5-2018

# Bovine Mastitis Resistance: Novel Quantitative Trait Loci and the Role of Bovine Mammary Epithelial Cells

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# BOVINE MASTITIS RESISTANCE: NOVEL QUANTITATIVE TRAIT LOCI AND THE ROLE OF BOVINE MAMMARY EPITHELIAL CELLS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Animal, Dairy, and Veterinary Sciences

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2017

# ABSTRACT

Novel Quantitative Trait Loci and the Role of Bovine Mammary Epithelial Cells in Bovine Mastitis Resistance

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Utah State University, 2017

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Bovine mastitis causes substantial economic losses and animal welfare issues in the dairy industry, making the pursuit of preventative strategies a major area of focus in the field of dairy science. Identification of genetic markers and the investigation of underlying mechanisms of the genetic basis of mastitis resistance facilitate the development of preventative and therapeutic approaches. The main objectives of this dissertation research were to identify genetic markers of mastitis resistance in Holstein dairy cattle and to define contributions of bovine mammary epithelial cells, the milk-secreting cells of the mammary gland, to mastitis and mastitis resistance.

A genome-wide association study of a population of Holstein dairy cattle was carried out to identify genetic markers for mastitis resistance. One hundred seventeen single nucleotide polymorphism genetic markers were detected suggestive of genome-wide significance. From these identified genetic markers, 27 regions within the bovine

genome suggestive of an association with mastitis resistance were defined, of which ten have not been reported previously.

An *in vitro* model was used to investigate contributions of bovine mammary epithelial cells to the mechanisms of mastitis resistance. Differential expression of 42 genes relevant to inflammation by primary bovine mammary epithelial cells from mastitis-resistant versus mastitis-susceptible cattle was observed following exposure to bacterial lipopolysaccharide, implicating the bovine mammary epithelial cell as an important cell type in mastitis resistance.

Comparisons between primary bovine mammary epithelial cells and primary bovine fibroblasts revealed both similarities and differences in pro-inflammatory gene expression responses to lipopolysaccharide. This finding emphasizes cell type-specific contributions to mastitis, which should be considered when selecting an *in vitro* model. To facilitate future mastitis studies, a method for the establishment of milk-derived bovine mammary epithelial cell lines with extended growth potential via transfection with the viral protein simian virus large T antigen is described.

Examination of the effects of exogenous administration of the enzyme phospholipase A2 on primary bovine mammary epithelial cells revealed altered expression of several pro-inflammatory genes in response to lipopolysaccharide. Because modulation of the inflammatory responses of bovine mammary epithelial cells has the potential to influence the course of mastitis, this finding highlights phospholipase A2 as a potential therapeutic candidate.

(217 pages)

# PUBLIC ABSTRACT

Novel Quantitative Trait Loci and the Role of Bovine Mammary Epithelial Cells in Bovine Mastitis Resistance

Jacqueline P. Kurz

Bovine mastitis, or inflammation of the mammary gland, has substantial economic and animal welfare implications. A genetic basis for mastitis resistance traits is recognized and can be used to guide selective breeding programs. The discovery of regions of the genome associated with mastitis resistance, and knowledge of the underlying molecular mechanisms responsible, can facilitate development of efficient mastitis control and therapeutic strategies. The objectives of this dissertation research were to identify sites of genetic variation associated with mastitis resistance, and to define the contributions of the milk-secreting epithelial cells to mammary gland immune responses and mastitis resistance. Twenty seven regions of the bovine genome potentially involved in mastitis resistance were identified in Holstein dairy cattle. Additionally, this research demonstrates a role of bovine mammary epithelial cells in mastitis resistance, and provides guidance for the use of an *in vitro* model for mastitis studies. Primary bovine mammary epithelial cells from mastitis-resistant cows have differential expression of 42 inflammatory genes compared with cells from mastitis-susceptible cows, highlighting the importance of epithelial cells in mastitis resistance. Bovine mammary epithelial cells display both similarities and differences in pro-inflammatory gene

expression compared to fibroblasts, and their expression of inflammatory genes is influenced by administration of the enzyme phospholipase A2. The growth potential of milk-derived bovine mammary epithelial cells *in vitro* can be extended, facilitating their use in mastitis studies, by transfection with a viral protein. Collectively, this research contributes to current knowledge on bovine mastitis resistance and *in vitro* models.

# ACKNOWLEDGMENTS

I would like to thank my committee members, Dr. Zhongde Wang, Dr. Thomas Baldwin, Dr. Lee Rickords, Dr. Kerry Rood, Dr. David Wilson, and Dr. Brett Adams, for their support and assistance throughout this project.

I would also like to thank the Utah Agriculture Experiment Station Grants Program for providing funding for this project.

Jacqueline P. Kurz

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A: adenine

bF: bovine fibroblast

bMEC: bovine mammary epithelial cell

BTA: *Bos taurus* autosome

C: cytosine

C5: complement component 5

CBP: CREB-binding protein

CCL: C-C motif chemokine ligand

CCR: C-C motif chemokine receptor

CD: cluster of differentiation

cDNA: complementary DNA

Chr: chromosome

CNV: copy number variation

$CO_2$: carbon dioxide

CXCL: chemokine (C-X-C motif) ligand

CXCL8: interleukin-8

DHIA: Dairy Herd Improvement Association

DNA: deoxyribonucleic acid

FBS: fetal bovine serum

FCS: fetal calf serum

FDR: false detection rate

G: guanine

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

gBLUP: genomic best linear unbiased prediction

GRO1: chemokine (C-X-C motif) ligand 2

GWAS: genome-wide association study

IL: interleukin

IL1A: IL-1 alpha

IL1B: IL-1 beta

IL1R1: IL1 receptor type 1

IL1RN: IL-1 receptor antagonist

IL2RG: IL2 receptor subunit gamma

KER8: cytokeratin 8

LB: lysogeny broth

LD: linkage disequilibrium

LPS: lipopolysaccharide

QTL: quantitative trait locus

MEC: mammary epithelial cell

MHC: major histocompatibility complex

MLMM: multi-locus mixed model

MMP: matrix metalloproteinase

NAPDH: nicotinamide adenine dinucleotide phosphate

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK: natural killer

$P_x$: passage *x*

PCR: polymerase chain reaction

PLA2: phospholipase A2

qPCR: quantitative PCR

RASGRP1: RAS guanyl releasing protein 1

RNA: ribonucleic acid

SCC: somatic cell count

SCS: somatic cell score

SE: standard error

SLMM: single-locus mixed model

SNP: single nucleotide polymorphism

SORCS3: sortilin related VSP10 domain containing receptor 3

sPLA2: secreted PLA2

SV40 L Tag: Simian virus 40 large T antigen

T: thymine

$T_H1$: T-cell helper 1

TLR: Toll-like receptor

TNF: tumor necrosis factor

UXT: ubiquitously expressed transcript protein

# CHAPTER 1
# INTRODUCTION

## Mastitis Definition and Pathogens

Mastitis is the costliest disease in the dairy industry [1,2] due to loss of production, decreased milk quality, discarded milk, labor, veterinary treatments, mastitis-related culls, and diagnostics [3]. Additionally, the widespread use of preventative measures against mastitis are an ongoing source of economic losses [4].

Mastitis is defined as inflammation of the mammary gland. Most commonly, mastitis is a result of invasion of mammary tissue by bacterial pathogens entering through the teat canal [5], but may also result from invasion by fungal or algal pathogens, mechanical trauma, chemical injury, or thermal insult [6]. Inflammation results in temporary or permanent loss of function due to direct microbial and/or inflammatory-mediated damage to anatomic components, including the milk-secreting mammary epithelial cells (MECs). Mastitis is endemic among dairy cattle worldwide, with prevalence within dairy herds ranging from 5-75%, and among mammary quarters from 2-40% [7].

Bacterial infection is by far the most common cause of mastitis among dairy cattle. Mastitis-causing bacteria are traditionally categorized as environmental pathogens or contagious pathogens [5,6,8]. Classification of bacteria into one of these two categories is based on the predominant source of infection, although overlap may be seen

with some bacterial strains such as environmental pathogens with host-adapted virulence factors [9,10].

Important environmental pathogens include *Escherichia coli*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Serratia* spp., *Klebsiella* spp., and *Enterobacter* spp. [10,11]. In comparison to contagious pathogens, a higher proportion of environmental pathogens cause clinical, rather than subclinical, mastitis [11]. The source of environmental pathogens is primarily the cows' surroundings, where these bacterial species are normally ubiquitous and capable of long-term survival and growth [6,10]. Manure, bedding, milking equipment, pre- and post-dip preparations, and flies are common sources [7,10]. Seasonal variation in the incidence of mastitis caused by environmental pathogens has been observed, with incidence increasing during hot or damp weather [12]. Entry of environmental pathogens into the teat canal occurs by propulsion during milking or by passive penetration of the teat canal. Environmental hygiene and pre-milking teat dipping are important management factors that impact levels of exposure to environmental pathogens. However, the prevalence of mastitis caused by environmental pathogens within individual herds is not consistently associated with management practices traditionally considered to reduce exposure to environmental pathogens [10]. Despite the widespread implementation of management practices that are successful in reducing the incidence of contagious mastitis, the control of environmental mastitis remains a substantial challenge within most dairy herds. As a consequence, environmental mastitis has become more common than contagious mastitis in the majority of well-managed herds [10]. Due to the ubiquitous nature of environmental

pathogens, antibiotic therapy, with the exception of treatment during the early dry period, is relatively ineffective in preventing mastitis caused by these pathogens [11].

The universally recognized contagious mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp., and *Corynebacterium bovis* [6,7,10]. Contagious pathogens are spread between cows and generally cannot persist long-term off the host [6,13]. Adherence factors allow colonization of the teat end or teat canal, and infection of the mammary gland occurs via the teat canal [13]. Hematogenous spread to the mammary gland or between mammary gland quarters is uncommon but has been reported for *Mycoplasma* spp. [14]. Infection most commonly occurs at milking, where milking equipment and workers' hands serve as fomites in the spread of the bacteria between cows.

Mastitis, whether caused by environmental or contagious pathogens, may be clinical or subclinical. Clinical signs of mastitis include mammary gland edema and hyperemia, changes to milk appearance or consistency, and/or systemic manifestations such as pyrexia and obtundation [6,15]. Intramammary infection without clinically detectable signs is termed subclinical mastitis. Because subclinical mastitis requires ancillary testing for diagnosis, it may not be readily detected on-farm and so may persist chronically, resulting in long-term detrimental effects on mammary gland function, milk quality, and milk somatic cell count (SCC). *Staphylococcus aureus*, a contagious mastitis pathogen, is a particularly important cause of chronic, subclinical mastitis in dairy cattle that is often accompanied by large increases in SCC [16,17]. Somatic cell count is a measure of the concentration of cells, particularly leukocytes, within milk, and is often

used as an indicator of intramammary infection status. Somatic cell count is discussed in further detail below. Strategies used to detect subclinical mastitis include regular monitoring of SCC, milk electrical conductivity testing to detect changes in ion concentrations, milk bacterial culture, polymerase chain reaction (PCR)-based pathogen detection, and assessment of milk pH [18–20]. Several methods exist for SCC monitoring, ranging from cowside tests (California Mastitis Test) to more precise methods such as nuclear staining and optical fluorescence (Fossometric SCC; Delaval cell counter) [20].

*Escherichia coli* is an environmental pathogen capable of causing mild to severe, usually clinical mastitis variably accompanied by systemic illness. *Escherichia coli* has been used in many bovine mastitis studies because of its importance as a mastitis pathogen as well as its capacity to induce a strong inflammatory response in the mammary gland [21]. In this dissertation, lipopolysaccharide (LPS) from *E. coli* was used in several studies to stimulate immune responses of bovine MECs. Pathogenicity of *E. coli* in intramammary infections generally is not strain-dependent [22,23], except in instances where host-specific virulence factors develop [9]. Pathogenicity is attributable predominantly to the presence of LPS, common to all *E. coli* strains, and is dose-dependent [22]. Recognition of the presence of LPS by host cells occurs predominantly through Toll-like receptor (TLR) 4. Bound TLR4 initiates transcription of various factors via the NFκB signaling pathway, ultimately resulting in production of cytokines, antimicrobial defense proteins, and lipid mediators. Excessive cytokine production induced by LPS is an important contributor to the severity and clinical signs of *E. coli*-

associated mastitis. For example, the degree of production of the cytokine tumor necrosis factor (TNF) α is an important factor in determining the severity of clinical signs [24]. High levels of TNFα induce both local and systemic effects contributing to the morbidity and mortality in *E. coli* mastitis. Locally, TNFα promotes leukocyte-mediated tissue damage via its effects on leukocyte recruitment, activation, and nitric oxide production. Tumor necrosis factor α-induced increases in vascular permeability contribute to mammary gland hyperemia, edema, and altered milk composition due to leakage of plasma components into the milk. Systemic effects of TNFα include cardiovascular compromise via decreased cardiac output, systemic vasodilation, and increased vascular permeability; promotion of a pro-thrombotic state via endothelial cell activation; induction of pyrexia; and metabolic disturbances [6,25]. Other important proinflammatory mediators in the pathogenesis of *E. coli* mastitis include interleukin (IL)-1, IL-6, IL-8, platelet-activating factor, prostaglandins, and complement components [25].

Mastitis caused by *E. coli* occurs most commonly in periparturient or early-lactation cattle, and tends to be most severe in these animals. Intramammary infection by *E. coli* is an important cause of acute toxic mastitis in early-lactation cows [26]. Periparturient immunosuppression contributes to the increased incidence and severity of mastitis during this time. Weak or delayed neutrophil influx following intramammary infection is of particular relevance in *E. coli* infection [11,27], particularly when compounded by bacterial capsule production by some strains. Encapsulation serves a

protective function in bacteria against neutrophil-dependent defenses and contributes to prolonged infection by these strains [11].

**Innate Immunity of the Bovine Mammary Gland**

The pathogenesis of mastitis involves complex interactions between the etiologic agent and host tissues, influenced by a wide variety of environmental and genetic factors that contribute to disease susceptibility and outcome of infection [28]. Innate immunity, comprising relatively non pathogen-specific host responses, plays a particularly important role in mastitis pathogenesis[29]. Defense mechanisms of innate immunity that are of particular importance in prevention and elimination of intramammary infection include the epithelial barrier, secretions in milk, and leukocytes [30].

