is the most economically devastating disease to the dairy industry, causing losses of over two billion dollars a year to the United States alone. Neutrophils play an essential role in the resolution of bacterial infections such as mastitis. If neutrophils do not get to the site of infection in a timely manner it is more likely that the inflammation will persist for longer and have more detrimental effects. In the case of mastitis, this can have devastating economic impacts because mastitis not only reduces milk yield but also causes losses due to discarded milk, replacement costs, extra labor, treatment and veterinary costs.

The first crucial step in resolution of mastitis is the migration of neutrophils to the site of infection, and secondly their functional activity once there (Paape, Shafer-Weaver et al. 2000; Kehrli and Harp 2001). Interleukin-8 (IL-8) is a crucial mediator of neutrophil function. Neutrophils recognize IL-8 through two cell surface receptors on their surfaces, CXCR1 and CXCR2. Research in our laboratory has identified a polymorphism in the bovine CXCR1 gene associated with mastitis susceptibility (Youngerman, Saxton et al. 2004). Cows with a CC genotype at position +777 had more subclinical mastitis when compared with cows with a GG genotype (Youngerman, Saxton et al. 2004; Youngerman, Saxton et al. 2004). This increase in susceptibility may be tied to impaired neutrophil function as cows with the CC genotype also have lower neutrophil migration in response to IL-8 and zymosan activated sera (ZAS), adhesion molecule upregulation in response to IL-8, and reactive oxygen species generation in response to PMA, as well as decreased apoptosis in response to

IL-8 (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006). Previous experiments revealed that neutrophil functions were not only impaired in response to IL-8 but also to ZAS and PMA indicating that the responses are not CXCR1 or CXCR2 specific, but more likely a common downstream signaling event is affected. Thus far two pathways have been implicated in neutrophil activation and subsequent migration: the phosphatidylinositol-3 kinase (PI3-K) and p38 mitogen activated protein kinase pathway (p38-MAPK) pathways (Hirsch, Katanaev et al. 2000).

Based on this information, we hypothesize that migration of neutrophils from cows with different CXCR1+777 genotypes are different in response varying ligands and doses and these differences are paralleled by differences in intracellular signaling pathways that initiate migration. To test this hypothesis, we had two objectives: 1) Identify the effect of ligand and dose on neutrophil migration in cows with different CXCR1+777 genotypes and 2) Evaluate if cows with different CXCR1 genotypes exhibit differences in intracellular signaling pathways. Evaluation of the different ligands will allow determination of the specificity of the response. Also determining if different signaling pathways are affected in neutrophils from cows with different genotypes will help further explain the functional differences previously seen.

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Part II
LITERATURE REVIEW

MASTITIS

Economic Importance

Mastitis is the most economically devastating disease to the dairy industry, causing losses of over two billion dollars a year to the United States alone. These losses are due to many factors including discarded milk, decreased milk yield, treatment costs, culling or death of affected cows (Hansen, Young et al. 1979; Short, Bell et al. 1990; Jones, Hansen et al. 1994; Fetrow 2000). The National Mastitis Council estimates that mastitis affects one-third of all dairy cows (NMC 1996), and also in 2002, producers reported 26.9% of culled cows were due to mastitis or mastitis related problems (N.A.H.M.S. 2002). Because of its impact, ongoing research in this area seeks to understand the exact cause and process of mastitis in order to prevent and control the disease.

Infection and Control

Mastitis is an inflammation of the mammary gland commonly caused by a variety of microorganisms that invade the udder, multiply and potentially cause harm to the tissue. Watts identified 137 different mastitis causing organisms, although most often bacteria, organisms as diverse as mycoplasma, yeasts and algae have also been implicated (Watts 1988). Two common categories of mastitis pathogens are contagious and environmental, based on their modes of transmission (Smith 1983; Bramley 1990). Contagious pathogens survive and multiply within the mammary gland. Transmission occurs from infected cow to uninfected cow primarily during milking and has been linked to unsanitized

milking equipment and poor hygienic practices (Neave, Dodd et al. 1969; Fox and Gay 1993). Common contagious pathogens include *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma spp.* and *Corynebacterium bovis* (Bramley and Dodd 1984; Fox and Gay 1993). On the other hand, environmental pathogens are found everywhere in the dairy environment and teat infection can occur at any time (Smith and Hogan 1993). Environmental pathogens include streptococci, coagulase-negative staphylococci (CNS), coliforms, and Gram-negative bacteria (Hogan, Gonzalez et al. 1999; Jayarao and Wang 1999; Jayarao and Wolfgang 2003). *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, and *Klebsiella spp.* are the most commonly isolated environmental bacteria (Smith and Hogan 1993). Stress and physical injuries can also cause inflammation of the gland, but the main cause of mastitis is microbial growth.

Mastitis is commonly divided into two types: clinical and subclinical cases. Clinical cases present visible signs of the disease such as flakes or clots in the milk and a swollen quarter or udder. In severe cases of clinical mastitis, the infection can be acute causing a fever, rapid pulse, loss of appetite and sharp decline in milk production. In contrast, subclinical cases have no visible signs of the disease, but bacteria are present. As such, subclinical mastitis is often difficult to detect and requires tests to identify quarters with infections (Ruegg and Reinemann 2002). Because of its difficulty to detect, subclinical mastitis causes reduced milk production persisting for long periods of time causing the greatest financial loss to dairy farmers (Hortet and Seegers 1998). In addition to

reduced milk production mastitis also causes changes in the milk composition reducing its quality, by reducing calcium, phosphorus, protein and fat content and increasing sodium and chloride content (Korhonen and Kaartinen 1995).

Reducing the incidence and subsequent costs of mastitis is necessary to keep milk producers economically stable.

Mastitis is currently controlled by proper milking hygiene, reduced exposure to environmental pathogens and dry cow antibiotic therapy. Even with these control measures, mastitis continues to be prevalent in dairy herds worldwide. The continued prevalence of mastitis in spite of these control measures indicates the complex nature of this disease. Mastitis susceptibility is influenced by three important factors: cow, pathogen and environmental. Whether or not a cow will become infected is based on a complex interplay of these three factors. Type of pathogen, climate, season, parity, stage of lactation and individual cow genetics all influence susceptibility to mastitis (Oliver and Mitchell 1983; Smith, Todhunter et al. 1985; Detilleux 2002; Ruegg 2003). For example during the summer months and during the first two weeks of lactation cows have been shown to have increased somatic cell counts which are associated with mastitis (Blackburn 1969; Miller, Owen et al. 1976; Simenson 1976). Older cows have also been shown to have increased somatic cell scores (Eberhart and Buckalew 1972; Natzke, Everett et al. 1972; Kehrli, Weigel et al. 1991). Often SCC are used as an indication of infection status because elevated scores are associated with infection (Berning and Shook 1992). The cow's ability to mount an immune response to infection is also an important cow factor. Measurement of this ability

has been suggested as a potential resistance trait (Kehrli, Weigel et al. 1991). Also the diversity of mastitis-causing pathogens contributes to the difficulty in controlling mastitis. Also good management practices have been shown to be critical for mastitis prevention and control (Jayarao and Wolfgang 2003; Ruegg 2003). In order to reduce and control the incidence of mastitis, cow factors in addition to the pathogen and environment must be considered.

IMMUNE RESPONSE

Innate Defenses

The mammary gland has many defenses against mastitis, including both innate and adaptive immunity. The innate immune response includes anatomic, soluble and cellular defenses and is most important at the onset of the disease. The teat end itself, an anatomical defense, is responsible for preventing the entrance of bacteria into the mammary gland. Normally the sphincter muscle closes the teat canal tightly to prevent bacteria and other organisms from gaining entrance into the udder. Milking causes the relaxation of this sphincter and it remains relaxed for one to two hours (Schultze and Bright 1983). This allows pathogens to enter the teat potentially colonize the area and cause inflammation

Once bacterial pathogens enter the teat, the invading organism can attach and colonize the mammary tissue, first in the large milk collecting ducts. Once in the gland, the organism encounters another important part of the innate immune response, the cellular defenses including neutrophils, macrophages, and other leukocytes. In an uninfected quarter the predominant cell type in milk is a

macrophage (Lee, Wooding et al. 1980). Macrophages are the first line of defense against invading pathogens and have the ability to ingest bacteria, cellular debris and accumulated milk debris (Sordillo and Nickerson 1988). Macrophages, through toll-like receptors, also have the ability to recognize pathogen-associated molecular patterns which allow discrimination between pathogens (Janeway 1992). Toll-like receptors have the capacity to recognize a wide variety of stimuli and once activated trigger a multi-step signaling pathway which results in activation of particular genes (O'Neil and Greene 1998). This includes the release of an array of cytokines which play role in the inflammatory response by recruiting neutrophils (Craven 1986). This influx of neutrophils requires adhesion to the endothelial wall of blood vessels, migration through the endothelial wall into the tissue, and subsequent migration to the site of infection. Recruitment is mediated by the secretion of chemoattractants by the epithelial cells and macrophages present in the milk at the time of infection (Sordillo, Shafer-Weaver et al. 1997).

In addition to these defenses, there are many soluble factors present in the milk that work in collaboration with the anatomic and cellular defenses. These include immunoglobulins (Ig), complement fractions, lactoferrin and lysozyme. Immunoglobulins are produced by local plasma cells or transported from serum (Sordillo and Nickerson 1988). Immunoglobulins and complement fragment C3b act as opsonins and aid neutrophils in the phagocytosis of bacteria (Howard, Taylor et al. 1980; Targowski 1983). Lactoferrin is an iron binding glycoprotein produced by epithelial cells and neutrophils that prevents the growth of bacteria

that have an iron requirement (Smith and Oliver 1981). Another important bactericidal protein is lysozyme which cleaves peptidoglycans from the cell wall of Gram-positive bacteria and from the outer membrane of Gram-negative bacteria (Reiter 1978).

If these defenses cannot resolve the infection then the disease causing microorganism continues to multiply and invades the small ducts and alveolar areas of the mammary gland. However with an effective immune response, microorganisms can be eliminated rapidly and the infection cleared quickly. Milk production and composition then return to normal in several days. Whether this resolution occurs quickly is based on a variety of factors, one of which is the presence of neutrophils.

Role of Neutrophils

Neutrophils play an essential role in the resolution of bacterial infections such as mastitis. Neutrophils develop and mature in the bone marrow before their release into blood. During their short life cycle, neutrophils require 10 to 14 days to mature in the bone marrow and may be stored for a few days before release into the blood (Bainton, Ulliyot et al. 1971). Once in the blood neutrophils circulate briefly with a half life of 8.9 hours (Carlson and Kaneko 1975), then migrate to tissues where the neutrophils function as phagocytes for 1-2 days. This process is tightly regulated which keeps levels in the blood and milk constant in healthy animals (Paape, Shafer-Weaver et al. 2000). There is a slow influx of neutrophils into the mammary gland for surveillance but this influx

increases exponentially when an infection occurs. Studies have shown that neutrophils make up only 3-23% of cells found in milk from healthy mammary glands (Lee, Wooding et al. 1980; Ostensson, Hageltorn et al. 1988; Miller, Paape et al. 1993), but they are the predominant cell type found in mammary tissues and secretions during early inflammation and can constitute greater than 90% of the total leukocytes (Paape 1981).

In bovine mastitis, circulating neutrophil number and effective adhesion, migration, phagocytosis, and killing ability control the outcome of intramammary infections and the severity of the infection (Gray, Knight et al. 1982; Burvenich, Paape et al. 1999). Many characteristics allow this cell to be so effective. The most prominent of these characteristics is its multilobulated nucleus, which allow it to migrate quickly between endothelial cells. Also aiding in migration are a variety of receptors on the neutrophil surface. These receptors include L-selectin and beta-2 integrin adhesion molecules, which aid in binding the neutrophil to the endothelial wall beginning adhesion, the first essential step for migration (Kishimoto, Jutila et al. 1989; Zimmerman 1992). Neutrophils also contain numerous membrane bound granules containing compounds important in killing bacteria.

The severity and duration of mastitis is related to the promptness of neutrophil migration to the site of infection (Hill 1981). Cows with low blood and milk leukocyte counts recruit cells more slowly on intramammary challenge and subsequently developed more severe mastitis (Van Werven, Noordhuizen-Stassen et al. 1997). Migration is initiated when macrophages and epithelial

cells interact with invading microorganisms and cause the release of a variety of inflammatory mediators including cytokines and complement fragments, which attract the neutrophils to the site of infection by a chemotatic gradient.

Potent chemoattractants for bovine neutrophils are C5a, an active cleavage product of the fifth component (C5) of complement, various lipopolysaccharide, interleukin-1 (IL -1), and interleukin-8 (IL -8) (Gray, Knight et al. 1982; Daley, Coyle et al. 1991; Lee and Zhao 2000). These chemoattractants, in addition to binding specific receptors and activating neutrophils, also have effects on local tissues. Chemoattractants also have other effects important in an inflammatory response such as fever, breakdown of the blood-milk barrier and increased bone marrow output of leukocytes. Once activated, neutrophils then begin migration by adhering to the endothelial lining of the blood vessel wall.

Adhesion and subsequent transmigration occur because of a series of coordinated interactions between leukocytes and endothelial cells regulated by adhesion molecules on their surfaces (Carlos and Harlan 1994). This process is illustrated below in figure 1. Unstimulated neutrophils are constantly rolling along the endothelial wall. Rolling slows when L-selectin (CD62L) receptors on neutrophil surfaces bind to target molecules on endothelial cells and allow neutrophils to detect the presence of E- and P-selectin on endothelial cell surfaces (Kansas 1996; Diez-Fraile, Meyer et al. 2002; Burton and Erksine 2003).

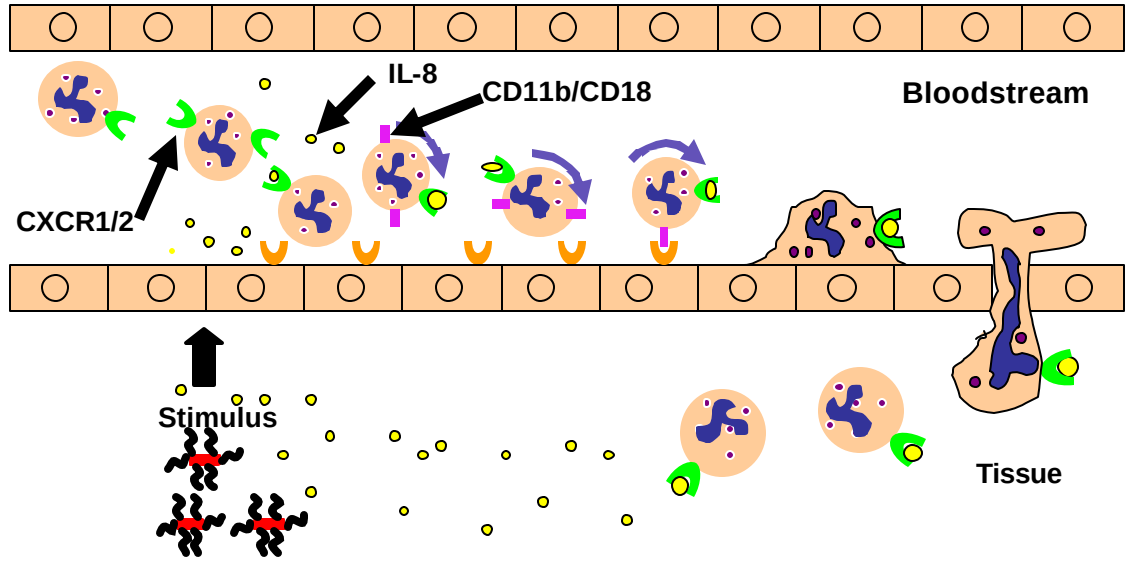


Figure 1: Process of Neutrophil Migration from binding of IL-8 to its receptor (CXCR1/CXCR2), activation of the neutrophil, rolling, expression of adhesion molecules (CD11b/CD18), tight adhesion, and finally transmigration following a chemotactic gradient to stimulus

E- and P-selectins are upregulated on the endothelial surface concentrating in sites of inflammation (Burton and Erksine 2003). Proinflammatory mediators, such as interleukin (IL) -1β , -6, -8, tumor necrosis factor (TNF), complement factor 5a, platelet activating factor and leukotriene B₄ (LTB₄), migrate to the surface of endothelial cells and interact with neutrophils, causing the downregulation of CD62L on the surface and upregulation of beta-2 integrins, and intercellular and vascular cell adhesion molecules (Shuster, Kehrli et al. 1993; Diez-Fraile, Meyer et al. 2002). This begins the process of tight adhesion mediated by beta-2 integrin complexes, CD11a/CD18 and CD11b/CD18 that have been upregulated on the surface.

Beta-2 integrins serve a dual role on neutrophils inducing firm adhesion of neutrophils to vessel endothelial cells and mediating back up signaling. Beta-2 integrins are heterodimers consisting of a common beta subunit, CD18, and a variable alpha subunit, CD11a, CD11b, CD11c, or CD11d (Arnaout 1990). These molecules bind to endothelial intercellular adhesion molecules (ICAM-1 and ICAM-2) and endothelial leukocyte adhesion molecules (ELAM-1) on the endothelial cell surface, which allow neutrophils to localize at the site of infection. In addition to these adhesion molecules, CD11b/CD18 binds to other ligands including fibrinogen, iC3b and factor X (Arnaout, Todd et al. 1983; Altieri, Bader et al. 1988; Wright, Weitz et al. 1988). The exact role of these molecules in vivo is unclear but it is possible that they aid in CD11b/CD18 dependent adhesion and subsequent transmigration by triggering a complex set of intracellular events that result in inside-out signaling (Dana and Arnaout 1988).

Once in the gland, neutrophils aid in eliminating infections by phagocytosis of opsonized and non-opsonized pathogens and subsequent killing of these pathogens (Gray, Knight et al. 1982; Burvenich, Paape et al. 1999). Opsonization promotes bacteria uptake by binding of immunoglobulins to the bacterium. Receptors on the neutrophil surface then bind the immunoglobulin which has coated the bacteria. Once bound the neutrophil will engulf the bacteria by phagocytosis effectively eliminating it in most cases (Paape, Miller et al. 1991). In addition to opsonization, complement activation also promotes phagocytosis. Complement protein C3b generated on the surface of bacteria following antibody union is recognized by receptors located on the surface of the neutrophil (DiCarlo, Paape et al. 1996). Once complement and immunoglobulins bind to receptors on the neutrophil surface, the cell becomes activated and generates reactive oxygen species (ROS) (Leino and Paape 1993). ROS generation accompanied by an increase in oxygen consumption has been termed the respiratory burst which is very effective for eliminating Gram negative bacteria (Burvenich, Guidry et al. 1996). Also ROS have been implicated recently in activation of proteases which can also play a role in killing (Reeves, Lu et al. 2002). Also when opsonins are not present, the neutrophil can still phagocytose bacteria by a process called non-opsonic phagocytosis. (Paape, Lillius et al. 1996).

Inflammatory Mediators

For neutrophils to be effective against bacterial infections, they must first travel to the site of infection. Inflammatory mediators, such as interleukin (IL)-1 α , -1 β , -6, -8, tumor necrosis factor (TNF)- α , complement factor 5a, and leukotriene B₄ (LTB₄), play an essential role in this process by activating and directing the movement of neutrophils (Shuster, Kehrli et al. 1993). Epithelial cells and macrophages react to bacterial products and toxins released by bacteria by synthesizing chemokines, including interleukin-8, which form a decreasing gradient of the chemokine up to the blood vessel (McCormick, Hofman et al. 1995). The endothelial cells of the blood vessels also react to this signal by expressing adhesion molecules important for CD11b/CD18 binding. This allows for anchoring and activation of the neutrophil (Hogg and Landis 1996). Chemokines and adhesion molecules work in concert to facilitate neutrophil movement from the circulation to the tissue.

Chemokines are relatively small proteins divided into four subfamilies based on the presence of a cysteine motif. The CXC family contains chemokines with two cysteines at the amino terminus of the protein separated by another amino acid, the CC subfamily has two cysteines side by side, the C subfamily has a single cysteine and the CX3C subfamily exhibit two cysteines separated by three amino acids (Rossi and Zlotnik 2000; Mackay 2001). These chemokines are often further subdivided based on the presence of a Glu-Leu-Arg (ELR) motif preceding the first cysteine residue. Chemokines that contain this motif bind

CXCR1 and CXCR2 receptors, whereas those chemokines that do not contain this motif bind CXCR3 and CXCR6 (Rossi and Zlotnik 2000).

IL-8 is an ELR+ chemokine important in the activation and migration of neutrophils as well as subsequent resolution of inflammatory diseases, such as mastitis (Harada, Sekido et al. 1994). Not only is IL-8 the most abundantly secreted cytokine by neutrophils, but neutrophils are also IL-8's primary cellular target (Gainet, Chollet-Martin et al. 1998). IL-8 has been found in large amounts in response *Escherichia coli* and *Streptococcus uberis* infections (Shuster, Kehrli et al. 1997; Rambeaud, Almeida et al. 2003). Neutrophils recognize IL-8 through two receptors, CXCR1 and CXCR2 and become activated, causing changes in neutrophil shape, regulation of adhesion molecule expression, adherence to cytokine-activated endothelial tissues, chemotaxis, phagocytose and foreign pathogens and generate reactive oxygen species (Peveri, Walz et al. 1988; Thelen, Peveri et al. 1988; Lee, Horuk et al. 1992; Lindley 1998; Riollot, Rainard et al. 2000; Feniger-Barish, Yron et al. 2003).

Chemokine Receptors- CXCR1 and CXCR2

Neutrophils have two cell surface receptors capable of recognizing IL-8, CXCR1 (IL-8 receptor A) and CXCR2 (IL-8 receptor B) (Moser, Schumacher et al. 1990). Both receptors belong to a family of large, G-protein linked serpentine receptors with seven transmembrane domains characterized by three intracellular loops and three extracellular loops (Murphy 1994). Both IL-8 receptors are structurally very similar as they share 77% amino acid homology, but have very different

binding characteristics. Of the two receptors, CXCR1 has greater ligand specificity only binding IL -8 and granulocyte chemotactic protein with high affinity (Devalaraja and Richmond 1999). In contrast, CXCR2 binds multiple chemokines in addition to IL -8, such as neutrophil activating peptide-2 (NAP-2), growth related oncogene (GRO) alpha, beta and gamma as well as epithelial neutrophil activating peptide-78 (ENA-78) (Ahuja, Murphy et al. 1996).

The two receptors are highly homologous at the amino acid level except at the N termini, fourth transmembrane domain and C termini (Lee, Horuk et al. 1992; Cerretti, Kozlosky et al. 1993). Ligand binding specificity has been tied to the amino terminus, whereas the ability of the receptor ligand complex to internalize and initiate signal transduction is related to the cytoplasmic carboxyl terminus of the receptor (LaRosa, Thomas et al. 1992; Suzuki, Prado et al. 1994). Damaj (1996) found five residues important in intracellular calcium signaling, four of which are found in the intracellular loop (Y136, L137, I139 and V140) and one in the third intracellular loop (M241). G proteins bind to the third intracellular loop of the IL -8 receptors and are responsible for initiating intracellular signaling (Damaj, McColl et al. 1996). G proteins are made up of three subunits, α , β , and γ . There are about 20 known α subunits which associate with 5 different types of β subunits and at least 11 types of γ subunits (Hermans 2003). This wide variety of G proteins are divided into four main classes: 1) G_s which activate adenylyl cyclase, 2) G_i , which inhibit adenylyl cyclase, 3) G_q , which activate phospholipase C and G_{12} and G_{13} , which are of unknown function (Neves, Ram et al. 2002). CXCRs have been shown to couple

with G_i which inhibit adenylyl cyclase (Wu, LaRosa et al. 1993; Damaj, McColl et al. 1996). The α subunit is thought to inhibit adenylyl cyclase whereas the $\beta\gamma$ subunit is thought to activate phospholipase C which is responsible for calcium mobilization (Morris and Scarlata 1996). There is also evidence to suggest that CXCR1 and CXCR2 interact with the G_q family because calcium mobilization was not completely inhibited with pertussis toxin suggesting that the receptors couple with the G_q family (Hall, Beresford et al. 1999). G proteins are activated on ligation of the receptor.