The teat canal is by far the most common portal of entry for mastitis pathogens [28]. Adaptations of the teat canal therefore comprise the first barrier to entry and establishment of pathogens [31,32]. Keratin and lipids continuously produced by keratinocytes lining the teat canal provide an impermeable barrier to most bacterial pathogens, and are of particular importance in forming a protective plug during mammary gland involution and the dry period [28,31,33]. In the lactating cow, the keratin plug is lost but the teat orifice closes between milkings due to the contraction of the teat sphincter muscle, which is responsive to acetylcholine and tension from milk fill [32]. Closure by the teat sphincter following milking takes up to two hours or longer [28,30], during which time the mammary gland is susceptible to entry by mastitis pathogens. The common management practice of feeding lactating cows directly

following milking is in part a measure to reduce mastitis incidence [34], as feeding activity delays lying behavior, reducing contact between the open teat end and sources of environmental contamination such as bedding material and manure. Continual shedding of teat canal keratinocytes as well as the flushing action of milk during milking provide additional defenses against bacterial colonization of the mammary gland [31].

Mammary epithelial cells lining the teat canal, ductal system, and gland acini provide a second line of defense. In addition to providing a dynamic physical barrier against bacterial entry, these cells contribute to inflammatory responses during intramammary infection [30]. Tight junctions between MECs are a major component of the physical barrier between the milk space (acinar and cisternal spaces and duct lumens) and the interstitial space of the mammary gland. The permeability of this barrier varies with lactational stage and intramammary infection status, and is influenced by factors such as epithelial growth factor and transforming growth factor β, which in turn are under hormonal control by prolactin, progesterone, and glucocorticoids [35]. Permeability is increased, for example, during the dry period in uninfected glands [30]. Impermeability is important during lactation to prevent microbial entry. However, during active intramammary infection, permeability is increased as a result of direct damage by bacterial toxins as well as the influence of pro-inflammatory mediators such as histamine, TNF α, and interferon γ [25,30]. Although increased permeability may compromise barrier function, it facilitates entry of effectors of innate immunity into the milk compartment. Natural (opsonic) antibodies, complement components, transferrin, and acute-phase proteins are some examples [30].

Mammary epithelial cells are capable of recognizing antigen from invading organisms through expression of pattern recognition receptors, including TLRs 2 and 4 [36]. A soluble form of CD14 (sCD14) which binds LPS and facilitates its interaction with TLR4, is present in milk and is produced by MECs [28,37]. Binding of pathogen components to TLRs initiates a cellular signal transduction pathway that ultimately leads to the production of inflammatory mediators. Toll-like receptor 2 binds components of Gram-positive bacteria, such as lipoteichoic acid and peptidoglycan, while TLR4 binds mainly LPS. Initially, LPS binding protein binds LPS, subsequently forming a complex with sCD14. Lipopolysaccharide is then transferred to the LPS receptor complex composed of TLR4 and lymphocyte antigen 96 (MD2), inducing homodimerization of TLR4. Transmembrane signaling results in activation of nuclear factors NF-ƙB and activated protein-1 and initiates production of pro-inflammatory cytokines and chemokines [6,24,30]. Specific cytokines generated as a result of intramammary infection vary by pathogen, as demonstrated by *in vivo* and *in vitro* studies examining cytokine expression profiles of MEC challenged with various bacterial species and/or bacterial components [36,38,39].

Alveolar and ductal MECs additionally produce non-cytokine secreted factors that contribute to innate immunity. Lactoferrin and citrate from MECs contribute to iron chelation, which exerts a bacteriostatic effect on bacterial species with high iron requirements, such as *E. coli* and *S. aureus*. Lactoferrin additionally has bactericidal effects (including synergism with other bactericidal proteins), anti-inflammatory effects via LPS binding, and modulatory effects on complement activation [30,40]. The enzyme

xanthine oxidase, derived from milk fat globule membranes produced by MECs, additionally has bactericidal effects through generation of reactive oxygen species [30,41].

Neutrophils and macrophages are the major effector leukocytes involved in the innate immune response against intramammary infection. In the uninfected mammary gland, macrophages are the predominant cell type in milk, comprising 66-88% of somatic cells, accompanied by low numbers of neutrophils ($<10^5$ cells/ml; 0-11% of milk somatic cells) [42,43]. Lymphocytes are also present, comprising 10-27% of milk somatic cells, and are predominantly CD8+ T-lymphocytes expressing the αβ receptor [43]. These populations shift with intramammary infection.

In the acute stages of inflammation, neutrophils become the predominant cell type, making up >90% of the total leukocyte population in milk [42]. Neutrophils are recruited from circulation by inflammatory mediators produced by MECs as well as by other leukocytes, predominantly macrophages. Inflammatory cytokines such as TNFα and interleukins (ILs) stimulate the expression of endothelial selectins (E-selectin, P-selectin) and adhesion molecule (intercellular adhesion molecule 1, vascular cellular adhesion molecule 1) as well as expression of neutrophil adhesion molecule Mac-1, enabling neutrophil migration from the circulation to sites of inflammation [6,43]. Migration of neutrophils in the extravascular compartment to target sites occurs along concentration gradients of a number of factors, including complement components C5a and C3a, LPS, IL-1, IL-2, and IL-8 [30]. Neutrophils act as effector cells of innate immunity via phagocytosis of bacteria as well as through bactericidal effects of secreted

molecules. Bactericidal effects are mediated mainly through the respiratory burst, in which nicotinamide adenine dinucleotide phosphate (NADPH) oxidases produce superoxide anions which then dismutate into hydrogen peroxide, hydroxyl, and other reactive oxygen species capable of destroying bacteria [44]. Other anti-microbial molecules released by neutrophils include defensins, lactoferrin, and bactenecins [30,42].

Although neutrophil numbers are low in the healthy mammary gland, the concentration and viability of neutrophils in the healthy mammary gland is inversely related to the risk of intramammary infection and the severity of coliform mastitis. Additionally, the degree and rapidity of neutrophil recruitment vary between individuals and are important factors in the outcome of infection [45]. Factors reported to affect the functionality of neutrophils within the mammary gland include cow parity, stage of lactation, nutritional/metabolic status, and genetic factors [25,30]. Neutrophils are highly effective at combating bacterial infection, but their responses secondarily contribute to mammary gland damage as a result of the release of lysosomal contents, reactive oxygen species, and tissue disruption associated with trans epithelial migration [25,46,47].

Macrophages comprise 9-32% of milk somatic cells in acutely infected quarters [43]. These cells are important in pathogen recognition, antigen presentation, and leukocyte recruitment. Pathogens are destroyed through phagocytosis and exposure to reactive oxygen species (superoxide, produced from $O_2$ by NADPH oxidase) and proteases. Phagocytosis is enhanced by opsonization of pathogens by complement components and by natural or pathogen-specific antibody that is recognized by macrophage Fc receptors [6,48]. The contribution of macrophages to phagocytosis during

mastitis is considered fairly minor in comparison with that of neutrophils [43]. More importantly, macrophages contribute to the innate and adaptive immune response through secretion of nitric oxide and inflammatory mediators as well as antigen processing and presentation via major histocompatibility complex (MHC) class II receptors. Activated macrophages secrete prostaglandins, leukotrienes, and cytokines to influence various aspects of the inflammatory response, including development and recruitment of other leukocytes. For example, IL-12 produced by macrophages enhances the development of CD8+ (cytotoxic) T-lymphocytes while simultaneously driving a $T_H1$ response, stimulating cell-mediated adaptive immune mechanisms [6,43]. As inflammation resolves, macrophages participate in phagocytosis and clearing of neutrophils [46,48]. Macrophage function is diminished during the periparturient period, contributing to an increased susceptibility to intramammary infection during this time [30].

Lymphocyte populations with a role in the response to intramammary infection include natural killer (NK) cells, CD4+ and CD8+ αβ T-lymphocytes, γδ T-lymphocytes, and B-lymphocytes. Total lymphocytes comprise 14-24% of milk somatic cells from infected quarters [43].

Natural killer cells are a subset of cytotoxic lymphocytes capable of exerting their effects independently of interaction with antigen presented by MHC molecules, and are an important component of innate immunity [6]. Contributions of NK cells during bovine mastitis have not yet been fully defined, but cells with NK-like activity have been demonstrated in the mammary gland [26] and may be an important component of innate immunity against intramammary infection [28–30]. In general, NK cells identify and

destroy injured or infected cells via recognition of decreased MHC I expression, increased expression of activating receptors, and decreased expression of inhibitory receptors by target cells, and can recognize antibody-bound cells via Fc receptor binding [6]. Natural killer cells are capable of secreting TNF-α and other factors that promote pro-inflammatory responses [29].

Besides NK cells, lymphocytes are primarily involved in the adaptive, rather than innate, immune response. The relative degree of expansion of CD8+ and CD4+ αβ T-lymphocyte populations in response to intramammary infection varies depending on the causal pathogen and the stage of infection [21,49]. For example, increased lymphocyte numbers during intramammary infection are due primarily to CD4+ T-lymphocyte expansion during staphylococcal infection, whereas both CD4+ and CD8+ T-lymphocyte expansion occurs during streptococcal infection [49]. In the uninfected mammary gland, B-cell levels remain fairly consistent throughout stages of lactation. In the infected mammary gland, B-cell differentiation and clonal expansion occurs in response to IL-2 secreted from CD4+ T-lymphocytes, constituting the humoral component of the immune response [29].

Depression of immunity against mastitis and other diseases during the periparturient and early lactation periods is a widely-recognized phenomenon in cattle [50,51]. Several mechanisms are involved, including decreased leukocyte function, altered leukocyte trafficking, changes in circulating and mammary gland leukocyte populations, altered cytokine production, and decreased leukocyte survival [51–53].

Important factors driving these changes include increased glucocorticoid levels associated with the stress of calving and nutritional and genetic factors [53].

**Damage to the Mammary Gland during Mastitis**

Mastitis-induced mammary gland damage and disruption of function are consequences of direct effects of pathogens as well as effects from the host immune response, namely those associated with leukocyte responses, proteases, and inflammatory mediators.

The presence of some bacterial components such as LPS can induce apoptosis in MECs via upregulation of pro-apoptotic factors (Bax, IL-1β-converting enzyme) and simultaneous downregulation of anti-apoptotic factors such as Bcl-2. Some bacteria, such as *S. aureus*, induce apoptosis of MECs and other cells through cellular injury associated with direct invasion [54]. Direct bacterial effects contribute also to necrosis during mastitis. Virulence factors of causative bacteria may include toxin production, inducing cell death through host cellular membrane damage or intracytoplasmic enzymatic activity. Some pathogens produce effector proteins such as proteinases, while others induce damage through the production of superantigens [55].

Reactive oxygen species, produced by neutrophils and macrophages during the respiratory burst, are an important cause of tissue injury and necrosis during mastitis. The purpose of the respiratory burst is to destroy bacterial pathogens, but local host cells frequently become casualties during this process. Reactive oxygen species are damaging

to lipids, proteins, carbohydrates, and nucleic acids, and therefore exert a wide range of detrimental effects on exposed host cells, including MECs [55].

In addition to production of reactive oxygen species, neutrophils initiate cellular damage through degranulation. Among other constituents, granules contain neutral and acidic proteases such as elastase, cathepsins G, B, and D, and matrix metalloproteinase (MMP). These proteases are capable of damaging mammary gland membrane proteins and interstitial matrix components, thereby exerting damaging effects on MECs and other mammary gland components [33].

Inflammatory cytokines such as TNFα produced during mastitis contribute to increased vascular and MEC barrier permeability. This, in turn, facilitates entry of potentially damaging plasma proteins into the mammary gland. For example, plasmin and plasminogen concentrations in the milk increase during mastitis as a result of increased MEC barrier permeability. The presence of these higher concentrations is further compounded by release of plasminogen-activating factors from neutrophils and some bacterial pathogens. Plasmin exerts damaging effects on MECs through direct degradation of matrix proteins (fibrin, laminin) as well as through activation of MMP precursors. Other potentially damaging plasma proteins increased in the mammary gland during mastitis include MMP-9, MMP-2, 120-kDa gelatinase, and stromelysin-1. Degradation of matrix proteins by these proteases contributes to compromise of MEC attachment to the extracellular matrix, and thereby to MEC damage [28,55].

Inflammatory cytokines produced during mastitis may also have direct damaging effects on host cells. Exposure to TNFα and IL-1 induces apoptosis in bovine endothelial cells and human MECs. A similar effect on bovine MECs is possible [55].

**Changes to Milk during Mastitis**

One of the most economically important consequences of mastitis is the decrease in milk production that accompanies the disease. Damage to MECs, and therefore reduced production capacity, is a result of direct damage by mastitis pathogens as well as injury secondary to the inflammatory response [6], as discussed above. Even with therapeutic intervention, milk production may be affected for several weeks following intramammary infection before returning to expected levels [56]. Irreversible damage can occur as a result of severe or prolonged intramammary infection, in which secretory cells may be sloughed or permanently replaced by fibrous tissue [6]. In these cases, milk production is affected throughout the current as well as all subsequent lactations.

In addition to lowering the volume of milk produced, mastitis results in substantial changes in milk composition. Decreases occur in lactose, α-lactalbumin, fat, and potassium. Increased vascular permeability as a result of inflammation results in leakage of sodium, chloride, and plasma proteins into the milk, particularly proteins associated with inflammatory responses. Additional proteins are released by mammary epithelial cells and leukocytes. Proteins increased in milk from infected quarters include whey, serum albumin, immunoglobulins, transferrin, lactoferrin, and various enzymes including xanthine oxidase, acid phosphatase, α1-antitrypsin, and n-Acetyl-*β*-D-

glucosaminidase [57,58]. Some of these enzymes, such as plasmin, may alter milk quality further via cleavage of milk components such as casein both prior to and following milking [28,58]. The pH of milk from mastitis quarters is frequently elevated due to the presence of plasma components [58]. During active infection, the causative organisms, usually bacteria, are present in variable numbers in the milk, and may be accompanied by bacterial toxins [58,59]. Milk SCC rises as a result of an influx of large numbers of leukocytes into the milk. In cases of clinical mastitis, milk from affected quarters may have altered color and/or consistency, often characterized by decreased viscosity with or without clots due to coagulation of leukocytes and clotting factors [58].