IL-8 induced cell migration is predominately mediated through CXCR1 (Quan, Martin et al. 1996). The differences in functional activity between receptors may be due to distinct primary or secondary signal transduction events. Secondary events could include priming of CXCR1 by CXCR2 activation or faster phosphorylation and downregulation of CXCR2 (Richardson, Pridgen et al. 1998; Schraugstatter, Burger et al. 1998; Hauser, Pekete et al. 1999).

Signaling Through Receptors to Enhance Migration

Chemoattractants including IL-8 and C5a are generated at inflammatory sites and are key factors in neutrophil migration and activation. When ligand, such as IL-8 binds to its receptor, it induces changes in the relative orientation of the 3rd and 6th transmembrane helices which uncovers the previously masked G protein binding sites in the intracellular loops (Altenbach, Yang et al. 1996; Farrens, Altenbach et al. 1996). This allows interaction of the receptor with G protein coupled receptors (GPCR) kinases, which specifically phosphorylate

GPCRs at serine and threonine residues found in their carboxyl terminal tails and/ or third intracellular loops (Premont, Inglese et al. 1995). Phosphorylation of these residues promotes β -arrestin binding, which uncouples the receptor from the G-protein (Ferguson, Zhang et al. 1998) Upon uncoupling from the receptor, the G protein is activated causing the exchange of GDP for GTP on the alpha subunit and subsequently the alpha subunit dissociates from the beta-gamma subunit. Phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3-K), adenylyl cyclase and other compounds are activated by the beta-gamma subunit. Phospholipase C catalyzes the hydrolysis of membrane phospholipids resulting in formation of inositol triphosphate and diacylglycerol (DAG) and release of arachidonic acid. Inositol triphosphate induces calcium release from intracellular stores while diacylglycerol in conjunction with calcium causes the activation of protein kinase C (PKC) (Bokoch 1995).

Both PI3-K and p38 MAPK pathways have been shown to be important for neutrophil activation, adhesion and migration (Capodici, Hanft et al. 1998). The PI3-K pathway has also been shown to play a role in the respiratory burst (Ahmed, Hazeki et al. 1995) and blockade of the apoptotic pathway through activation of PKB (Klein, Rane et al. 2000). The pathway from activation of receptor by the chemoattractant to the functional of neutrophil migration is diagramed below (figure 2).

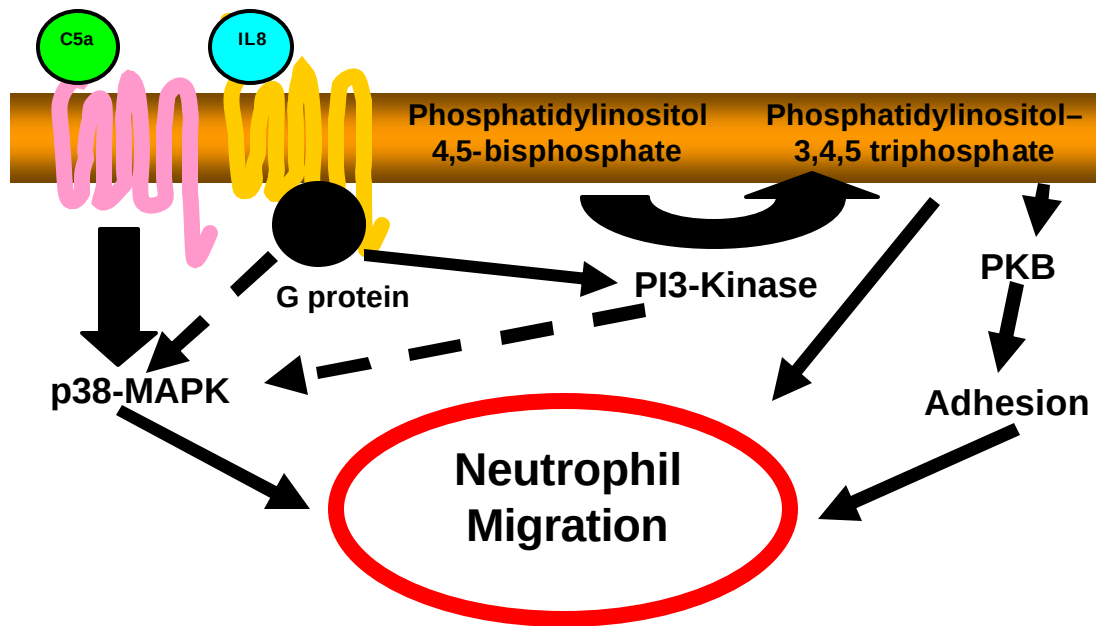


Figure 2: Intracellular signaling pathways in neutrophils leading to migration when activated with IL-8 and C5a

PI3-Kinase

Phosphatidylinositol 3-kinase (PI3-Ks) are a group of heterodimeric enzymes activated in response to various growth factors, hormones, chemokines and chemoattractants (Cantley 2002). All PI3Ks have a regulatory subunit and a catalytic subunit of which there are four recognized isoforms, p110- α , - β , - γ and - δ . There are three classes of PI3K based on structure and substrate specificity: classes I, II, and III. Class I PI3K are subdivided into two classes; IA and IB. Class IA have a p110- α , - β or - δ catalytic subunit, are regulated primarily by tyrosine kinases, can be activated by integrin engagement (Axelsson, Hellberg et al. 2000) and associate primarily with the p85 α regulatory subunit. p110 α

and p110 β are expressed in many tissues whereas p110 δ is expressed primarily in leukocytes (Koyasu 2003). Neutrophils express all class IA isoforms, α , β , γ , and δ . Class IB PI3-K have a p110 γ catalytic subunit, bind a p101 regulatory subunit, and are activated by heterotrimeric GTP binding proteins (Hannigan, Zhan et al. 2002).

This class is primarily expressed in leukocytes. In mice lacking the p110 γ subunit, phosphorylation of PKB in neutrophils was prevented upon activation of chemotactic receptors. In turn, PKB activation was also prevented which significantly impaired neutrophil chemotaxis (Hirsch, Katanaev et al. 2000). However, neutrophils from these mice were still able to migrate suggesting another pathway also must be important in the chemotaxis of neutrophils. In particular, in p110 γ deficient mice the p38 MAPK pathway is active and has been demonstrated to play an important role chemotaxis (Nick, Avdi et al. 1997; Sasaki, Jones et al. 2000). Class II and III PI3K are expressed ubiquitously, but the specifics of their activation are still being studied (Koyasu 2003).

PI3K upon activation phosphorylates plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP₂), yielding phosphatidyl-3,4,5-trisphosphate (PIP₃). PIP₃ once formed can activate a number of cytosolic proteins including protein kinase B (PKB/Akt) and Rac (Cantley 2002). PKB is recruited to the plasma membrane by products of PI3K, PI(3,4)P₂ and PI(3,4,5)P₃ and becomes phosphorylated at threonine-308 and serine-473 which fully activates PKB. There are three known

members of the PKB/Akt family, Akt1, Akt2, and Akt3. They are all highly related, exhibiting more than 80% sequence homology (Nicholson and Anderson 2002).

PKB acts on a plethora of substrates and is a key mediator of cell proliferation, differentiation and survival as well as neutrophil adhesion and thus migration. PI3K has been shown to play a role in neutrophil adhesion to the endothelial wall. Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) which is an adhesion molecule on neutrophils causes the upregulation of β 1 and β 2 integrins through PI3-K dependent signaling (Pellegatta, Chierchia et al. 1998). The PI3-K inhibitor LY294002 blocks IL-8 mediated adhesion by 75% (Takami, Terry et al. 2002). Adhesion was largely unaffected by inhibitors of tyrosine kinase, and protein kinase C or by chelation of intracellular calcium (Chen, Powell et al. 2003).

p38-MAPK

Many stimuli including cytokines, growth factors, stress factors and the PI3-kinase pathway have been shown to stimulate the p38 MAPK pathway. Cross-linking of L-selectin on neutrophils and stimulation of neutrophils by chemotactic factors result in phosphorylation and activation of p38 MAPK (Zu, Qi et al. 1998; Smolen, Petersen et al. 2000). Also Ras activation triggers the MAPK/ERK cascade, which appears to be important in many chemoattractant induced neutrophil functions.

MAPKs are serine/threonine kinases including p38, ERK 1 and 2, and JNK that participate in cell signaling. Based on their ability to respond to stimuli, p38 MAPKs are divided into two families p38 α / β and p38 γ / δ (Kumar, McDonnell et al. 1997). All MAPK isoforms become phosphorylated on threonine and tyrosine residues by a dual specificity serine threonine MAPK kinase (MKK). MKK becomes phosphorylated and activated by an upstream MKK-kinase (MKKK). The amino acid sequence Thr-X-Tyr, which is located in the activation loop near the ATP and substrate binding sites, is shared between all of the isoforms in which X differs between the isoforms. In p38-MAPK the residue is glycine. MKK phosphorylates MAPKs by addition of phosphate to tyrosine followed by dissociation and then subsequent addition to threonine (Gum, McLaughlin et al. 1998). In most inflammatory cells, p38 α is the major isoform. Each isoform's activation is regulated by a variety of mechanisms which result in either phosphorylation of different substrates or activation of the same substrate but on different phosphorylation sites leading to different biological responses. Phosphorylation of Tyr 182 and Thr 180 is required for p38 α to be fully functional (Hale, Trollinger et al. 1999).

Activated p38 MAPK can activate other kinases by binding protein in its substrate pocket and ATP in its ATP binding pocket where the kinase becomes phosphorylated and activated (Herlaar and Brown 1999). p38 MAPK can activate many downstream targets including MAPK activated protein kinases 2,3 and 5 and transcription factors ATF-2 and CHOP-1,

which are involved in the production of inflammatory mediators such as TNF- α , as well as the process of leukocyte recruitment and extravasation into inflamed tissues (Foxman, Campbell et al. 1997; Goedert, Cuenda et al. 1997; Read, Whitley et al. 1997; Deak, Clifton et al. 1998; Detmers, Zhou et al. 1998). p38 MAPK has a role in neutrophil migration because some of these downstream targets are involved in the production and action of inflammatory mediators, as well as the process of leukocyte recruitment and extravasation into inflamed tissues.

SB-203580 and SKF86002 are widely used as specific inhibitors of p38 MAPK α and β isoforms (Hale, Trollinger et al. 1999). These inhibitors do not effect selectin dependent leukocyte rolling or integrin dependent leukocyte adhesion, but significantly effected downstream events such as neutrophil migration and chemotaxis (Cara, Kaur et al. 2001). This inhibition in chemotaxis was shown previously with inhibitors to the p38-MAPK pathway (Zu, Qi et al. 1998). In vivo studies have also shown that p38 MAPK inhibitors reduce neutrophil infiltration (Griswold, Hoffstein et al. 1989).

Interaction of PI3-K and p38 MAPK pathways

Both the PI3-kinase and p38-MAPK pathways have been shown to be important in neutrophil chemotaxis. It has been suggested that each pathway is activated by different chemoattractants and interact through a signaling hierarchy (Heit, Tavener et al. 2002). In this study, neutrophils

were allowed to migrate in the presence of end target chemoattractants (C5a/zymosan activated sera) and intermediary chemoattractants (IL-8), and the neutrophils migrated preferentially toward end target chemoattractants. In a separate study, activation of the p38 MAPK pathway through preincubation of neutrophils with fMLP or β -glucan, a lectin that binds CD11 also reduced migration to IL-8 by downregulation of the PI-3 kinase pathway (Tsikitis, Albina et al. 2004). When p38 MAPK was blocked with inhibitors neutrophils migrated toward the intermediary chemoattractants and also increased PKB activation (Heit, Tavener et al. 2002). Neutrophils migrate preferentially toward end target chemoattractants (e.g. C5a/ZAS) coming from the site of infection over intermediary chemoattractants (e.g. IL-8) encountered en route to sites of infection. End target chemoattractants primarily cause neutrophil migration via p38-MAPK whereas intermediary chemoattractants induce migration via the phosphoinositide 3-kinase/PKB pathway. This suggests that either a reduction in the PI-3 kinase pathway or an increase in the p38 MAPK pathway, or a combination of the two could potentially impair neutrophil migration.