**Somatic Cell Count as an Indicator of Intramammary Infection**

Measurement of milk SCC is frequently used as a diagnostic indicator of mammary gland health status. As an indicator of intramammary infection, SCC is used in the dairy industry in public health regulatory programs as a factor in determining the suitability of milk for human consumption. In the US dairy industry, for example, requirements of the Pasteurized Milk Ordinance place a limit on the SCC of milk (<750,000 cells/ml in Grade A raw milk) [60]. Additionally, SCC limits are used in some milk quality incentive programs, since mastitis can result in altered milk composition (discussed previously) and decreased shelf-life [60]. These applications of SCC measurement demonstrate aspects of the economic importance of minimizing mastitis in dairy cattle, as high SCC can potentially result in decreased milk value or even exclusion of milk from the food supply.

Intramammary infection is by far the largest contributor to an increased SCC, but a number of other factors can also affect SCC. Variation exists in the degree of SCC increase between pathogens. Bacteria that elicit marked cellular responses, with resultant large increases in SCC, are known as major pathogens. Common examples include coliforms and *S. aureus*. Infection by major pathogens results in an average SCC of >600,000 cells/ml [61]. Minor pathogens result in a less pronounced cellular response and thus a smaller increase in SCC. Minor pathogens include *Corynebacterium bovis* and coagulase-negative staphylococci. Infection by minor pathogens can result in SCCs of near or even less than 200,000 cells/ml [61]. Therefore, detection of cattle infected with minor pathogens may be poor unless additional testing is not carried out [16].

The stage of infection additionally affects SCC. In experimentally infected cows, SCC was highest during the acute stages of infection. Variation due to the challenge organism existed in the time to peak SCC after infection, but occurred within hours to days [62]. Somatic cell count may be elevated 10 days prior to clinical mastitis in naturally-infected cows [63] and can persist for variable amounts of time following clearance of intramammary infection [64,65]. As elevated SCC may persist after an infection is cleared, a cow in this stage may be falsely classified as actively infected despite having cleared the causative organism.

The stage of lactation also influences SCC. In uninfected cows, the highest SCC occurs at drying off, followed by directly after calving, and the lowest occurs from peak to mid-lactation. This trend is the inverse of milk volume production, suggesting a

dilution effect [16]. Variation in this trend may be influenced by parity, as somatic cell linear scores in first-lactation cows may conversely be higher in early lactation [66].

Hourly and daily variations in SCC occur. Hourly variations are influenced by the milking schedule, with the highest SCC generally detected at milk stripping and within 1-3 hours after milking. Differences between daily high and low SCC ranges from 4- to 70-fold for individual quarters [58]. Therefore, a single daily SCC measurement may not be representative of overall SCC. Day-to-day variation is particularly of relevance among infected cows [16].

Because milk somatic cells represent a component of the immune defense against mastitis pathogens, a low SCC (<200,000 cells per ml during intramammary infection) in some cattle may indicate a depressed or ineffective immune response to intramammary infection rather than an absence of intramammary infection. In fact, low SCC has been reported as a risk factor in the subsequent development of clinical mastitis [67,68].

Somatic cell count is a widely used but imperfect indicator of intramammary infection status, with the potential to be influenced by the above factors. Measures other than SCC have been used to determine the infection status of cows. Milk bacterial culture is useful and can be carried out on-farm [69]. Identification of mastitis pathogens via PCR or immunoassays provides an alternative to bacterial culture for detection of specific pathogens, and is becoming more widely available in recent years but may still be cost-prohibitive [20]. The potential for sample contamination as well as the potential for a lack of bacterial shedding during intramammary infection must be considered in the interpretation of pathogen detection methods. Regular observation of milk and the

mammary gland for changes consistent with mastitis (e.g. altered milk consistency, visibly inflamed mammary gland tissue) allows for detection of clinical mastitis, but gives no indication of subclinical mastitis. Tests for biomarkers of mastitis, such as measurement of milk lactate dehydrogenase levels [70], have been proposed, but the use of these for commercial purposes has limitations [20]. Reliable determination of infection status is best achieved through a combination of SCC measurement, pathogen detection, and examination for clinical mastitis [71].

**Factors Influencing Mastitis Resistance**

An individual's phenotype for any given trait, including disease resistance, may be influenced by genetic and environmental factors. The relative contribution of each of these factors varies by trait [6,72]. In disease resistance, environmental factors may include nutritional and metabolic status, age, concurrent disease, hormonal status, environmental temperatures, and, for infectious diseases, the type and dose of pathogen exposure. Genetic factors that impact disease severity vary greatly between different diseases, but by definition include any variation within the genome that influences an individual's susceptibility. Many traits are considered quantitative, meaning that the cumulative influence of and interaction between multiple genes (polygenes) determines the phenotype. These traits display polygenic inheritance, yielding a continuous gradient of phenotypes among individuals rather than distinct phenotypic categories [73]. Environmental factors often have substantial influences on expression patterns of the genotype, thereby contributing to the phenotype (multifactorial inheritance) [28,74].

Mastitis resistance is a multifactorial, quantitative trait [28,72,75]. In dairy cattle, recognized external environmental factors influencing mastitis susceptibility are many, including nutritional management, housing type and management, bedding material, milking routine, maintenance and performance of milking equipment, season, dry cow management, and the use of preventative disease programs [12,76–78]. Additional, cow-dependent, non-genetic factors include stage of lactation, reproductive status, concurrent disease, and metabolic status [53,76,79,80]. These factors have variable but often substantial effects on mastitis traits, and much research has focused on optimizing the management of environmental factors and milking practices in an effort to decrease mastitis incidence.

Genetic factors are also known to contribute to mastitis susceptibility. Heritability for clinical mastitis is reported by most studies to be within the range of 0.003 to 0.17 [81–88], but has been reported to be as high as 0.42 [89]. Heritability for subclinical mastitis is reported within the range of 0.04 to 0.14 [87,88], with one study reporting differences in heritability to subclinical mastitis based on the causative agent [88]. Heritability for somatic cell score (SCS; a derivative of SCC) is reported within the range of 0.01 to 0.187 [84,90–93]. Although the heritability of mastitis traits appears to be relatively low, genetic selection for mastitis resistance nevertheless has been demonstrated to reduce the incidence of mastitis, particularly when genetic markers of mastitis traits are used [89,94]. Because of the potentially substantial benefits of marker-assisted selection, discovery of genetic markers of mastitis resistance is an ongoing pursuit within the field of dairy science.

**An Introduction to Genome-Wide Association Studies**

Regions of the genome associated with quantitative traits, such as mastitis susceptibility, are termed quantitative trait loci (QTLs). Identification of QTLs, and the specific variants within them associated with phenotype for a given trait, provide the basis for marker-assisted selection in animal breeding.

Within QTLs, a number of recognized types of variation can influence phenotype. These include differences in copy numbers of genes, gene segments, or non-coding sequences (copy number variations, CNVs); micro- and minisatellites; and sequence polymorphisms [95]. Single nucleotide polymorphisms (SNPs), the most common form of variation [96], are sites within the genome where allelic variations in a single nucleotide base are present in greater than 1% of the population. They may occur in coding or non-coding regions, and can affect gene expression or gene products [95,97]. Single nucleotide polymorphisms have been associated with variations in specific phenotypic traits in many species, including mastitis traits in cattle.

Single nucleotide polymorphisms significantly associated with a particular trait can be identified through candidate gene studies or genome-wide association studies (GWAS). In candidate gene studies, specific genes or genomic regions hypothesized or known to be relevant to the phenotype of interest are selected and genotyped. These genotyped regions are then examined for SNPs, and whether specific alleles of these SNPs vary significantly among phenotypically divergent individuals (e.g. cattle with a high level of susceptibility versus those with high level of resistance to a disease). Selection of candidate genes may be based on knowledge of biological function or on

results of previous genomic studies [97,98]. Genome-wide association studies, on the other hand, allow genotyping of large numbers of SNPs across the genome. Detection of SNPs that are significantly associated with the trait of interest is not limited to previously-identified regions known to be associated with the trait, thus allowing for an expanded potential to detect novel variants (previously unidentified SNPs) [97]. In the context of disease, GWAS can potentially highlight previously unknown contributions of specific genes or regions to disease susceptibility, thereby contributing to elucidation of disease mechanisms in addition to detection of novel genetic markers. Genome-wide association studies are appropriate if the common disease/common variant hypothesis is true, in which a common, complex disease is influenced by genetic variants that are common within the population. Complex diseases influenced by multiple loci, each with a small effect, are most well suited to GWAS [98,99].

Using GWAS, the number of SNPs genotyped varies by assay. Low-density (several thousand SNPs), medium-density, and high-density (hundreds of thousands of SNPs) arrays are available for GWAS in cattle. Increased marker density increases the power to detect significant associations [100], and is particularly important when numerous loci with small effects are studied. Even with high-density arrays, however, only a small proportion of the total SNPs in the genome are genotyped. Genome-wide association studies rely on the concept of linkage disequilibrium (LD), defined as the non-random association of alleles at different loci due to non-random patterns of genetic recombination [101]. Single nucleotide polymorphisms located together between two areas in the genome where recombination commonly occurs may be inherited together

more frequently than would be expected by chance, and thus would be considered in LD with each other [96]. The degree of LD is commonly expressed as an $r^2$ statistic, defined as the square of the correlation coefficient between the presence or absence of a specific allele at one locus and the presence or absence of a specific allele at a second locus [102]. The $r^2$ values range from 0 to 1, where $r^2 = 1$ indicates that specific alleles of the SNPs are inherited together in all individuals of the study population, whereas $r^2 = 0$ indicates that the alleles are independently inherited and thus in linkage *equilibrium* [96]. Linkage disequilibrium among some groups of SNPs is strong enough that a relatively small number of associated allele sets, or haplotypes, account for the majority of the variation at that genomic region within a population. Because of LD, the causal SNP of the phenotypic variation does not itself need to be genotyped so long as a SNP with which it is in strong LD is genotyped. Genotyped SNPs in high LD with a causal SNP are called tag SNPs, and can be used to predict phenotype due to their LD relationship with the causal SNP [96,98]. In the human genome, for example, LD allows for the majority of genomic variation to be detected in subjects of European ancestry using 300,000 well-chosen tag SNPs. Single nucleotide polymorphisms can be chosen for GWAS arrays based on existing knowledge of SNP-phenotype associations, such as that provided by the human HapMap project, or, more commonly, are an unbiased selection distributed uniformly across the genome [98].

**Genome-Wide Association Study Methodology**

The first step in a successful GWAS is study population selection. Sample size is one factor that influences the power of detection of significant SNPs, and is particularly important in detection of rare variants [95,99]. Additionally, robust methods for phenotypic classification of individuals within the sample population are essential in order for true associations to be detected [99]. Due to the high expense of GWAS arrays and data analysis, studies may be carried out as two-stage designs, in which a relatively small sample size is used initially. Single nucleotide polymorphisms found to be significantly associated with the trait of interest within the initial sample population can be selected for further analysis in the second stage of the study, within an expanded population. Thus, the number of SNPs to be genotyped in the second stage of the study can be greatly reduced by eliminating SNPs with no evidence of association to the trait. In some studies, the initial stage is used to select specific areas of the genome to examine in more detail by increasing marker density at that area for the second stage of the study [95,98]. For single-stage studies, an approach to reduce sample size while retaining statistical power has been described ("selective genotyping"). In this approach, only the phenotypically extreme individuals (e.g. the most highly resistant and most highly susceptible individuals to a disease) are genotyped; the number of individuals characterized as phenotypically extreme should not exceed 20-25% of the population [103]. This approach is based on the assumption that allelic frequencies of causal variants are most highly divergent between the high and low phenotypic extremes within a

population. Selective genotyping therefore provides an enrichment effect of causal alleles within the genotyped population [104].

Genotyping is carried out using a SNP microarray following genomic DNA isolation and purification. Several SNP array platforms exist, with widely used arrays available from Affymetrix (Santa Clara, CA) and Illumina (San Diego, CA). Both are based on hybridization of DNA fragments containing a SNP of interest to a complimentary oligonucleotide probe on the array, combined with a detection chemistry to generate nucleotide-specific signals at each SNP site. Affymetrix arrays consist of short oligonucleotides affixed directly to the array chip surface. Illumina arrays are microbead-based, with oligonucleotide probes affixed to microbeads on the chip. Illumina chips are more expensive to manufacture, but have a higher specificity than Affmetrix arrays due to the use of longer oligonucleotide probes [99].

For analysis using Illumina arrays, genomic DNA is first processed by fragmentation and whole-genome amplification before hybridization to the array. Each probe on the array consists of an oligonucleotide sequence complementary to a sample genomic DNA sequence, with its 3’ end directly adjacent to a SNP site. Hybridization of genomic DNA to its complementary probe results in overhang of the genomic DNA fragment directly at the SNP site. Subsequently, single base extension at the 3’ end of the probe incorporates a labeled nucleotide complementary to the SNP allele. A wash step then eliminates remaining, unbound labeled nucleotides from the array. The chip is then scanned, and signals emitted by the labeled nucleotides are recorded. Because each of the four nucleotides (A, C, T, G) are differentially labeled, and the positions of the

oligonucleotide probes on the array are known, the signal emitted at each microbead position is used to determine the allele(s) present at each specific SNP site [105,106]. Pixel values obtained from the array scan are first converted to signal intensity values at each position. Signal intensities are then subjected to intensive quality control measures, also called low-level analyses, before the genotype (the allele present at each SNP site) can be called. Many factors affect signal intensities, including DNA concentration and differences in probe affinities between individual alleles. Genotype calling algorithms account for these factors, normalizing signal intensities to facilitate accurate genotype calling. A number of algorithms have been developed, reviewed elsewhere [98], and commonly employ a Bayesian approach. Genotypes that cannot be accurately called following these quality control measures are excluded from the data set. Ultimately, three signal intensities are possible, indicating that an individual is homozygous for the major (more common) allele, homozygous for the minor (less common) allele, or heterozygous [98].

The data are filtered by a number of further quality control steps after genotype calling. Individual samples for which more than a few SNPs could not be called are assumed to be of poor quality and are excluded from the data set. In most studies, samples with a call rate of less than 97% of SNPs are excluded. Individual SNPs for which genotyping failed in multiple animals are also excluded from the dataset. A threshold of 2 to 3% is usually set, such that any SNP with a failed call rate above this is excluded. Additionally, SNPs for which the minor (less common) allele frequency is low are excluded from the data set, as there is insufficient statistical power to detect a true

association of these SNPs to the trait using GWAS. Single nucleotide polymorphisms with minor allele frequencies of less than 1 to 5% are usually excluded [98].

Following quality control measures, GWAS require adjustment for familial relatedness between animals in order to avoid artificial inflation of significance values. One method to account for familial relatedness is the genomic best linear unbiased prediction (gBLUP) method, which generates a marker-based genomic kinship matrix for integration into the analysis. Covariance between individuals is determined based on comparison between samples of a large number of genetic markers such as SNPs, and is used to determine relatedness with more accuracy than pedigree-based methods [107]. The kinship matrix generated by gBLUP is then incorporated into mixed linear model analysis, with relatedness corrected for as a random effect [108].

Once genotype calling, data filtering, and generation of a kinship matrix have been completed, data analysis can proceed. Linear regression, the single locus mixed model (SLMM), and the multi-locus mixed model (MLMM) are three alternative methods commonly used in GWAS. Of these, the mixed models incorporate both fixed and random effects, and thus are able to take into account familial relatedness using a kinship matrix, as discussed above [109,110]. The SLMM detects associations between a trait and individual markers, examining the relationship of each SNP to the phenotype independently of other SNPs [110]. The MLMM is used for complex traits influenced by multiple moderate- to large-effect loci, and examines interaction effects between SNPs throughout the genome. It employs a stepwise mixed model regression and a forward inclusion and backward elimination of multiple loci as cofactors, thereby reducing

confounding effects of background loci across the genome. Compared with the single locus MLM, the MLMM generally yields lower false detection rates (FDR) and higher power [108,110].

In mixed model analysis, a genetic model must be specified. An additive, dominant, recessive, or multiplicative inheritance pattern is selected based on knowledge from previous studies, if available [99,108]. Frequently, the inheritance pattern of the SNPs of interest is unknown. In these cases, the additive inheritance model is selected, as this has the power to detect both additive and dominant effects. Additive inheritance assumes a linear, uniform increase in risk for expression of the phenotype in question (e.g. disease susceptibility) for each copy of the risk-associated allele. In other words, if two alleles, *a* and *A*, exist for a particular SNP, and the *A* allele is associated with increased disease susceptibility, an animal with the genotype *AA* will be twice as disease-susceptible as an animal with the *Aa* genotype. If an animal with the *Aa* genotype is three times more susceptible to developing the disease than an animal with the *aa* genotype, an animal with the *AA* genotype will be twice as susceptible than the *Aa* animal, or six times more susceptible than the *aa* animal. The dominant model, on the other hand, assumes equal risk for *Aa* and *AA* animals, while the recessive model assumes that an animal must have two copies of the causative allele (*AA*) in order to have increased susceptibility. The multiplicative model assumes that an animal with the genotype *AA* is $x^2$ more susceptible than an animal with the *Aa* genotype. [99].

Depending on the study population, additional covariates (other known factors that may influence the trait) must be considered and incorporated into the model if

appropriate. These may include population stratification (e.g. different ethnic groups), sex, age, and study site [99,108].

Due to the large number of individual statistical tests carried out in mixed model analysis, p-values generated by the analysis must be corrected for multiple testing. The Bonferroni and FDR methods are two commonly used methods. The Bonferroni correction adjusts the false positive rate for the number of statistical tests; the alpha value (significance value) is divided by the number of statistical tests run. This method assumes that each association test for every SNP is independent of all other tests, and therefore does not account for LD. The FDR method allows adjustment of the false positive rate through providing an estimate of the proportion of significant results that are false positives [99]. This method has increased power over the Bonferroni method, with an increasing advantage as the number of tests increase, but may overestimate false positives [111].

The corrected p-values indicate the level of association between each SNP and the trait of interest. A universal threshold for genome-wide significance has not been established, although $5.5 \times 10^{-8}$ has been suggested by the International HapMap Consortium [112] and has been used in many GWAS [98,113]. However, less stringent thresholds have been proposed to account for factors such as allele frequency [113] and LD [98]. Recently, guidelines for determining appropriate significance thresholds adapted for individual GWAS have been suggested [113].

Although GWAS provide a powerful tool for genetic marker discovery, the risk of falsely identifying associations is a notable limitation [114]. Follow-on studies are

therefore required to validate GWAS findings, and should include replication of results in a separate but comparable and ideally expanded population [114]. Consideration of the functional relevance (known or demonstrable influences on the phenotype of interest) of candidate genes identified by GWAS can also assist in distinguishing false positives from true associations and serve as a guide for the direction of follow-on studies [115,116].

**The Application of Marker-Assisted Selection for Mastitis Traits**

In livestock species, selection for health or production traits may be based on phenotypic measures or the use of genetic marker-assisted methods for single-gene or quantitative traits [117]. The potential for marker-assisted methods to improve the efficiency and precision of selection over conventional methods has been demonstrated in a number of production species. In dairy cattle, for example, marker-assisted selection for decreased SCS in first-lactation heifers resulted in a higher level of discrimination between high and low SCS heifers than did a conventional strategy relying on parental relative estimated breeding values for SCS [94]. Similar conventional and marker-assisted selection strategies were used in a separate study to identify mastitis-resistant and mastitis-susceptible cattle [118]. In that study, primary MECs from cattle within the marker-assisted groups displayed marked differences in inflammatory gene expression between resistant and susceptible cattle following challenge by *E. coli* and *S. aureus*. In contrast, differences in inflammatory gene expression by MECs between resistant and susceptible cattle within the conventionally selected group were limited to a single gene,

at a single timepoint, after challenge by *E. coli* only. These and other studies illustrate the potential of marker-assisted selection in improving mastitis traits in dairy cattle.

The search for genetic markers of mastitis traits for use in selection programs has focused on identification of QTLs and candidate genes. As of March 2017, the cattle QTL database (http://animalgenome.org/cgi-bin/QTLdb/BT/index), which catalogues publicly available QTLs in cattle [119], lists 244 distinct QTLs of mastitis traits. These include 69 QTLs for clinical mastitis, 28 QTLs for SCC, and 147 QTLs for SCS. Numerous candidate genes with recognized genetic variants have been proposed as likely contributors to mastitis traits due to their roles in the pathogenesis of mastitis. Examples of promising candidate genes proposed by multiple independent studies include C-X-C motif chemokine receptor 1 (CXCR1) [120–127], TLR4 [128–131], and TLR2 [132–134]. C-X-C motif chemokine receptor 1 is a high-affinity receptor for IL-8. Receptor-ligand interaction promotes cell migration, production of reactive oxygen species, and phagocytosis, and affects cell survival regulation and cytokine production [135]. Significant upregulation of CXCR1 expression occurs rapidly in the bovine mammary gland following intramammary infusion of bacteria [136]. Toll-like receptors 2 and 4 are involved in pathogen recognition [6], and are upregulated in the bovine mammary gland during mastitis [137].

Before any selection method, including marker-assisted selection, is implemented, the effects of selection on other traits must be considered. In some instances, selection for a single trait can have detrimental effects on the selection progress of separate traits [117]. For example, an unfavorable genetic correlation for mastitis traits and milk production

traits has been demonstrated [93,138,139]. Linkage disequilibrium between causal variants of these two traits exists in some regions of the genome, with common haplotypes containing unfavorable alleles for one trait and favorable alleles for the other [138,140]. Because these two traits are both considered important in dairy cattle, care should be taken not to unacceptably compromise one trait for improvement of the other. However, selection for both traits simultaneously is possible by, for example, selecting for less common haplotypes that confer favorable alleles for both traits [140,141].

The identification and validation of genetic markers of mastitis traits, as well as their implementation in selective breeding programs, are not without challenges. Genome-wide association studies provide a powerful tool for discovery of genetic markers and QTLs, but results must be repeatable for validation of true associations [114]. Identification of candidate genes can serve as a guide for follow-on studies to define the functional relevance of variants and can advance knowledge of disease pathogenesis. Additionally, in applying marker-assisted techniques to livestock improvement, the effects of selection for a single trait on other important health or production traits must be considered.

**Research Objectives and Findings**

The first objective of this dissertation was to identify QTLs of mastitis resistance based on SNPs in Holstein dairy cattle. Phenotypic characterization of lactating Holstein cattle was based on monitoring for clinical mastitis and milk bacterial culture, supplemented by monthly SCC measurements, over an eight-month period. Phenotypic

extremes (highly mastitis-resistant cattle and highly mastitis-susceptible cattle) were identified and included in a GWAS using a selective genotyping approach. Using a SLMM analysis, 117 SNPs suggestive of genome-wide significance ($p<1E-04$) for mastitis resistance were identified. From these, 27 QTLs, including 10 novel QTLs, of mastitis resistance were identified.

The remaining objectives of this dissertation involved assessment of bovine MEC inflammatory responses. In order to accomplish these objectives, a method to establish primary bovine MEC lines from a small volume of bovine milk was developed. The first objective was to compare expression of inflammatory genes between bMECs from mastitis-resistant cows, before and after stimulation of an inflammatory response, to those of bMECs from mastitis-susceptible cows. The expression of genes relevant to inflammation by unchallenged and LPS-challenged bovine MECs from mastitis-resistant and mastitis-susceptible cattle were identified. An enhanced ability of bovine MECs from mastitis-resistant cattle over those from mastitis-susceptible cattle to rapidly respond to LPS challenge by differential expression of inflammatory genes was demonstrated. Secondly, the expression of pro-inflammatory genes by unchallenged and LPS-challenged bovine MECs and bovine fibroblasts was compared, and a similar expression pattern of interleukin 1 beta between the two cell types but a divergent expression pattern of C-C motif chemokine ligand 20 was demonstrated. Next, the effect of exogenous phospholipase A2 (PLA2), an enzyme involved in inflammatory mediator generation, on the expression of pro-inflammatory genes by unchallenged and LPS-challenged bovine MECs was examined. It was determined that PLA2 influences bMEC pro-inflammatory

gene expression in response to LPS challenge but does not influence constitutive expression. Finally, a milk-derived primary bovine MEC line with an extended capacity for division through transfection with simian virus 40 large T antigen was established. This cell line, as well as the methods used in its establishment, may be used to facilitate future studies that require large numbers or extended growth of primary bMECs.

# CHAPTER 2

# A GENOME-WIDE ASSOCIATION STUDY FOR MASTITIS RESISTANCE IN PHENOTYPICALLY WELL-CHARACTERIZED HOLSTEIN DAIRY CATTLE USING A SELECTIVE GENOTYPING DESIGN

**Abstract**

Background

A decrease in the incidence of bovine mastitis, the costliest disease in the dairy industry, can be facilitated through genetic marker-assisted selective breeding programs. Identification of genomic variants associated with mastitis resistance is an ongoing endeavor for which genome-wide association studies (GWAS) using high-density arrays provide a valuable tool.

Results

We identified single nucleotide polymorphisms (SNPs) in Holstein dairy cattle associated with mastitis resistance in a GWAS by using a high-density SNP array. Mastitis-resistant (15) and mastitis-susceptible (28) phenotypic extremes were identified from 224 lactating dairy cows on commercial dairy farm located in Utah based on multiple criteria of mastitis resistance over an 8-month period. Twenty-seven quantitative trait loci (QTLs) for mastitis resistance were identified based on 117 SNPs suggestive of genome-wide significance for mastitis resistance ($p<0.0001$), including 10 novel QTLs. Seventeen QTLs overlapped previously-reported QTLs of traits relevant to mastitis, including four

QTLs for teat length. One QTL includes the RAS guanyl releasing protein 1 (RASGRP1) gene, a candidate gene for mastitis resistance.

Conclusions

This GWAS identifies 117 candidate SNPs and 27 QTLs for mastitis resistance using a selective genotyping approach, including ten novel QTLs. Based on overlap with previously-identified QTLs, teat length appears to be an important trait in mastitis resistance. The RASGRP1 gene, overlapped by one QTL, is a candidate gene for mastitis resistance.

**Key words**

Genome-wide association study; bovine mastitis resistance; selective genotyping; cattle

## Background

Mastitis, defined as inflammation of the mammary gland, is the costliest disease in the dairy industry [1,2]. In the United States, the cost of bovine mastitis is estimated at a value of approximately 10% of total milk sales [89]. Associated costs include loss of production, decreased milk quality, discarded milk, labor, veterinary treatments, mastitis-related culls, diagnostics, and preventative measures [3].

Conventional methods to reduce the incidence of mastitis within a herd encompass both management practices and selection for mastitis-resistant phenotypes. Recent technical advancement in cattle genomics, such as genome-wide association

studies (GWAS), has led to the identification of quantitative trait loci (QTLs) associated with mastitis traits [75,142,143]. Genetic selection for mastitis resistance traits provides a valuable tool for decreasing mastitis incidence. Genetic marker-assisted selection for mastitis traits results in a higher level of discrimination between phenotypes and a greater uniformity than does conventional selection [94], highlighting the importance of identifying robust genetic markers for mastitis resistance. Genome-wide association studies are well-suited to identifying genetic markers of complex traits such as mastitis, enabling genotyping of large numbers of potential genetic markers, such single nucleotide polymorphisms (SNPs), across the genome [98,99]. Indeed, GWAS carried out over the past several years have identified genetic markers, candidate genes, and QTLs for individual mastitis traits such as somatic cell count (SCC), somatic cells score (SCS) and clinical mastitis [142,144,145]. Many of these studies use low- or medium-marker density arrays to detect genetic markers [75,145,146]. High-density bovine arrays capable of genotyping close to one million SNPs are available for cattle and offer the advantage of increased genomic coverage and statistical power [144]. Studies using such high-density arrays have the potential to identify novel genetic markers as well as verify the significance of previously identified markers.

In this study, we performed a GWAS using a high-density array to identify SNP genetic markers and define QTLs of mastitis resistance in Holstein dairy cows. We used a selective genotyping approach, identifying the most mastitis-resistant and mastitis-susceptible animals within the sample population. This approach facilitated detection of causative alleles due to an enrichment effect of these alleles among phenotypically

extreme individuals [104]. Phenotypic characterization was based on multiple criteria of intramammary infection status in order to achieve more accurate characterization of phenotypic extremes of mastitis resistance and mastitis susceptibility than could be achieved with use of a single measure of mastitis alone.

## Methods

The aim of this study is to identify SNP genetic markers and QTLs of mastitis resistance in dairy cattle. A genome-wide association study was performed using a selective genotyping approach.

### Selection of phenotypically extreme cattle

Cattle used in the study were adult lactating Holstein cattle from a single farm, and phenotypically extreme individuals of mastitis resistance and mastitis susceptibility were identified and selected for genotyping. Phenotypic characterization was based on a combination of milk bacterial culture, observation for clinical mastitis, and SCC evaluation over an eight-month period. Subclinical mastitis was defined as cases in which intramammary infection was detected by bacterial culture of milk but no changes were detected in the appearance of the mammary gland or milk. Clinical mastitis was defined as intramammary infection accompanied by clinically detectable inflammatory changes in the mammary gland and/or changes in the consistency or color of the milk.