CHEMOKINE RECEPTORS, SNPs, AND DISEASE ASSOCIATION

Single nucleotide polymorphisms (SNPs) are single nucleotide variations in the DNA. SNPs in chemokine receptors have been associated with decreased function or expression, as well as with disease prevalence and progression.

SNPs in the CCR2 and CCR5 genes have shown association with HIV disease progression in Japanese individuals (Kageyama, Mimaya et al. 2001). Also polymorphisms in the CXCR1 gene have been associated with decreased chemokine binding, coronary artery disease risk and HIV disease progression (Faure, Meyer et al. 2000; Moatti, Faure et al. 2001). Polymorphisms in the CXCR2 gene have also been identified in humans. Two polymorphisms at positions +785 CC and position +1208 TT had higher frequency in individuals with systemic sclerosis when compared to control (Kato, Tsuchiya et al. 2000; Renzoni, Lympant et al. 2000).

Polymorphisms within the CXCR1 gene have recently been identified in beef cattle and dairy cattle (Grosse, Kappes et al. 1999; Kato, Tsuchiya et al. 2000; Youngerman, Saxton et al. 2004). Youngerman et al. (2004) found five SNPs at positions 612, 684, 777, 858, and 861 of the CXCR1 gene in both Holstein and Jersey dairy cattle. Four SNPs resulted in synonymous substitutions, while a non-synonymous switch at position +777 results in a glutamine to histidine substitution at amino acid residue 245. Previously this polymorphism was reported in the CXCR2 gene, however comparison of a recently available bovine genome sequence to the human CXCR1 and CXCR2 sequences has revealed this gene to be incorrectly annotated and should be CXCR1 (Pighetti and Rambeaud 2006). Holsteins with a GG genotype at CXCR1 position +777 had decreased percentages of subclinical mastitis, but genotype CC cows had increased percentages of subclinical mastitis (Youngerman, Saxton et al. 2004). Because of the location of this polymorphism, there are potential effects on

CXCR1 function and signal transduction. Damaj et al. (1996) determined that Met 241 is important in G protein binding and also that other amino acids in the third intracellular loop were moderately involved in mediating intracellular signaling (Damaj, McColl et al. 1996). As the polymorphism at +777 results in an amino acid change at amino acid position 245 in the third intracellular loop, it has potential to interfere with G protein mediated signaling and subsequent neutrophil functions (Youngerman, Saxton et al. 2004)

Rambeaud and Pighetti (2005) evaluated in vitro neutrophil migration and adhesion molecule expression from cows with different CXCR1 +777 genotypes. Cows with a CC and GC genotype at CXCR1 +777 showed significantly lower neutrophil migration to recombinant human IL-8 and zymosan activated sera (ZAS) than cows with a GG genotype. Also observed was decreased neutrophil upregulation of CD18 expression and a similar trend also was seen for CD11 in cows with a CC genotype when compared to cows with a GG genotype. However no differences were seen with CD62 downregulation. Subsequent studies by Rambeaud revealed that neutrophils from cows with a CC genotype produced significantly less reactive oxygen species and intracellular calcium release than neutrophils from GG cows, but exhibited increased survival from spontaneous apoptosis when incubated with IL-8 (Rambeaud, Clift et al. 2006). Additionally, Rambeaud et al. discovered reduced CXCR1 affinity and/or a reduction in IL-8R number which may partly account for the IL-8 dependent effects observed. However as differences in migration and ROS generation were independent of IL-8 other downstream signaling events may be responsible.

SUMMARY AND STATEMENT OF PROBLEM

Neutrophils play an essential role in the resolution of bacterial infections such as mastitis. The first important step for neutrophils to be effective in the elimination of an infection is their migration from blood to the site of infection. The severity and duration of mastitis has been shown to be related to the promptness of neutrophil migration to the site of infection (Hill 1981). Interleukin-8 (IL-8) is an important chemokine in the recruitment of neutrophils to the site of infection and subsequent resolution of an infection. IL-8 has been found in large amounts in response *Escherichia coli* and *Streptococcus uberis* infections (Shuster, Kehrli et al. 1997; Rambeaud, Almeida et al. 2003). IL-8 binds two related receptors on the neutrophil surface, CXCR1 and CXCR2. When the chemoattractant binds to either of these receptors, it causes signal transduction pathways to be activated through the associated G-protein. From this point, two pathways have been shown to be important in neutrophil activation and subsequent migration: the phosphatidylinositol-3 kinase (PI3-K) and the mitogen activated protein kinase pathway (MAPK) pathways. Both of these pathways have been shown to play a role in adhesion and migration in neutrophils (Hirsch, Katanaev et al. 2000).

Research in our laboratory has identified a polymorphism in the bovine CXCR1 gene associated with mastitis susceptibility. Cows with a CC genotype at this position had more subclinical mastitis when compared with cows with a GG genotype (Youngerman, Saxton et al. 2004; Youngerman, Saxton et al. 2004). Cows with a CC genotype also have impaired neutrophil migration, adhesion molecule upregulation, reactive oxygen species generation and calcium

signaling as well as decreased apoptosis (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006). These differences were not only seen in response to IL-8 and ENA-78, but also in response to ZAS and PMA, which are activated independent of CXCR1 and CXCR2. Possibly this indicates that differences in function are primarily caused by a common downstream signaling pathway, not a defect in the CXCR1 receptor. Based on these observations, we hypothesized that cows with different CXCR1+777 genotypes have differing levels of intracellular signaling necessary for neutrophil migration. In order to test this hypothesis, the following research objectives were conducted:

- 1) Identify the effect of ligand and dose on neutrophil migration in cows with different CXCR1+777 genotypes
- 2) Evaluate if cows with different CXCR1 genotypes exhibit differences in intracellular signaling pathways

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Part III

RELATIONSHIP OF INTRACELLULAR SIGNALING PATHWAYS WITH MIGRATION IN BOVINE NEUTROPHILS WITH DIFFERENT CXCR1+777 GENOTYPES

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

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

ABSTRACT

Prior research in our lab has demonstrated a significant association between the incidence of subclinical mastitis and neutrophil functions with polymorphisms of the CXCR1 gene in Holstein dairy cattle. The objective of this study was to evaluate the specificity of the response relative to CXCR1 and potential involvement of intracellular signaling pathways. Migration was evaluated in cows of both homozygous genotypes (n=7 each) in response to high and low doses of interleukin-8 (IL-8; specific for CXCR1 and CXCR2), epithelial neutrophil activating peptide (ENA-78; specific for CXCR2) or zymosan activated sera (ZAS; complement receptors). Cows with a GG genotype had significantly more migration in response to both 12.5 and 50 nM doses of IL-8 ($p<0.05$) compared to cows with a CC genotype. Although a similar trend was observed for 50 nM ENA-78 ($p<0.07$), this was not evident with the 12.5 nM dose. This result was comparable to ZAS, where the 5% dose caused significantly more neutrophil migration in cows with a GG genotype than those with a CC genotype ($p<0.03$), while the response to the 1% dose of ZAS was similar between genotypes. p38 MAPK and PKB phosphorylation were evaluated prior to and following stimulation with the high doses of each ligand. Relative increases in p38 MAPK

phosphorylation were greater in neutrophils from cows with a GG genotype when compared to those with a CC genotype regardless of treatment with rhu-IL-8, rhu-ENA-78, and ZAS or not ($p < 0.001$). But CC cows had higher baseline levels of p38-MAPK phosphorylation than GG cows. Therefore, the lower increase in phosphorylation in neutrophils from CC cows may have been due to higher starting levels. Increases in PKB phosphorylation also were greater in neutrophils from cows with a GG genotype when compared to a CC genotype when treated with ZAS, but not other ligands. Because no differences between genotypes were observed at time zero this may be tied to the altered p38 MAPK pathway. Future research will be aimed at further elucidating the signaling pathways causing functional differences in neutrophils from cows with different CXCR1 genotypes.

INTRODUCTION

Neutrophils play an essential role in the resolution of bacterial infections such as mastitis. The severity and duration of mastitis has been shown to be related to the promptness of neutrophil migration to the site of infection (Hill 1981). Migration is initiated when macrophages, epithelial cells, endothelial cells, and soluble factors interact with invading microorganisms and cause the release of a variety of inflammatory mediators including cytokines and complement fragments that attract neutrophils to the site of infection by a chemotactic gradient (Craven 1983; Riollot, Rainard et al. 2000). Chemoattractants including C5a, an active cleavage product of the fifth component (C5) of complement,

various lipopolysaccharide, interleukin-1 (IL-1), and interleukin-8 (IL-8) bind to their specific receptors on the neutrophil surface and initiate migration by causing the cell to express adhesion molecules that bind the endothelial lining of the blood vessel wall (Gray, Knight et al. 1982; Daley, Coyle et al. 1991; Lee and Zhao 2000). C5a and IL-8 have both been shown to be important in the resolution of mastitis (Shuster, Kehrli et al. 1997; Riollot, Rainard et al. 2000; Rambeaud, Almeida et al. 2003). After tight adhesion, neutrophils then transmigrate to the site of infection following the chemotactic gradient that guides neutrophils through the tissue to the site of infection.

Interleukin-8 is an important chemokine in the recruitment of neutrophils to the site of infection and subsequent resolution of an infection (Harada, Sekido et al. 1994; Shuster, Kehrli et al. 1997; Rambeaud, Almeida et al. 2003). IL-8 binds two receptors on the neutrophil surface, CXCR1 and CXCR2 (Moser, Schumacher et al. 1990). These two cell surface receptors are structurally very similar, but have different binding characteristics (Devalaraja and Richmond 1999). CXCR1 has greater ligand specificity only binding IL-8 and granulocyte chemotactic protein with high affinity whereas CXCR2 binds other chemokines in addition to IL-8 including neutrophil activating peptide-2 (NAP-2), growth related oncogene (GRO) alpha, beta and gamma, as well as epithelial neutrophil activating peptide-78 (ENA-78) (Ahuja, Murphy et al. 1996).

Research in our laboratory has identified a polymorphism in the bovine CXCR1 gene associated with mastitis susceptibility (Youngerman, Saxton et al. 2004). Previously this polymorphism was reported in the CXCR2 gene, however

recent comparison of the bovine and human genomes has revealed this gene to be CXCR1 (Pighetti and Rambeaud 2006). Cows with a CC genotype at position +777 had more subclinical mastitis when compared with cows with a GG genotype (Youngerman, Saxton et al. 2004; Youngerman, Saxton et al. 2004). This increase in susceptibility may be tied to impaired neutrophil function as cows with the CC genotype also have lower neutrophil migration in response to IL-8 and zymosan activated sera (ZAS), adhesion molecule upregulation in response to IL-8, and reactive oxygen species generation in response to PMA, as well as decreased apoptosis in response to IL-8 (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006). Previous experiments revealed that neutrophil functions were not only impaired in response to IL-8 but also to ZAS and PMA indicating that the responses are not CXCR1 or CXCR2 specific, but more likely a common downstream signaling event is affected. Thus far two pathways have been implicated in neutrophil activation and subsequent migration: the phosphatidylinositol-3 kinase (PI3-K) and p38 mitogen activated protein kinase pathway (p38-MAPK) pathways (Hirsch, Katanaev et al. 2000).

PI3K upon activation phosphorylates plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP₂), yielding phosphatidyl-3,4,5-triphosphate (PIP₃). PIP₃ once formed can activate a number of cytosolic proteins including protein kinase B (PKB) and Rac (Cantley 2002). Following activation of PI3-K, PKB becomes phosphorylated at threonine-308 and serine-473 allowing it to become fully activated (Nicholson and Anderson 2002). The PI3-K inhibitor LY294002 blocks IL-8 mediated adhesion by 75% (Takami, Terry

et al. 2002). In addition PKB acts on a plethora of substrates and is a key mediator of cell proliferation, differentiation and survival as well as neutrophil chemotaxis (Hirsch, Katanaev et al. 2000).

p38-MAPK is phosphorylated on threonine and tyrosine residues by a dual specificity serine/threonine MAPK kinase (MKK) that is phosphorylated and activated by an upstream MKK-kinase (MKKK) (Gum, McLaughlin et al. 1998). Many stimuli including cytokines, growth factors, stress factors and the PI3-kinase pathway have been shown to stimulate the p38 MAPK pathway. Cross-linking of L-selectin on neutrophils and stimulation of neutrophils by chemotactic factors including interleukin-8, fMLP, and TNF- α result in the phosphorylation and activation of p38 MAPK (Zu, Qi et al. 1998; Smolen, Petersen et al. 2000). The downstream targets include MAPK activated protein kinases 2,3 and 5 and transcription factors ATF-2, CHOP-1 and others, which are involved in the production of inflammatory mediators such as TNF- α , as well as the process of leukocyte recruitment and extravasation into inflamed tissues (Foxman, Campbell et al. 1997; Goedert, Cuenda et al. 1997; Read, Whitley et al. 1997; Deak, Clifton et al. 1998; Detmers, Zhou et al. 1998). In vivo studies have also shown that p38 MAPK inhibitors reduce neutrophil infiltration in numerous inflammatory processes (Griswold, Hoffstein et al. 1989).

Based on our previous observations neutrophil migration was affected in response to both IL-8 and ZAS, we hypothesized that cows with different CXCR1+777 genotypes have differing levels of intracellular signaling necessary for neutrophil migration. Previous experiments revealed that neutrophil functions

were not only impaired in response to IL-8 but also to ZAS and PMA indicating that the responses are not CXCR1 or CXCR2 specific, but more likely differences in a common downstream signaling pathway. Therefore, the objective of this study was to evaluate the specificity of the migratory response in relation to two common signaling pathways known to play a role in neutrophil migration, the phosphatidylinositol-3 kinase pathway and the p38 mitogen activated pathway.

MATERIALS AND METHODS

Animal Selection and Sample Collection

Holstein cows in mid to late lactation (at least 60 days in milk) and housed at the East Tennessee Research and Education Center were used for this study. Cows were chosen based on their CXCR1+777 genotype, which was determined by MALDI-TOF mass spectroscopy at a commercial facility (Geneseek, Lincoln, NE) or by PCR amplification and sequencing at the University of Tennessee molecular biology core facility (Youngerman, Saxton et al. 2004). All cows were free from clinical signs of disease including mastitis. Blood was collected by jugular venipuncture into 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). White blood cell count in any cow did not exceed 12×10^3 cells/mm³. In addition, blood smears were evaluated to determine differential leukocyte counts.

Neutrophil Isolation

Neutrophils were isolated from blood as described previously (Rambeaud and Pighetti, 2005). Briefly the blood was centrifuged at 4°C, then the plasma and buffy coat were removed. After repeating this step, erythrocytes were lysed by the addition of ultrapure water for 30 seconds and then a 3X concentration of RPMI-1640 medium (Sigma, St. Louis, MO) was added to regain isotonicity. After repeating this step, remaining neutrophils 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 different assays described below. Viability was assessed by trypan blue dye exclusion and always exceeded 95%, while mean neutrophil purity was 99%.

Zymosan Activated Serum Preparation

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

Neutrophil Migration

The ability of neutrophils to migrate towards recombinant human IL -8 (rhIL-8) (12.5 nM and 50nM), recombinant human ENA-78 (rhuENA-78) (12.5

nM and 50nM), and zymosan activated serum (1% and 5%) were evaluated using Costar transwell inserts (Corning, Acton, MA) in a 24 well plate. Briefly, ligands were added to triplicate wells of a 24 well plate. Then 200 μ l of neutrophils resuspended at 1×10^7 cells per ml in HBSS supplemented with 2mM calcium chloride, 2mM magnesium sulfate and 5% bovine serum albumin were added to the insert and the plates were incubated at 39°C for one hour. Excess liquid was then aspirated from the top of each insert to remove non-migrated cells and plates were centrifuged to remove migrated cells adhered to the bottom of the insert. Migrated cells were then counted using a hemacytometer.

Protein Isolation and Preparation of Cell Lysates

Isolated neutrophils resuspended in HBSS were pelleted by centrifugation, the supernatant was removed and the cells were resuspended in mammalian protein extraction reagent (Pierce, Rockford, IL) with complete, EDTA-free protease inhibitor cocktail added (Roche, Indianapolis, IN). Cell debris was then pelleted, and the protein containing supernatant was transferred to another tube. Samples for SDS-PAGE analysis were then concentrated using Microcon centrifugal filter devices, molecular weight cutoff of 10 kDa (Millipore, Bedford, MA). After concentration, protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), and sample buffer consisting of 63mM Tris-HCl pH 6.8, 26% glycerol, 2.1% SDS, 0.01% bromophenol blue, and 5% β -mercaptoethanol was added and samples were boiled at 95°C for 10 minutes and stored at -20°C until analyzed.

SDS-PAGE Gel Electrophoresis, Electrophoretic Transfer and Western Blotting

Proteins (20µg total protein per lane) were separated on a SDS-PAGE gel made of a 12% running gel and 4% stacking gel using the mini-protean 3 electrophoresis apparatus (Bio-Rad, Rockford, IL) at 150V. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) using the Bio-Rad trans blot SD Semi Dry electrophoresis cell at 5.5mA/cm². The PVDF membrane was blocked overnight in 5% bovine serum albumin (BSA) and then incubated with primary antibodies for 2 hours at room temperature. Antibodies raised against human p38 (KAS-MA009, Stressgen, Ann Arbor, MI) and protein kinase B (PKB) (sc-1619, Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:1000 in 5% BSA in tris buffered saline with 0.05% tween-20, pH 7.5 (TBST). Excess primary antibody was then washed away by shaking in TBST three times for 20 minutes. Membranes were then incubated with goat anti-rabbit IgG HRP diluted at 1:5000 (Pierce Biotechnology, Rockford, IL) for two hours at room temperature. After incubation, membranes were washed three times for 20 minutes each to remove excess secondary antibody. Membranes were then incubated in West Dura chemiluminescent substrate for 5 minutes at room temperature. The chemiluminescence was detected using an Alpha Innotech Imaging System and band intensity was determined by spot densitometry.

Fluorescent Evaluation of Intracellular Signaling

Phosphorylation of signaling molecules in neutrophils was stimulated by treating 1×10^7 cells/ml with either HBSS, rhuIL-8 (50nM), rhuENA-78 (50nM) or ZAS (5%) in a 96 well plate and incubated at 39°C for either 0, 2.5, 5, 10, or 30 minutes. Each treatment was performed in triplicate. After incubation, cells were fixed with 2% paraformaldehyde for 10 minutes at 39°C, permeabilized with 100% methanol for 30 minutes at 4°C, and blocked with 1% goat serum for 10 minutes at room temperature. Primary polyclonal antibodies raised in rabbit against dual phospho-p38 (KAP-MA022) and phospho-PKB (KAP-PK006) were diluted 1:2000 (Stressgen, Ann Arbor, MI) added to the appropriate wells and incubated for 30 minutes at room temperature. The degree of non-specific binding by rabbit IgG was assessed by incubating cells with normal rabbit IgG sera (Caltag, Carlsbad, CA) diluted 1:200 in 5% BSA in TBST. Plates were then washed three times with phosphate buffered saline (PBS). Goat anti-rabbit IgG (H &L)-FITC conjugated secondary antibody (Caltag, Carlsbad, CA) was then added and plates were incubated for 30 minutes at room temperature. After washing three times, the cells were transferred to a black fluorescent plate (Costar, Corning, NY) and read on Synergy HT Multi-Detection Microplate Reader (Biotek, Winooski, Vermont). Specific binding to phosphorylated p38 and PKB was calculated by subtracting the fluorescent value obtained following incubation with rabbit IgG from all wells with specific antibodies. If background fluorescence was greater than specific binding, the fluorescent value was set to 1. Data was normalized to time 0 by two methods: 1) Subtraction of fluorescence

at time 0 from all other time points to determine absolute changes in fluorescence and 2) division of fluorescence at all time points by fluorescence at time 0 to provide a stimulation index.

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 blocked on day was used to determine the effect of genotype on migration and intracellular signaling. The statistical model was $y_{ij} = \mu + D_i + G_j + D \times G_{ij}$ (D= day, G= genotype). Data for migration in response to IL-8 and fluorescent evaluation of phosphorylation were log transformed in order to normalize the data and pre-transformed means were reported. Data are presented as least square means with associated standard error. Statistical significance was declared at $P < 0.05$, and trend towards significance was declared at $P < 0.10$.

RESULTS

Neutrophil Migration in Response to Ligands

Neutrophils from all cows were capable of migrating towards high and low doses of IL-8, ENA-78 and ZAS when compared to the negative control, HBSS (figure 3). Those cows with a GG genotype had significantly more neutrophil migration in response to both low (12.5 nM) and high (50 nM) doses of the CXCR1 and CXCR2 ligand, IL-8 ($p < 0.05$) when compared to neutrophils from cows with a CC genotype. Although a similar trend was observed for a high dose

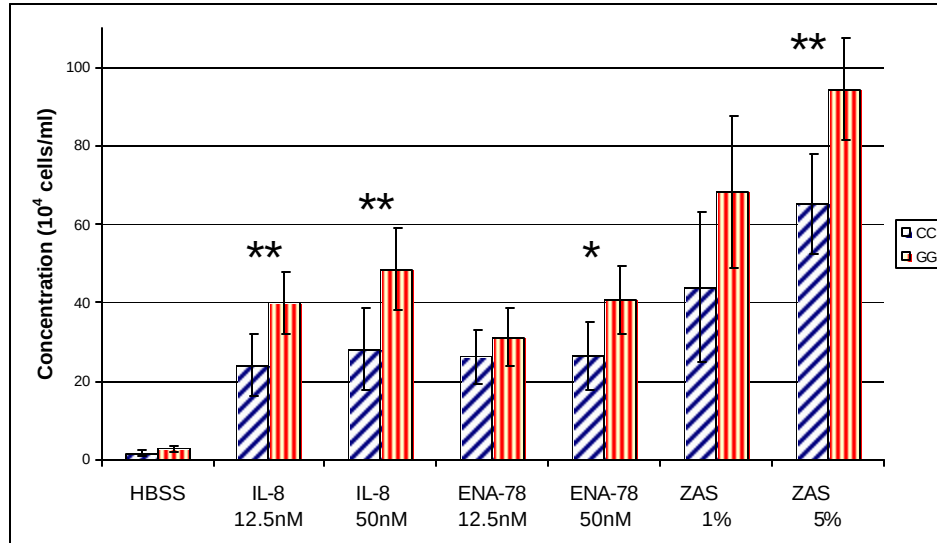


Figure 3: Migration of neutrophils from cows with different CXCR1+777 genotypes (n=7/ genotype) in response to different ligands and doses. Data presented as least square means $\pm$ SE. ** Indicates a significant difference ($p < 0.05$) between genotypes within a treatment. * Indicates a trend towards significance ($p < 0.10$) between genotypes within a treatment.

(50 nM) of the CXCR2 specific ligand ENA-78 ($p < 0.07$), this was not evident with the low dose (12.5 nM). This result was comparable to ZAS which primarily activates the C5a receptor, where a high dose (5%) caused significantly more migration in cows with a GG genotype than in cows with a CC genotype ($p < 0.03$), while a low dose (1%) of ZAS had no significant effect on migration among genotypes.