To detect of clinical and subclinical mastitis, monthly bacterial cultures were performed by using aseptically collected composite milk samples. Milk microbial culture

was carried out according to the guidelines outlined by the National Mastitis Council [147]. Isolation of at least one bacterial colony from a 0.01 ml inoculum of a single milk culture sample was considered sufficient to diagnose intramammary infection, as proposed by the Mastitis Research Workers [71]. Detection of clinical and subclinical mastitis was also based on bi-monthly veterinary clinical evaluations to detect abnormal mammary gland quarters or secretions; continuous monitoring for clinical mastitis by farm staff; and individual quarter bacterial culture of milk from suspect intramammary infection cases at the time of detection. Monthly SCCs were used as supplementary evidence for the absence of intramammary infection in animals from which no bacteria were isolated from milk samples and no clinical mastitis was detected. Monthly SCC measures were obtained from the Dairy Herd Improvement Association (DHIA).

Chronic (continuing) cases of clinical and subclinical mastitis were distinguished from new cases on the basis of time elapsed between detection of sequential episodes, time elapsed after treatment from a previous episode, quarter(s) affected, and etiologic agent [148–150]. For the purposes of this study, cattle with multiple new episodes of mastitis were selected as mastitis-susceptible over those with few but chronic episodes. Criteria for classification as mastitis-resistant included an absence of clinical mastitis, an absence of bacteria cultured from milk samples throughout the eight-month period, and consistently low SCCs (<250,000 cells/ml). The criterion for classification as mastitis-susceptible was detection of at least four separate episodes of mastitis. Episodes of mastitis were defined by any of: isolation of one or more mastitis pathogens from a milk sample, detection of clinical mastitis, and/or elevated composite SCC >250,000 cells/ml.

Isolates from more than one quarter on one date contributed as many mastitis episodes as there were culture-positive quarters. Clinical mastitis detected in more than one quarter on one date contributed as many mastitis episodes as there were clinically mastitic quarters.

DNA isolation

Genomic DNA of cows characterized as mastitis-resistant or mastitis-susceptible was isolated from ear notches or hair follicles. Isolation and purification of DNA was carried out using the Gentra Puregene Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

SNP genotyping

Genotype calling was carried out by the Core Facility at the University of Utah for SNP genotyping using the Illumina BovineHD BeadChip (part # WG-450-1002; Illumina Inc., San Diego, CA), an array with 777,962 SNPs that uniformly span the entire bovine genome. Bead chips were processed according to the Infinium protocol from Illumina, and scanning carried out by the iScan scanner (Illumina Inc., San Diego, CA). Quality control measures included removal of animals with low call rates (<96%), SNPs with low call rates (<0.95), and SNPs with low minor allele frequencies (<5%).

Statistical analysis

Significant associations between SNPs and mastitis resistance were detected using a single locus mixed model approach as implemented by the SNP and Variation Suite software (SVS version 8.4, Golden Helix, Bozeman, MT). Familial relatedness was corrected for as a random effect by incorporation of a genomic best linear unbiased prediction (gBLUP) kinship matrix [107] into the model, constructed from genome-wide SNPs after pruning for linkage disequilibrium (LD). Genome-wide association mapping used a mixed linear model analysis [110] using the gBLUP matrix to correct for cryptic relatedness, with mastitis resistance/susceptibility coded as a binary phenotype. A genome-wide suggestive threshold was set at an uncorrected p-value of $p < 0.0001$, with $p < 0.001$ considered nominal.

Defining QTLs

Quantitative trait loci were defined as described previously [142]. A QTL surrounding each SNP detected as significant ($p < 0.0001$) was defined based on local LD structure. Pairwise LD between the target SNP and all individual genotyped SNPs within 1 Mb upstream and downstream was calculated using PLINK [151]. Within this region, visualized using the *ggplot* function of the R Studio statistical package [152], the furthest upstream and downstream SNPs in strong LD with the target SNP ($r^2 \geq 0.8$) were used to define QTLs. Quantitative trait loci comprised of a single SNP only were excluded. Overlapping QTLs were combined into a single QTL, defined by the furthest upstream and downstream SNPs for the combined region. Once defined, QTLs were aligned to the

bovine genome (Bos_taurus_3.1.1/bosTau8 assembly; [153]) using the University of California Santa Cruz (UCSC) Genome Browser tool (https://genome.ucsc.edu/) and dbSNP [154] to identify genes overlapping these regions. These QTLs were checked for overlap with known bovine QTLs using the cattle QTL database (http://animalgenome.org/cgi-bin/QTLdb/BT/index) as of April 2017 [155].

## Results

### Sample population

Mastitis-resistant and mastitis-susceptible cows in a commercial dairy herd with 224 lactating Holstein cows were identified following monitoring for clinical and subclinical mastitis over an eight-month period. From this rigorous mastitis-screening program, 15 animals were characterized as mastitis-resistant (Table 1A) and 28 animals as mastitis-susceptible (Table 1B). All mastitis-susceptible cows with the exception of one had four confirmed cases of mastitis. One cow had three cases of mastitis confirmed by isolation of three separate pathogens, and one tentative case where sample contamination precluded definitive pathogen isolation. Cattle within the mastitis-resistant group ranged from second to sixth lactation, and cattle within the mastitis-susceptible group ranged from first to sixth lactation. Among the mastitis-resistant group, individual composite milk SCCs over the eight-month period ranged from 5,000 to 220,000 cells/ml, with an average of 56,300 cells/ml. Among the mastitis-susceptible group, the number of clinical mastitis episodes ranged from none to three. Individual quarter and composite milk SCCs ranged from 6,000 to 2,676,000 cells/ml, with an average of

303,000 cells/ml. Commonly isolated bacterial species from composite and individual quarter milk samples from mastitis-susceptible cattle included coagulase-negative staphylococci, *Streptococcus* sp., *Corynebacterium* sp., and *Escherichia coli*.

Genome-wide associations

In order to identify SNP genetic markers and QTLs of mastitis resistance, we carried out a GWAS using a selective genotyping approach. Of 224 lactating Holstein dairy cows, we selected the top 15 most mastitis-resistant and the top 28 most mastitis-susceptible cows for genotyping.

Following data quality control measures, 585,949 SNPs remained for association testing using data from 43 animals (28 mastitis-susceptible, 15 mastitis-resistant), represented in Figure 1A as a Manhattan plot. Based on deviation from a linear relationship between observed and expected p-values, as illustrated in Figure 1B in a quantile-quantile plot, a p-value threshold of 0.0001 was set as suggestive of genome-wide significance and a p-value of $> 0.0001, \leq 0.001$ was considered nominal for association.

One hundred and seventeen SNPs were suggestive of genome-wide significance (Table 2). Based on these 117 SNPs, we identified 27 QTLs of mastitis resistance, distributed across 14 chromosomes (2, 3, 7, 8, 10, 11, 12, 15, 16, 17, 18, 26, 27, and 28) and overlapping a total of 29 genes (Table 3). Of these QTLs, 10 have not been reported previously. Thorough phenotypic characterization using both direct (clinical mastitis and milk bacterial culture) and indirect (SCC) measures to detect mastitis over an eight-

month period, as well as use of a selective genotyping approach, facilitated identification of these 10 novel QTLs.

The three QTLs most highly suggestive of genome-wide significance (-log10(p-value) ≥ 5.41) are located on *Bos taurus* autosome (BTA) 26 and overlap the sortilin related VSP10 domain containing receptor 3 (SORCS3) gene as well as a previously-identified QTL for teat length. The SORCS3 gene has no known function in bovine mastitis. Another QTL suggestive of genome-wide significance overlaps the RAS guanyl-releasing protein 1 (RASGRP1) gene, a candidate gene for mastitis resistance. Seven hundred and sixty-three SNPs were nominal for genome-wide significance (Table 12, Appendix), distributed across all autosomal and the X chromosome.

Seventeen of the QTLs we identified overlap with previously identified QTLs of mastitis traits (somatic cell score, SCC, and clinical mastitis) and/or udder conformation traits (teat length, teat number, udder attachment, and udder depth; Table 4). Our findings reinforce the discovery of these 17 QTLs and provide supporting evidence that these QTLs may influence mastitis resistance. The top 3 QTLs overlap with a known QTL for teat length, which may provide the basis of mastitis resistance at these regions.

**Discussion**

We carried out a GWAS using a selective genotyping approach and a high-density bovine SNP array, and identified 117 SNPs suggestive of genome-wide association for mastitis resistance in Holstein dairy cattle. Based on these 117 SNPs, we identified 27 QTLs of mastitis resistance, including 10 novel QTLs.

The RASGRP1 gene is located within a QTL we identified on BTA10, defined by 8 SNPs genotyped in our GWAS. *RASGRP1* is involved in the regulation of lymphocyte development, activation, and function and in T-cell receptor signaling [156]. Differential expression of *RASGRP1* as a result of pathogen challenge occurs in primary bMECs [157] and in ovine milk somatic cells [156], indicating a potential role in mastitis in ruminants. Overlap of *RASGRP1* by one of the QTLs indicates this gene as a strong candidate for mastitis resistance, warranting further investigation.

In dairy cattle, both immune functions and udder conformation traits are recognized factors affecting mastitis resistance [158]. Udder attachment and udder depth have been associated previously with SCC and clinical mastitis [159,160]. Teat placement has been associated with SCC [160], and various studies show conflicting results of the association between teat length and SCC and clinical mastitis [161]. The presence of supernumerary teats is considered a risk factor in bovine mastitis, and their surgical removal may affect subclinical mastitis incidence in heifers [162]. Seventeen QTLs that overlap with previously identified QTLs of udder conformation traits (teat length, teat number, udder attachment, and udder depth) as well as mastitis traits (somatic cell score, SCC, and clinical mastitis) were identified. Overall, 11 of these 17 QTLs overlap with QTLs for mastitis traits, and 13 overlap with QTLs for udder conformation traits.

Ten of these 17 QTLs overlap with previously identified QTLs for teat length. Six of these, including the top 3 where the strongest association signals were detected overall, are located on BTA26 and overlap with a single previously-identified QTL for teat length

[158]. The remaining overlap with QTLs for teat length on BTA16 [158], BTA18 [163], and BTA10 [163]. This finding provides strong supportive evidence for an effect of teat length on bovine mastitis resistance, highlighting the importance of udder conformation traits as factors in the pathogenesis of this disease.

A notable strength of this study lies in the methods used for phenotypic classification, wherein multiple measures were used to determine intramammary infection status over time and identify mastitis-resistant and mastitis-susceptible phenotypic extremes. The effectiveness of selection for mastitis resistance increases when more than a single trait is measured for determination of intramammary infection status. For example, the use of SCC and clinical mastitis together is approximately 20% more effective than the use of either of these traits alone in selecting for mastitis resistance [164,165]. The use of multiple measures to detect mastitis helps to overcome limitations of any one method. For example, patterns of bacterial shedding in milk during the course of infection may affect the sensitivity of milk bacterial culture to detect intramammary infection [166]. Examination for clinical mastitis alone by definition excludes cases of subclinical mastitis, potentially excluding a substantial number of intramammary infections from being detected. Indirect measures such as SCC or its derivatives (linear score and estimated breeding values for these traits) can be influenced by a number of management and cow-dependent factors such as immune status, parity, lactation stage, diurnal variation, and sudden changes in feed or water management [62,65,167]. Additionally, although low SCC is commonly accepted as indicative of an absence of intramammary infection, some studies have demonstrated low SCC as a risk

factor in the subsequent development of clinical mastitis [67,68]. Thus, the use of SCC to indicate mastitis resistance could be misleading in some cases if not supplemented by additional measures.

In consideration of the above limitations, phenotypic characterization was based on multiple criteria in order to accurately identify phenotypic extremes of mastitis resistance and susceptibility. Reliable determination of intramammary infection status is best achieved through a combination of SCC measurement, bacterial culture, and clinical detection [71], as used in this study. Regular monitoring using these three parameters facilitates detection of clinical and subclinical mastitis, including infections resulting in minor increases in SCC. Additionally, identification of the causative bacteria allows distinction between continuing and new intramammary infections, yielding a more accurate picture of the frequency of intramammary infection in individual cows (i.e., whether increased SCC or clinical mastitis over time represents an ongoing infection or multiple separate infections).

All cows within the current study were within the same herd and were subjected to the same management conditions. Thus, effects of environmental variables on mastitis susceptibility are expected to be low relative to studies in which cattle from different farms and thereby under different environmental and management conditions are included. Identification of 10 novel QTLs of mastitis resistance was likely facilitated by particularly stringent phenotypic characterization methods and sample population selection.

A potential limitation to the current study is the relatively small sample size. Out of 224 lactating cows, 43 were characterized as phenotypic extremes for mastitis resistance or susceptibility. In GWAS, sample size is one of the factors influencing statistical power, and sample sizes in the thousands are often used [96]. In this study, meticulous phenotypic characterization was chosen at the expense of large sample size in order to identify individual cattle representative of phenotypic extremes. Genotyping only individuals that represent phenotypic extremes for a trait (no more than 20-25% of the sample population) can be used to detect QTLs for single traits among a small sample size while preserving statistical power in a selective genotyping approach [103,168]. Out of 224 cows, only the highest and lowest extremes for mastitis resistance of the population at 6.7% and 12.5%, respectively, were genotyped. The use of selective genotyping provides an enrichment effect, as causal and protective variants are more likely to be concentrated in these individuals as compared with individuals sampled randomly from the population. Thus, the power to detect causal and protective variants, particularly rare variants, is increased, although the effect size will be overestimated [104]. Follow-on studies to replicate results are therefore important [104]. We believe that, in addition to phenotypic characterization methods, the use of selective genotyping along with a high-density SNP array facilitated identification of the 10 novel QTLs.

**Conclusions**

One hundred seventeen candidate SNPs and 27 QTLs for mastitis resistance within a population of phenotypically well-characterized dairy cattle were identified. The

three QTLs most suggestive of genome-wide significance are located on BTA26 and overlap *SORCS3* and a previously identified QTL for teat length. Ten of the 27 QTLs have not been reported previously, while 17 overlap previously identified QTLs for mastitis or udder conformation traits relevant to mastitis. One QTL on BTA10 overlaps *RASGRP1*, considered a candidate gene of mastitis resistance requiring further study. Validation of these QTLs as genetic markers of mastitis resistance in an expanded population is required.