Phosphorylation of p38 MAPK and PKB

Neutrophils from cows with a CC genotype had significantly higher starting levels of phosphorylation than neutrophils from cows with GG genotype ($p < 0.0001$) (figure 4). Because the different genotypes varied with respect to the

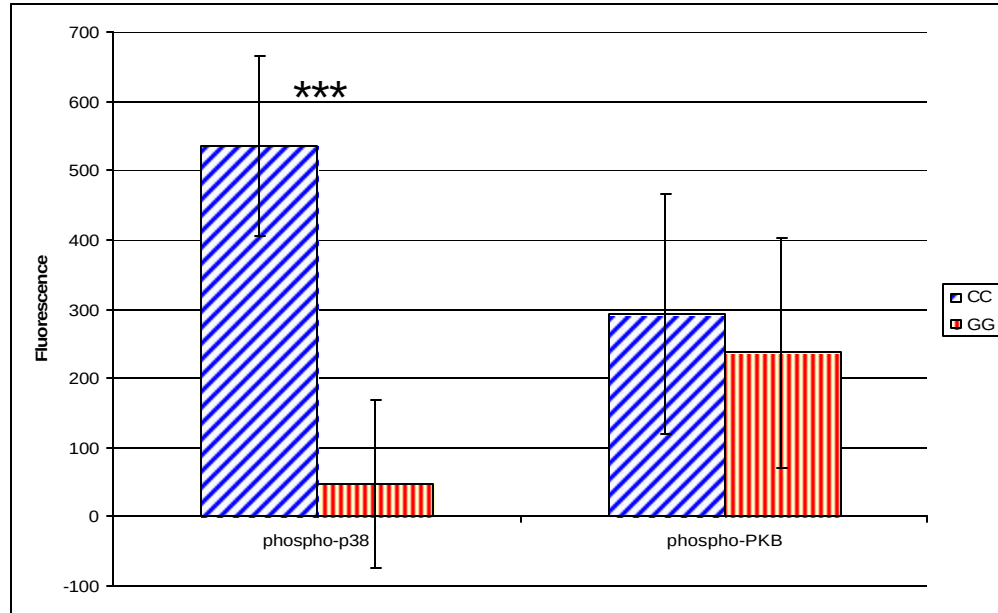


Figure 4: Phosphorylation of p38 MAPK and PKB at Time 0 in neutrophils from cows with different CXCR1+777 genotype (n=9/ genotype). Data is presented as least square mean fluorescent intensity +/- SE. *** Indicates a significant difference ($p < 0.001$) between genotypes within a treatment.

base level of phosphorylation, the effect of treatment on p38 MAPK

phosphorylation with CXCR1 and CXCR2 dependent and independent activators

was evaluated relative to time zero. Once normalized to time zero, no significant differences were observed with respect to time or genotype x time interactions.

However significant differences were observed relative to genotype, therefore

data is presented as least square means for genotype and includes all time

points but zero. Neutrophils not receiving stimulation had differing levels of p38-

MAPK phosphorylation with GG cows having a greater increase in

phosphorylation than CC cows ($p < 0.01$) (table 1). This difference further

increased when neutrophils were treated with IL-8 (50nM) a CXCR1 and CXCR2

activator, ENA-78 (50nM) a CXCR2 specific activator, and ZAS (5%) a CXCR1

Table 1- Relative increase over time 0 in p38 MAPK and PKB phosphorylation measured by fluorescence in neutrophils from cows with different CXCR1+777 genotypes (HBSS & IL-8,n=9/ genotype, ENA-78- n=7/genotype, ZAS- n=8/genotype) Data was normalized to time 0, log transformed for analysis and is presented as pre-transformed least square means +/- SE. ***Indicates a significant difference (p<0.001) between genotypes within a treatment. ** Indicates a significant difference (p<0.05) between genotypes within a treatment. * Indicates a trend towards significance (p<0.10) between genotypes within a treatment.

	phospho-p38 MAPK		phospho-PKB	
	CC	GG	CC	GG
HBSS	*** 7.77	296.14	* 773.04	185.53
+/- SE	70.11	63.46	324.42	313.27
IL-8	*** 9.56	1065.36	1181.44	506.50
+/- SE	207.11	196.75	343.03	328.31
ENA-78	*** 11.78	1132.87	797.94	1019.22
+/- SE	214.90	201.02	254.05	237.66
ZAS	*** 109.90	8161.25	** 5816.00	7230.85
+/- SE	1502.61	1521.22	2568.84	2576.38

and CXCR2 independent activator ($p<0.001$). However no differences in levels of unphosphorylated p38 MAPK were observed between genotypes when examined by western blot analysis, suggesting variation in baseline levels of p38 MAPK were not a factor (figure 5).

Unlike p38 MAPK, phospho-PKB levels at time zero were similar between genotypes (figure 4). The starting levels of unphosphorylated PKB were similar between genotypes (figure 5). Additionally, no significant differences were observed for phospho-PKB phosphorylation over time with any treatment or for genotype x time interactions. Neutrophils from CC cows had significantly less PKB phosphorylation when treated with ZAS (5%) than did those neutrophils from cows with a GG genotype ($p<0.01$) (table 1). However this genotype effect was not observed following stimulation with IL-8 and ENA-78. A trend towards increased phosphorylation of PKB was also noted between genotypes when neutrophils were not stimulated ($p<0.10$).

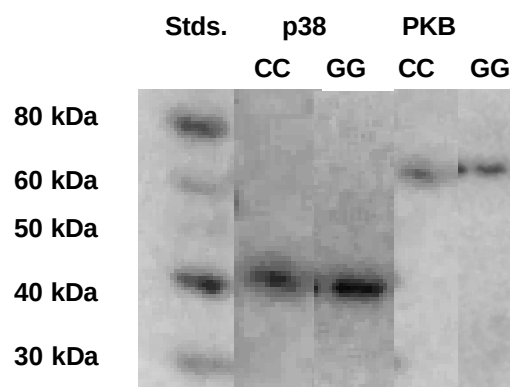


Figure 5: Representative western blot on neutrophil whole cell lysates from cows with different CXCR1+777 genotypes with anti-p38 and anti-PKB ($n=3$ / genotype)

DISCUSSION

Previous research in our lab indicated that cows with a CC genotype at CXCR1+777 had increased susceptibility to mastitis and decreased neutrophil migration when compared to GG cows (Youngerman, Saxton et al. 2004; Rambeaud and Pighetti 2005). The present study was conducted to evaluate the specificity of this response and its relationship to critical neutrophil signal transduction pathways. In prior experiments IL-8 was the only chemokine evaluated. Because this chemokine binds both CXCR1 and CXCR2 it was not possible to determine if altered neutrophil responses were CXCR1 or CXCR2 specific. Therefore in the present study, ENA-78, a CXCR2 specific activator, was included at high and low concentrations in order to determine specificity of the response relative to a cows' genotype. In addition, ZAS was used because it contains C5a which activates neutrophils through a separate G protein-linked seven transmembrane domain receptor (Hopken, Lu et al. 1997).

Cows with a CC CXCR1+777 genotype had significantly less neutrophil migration in response to both low (12.5nM) and high doses (50nM) of rhIL-8 ($p<0.05$). Whereas CC cows had significantly less migration in response to only the high dose (5%) of ZAS ($p<0.05$). These results are consistent with our previous study where neutrophil migration also was significantly lower in cows with a CC genotype following stimulation with 100 ng/ml (~12.5nM) IL-8 and 5% ZAS (Rambeaud and Pighetti, 2005). The present study also observed a trend ($p<0.10$) demonstrating that neutrophils from cows with a CC genotype also had

decreased migration in response to the high dose (50nM) of ENA-78. The greater impact of genetic background on neutrophil responses to IL -8 may be tied to specific defects in CXCR1. In a recent study, cows with a CC genotype appeared to have reduced CXCR1 affinity for IL -8 when compared to GG cows, which could in part explain why differences in migration were seen with the low dose of IL-8 but not other ligands (Rambeaud and Pighetti 2006). If there is less binding of IL -8 to CXCR1 in neutrophils of cows with a CC genotype, then migration initiated by this binding would be altered more significantly than migration initiated by other pathways. Another possibility is that signaling pathways initiated at the G protein of seven transmembrane domain receptors are altered in cows with a CC genotype when compared with a GG genotype because of the polymorphism at position +777. This polymorphism at position +777 leads to an amino acid substitution in the receptor's third intracellular loop where G proteins bind and cannot be ruled out at this point in time (Damaj, McColl et al. 1996).

Because differences in migration were observed with high levels of all three ligands, this suggests a common mechanism, possibly an intracellular signaling pathway, is involved. Both the PI3-K pathway and p38 MAPK pathways have been shown to be important for neutrophil activation, adhesion and migration (Capodici, Hanft et al. 1998). Therefore an alteration in either pathway would have the potential to affect neutrophil responses to IL-8, ENA-78 and ZAS. Closer examination of the p38 MAPK pathway by examining phosphorylation of p38 MAPK itself revealed several significant findings. Regardless of whether or

not neutrophils were stimulated with IL -8, ENA-78 or ZAS the relative increase in phosphorylated p38-MAPK was greater in cows with a GG genotype than those with a CC genotype ($p < 0.01$). However, when the levels of p38-MAPK phosphorylation at time 0 were evaluated, we discovered that neutrophils from cows with a CC genotype had significantly higher starting levels of p38-MAPK phosphorylation than those neutrophils from cows with GG genotype ($p < 0.0001$). This was not related to differences in expression of unphosphorylated p38 MAPK as cows with a CC genotype and GG genotype have similar levels at time 0. Phosphorylation of p38 MAPK is regulated by various protein phosphatases and kinases such as mitogen-activated protein kinase kinase kinases (MAPKKKs) including MTK1, TAK1, and ASK1 and serine/threonine specific phosphatases, PP2A and PP2Ca (Keyse 2000; Ashwell 2006). Therefore it is possible that neutrophils from CC cows have higher base levels of p38 MAPK because of a polymorphism located in one of these regulatory phosphatases or kinases. This proposed regulatory gene most likely is in linkage disequilibrium with the CXCR1+777 genotype.

The higher base level of p38-MAPK phosphorylation may have limited the ability of neutrophils to respond to ligands such as IL -8, ENA-78 and ZAS. First, neutrophils preferentially migrate toward end target chemoattractants such as C5a through p38 MAPK when faced with competing gradients of end target and intermediary chemoattractants such as IL-8 which primarily uses the PI3-K pathway (Heit, Tavener et al. 2002). So the greater basal level of p38 MAPK phosphorylation suggests the p38 MAPK pathway is pre-activated in neutrophils

from cows with a CC genotype and may prevent or impair migration relative to neutrophils from cows with a GG genotype when exposed to intermediary chemoattractants such as IL-8. Second, downregulation of CXCR1 and CXCR2 occurs following LPS stimulation via a p38 MAPK pathway. Blocking p38 MAPK phosphorylation with a kinase inhibitor also prevented this from occurring (Van Den Blink, Branger et al. 2004). Therefore high starting levels of p38 MAPK in neutrophils from cows with a CC genotype may mimic this situation and reduce CXCR1 and CXCR2 expression. As a consequence, intracellular signaling and neutrophil migration will be less. Third, Lokuta and coworkers have demonstrated that p38 MAPK phosphorylation and impaired migration of neutrophils towards IL-8 and C5a (Lokuta and Huttenlocher 2005). Overall, this evidence suggests the high baseline levels of p38 MAPK phosphorylation in neutrophils from cows with a CC genotype could cause neutrophils to migrate less.

In addition to p38-MAPK, the PI3-K pathway is also important in neutrophil migration by initiating adhesion and causing changes in the actin cytoskeleton essential for migration (Heit, Tavener et al. 2002; Takami, Terry et al. 2002). PKB, a downstream target of PI3-kinase upon activation phosphorylates plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP₂), yielding phosphatidyl-3,4,5-trisphosphate (PIP₃). PIP₃ in turn activates PKB, by recruiting it to the plasma membrane where it becomes phosphorylated. Similar to that observed with p38 phosphorylation, significantly lower PKB phosphorylation occurred in ZAS stimulated neutrophils from cows with a CC genotype versus a

GG genotype. One possible explanation is that activated p38 MAPK phosphorylates and activates MAPKAPK-2 which has been implicated as 3-phosphoinositide-dependent kinase-2 which phosphorylates Ser473 of PKB, thus activating it (Alessi, James et al. 1997; Rane, Coxon et al. 2000). Therefore when there is less of an increase in p38 MAPK phosphorylation, as occurs in neutrophils from CC cows when neutrophils are treated with ZAS, then potentially there would be less activated MAPKAPK-2 and subsequently less PKB phosphorylation (Rane, Coxon et al. 2000). This decrease in PKB phosphorylation could potentially decrease migration, but not completely stop migration because ZAS causes migration primarily through p38 MAPK (Heit, Tavener et al. 2002). Surprisingly, there were no differences seen in phosphorylation of PKB between neutrophils from cows with a CC genotype and those with a GG genotype when treated with IL-8 or ENA-78 ($p>0.10$). These results would indicate that differences seen in phosphorylation are not dependent on CXCR1 or CXCR2 activation and the +777 polymorphism is a marker for a mutation on a separate gene.

CONCLUSION

In conclusion, this study provides insight into the potential signaling pathways that may be affected in neutrophils of cows with different CXCR1+777 genotypes. Cows with a CC genotype had significantly less neutrophil migration in response to rhu-IL-8, rhu-ENA-78 and ZAS and indicate a common signaling pathway is affected. In addition, the increase in p38-MAPK phosphorylation was

lower in cows with a CC genotype when compared to those with a GG genotype, but this may be caused by CC cows have higher starting levels of p38-MAPK phosphorylation. Differences in phosphorylation of p38 MAPK in response to all three ligands parallel those differences seen in neutrophil migration. These variations in signaling may partially explain the differences in migration and mastitis susceptibility previously seen in cows with these genotypes. Future studies will be aimed at determining the profile of phosphorylated proteins to better understand the intracellular signaling pathways of bovine neutrophils and determine if there are differences based on CXCR1+777 genotype.

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Part IV
SUMMARY

The final objectives of this study were to identify the effect of ligand and dose on neutrophil migration and to evaluate if cows with different CXCR1+777 genotypes exhibit differences in intracellular signaling pathways. The CXCR1 gene is crucial for neutrophil activation and migration and has been associated with mastitis susceptibility (Youngerman, Saxton et al. 2004; Youngerman, Saxton et al. 2004). Previous research also indicated that neutrophil adhesion molecule upregulation and migration is impaired in cows with a CC genotype when compared to cows with a GG genotype (Rambeaud and Pighetti 2005). However the mechanism responsible for altered neutrophil function was not readily apparent. The questions left were whether 1) differential adhesion molecule upregulation would in turn cause differential neutrophil migration, 2) the specificity of the response was CXCR1 or CXCR2 specific, and 3) a common downstream signaling event was affected. Therefore, the goal of this research was to start answering these questions.

CD11b and CD18 adhesion molecule upregulation was greater in neutrophils from cows with a GG genotype when compared to neutrophils from cows with a CC genotype, which could have a positive effect on neutrophil migration by increasing tight adhesion. Neutrophil migration was determined in the presence and absence of fibrinogen, a ligand for CD11b/CD18, in order to determine if migration was altered when CD11b/CD18 was engaged (appendix 1). Unexpectedly, migration in the presence and absence of fibrinogen was determined to be similar. One possible explanation was that the fibrinogen was immobilized on the insert and it might have been more effective if it was soluble.

To test this we performed the same experiment with soluble fibrinogen and had similar results. The presence of fibrinogen appears to have no effect on increasing migration in this system. This could occur because the inserts are not effective at mimicking an endothelial wall so the neutrophils are following a chemotactic gradient without interaction of CD11b/CD18.

Previous data showed that neutrophils from CC cows had less upregulation of CD11b and CD18 when compared to neutrophils from cows with a GG genotype when treated with IL -8 (Rambeaud and Pighetti 2005), so CD11b and CD18 expression was also evaluated before and after migration to determine if upregulation of CD11b and CD18 was altered in neutrophils from cows with different genotypes after migration. The values that were seen in the study were low compared to those seen previously and were similar to those levels observed in neutrophils that have not been stimulated. One possible reason is that adhesion molecule levels were downregulated after migration and masked any potential differences that may have occurred. This type of downregulation in CD11b/CD18 expression has been tied to cytoskeletal rearrangement during migration (Anderson, Hotchin et al. 2000). In order to evaluate differential adhesion molecule expression, a method would have to be developed to harvest the neutrophils in mid-migration so that they would still be expressing CD11b/CD18 molecules.

The second question, the specificity of the response to CXCR1 and CXCR2, arose because IL -8 was the only chemokine evaluated and it binds both CXCR1 and CXCR2. As such, it was not possible to determine if altered

neutrophil responses were CXCR1 or CXCR2 specific. Therefore in the present study, ENA-78, a CXCR2 specific activator, was included in order to determine specificity of the response relative to a cows' genotype. In addition, ZAS was used because it contains C5a which activates neutrophils through a separate G protein-linked seven transmembrane domain receptor (Hopken, Lu et al. 1997). Migration in response to IL-8, ENA-78 and ZAS was significantly less in neutrophils from cows with a CC genotype when compared to neutrophils from cows with a GG genotype. Because differences in migration were observed for all three ligands between genotypes, this indicates that a common mechanism to all three ligands, IL-8, ENA-78 and ZAS is affected.

Because neutrophil migration was altered to all three ligands indicating that a common pathway is altered, we examined the intracellular signaling pathways important in migration and could potentially be affected and cause differential migration. p38 MAPK and PKB phosphorylation, represent two signaling pathways indicated as being important to migration were evaluated. The relative increase in p38 MAPK phosphorylation was significantly lower in neutrophils from cows with a CC genotype when compared to neutrophils from cows with a GG genotype when treated with all three ligands. But levels of phosphorylation at time zero were higher in neutrophils from cows with a CC genotype when compared to those from cows with a GG genotype and could account for the relatively lower increase in phosphorylation observed. Similar to that observed with p38 phosphorylation, significantly lower PKB phosphorylation occurred in ZAS stimulated neutrophils from cows with a CC genotype versus a

GG genotype. Because responses to all ligands were affected, it indicates that phosphorylation differences are not dependent on CXCR1 or CXCR2 activation and the +777 polymorphism is a marker for a mutation on a separate gene.

Phosphorylated proteins were detected by a fluorescent intracellular detection system, but we had difficulty detecting these proteins by western blotting. It is possible that our chemiluminescence substrate and imaging system was not sensitive enough to detect the small amount of phosphorylated protein present. Others have reported loading 75 µg total protein per lane for bovine samples (Yamamori, Inanami et al. 2000), which is a high concentration compared to 30 µg, the typical amount of protein loaded for human samples. Also when loading a large amount of protein such as 75µg, the nonspecific chemiluminescence increases making it even more difficult to detect a small band. The inability of the proteins to be detected by western blotting, and the high level of protein needed for detection reported by others, indicates that possibly these two pathways are not the primary signaling targets in bovine neutrophils. Therefore in an attempt to verify differential phosphorylation between genotypes all phosphorylated proteins were evaluated using a specific stain- Pro-Q Diamond (Invitrogen, Carlsbad, CA, appendix 2). One protein that stood out was at 84 kDa and was only present when neutrophils were treated with IL-8 and ENA-78, but was not present in untreated cells indicating its possible importance in CXCR1 and CXCR2 signaling or as a final target.

This research has provided some explanations to differences previously seen in neutrophil migration. One, it indicates that migration is affected by a

common pathway activated via CXCR1, CXCR2 and another 7TMD receptor which would argue that the polymorphism at position +777 in CXCR1 serves as a marker for a causative polymorphism in another gene. Two, this gene is related to the p38 MAPK pathway as elevated basal levels of p38 MAPK were evident in neutrophils from cows with a CC genotype versus those with a GG genotype. Future research will be aimed at elucidating the complete phospho-proteomic profile of bovine neutrophils and determining if there are differences in expression of other proteins important in signaling between genotypes.

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APPENDICES

APPENDIX 1: Neutrophil Migration in the Presence of Fibrinogen and Adhesion Molecule Expression

Introduction

Tight adhesion of neutrophils to the endothelial wall to initiate migration is mediated by the beta-2 integrin complexes, CD11a/CD18 and CD11b/CD18. Beta-2 integrins are heterodimers consisting of a common beta subunit, CD18, and a variable alpha subunit, CD11a, CD11b, CD11c, or CD11d (Arnaout, 1990). Binding of chemoattractants, such as IL -8, to its cell surface receptor rapidly increase beta-2 integrin mediated neutrophil adhesion by triggering a complex set of intracellular events that result in inside-out signaling (Hynes, 1992). Inside-out signaling causes translocation of CD11b/CD18 (Mac-1) from internal stores in cytoplasmic granules to the cell surface (Miller et al., 1987). CD11b/CD18 molecules on the neutrophil surface then bind to endothelial intracellular adhesion molecules (ICAM-1 and ICAM-2) and endothelial leukocyte adhesion molecules (ELAM-1) on the endothelial cell surface, which allow neutrophils to localize at the site of infection. In addition to these adhesion molecules, CD11b/CD18 binds to other ligands including fibrinogen, iC3b, heparin and factor X. This adhesion allows neutrophils to begin to traverse the endothelial wall to the tissue where these cells are needed to fight infection. In addition to this, CD11b/CD18 ligation also begins a series of signaling events that mediate neutrophil adhesion as well as other functions that help fight bacterial infection (Miller et al., 1987; Berton and Lowell, 1999; Larsson et al.,

2000). Previous research in our lab has determined that upregulation of CD11b/CD18 was lower in neutrophils with cows with a CC genotype when compared with neutrophils from cows with a GG genotype (Rambeaud and Pighetti, 2005). The objective of this experiment was to determine if upregulation of CD11b/CD18 differed between genotypes after migration and if cross-linking of CD11b/CD18 on fibrinogen had any effect on altered neutrophil migration.

MATERIALS AND METHODS

Coating of Inserts with Fibrinogen

Costar mesh tissue culture inserts with 3µm pores (Corning, Acton, MA) were placed in the wells of a 24 well plate containing bovine fibrinogen at 0.5µg/ml. Additional fibrinogen was placed in the inserts to ensure both sides of the insert were coated. The plates were incubated overnight at 4°C. After incubation the fibrinogen was aspirated from the inserts and the inserts were washed twice with phosphate buffered saline.

Neutrophil Migration

The ability of neutrophils to migrate towards different ligands and ligand concentrations, rhIL-8 (12.5 nM and 50 nM), rhENA-78 (12.5 nM and 50 nM), and zymosan activated serum (1% and 5%) were evaluated using a 24-well plate and fibrinogen coated inserts (Costar). Briefly, ligands were added in triplicate to two 24 well plates. Then 200µl of neutrophils at 1×10^7 cells per ml were added to the top of the fibrinogen coated inserts and incubated at 39°C for one hour.

Excess liquid was then aspirated from the top of the inserts to remove non migrated cells and then centrifuged to remove migrated cells that were adhered to the bottom of the insert. Migrated cells were then counted with a hemacytometer.

Adhesion Molecule Expression

Indirect immunofluorescent analysis of CD11b and CD18 expression on migrated neutrophils in the presence and absence of fibrinogen was performed using murine monoclonal antibodies and fluorescein- conjugated antibody to mouse IgG. After migration in response to HBSS (negative control), rhIL-8, rhENA-78, or ZAS, 50 µl of neutrophils were added to 96-well round bottom plates containing 50 µl IgG as a negative control (Caltag Laboratories, Burlingame, CA), monoclonal antibodies to bovine CD11b (5µg/ml; VMRD Inc., Pullman, WA), or bovine CD18 (10µg/ml; VMRD Inc.). Plates were incubated at 4°C for 30 minutes, washed twice with HBSS, and 100µl of fluorescently labeled goat anti-mouse IgG (Calbiochem, La Jolla, CA) were added to each well. Samples were incubated at 4°C for 30 minutes, washed as before, and resuspended in 2% formaldehyde in 0.15M phosphate buffered saline (PBS). Samples were stored at 4°C in the dark until analyzed by flow cytometry using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Data are expressed as median fluorescence intensity.

RESULTS & DISCUSSION

Neutrophil Migration

Within a treatment neutrophil migration was similar regardless of the presence or absence of fibrinogen (figure 6).

Adhesion Molecule Expression

Because, no differences were seen in the presence and absence of fibrinogen, CD11b and CD18 upregulation data is shown combined for each treatment. CD11b upregulation did not differ between neutrophils that migrated and those that did not undergo migration (figure 7). This trend also held for CD18 expression (figure 8). There were also no differences seen between genotypes for CD11b or CD18 upregulation.