Table 1A: Lactation number and phenotypic classification data for mastitis-resistant cows.

| Lactation Number | Mean SCC, x1000 cells/ml | SCC Range, x1000 cells/ml | Clinical Mastitis Episodes | Pathogens Isolated |
|---|---|---|---|---|
| 2 | 24.7 | 16-33 | 0 | None |
| 6 | 57.8 | 32-84 | 0 | None |
| 5 | 84.5 | 22-155 | 0 | None |
| 4 | 48.0 | 21-125 | 0 | None |
| 4 | 31.3 | 16-50 | 0 | None |
| 3 | 86.3 | 20-150 | 0 | None |
| 3 | 69.7 | 21-123 | 0 | None |
| 3 | 70.5 | 19-107 | 0 | None |
| 3 | 40.5 | 5-61 | 0 | None |
| 3 | 21.4 | 9-47 | 0 | None |
| 2 | 23.8 | 20-29 | 0 | None |
| 2 | 100.3 | 35-167 | 0 | None |
| 2 | 15.0 | 5-27 | 0 | None |
| 2 | 33.6 | 9-69 | 0 | None |
| 2 | 123.0 | 30-220 | 0 | None |

Table 1B: Lactation number and phenotypic classification data for mastitis-susceptible cows.

| Lactation Number | Average SCC, x1000 cells/ml | SCC Range, x1000 cells/ml | Clinical Mastitis Episodes | Pathogens[1] Isolated (Number of Times) |
|---|---|---|---|---|
| 2 | 519.7 | 21-2406 | 1 | CNS (2); CNS and St (2) |
| 6 | 217.6 | 146-1327 | 0 | CNS (1); St (2): CNS and St (3) |
| 4 | 136.1 | 88-197 | 0 | CNS (1); C (1); Y (1) |
| 4 | 617.3 | 8-1464 | 3 | St (2); CNS and St (1); C and St (3) |
| 3 | 298.3 | 120-622 | 0 | CNS (4); CNS and St (2) |
| 4 | 477.4 | 86-1899 | 0 | CNS (5); CNS and E (1) |
| 4 | 391.0 | 210-868 | 2 | CNS (2); CNS and St (3) |
| 3 | 463.0 | 270-591 | 2 | St (6) |
| 3 | 1292.7 | 401-2945 | 0 | CNS (2); CNS and St (4); E and St (1) |
| 3 | 144.2 | 26-332 | 0 | St (4) |
| 2 | 49.0 | 16-84 | 0 | CNS (5); CNS and C (1) |
| 2 | 704.6 | 6-1605 | 0 | CNS (2); St (5) |
| 2 | 154.0 | 14-937 | 0 | CNS (1); CNS and St (2); C (1) |
| 2 | 258.8 | 9-981 | 3 | CNS and St (1); St (1); C (2); E (1) |
| 2 | 20.3 | 10-30 | 0 | CNS (2); C and St (1); C (1) |
| 2 | 81.0 | 10-248 | 1 | CNS (1); CNS and St (2); CNS and C (1) |
| 1 | 33.7 | 7-58 | 0 | CNS (2); CNS and St (2); CNS and C (1) |
| 1 | 108.9 | 55-191 | 0 | CNS (5) |
| 1 | 60.0 | 48-88 | 0 | CNS (4); E (1) |
| 1 | 112.9 | 76-139 | 0 | CNS (6) |
| 1 | 32.0 | 17-58 | 0 | CNS (4) |
| 1 | 97.3 | 55-238 | 0 | CNS (5) |
| 1 | 129.4 | 79-287 | 0 | CNS (4) |
| 2 | 615.2 | 13-2676 | 1 | CNS (4); Y (1) |
| 1 | 43.3 | 27-74 | 0 | CNS (3); St (1) |
| 1 | 197.0 | 35-568 | 0 | CNS (5); CNS and St (1) |
| 1 | 18.1 | 7-33 | 0 | CNS (3); C (2) |
| 1 | 115.4 | 60-175 | 1 | CNS (2); St (2); CNS and St (1) |

[1]C: *Corynebacterium* sp.; CNS: coagulase-negative staphylococci; E: *Escherichia coli*; St: *Streptococcus* sp.; Y: yeast.

Table 2: Single nucleotide polymorphisms suggestive of genome-wide significance for bovine mastitis resistance. The allele associated with mastitis resistance is shown.

| Marker | Chr[1] | Position | -log10(p-value) | Protective allele |
|---|---|---|---|---|
| rs43503386 | 7 | 31648926 | 6.33 | A |
| rs110130285 | 26 | 26080988 | 5.81 | G |
| rs110925919 | 26 | 26081853 | 5.81 | T |
| rs135137805 | 26 | 26083915 | 5.81 | C |
| rs109051904 | 26 | 26085037 | 5.81 | A |
| rs134424973 | 26 | 26086114 | 5.81 | G |
| rs136355517 | 26 | 26202415 | 5.55 | A |
| rs137057269 | 26 | 26207987 | 5.55 | G |
| rs109151150 | 7 | 31002352 | 5.51 | T |
| rs135679846 | 26 | 26213600 | 5.42 | C |
| rs136832332 | 26 | 26214187 | 5.42 | T |
| rs135349914 | 26 | 26216213 | 5.42 | C |
| rs135745332 | 26 | 26170699 | 5.41 | C |
| rs29026516 | 26 | 26171235 | 5.41 | G |
| rs133973225 | 26 | 26190210 | 5.41 | A |
| rs42094305 | 26 | 26078080 | 5.14 | C |
| rs42094275 | 26 | 26097110 | 5.14 | C |
| rs110448143 | 8 | 103092247 | 4.98 | G |
| rs110566862 | 8 | 103096670 | 4.98 | T |
| rs134258818 | 26 | 26093838 | 4.92 | T |
| rs109674792 | 17 | 41771455 | 4.85 | G |
| rs110306521 | 17 | 41773340 | 4.85 | A |
| rs109747092 | 17 | 41775569 | 4.85 | C |
| rs110239244 | 17 | 41777130 | 4.85 | C |
| rs109757388 | 17 | 41785932 | 4.85 | G |
| rs41837662 | 26 | 28202019 | 4.80 | T |
| rs109555679 | 24 | 53848687 | 4.73 | C |
| rs41257394 | 18 | 49690172 | 4.64 | A |
| rs110711227 | 15 | 47742405 | 4.57 | A |
| rs109993951 | 15 | 47747052 | 4.57 | A |
| rs137210653 | 15 | 47752356 | 4.57 | C |
| rs109366311 | 15 | 47769743 | 4.57 | C |
| rs110973322 | 15 | 47771595 | 4.57 | A |
| rs110039012 | 15 | 47774554 | 4.57 | C |
| rs110259421 | 15 | 47775426 | 4.57 | C |

| | | | | |
|---|---|---|---|---|
| rs136099077 | 7 | 32661575 | 4.55 | G |
| rs133992636 | 7 | 32662549 | 4.55 | G |
| rs135340284 | 7 | 32667624 | 4.55 | A |
| rs29016545 | 7 | 32677080 | 4.55 | T |
| rs134516100 | 7 | 32678638 | 4.55 | T |
| rs41836660 | 26 | 28154738 | 4.55 | C |
| rs41604819 | 26 | 28155692 | 4.55 | T |
| rs41837669 | 26 | 28204944 | 4.55 | G |
| rs133596831 | 16 | 20614247 | 4.48 | C |
| rs133973886 | 2 | 118870124 | 4.47 | T |
| rs41858359 | 18 | 5268101 | 4.44 | T |
| rs41858365 | 18 | 5268998 | 4.44 | T |
| rs109361888 | 26 | 26164774 | 4.42 | T |
| rs135248266 | 9 | 93691403 | 4.42 | C |
| rs135549815 | 15 | 51302501 | 4.40 | C |
| rs136596564 | 15 | 51303719 | 4.40 | C |
| rs110825365 | 17 | 41777826 | 4.37 | A |
| rs137547715 | 3 | 91427052 | 4.35 | T |
| rs136877205 | 17 | 10393778 | 4.35 | T |
| rs110090917 | 11 | 96629841 | 4.32 | T |
| rs381266606 | 11 | 96715963 | 4.32 | T |
| rs136634740 | 11 | 96720014 | 4.32 | G |
| rs133879444 | 11 | 96730359 | 4.32 | T |
| rs134973228 | 11 | 96734171 | 4.32 | T |
| rs132794203 | 11 | 96739382 | 4.32 | G |
| rs134297845 | 11 | 96745000 | 4.32 | G |
| rs109277843 | 11 | 96758826 | 4.32 | C |
| rs135608670 | 11 | 96767134 | 4.32 | T |
| rs134694194 | 17 | 10162372 | 4.31 | T |
| rs109623385 | 10 | 34452835 | 4.29 | A |
| rs109758936 | 10 | 34455599 | 4.29 | T |
| rs133303871 | 11 | 86660988 | 4.28 | T |
| rs42434953 | 26 | 28796634 | 4.28 | T |
| rs42434958 | 26 | 28799734 | 4.28 | A |
| rs42434984 | 26 | 28813937 | 4.28 | A |
| rs42349819 | 3 | 91442018 | 4.27 | A |
| rs42349795 | 3 | 91449020 | 4.27 | A |
| rs109782486 | 7 | 31997138 | 4.25 | C |
| rs109397365 | 7 | 31999677 | 4.25 | T |
| rs135897745 | 7 | 32000505 | 4.25 | T |

| | | | | |
|---|---|---|---|---|
| rs109305062 | 7 | 32001031 | 4.25 | C |
| rs135287427 | 7 | 32001859 | 4.25 | A |
| rs132918628 | 7 | 32002610 | 4.25 | C |
| rs109653519 | 7 | 32003545 | 4.25 | T |
| rs109153790 | 7 | 32004528 | 4.25 | C |
| rs133045718 | 7 | 32005033 | 4.25 | T |
| rs137193453 | 7 | 32005542 | 4.25 | G |
| rs133716861 | 3 | 91429028 | 4.23 | A |
| rs42704013 | 12 | 76820530 | 4.22 | A |
| rs136350185 | 10 | 27915567 | 4.19 | A |
| rs41840890 | 26 | 27751543 | 4.17 | G |
| rs41840882 | 26 | 27754542 | 4.17 | T |
| rs41840873 | 26 | 27756172 | 4.17 | G |
| rs41840864 | 26 | 27762180 | 4.17 | A |
| rs137165178 | 26 | 27763096 | 4.17 | T |
| rs41840922 | 26 | 27779611 | 4.17 | A |
| rs41840912 | 26 | 27790973 | 4.17 | G |
| rs135563166 | 26 | 28157430 | 4.16 | G |
| rs41636626 | 26 | 28159060 | 4.16 | C |
| rs133999463 | 26 | 28159800 | 4.16 | C |
| rs133395250 | 26 | 28161303 | 4.16 | G |
| rs135413917 | 26 | 28161892 | 4.16 | G |
| rs133282066 | 26 | 28163345 | 4.16 | A |
| rs135170589 | 26 | 28165109 | 4.16 | T |
| rs136506930 | 26 | 28165749 | 4.16 | G |
| rs134913097 | 26 | 28167337 | 4.16 | A |
| rs137741079 | 26 | 28168568 | 4.16 | C |
| rs133840132 | 26 | 28170114 | 4.16 | A |
| rs135204195 | 26 | 28172302 | 4.16 | A |
| rs133679609 | 17 | 42208979 | 4.14 | C |
| rs41645946 | 11 | 96814663 | 4.12 | C |
| rs133086162 | 27 | 24405764 | 4.11 | C |
| rs132797061 | X | 10123521 | 4.10 | T |
| rs110373429 | 26 | 27935893 | 4.10 | C |
| rs110554155 | 4 | 41207992 | 4.08 | T |
| rs43506093 | 7 | 32187800 | 4.07 | C |
| rs134956968 | 28 | 30879841 | 4.07 | G |
| rs110413607 | 10 | 34258059 | 4.06 | T |
| rs41668080 | 12 | 76870470 | 4.05 | T |
| rs110442181 | 11 | 102314941 | 4.02 | C |

| | | | | |
|---|---|---|---|---|
| rs41879775 | 18 | 43568128 | 4.00 | G |
| rs135753929 | 18 | 43596859 | 4.00 | A |

[1]Chr: chromosome.

Table 3: Quantitative trait loci identified for bovine mastitis resistance. Overlapped genes are shown.

| Chr | QTL start | QTL end | QTL length (bp) | No. SNPs | Tag SNP | -log10(p-value) | No. Genes | Genes |
|---|---|---|---|---|---|---|---|---|
| 26 | 26078080 | 26097110 | 19030 | 7 | rs110130285 | 5.8056407 | 1 | SORCS3 |
| 26 | 26202415 | 26216213 | 13798 | 5 | rs136355517 | 5.5513441 | 1 | SORCS3 |
| 26 | 26170699 | 26190210 | 19511 | 3 | rs135745332 | 5.4109618 | 1 | SORCS3 |
| 8 | 103092247 | 103096670 | 4423 | 2 | rs110448143 | 4.9800087 | 0 | None |
| 17 | 41733436 | 41785932 | 52496 | 10 | rs109674792 | 4.851678 | 1 | FAM198B |
| 26 | 28154738 | 28204944 | 50206 | 15 | rs41837662 | 4.79832714 | 1 | SORCS1 |
| 18 | 49684020 | 49690172 | 6152 | 2 | rs41257394 | 4.6439513 | 2 | FBL, PSMC4 |
| 15 | 47742405 | 47775426 | 33021 | 7 | rs110711227 | 4.5675268 | 1 | OR52E4 |
| 7 | 32661575 | 32678638 | 17063 | 5 | rs136099077 | 4.5538269 | 0 | None |
| 16 | 20608750 | 20623978 | 15228 | 12 | rs133596831 | 4.4783498 | 1 | ESRRG |
| 2 | 118870124 | 118870999 | 875 | 2 | rs133973886 | 4.4696943 | 1 | FBXO36 |
| 18 | 5268101 | 5268998 | 897 | 2 | rs41858359 | 4.4439869 | 0 | None |
| 15 | 51068247 | 51303719 | 235472 | 3 | rs135549815 | 4.40396843 | 1 | LOC618050 |
| 17 | 10393778 | 10411003 | 17225 | 2 | rs136877205 | 4.3453674 | 1 | ARHGAP10 |
| 11 | 96629841 | 96777054 | 147213 | 11 | rs110090917 | 4.32303429 | 1 | PBX3 |
| 10 | 34258059 | 34455599 | 197540 | 8 | rs109623385 | 4.2941828 | 2 | RASGRP1, LOC104973119 |
| 26 | 28796634 | 28813937 | 17303 | 3 | rs42434953 | 4.276744 | 0 | None |
| 3 | 91429028 | 91852910 | 423882 | 4 | rs42349819 | 4.27157104 | 1 | USP24 |
| 7 | 31997138 | 32005542 | 8404 | 10 | rs109782486 | 4.2477407 | 1 | PRDM6 |
| 10 | 27798183 | 28002566 | 204383 | 31 | rs136350185 | 4.1880203 | 3 | LOC784925, LOC785050, LOC785050 |
| 26 | 27751543 | 27790973 | 39430 | 7 | rs41840890 | 4.1697935 | 0 | None |
| 17 | 42046346 | 42208979 | 162633 | 7 | rs133679609 | 4.13810535 | 0 | None |
| 27 | 24405764 | 24803258 | 397494 | 2 | rs133086162 | 4.1081123 | 1 | TNKS |
| 28 | 30834105 | 30879841 | 45736 | 8 | rs134956968 | 4.07061418 | 2 | MIR584-3, KAT6B |
| 12 | 76826267 | 76870470 | 44203 | 3 | rs41668080 | 4.0468275 | 2 | CLDN10, DZIP1 |
| 11 | 102314941 | 102336231 | 21290 | 8 | rs110442181 | 4.0153069 | 3 | NTNG2, SETX, LOC101906746 |
| 18 | 43568128 | 43596859 | 28731 | 5 | rs135753929 | 4.00357851 | 3 | FAAP24, RHPN2, CEP89 |

Table 4: Overlap of QTLs for bovine mastitis resistance with previously-reported QTLs[1] for bovine mastitis or udder conformation traits.

| Chr | QTL position (bp) | Tag SNP | -log10(p-value) | Known QTLs | Known QTL ID[1] | Known QTL position (bp) |
|---|---|---|---|---|---|---|
| 26 | 26078080-26097110 | rs110130285 | 5.8056407 | Teat length | 1651 | 25267910-30988113 |
| 26 | 26202415-26216213 | rs136355517 | 5.5513441 | Teat length | 1651 | 26:25267910-30988113 |
| 26 | 26170699-26190210 | rs135745332 | 5.4109618 | Teat length | 1651 | 25267910-30988113 |
| 8 | 103092247-103096670 | rs110448143 | 4.9800087 | None | | |
| 17 | 41733436-41785932 | rs109674792 | 4.851678 | Teat number | 20841 | 34618653-44087629 |
| 26 | 28154738-28204944 | rs41837662 | 4.79832714 | Udder attachment | 4995 | 27602977-30988113 |
| | | | | Clinical mastitis | 4994 | 27602977-30988113 |
| | | | | Teat length | 1651 | 25267910-30988113 |
| | | | | Somatic cell score | 2785 | 27602977-30988113 |
| | | | | Somatic cell score | 2736 | 27602977-30988113 |
| 18 | 49684020-49690172 | rs41257394 | 4.6439513 | Somatic cell score | 18471 | 46178647-52998234 |
| | | | | Somatic cell score | 18470 | 46178647-52983181 |
| | | | | Teat length | 1703 | 44616854-55337025 |
| 15 | 47742405-47775426 | rs110711227 | 4.5675268 | None | | |
| 7 | 32661575-32678638 | rs136099077 | 4.5538269 | Somatic cell score | 2667 | 27358606-42831622 |
| 16 | 20608750-20623978 | rs133596831 | 4.4783498 | Teat length | 1608 | 12209667-26166559 |
| 2 | 118870124-118870999 | rs133973886 | 4.4696943 | None | | |
| 18 | 5268101-5268998 | rs41858359 | 4.4439869 | Somatic cell score | 3554 | 4992421-18045667 |
| | | | | Udder attachment | 1701 | 1891819-7214579 |
| 15 | 51068247-51303719 | rs135549815 | 4.40396843 | None | | |
| 17 | 10393778-10411003 | rs136877205 | 4.3453674 | None | | |
| 11 | 96629841-96777054 | rs110090917 | 4.32303429 | None | | |
| 10 | 34258059-34455599 | rs109623385 | 4.2941828 | Udder attachment | 10294 | 10323420-79980762 |
| | | | | Teat length | 10296 | 10323420-79980762 |
| | | | | Udder attachment | 44454 | 34275633-34275673 |
| | | | | Somatic cell score | 44457 | 34275633-34275673 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Udder depth | 44459 | 34275633-34275673 |
| | | | | Somatic cell count | 2701 | 22939631-40797089 |
| 26 | Chr26:28796634-28813937 | rs42434953 | 4.276744 | Udder attachment | 4995 | 27602977-30988113 |
| | | | | Clinical mastitis | 4994 | 27602977-30988113 |
| | | | | Teat length | 1651 | 25267910-30988113 |
| | | | | Somatic cell score | 2785 | 27602977-30988113 |
| | | | | Somatic cell score | 2736 | 27602977-30988113 |
| 3 | 91429028-91852910 | rs42349819 | 4.27157104 | None | | |
| 7 | 31997138-32005542 | rs109782486 | 4.2477407 | Somatic cell score | 2667 | 27358606-42831622 |
| 10 | 27798183-28002566 | rs136350185 | 4.1880203 | Udder attachment | 10294 | 10323420-79980762 |
| | | | | Teat length | 10296 | 10323420-79980762 |
| | | | | Somatic cell count | 2701 | 22939631-40797089 |
| 26 | 27751543-27790973 | rs41840890 | 4.1697935 | Udder attachment | 4995 | 27602977-30988113 |
| | | | | Clinical mastitis | 4994 | 27602977-30988113 |
| | | | | Teat length | 1651 | 25267910-30988113 |
| | | | | Somatic cell score | 2785 | 27602977-30988113 |
| | | | | Somatic cell score | 2736 | 27602977-30988113 |
| 17 | 42046346-42208979 | rs133679609 | 4.13810535 | Teat number | 20841 | 34618653-44087629 |
| 27 | 24405764-24803258 | rs133086162 | 4.1081123 | Clinical mastitis | 2786 | 24311474-24427274 |
| 28 | 30834105-30879841 | rs134956968 | 4.07061418 | None | | |
| 12 | 76826267-76870470 | rs41668080 | 4.0468275 | None | | |
| 11 | 102314941-102336231 | rs110442181 | 4.0153069 | None | | |
| 18 | 43568128-43596859 | rs135753929 | 4.00357851 | Somatic cell score | 9904 | 33939994-43945245 |
| | | | | Somatic cell score | 18469 | 11438802-46178647 |

[1]QTLs as listed on the cattle QTL database (http://animalgenome.org/cgi-bin/QTLdb/BT/index).

Figure 1A: Manhattan plot of genome-wide associations for mastitis resistance in 43 Holstein cows. The genome-wide significance threshold is indicated by the solid line ($p < 0.0001$). Bovine chromosome position is shown on the x-axis. Strength of association for a single-locus mixed model GWAS is shown on the y-axis.

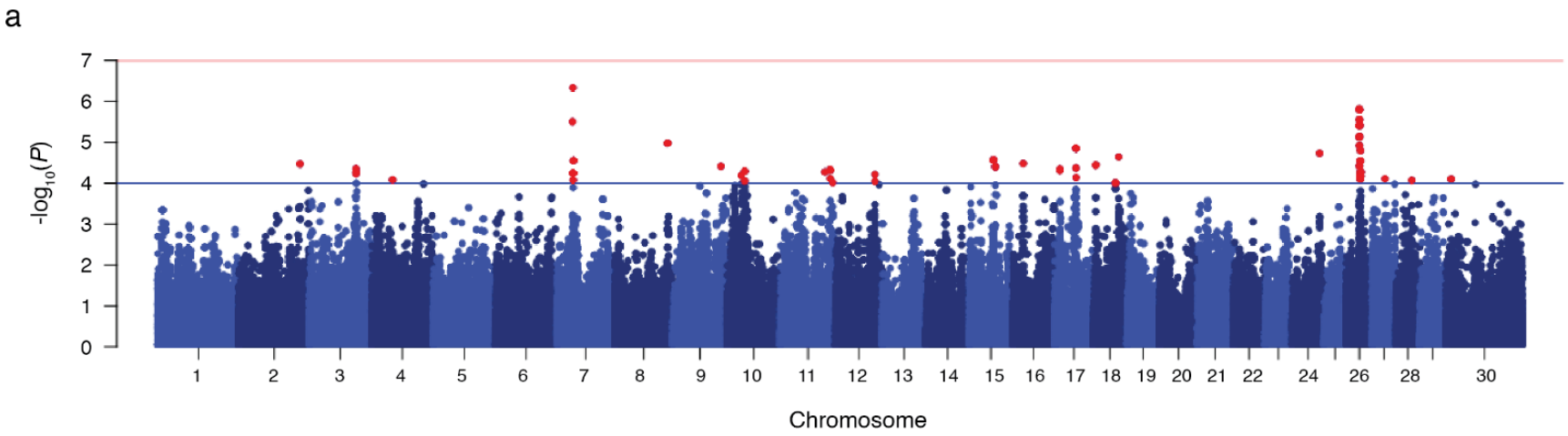
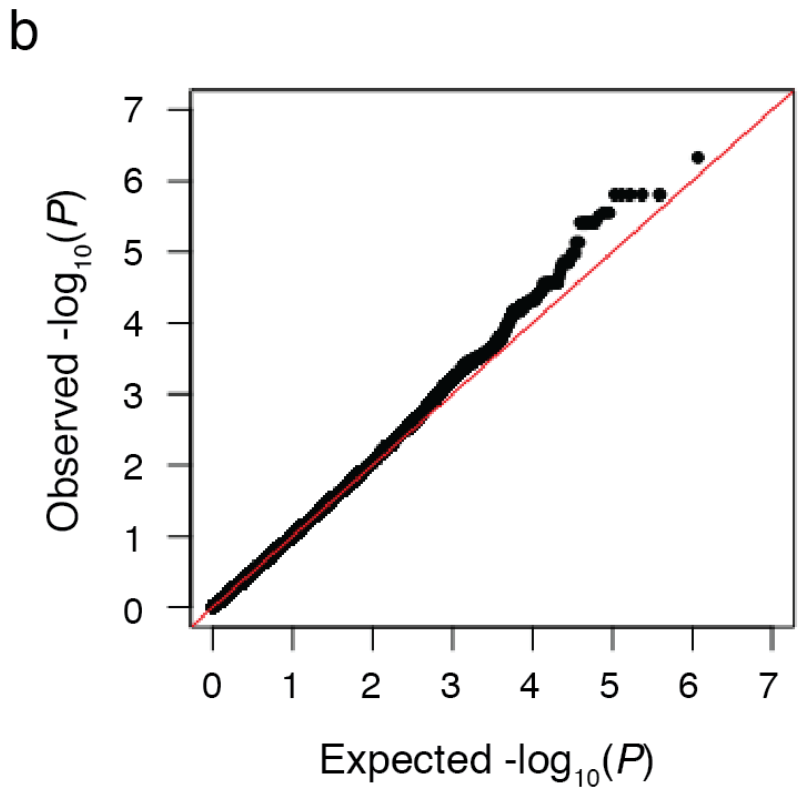


Figure 1B: Quantile-quantile plot of observed and expected p-values.



# CHAPTER 3

# BOVINE MAMMARY EPITHELIAL CELLS FROM MASTITIS-RESISTANT AND MASTITIS-SUSCEPTIBLE LACTATING HOLSTEIN COWS DISPLAY DIFFERENTIAL EXPRESSION OF INFLAMMATORY GENES IN RESPONSE TO LIPOPOLYSACCHARIDE


## Abstract

Bovine mammary epithelial cells (bMECs) are the milk-producing cells of the mammary gland, and are important contributors to innate immunity against intramammary infection. As such, differences in their innate immune responses against mastitis-causing pathogens may contribute to inherent differences in susceptibility to mastitis among individual cattle. In order to understand the immunogenetics of mastitis resistance conferred by bMECs, we carried out a gene expression polymerase chain reaction (PCR) array to characterize and compare the expression of 84 genes relevant to inflammation by bMECs isolated from mastitis-resistant and mastitis-susceptible lactating cows in response to lipopolysaccharide (LPS) from *Escherichia coli*. Primary bMEC lines were established from lactating Holstein cows characterized as mastitis-resistant or mastitis-susceptible based on clinical mastitis incidence, milk bacterial culture results, and monthly milk somatic cell counts (SCC) over an eight-month period. Exposure to LPS for 6 hours *in vitro* stimulated two-fold or greater differential expression of 43 genes ($p<0.05$) in bMECs from mastitis-resistant cows, but only one gene in bMECs from mastitis-susceptible cows. These results suggest that the ability of

bMECs to respond rapidly to mastitis pathogens may be a mechanism by which bMECs contribute to mastitis resistance. Among upregulated genes, chemokine (C-X-C motif) ligand 5 (*CXCL5*) was upregulated in bMECs from both mastitis-resistant and mastitis-susceptible cows, while chemokine (C-X-C motif) ligand 2 (*GRO1*) and interleukin 8 (*CXCL8*) were upregulated in bMECs from mastitis-resistant cows only. Tumor necrosis factor (*TNF*) was among the genes downregulated in bMEC from mastitis-resistant cows. In contrast, no significant differences in gene expression were noted between mastitis-susceptible and mastitis-resistant bMECs under unchallenged conditions, indicating that constitutive expression of inflammatory genes is unlikely to be a mechanism for mastitis resistance mediated by bMECs.

**Background**

Bovine mastitis is a major source of economic loss in the dairy industry worldwide. Strategies to reduce the prevalence of mastitis among dairy cattle are based on management of environmental factors as well as selection for cattle with increased resistance to mastitis. A genetic basis for between-cow variation in mastitis traits, such as clinical mastitis and somatic cell count (SCC), is widely recognized and is reflected in the use of estimated breeding values for these traits to guide selective breeding programs [169]. Many quantitative trait loci (QTLs) for mastitis traits have been identified (catalogued on the cattle QTL database, http://animalgenome.org/cgi-bin/QTLdb/BT/index; [155]), and genetic marker-based selection methods are

increasingly recognized as valuable tools in reducing the prevalence of mastitis within dairy herds. Cellular mechanisms underlying bovine mastitis resistance are complex, involving multiple cell types and pathogen-specific immune responses [79], and remain incompletely defined.