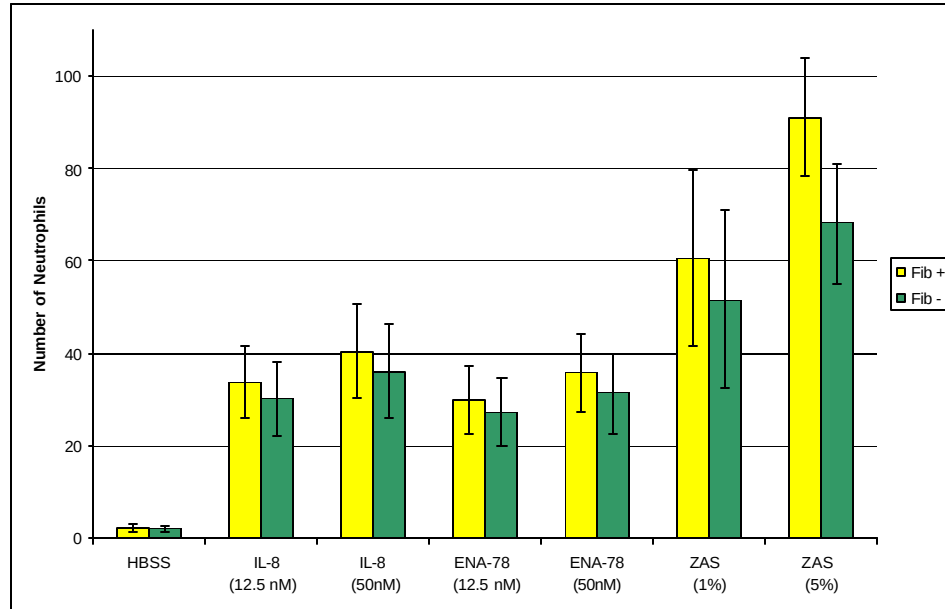


Figure 6: Number of neutrophils migrated in response to different ligands and doses in the presence and absence of fibrinogen (n=7/genotype). Data presented as least square mean $\pm$ SE.

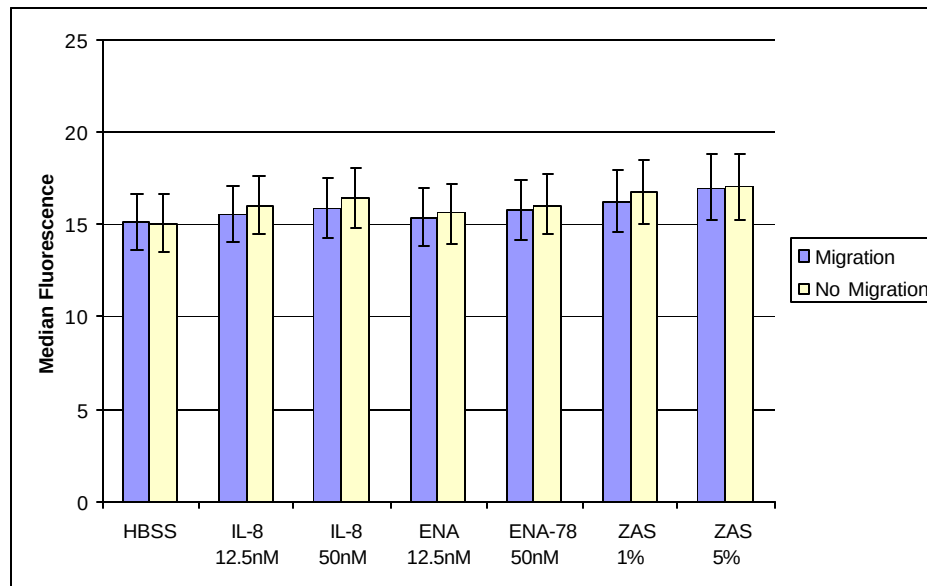


Figure 7: Expression of CD11b in bovine neutrophils before and after migration in response to different ligands and doses (n=7/genotype). Data presented as least square mean $\pm$ SE.

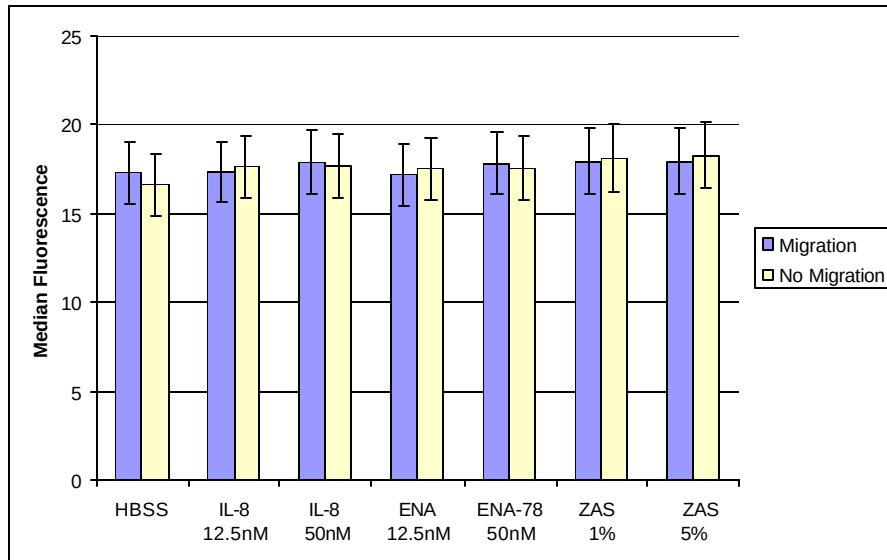


Figure 8: Expression of CD18 in bovine neutrophils before and after migration in response to different ligands and doses (n=7/genotype). Data presented as least square mean $\pm$ SE.

Migration toward chemoattractants in the presence and absence of fibrinogen was determined to be similar. Because fibrinogen is a ligand of CD11b/CD18, we expected that migration would be higher in the presence of fibrinogen by increasing tight adhesion. One possible explanation was that the fibrinogen was immobilized on the insert and it might have been more effective if it was soluble. To test this we performed the same experiment with soluble fibrinogen and had similar results. The presence of fibrinogen appears to have no effect on increasing migration in this system. This could occur because the inserts are not effective at mimicking an endothelial wall so the neutrophils are following a chemotactic gradient without interaction of CD11b/CD18.

Adhesion molecules are upregulated in response to chemoattractants and aid in initiating migration. The present study evaluated adhesion molecule

expression following migration through inserts coated with and without fibrinogen and found adhesion molecule levels to be similar to those seen without stimulation. The values observed in this study were low compared to those seen previously, between 20 and 30 median fluorescence intensity (MFI) units for CD11b and between 15 and 20 MFI units for CD18 (Rambeaud and Pighetti, 2005). The values we observed are similar to those levels observed in neutrophils that have not been stimulated. One possible reason is that adhesion molecule levels were downregulated after migration and masked any potential differences that may have occurred. This type of downregulation in CD11b/CD18 expression has been tied to cytoskeletal rearrangement during migration (Anderson et al., 2000). In order to evaluate differential adhesion molecule expression, a method would have to be developed to harvest the neutrophils in mid-migration so that they would still be expressing CD11b/CD18 molecules.

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APPENDIX 2: Phosphoprotein Evaluation

INTRODUCTION

Detection of phosphorylated p38 MAPK and PKB proteins was attempted with western blotting. Unfortunately, the phosphorylated proteins were not detected with western blotting system. Others have reported loading 75 µg total protein per lane for bovine samples (Yamamori, Inanami et al. 2000), which is a high concentration compared to the typical amount of protein loaded. The inability of the proteins to be detected by western blotting, and the high level of protein needed for detection reported by others, indicates that possibly these two pathways are not the primary signaling pathways in bovine neutrophils, so future studies will focus on determining the phospho-proteomic profile.

MATERIALS AND METHODS

Protein Isolation and Preparation of Cell Lysates

Isolated neutrophils resuspended at 1×10^7 cells/ml in HBSS were pelleted by centrifugation, the supernatant was removed and the cells were resuspended in mammalian protein extraction reagent (Pierce Rockford, IL) with complete, EDTA-free protease inhibitor cocktail added (Roche, Indianapolis, IN). Cell debris was then pelleted, and the protein containing supernatant was transferred to another tube. A small aliquot was removed at this point to determine protein concentration using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Supernatant containing the protein was delipidated and

desalted according to manufacturer's instructions (Pro-Q Diamond, Invitrogen, Carlsbad, CA), and then sample buffer was added and samples were boiled at 95°C for 10 minutes and stored at -20°C until analyzed.

Pro-Q Diamond Phosphoprotein

Proteins (20 µg per lane) were separated on SDS-PAGE gel, gels were fixed with a solution of 50% methanol and 10% acetic acid initially for 30 minutes and then overnight to ensure all SDS was removed from the gel. The gel was then rinsed in ultrapure water for 10 minutes before staining with Pro-Q Diamond phosphoprotein stain for 90 minutes in the dark. The gel was then destained with a solution of 20% acetonitrile and 50mM sodium acetate pH 4.0 three times for 30 minutes each before imaging on an Alpha Innotech Imaging System. Banding patterns were analyzed using software provided by the manufacturer.

RESULTS

The presence of phosphoproteins was detected with Pro-Q Diamond stain (figure 9). One protein band with an approximate molecular weight of 84 kDa stood out because it was only present when the cells were treated with IL-8 and ENA-78. So it is possible that this protein is important for CXCR1 and CXCR2 induced signaling or function. The amount of the protein is about twice as high following treatment with IL-8 for 2.5 minutes when compared to ENA-78 but reach levels similar by 30 minutes is reached (table 2). Neutrophils from cows with a CC genotype had greater phosphorylation of this protein when treated with

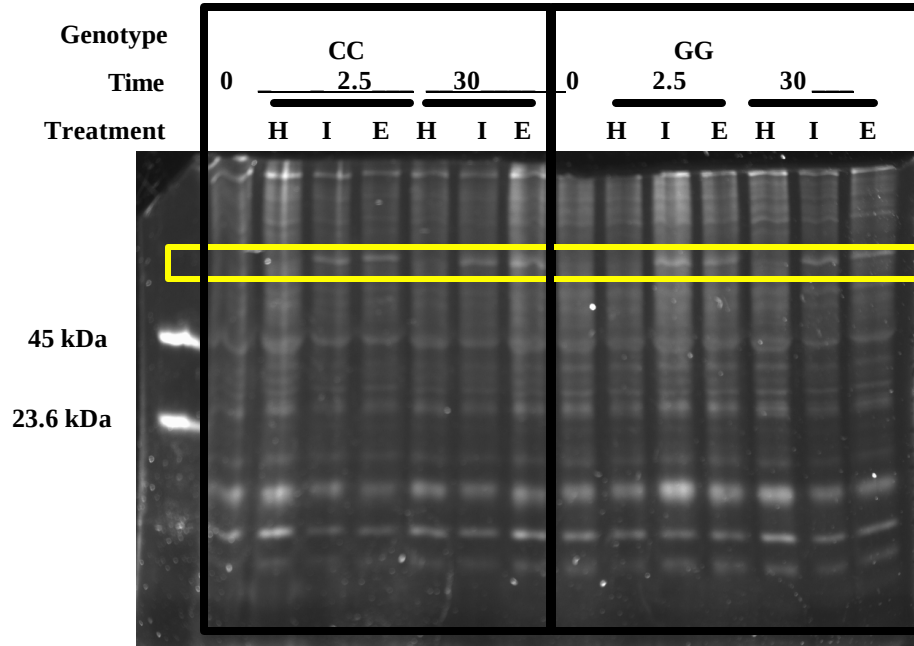


Figure 9: Representative gel of neutrophil whole cell lysates from cows with different CXCR1+777 genotypes stained with Pro-Q Diamond stain to detect total phosphoproteins (n=3/genotype) Treatments- H=HBSS, I=IL-8, E=ENA-78

Table 2: Average optical density measurements for the 84 kDa band from Pro-Q Diamond stained gels (n=3/genotype)

	2.5 IL-8	2.5 ENA	30 IL-8	30 ENA
CC	357611	190545	174723	229469
GG	321292	99819	112992	91724

both IL-8 and ENA-78 when compared to neutrophils for GG cattle, regardless of time. This protein may offer a promising candidate important for signaling and or function of CXCR1 and CXCR2 and could potentially explain functional differences that have previously been seen between cows with a CC genotype and cows with a GG genotype.

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VITA

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