An important cell type involved in immune responses to intramammary infection and mastitis resistance is the bovine mammary epithelial cell (bMEC). Bovine MECs are the milk-producing cells of the mammary gland, and also have roles in innate immunity through barrier functions, pathogen recognition, and production of inflammatory mediators, including leukocyte chemotactic factors [30]. Primary bMECs have been used as a model for studies of immune responses of the mammary gland [39,170,171]. *In vitro* models based on epithelial cell culture provide several notable advantages over *in vivo* systems for studying disease processes. *In vitro* systems allow for greater control over environmental variables, and provide economic and ethical advantages over *in vivo* systems [172]. Additionally, monoculture-based systems facilitate the study of the roles of individual cell types in disease processes, whereas differentiation between the contributions of multiple cell types in *in vivo* systems may present substantial challenges [170,172]. Epithelial monoculture systems have been used extensively in the study of host-pathogen interactions in infectious disease processes [172]. The capacity of primary bMECs to mount pathogen-specific immune responses has been clearly demonstrated [38,157,170,171,173]. Expression of many inflammatory genes involved in these responses are also demonstrated in bMECs *in vivo* in response to inflammatory stimuli, reflecting the usefulness of primary bMECs as a model for the bovine mammary gland in mastitis studies [36,174,175].

Variation in the immune responses of bMECs from different individual cows have been demonstrated, including significant differences between the responses of bMECs from mastitis-resistant and mastitis-susceptible cows. For example, when primary bMECs isolated from animals classified as exhibiting high and low susceptibility to mastitis with the QTL on *Bos taurus* autosome (BTA) 18 were challenged by mastitis pathogens, differential expression of several key innate immune genes was observed, including Toll-like receptor 2, tumor necrosis factor-alpha, interleukin-8 (IL-8), IL-6, chemokine (C-C motif) ligand 5, complement component C3, and lactoferrin [176]. In primary bMECs isolated from mastitis-resistant cows, compared to those isolated from mastitis-susceptible cows, a number of genes involved in leukocyte migration and acute-phase response signaling were expressed earlier and at higher levels following pathogen challenge [157]. These studies indicate an important role of bMECs in mastitis resistance that warrants further study.

The objective of this study was to compare immune responses of bMECs from mastitis-resistant and mastitis-susceptible cows in order to identify potential contributions of bMECs to mastitis resistance. The relative expression of 84 immune-related genes was determined by polymerase chain reaction (PCR) array, and compared between the two cell lines before and after challenge by lipopolysaccharide (LPS) from *Escherichia coli*.

## Methods

### Cow characterization

Lactating Holstein cows were classified as resistant or susceptible to mastitis based on individual incidence of clinical and subclinical mastitis over an eight-month period. Detection of clinical and subclinical mastitis was based on monthly bacterial and mycoplasma cultures of aseptically collected composite milk samples; bi-monthly clinical evaluations to detect abnormal mammary gland quarters or secretions; continuous monitoring for clinical mastitis by farm staff; bacterial culture of milk from suspect intramammary infection cases; and monthly SCC measurements obtained from the Dairy Herd Improvement Association. Milk microbial cultures were carried out according to the guidelines outlined by the National Mastitis Council [147].

The criterion for classification as mastitis-susceptible was detection of at least four separate episodes of mastitis. Episodes of mastitis were defined by any of: isolation of one or more mastitis pathogens from a milk sample, detection of clinical mastitis, and/or elevated composite SCC >250,000 cells/ml. Isolates from more than one quarter on one date contributed as many mastitis episodes as there were culture-positive quarters. Clinical mastitis detected in more than one quarter on one date contributed as many mastitis episodes as there were clinically mastitic quarters. Cattle with no clinical mastitis detected, consistently negative milk bacterial culture results, and consistently low (<250,000 cells/ml) milk SCC were characterized as mastitis-resistant.

Bovine MEC collection, isolation, and establishment

Primary bMEC lines were established from bovine milk. Four hundred milliliters of hand-stripped milk were aseptically collected into an equal volume of collection media consisting of Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), and nystatin (50 mg/L). Samples were transported on ice to the laboratory.

Bovine MEC isolation was carried out as previously reported [39,177], with some modifications. Briefly, 400 ml of hand-stripped milk were subjected to a series of wash and centrifugation steps (3000 rpm for 10 minutes each) followed by passage of the resuspended cell pellet through a 100 μm pore size cell strainer to separate cell pellets from debris and other milk components. Following a final centrifugation step, the cell pellet was resuspended in 5 ml of growth media consisting of HuMEC Ready Medium (Life Technologies, Grand Island, NY) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), nystatin (50 μg/ml), and 10% fetal bovine serum, and seeded into a T-25 culture flask. Cells were incubated at 38.5°C with 5% $CO_2$. After 12-18 hours, media was exchanged for fetal bovine serum-free growth media. Growth media was changed every 2-3 days thereafter.

Cells were passaged when confluency was reached using trypsin 0.05%/EDTA 0.02% (product 59417C, Sigma-Aldrich, St. Louis, MO). Cells were maintained to the second to third passage, at which point LPS challenge was carried out.

Cell lineage verification

Cell lines were evaluated at all passage numbers for the cobblestone morphology typical of epithelial cells on monolayer culture. Epithelial lineage was verified using PCR to detect expression of cytokeratin 8 (KER8) [178]. Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on NCBI reference sequence NM_001033610.1 and were as follows: forward primer AATCAAGTATGAGGAGCTGC; reverse primer CATCCTTAACAGCCATCTCA. Polymerase chain reaction conditions were as follows: initial denaturation at 98˚C for 30 seconds followed by 32 cycles of 98˚C for 10 seconds, 57 ˚C for 15 seconds, and 72˚C for 30 seconds. Gel electrophoresis was used to confirm the presence of PCR product. Sequencing of PCR product to confirm KER8 amplification was carried out by the Center for Integrated Biotechnology, Utah State University (Logan, UT).

LPS challenge and RNA isolation

Second to third passage bMECs were exposed to LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) to mimic exposure to a mastitis-causing pathogen. Lipopolysaccharide challenge methods were similar to those described previously [173]. Briefly, bMECs were split after the first or second passage into two T-25 culture flasks. Once 50-100% confluence was reached, media was exchanged for growth media with or without (unchallenged) 50 µg/ml LPS. Cells were incubated at culture conditions for 6 hours, after which media was removed.

Following LPS challenge, bMECs were lysed and total RNA was isolated and purified using the Purelink RNA Mini Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Spectrophotometric verification of RNA quality and generation of cDNA was carried out by QIAGEN Genomic Services (Frederick, MD).

PCR Array

The QIAGEN RT$^2$ Profiler$^{TM}$ PCR Array Cow Inflammatory Cytokines and Receptors array (product PABT-011Z, QIAGEN, Frederick, MD) was used to profile the expression of 84 genes involved in inflammation. Gene expression profiling and data analysis were carried out by QIAGEN Genomic Services (Frederick, MD). The delta-delta CT method was used for fold-change/regulation analysis, with data normalized using four internal control genes (glyceraldehyde-3-phosphate dehydrogenase; actin, beta; hypoxanthine phosphoribosyltransferase 1; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide). P-values were calculated using a Student's t-test of the replicate 2^ (- Delta CT) values for each gene in the groups being compared. Fold change/regulation values and p-values were generated for comparison of the following groupings: 1) unchallenged and LPS-challenged mastitis-resistant bMEC lines; 2) unchallenged and LPS-challenged mastitis-susceptible bMEC lines; and 3) unchallenged mastitis-resistant and mastitis-susceptible bMEC lines.

## Results

### Sample population

Bovine MEC lines were established from five mastitis susceptible and three mastitis resistant cattle. Cattle within the mastitis-resistant group ranged from second to third lactation, and cattle within the mastitis-susceptible group ranged from second to sixth lactation. All bMEC lines were morphologically consistent with epithelial cells and expressed KER8.

Among the mastitis-resistant group, composite milk SCC ranged from 16,000 to 220,000 cells/ml, with an average of 75,000 cells/ml. Among the mastitis-susceptible group, pathogens isolated from composite and individual quarter milk samples included *Streptococcus* sp., coagulase-negative staphylococci, and *Corynebacterium* sp. In this group, composite milk SCC ranged from 14,000 to 2,406,000 cells/ml, with an average of 290,000 cells/ml.

### PCR Array

*Bovine MECs from mastitis-resistant and mastitis-susceptible cows respond to LPS differently.* Gene expression following LPS challenge varied between mastitis-susceptible and mastitis-resistant bMEC lines. Among mastitis-resistant cell lines, three genes, C-X-C motif chemokine ligand 5 (CXCL5), chemokine (C-X-C motif) ligand 2 (GRO1), and interleukin 8 (CXCL8), were significantly ($p < 0.05$) upregulated by at least two-fold, while 40 genes were significantly downregulated by at least two-fold in LPS-challenged versus unchallenged bMEC lines (Table 5). Among the latter, complement

component 5 (C5), chemokine (C-C motif) receptor 5 (CCR5), and interleukin 13 (IL13) showed the highest magnitudes of downregulation, at -18.93-fold, -18.89-fold, and -18.74-fold, respectively. In contrast to the mastitis-resistant group, among mastitis-susceptible bMEC lines, only one gene, CXCL5, was differentially expressed as a result of LPS challenge. C-X-C motif chemokine ligand 5 was upregulated by 8.25-fold in LPS-challenged versus unchallenged bMECs. Upregulation of CXCL5 in both mastitis-resistant and mastitis-susceptible bMECs following LPS challenge suggests that this is a particularly important gene involved in the innate immune response of bMECs. A lack of differential expression of additional genes among mastitis-susceptible bMECs following LPS challenge contrasts with the differential expression of 42 additional genes by mastitis-resistant bMECs. This finding indicates inherent differences in the immune response capabilities of bMECs from the two groups.

*Mastitis-resistant and -susceptible bMECs show similar expression under unchallenged conditions.* Gene expression between the mastitis-susceptible and mastitis-resistant groups were investigated under unchallenged conditions. No genes were significantly differently expressed between these two groups (Table 6). Such data indicate that bMECs isolated from mastitis-resistant and -susceptible cows, while displaying distinct gene expression patterns in responding to LPS, have similar innate immune activities under unchallenged conditions. Therefore, it is likely that the contribution of bMECs to mastitis resistance depends on their capability of mounting

successful innate immune responses to mastitis pathogens, but not on the basal level activities of the inflammatory genes.

**Discussion**

The objective of this study was to investigate the molecular and cellular mechanisms by which bMECs contribute to mastitis resistance. To accomplish this, we established multiple bMEC lines from mastitis-resistant and mastitis-susceptible cows and compared the expression profiles of genes relevant to inflammation between these two groups of bMECs using the QIAGEN's $RT^2$ Profiler$^{TM}$ PCR Array before and after LPS challenge.

No significant differences in gene expression between mastitis-resistant and mastitis-susceptible bMECs under unchallenged conditions were observed. This is in agreement with other studies that demonstrate no significant differences in the expression of genes relevant to inflammation between bMECs from mastitis-susceptible and mastitis-resistant cattle under unchallenged conditions [118,157]. Such data suggest that constitutive expression of genes relevant to inflammation does not drive contributions of bMECs to mastitis resistance.

In contrast, expression of genes related to inflammation differed between mastitis-resistant and mastitis-susceptible bMECs following LPS challenge. Three genes relevant to inflammation were significantly upregulated and 40 were downregulated in response to LPS challenge in mastitis-resistant bMECs, while only one gene was significantly upregulated and none downregulated in mastitis-susceptible bMECs. This data

demonstrates a clear difference between the responses of mastitis-resistant and mastitis-susceptible bMECs after a 6-hour exposure to LPS. A similar effect has been previously demonstrated in bMECs from cattle with high and low mastitis susceptibilities based on a QTL and molecular marked-based selection strategy for milk somatic cell score [157]. In that study, bMEC lines from cattle with low mastitis susceptibility demonstrated more rapid and robust differential regulation of genes involved in cell death, neutrophil chemotaxis, complement system, leukocyte migration, and cell trafficking after challenge with *E. coli* or *S. aureus* than did bMECs from cattle with high mastitis susceptibility. Changes in the expression of some genes which were ultimately upregulated in both groups in response to pathogen challenge were not apparent until 24 hours after pathogen inoculation in bMECs from cattle with high mastitis susceptibility. In contrast, these genes were upregulated in bMECs from cattle with low mastitis susceptibility by 6 hours after pathogen inoculation, thereby demonstrating more rapid responses to pathogen challenge [157]. Differences in gene expression noted at 6 hours in this study may reflect variation in response times between mastitis-resistant and mastitis-susceptible bMECs, with bMECs from mastitis-resistant cattle displaying more rapid changes in expression of genes relevant to inflammation. This study provides further evidence that an ability for rapid inflammatory responses to mastitis pathogens may be an important mechanism for mastitis resistance mediated by bMECs.

Three genes, *CXCL5*, *GRO1*, and *CXCL8*, were upregulated in either mastitis-resistant bMECs only (GRO1 and CXCL8) or in both mastitis-resistant and mastitis-susceptible bMECs (*CXCL5*) following LPS challenge. Among other functions, these

three genes are involved in neutrophil chemotaxis and activation [179–181]. During the acute stages of mastitis, neutrophils surpass macrophages as the most numerous leukocyte type in the mammary gland, comprising >90% of the total leukocyte population in milk [42]. Neutrophils act as effector cells of innate immunity via phagocytosis of bacteria as well as through bactericidal effects of reactive oxygen species and other secreted factors [30,42,44]. The rapid upregulation of genes such as *CXCL5*, *GRO1*, and *CXCL8* is therefore an expected response following LPS challenge, and demonstrates the ability of primary bMECs to retain pathogen recognition and inflammatory response capabilities, as reported by others [39,173].

Upregulation of *CXCL5* was noted in both mastitis-resistant and mastitis-susceptible bMECs following LPS challenge. In addition to its roles as a chemoattractant and activator of neutrophils, *CXCL5* promotes angiogenesis [182] and fibrosis [183], two processes involved in tissue repair following inflammation. Expression of *CXCL5* is upregulated in *E. coli*-infected bovine mammary gland tissue [184] and in primary bMECs exposed to LPS [173], *E. coli* [185,186], *S. aureus* [186], or *Klebsiella pneumoniae* [186]. These observations, supported by our findings, highlight CXCL5 as an important chemokine in bovine mastitis. Interestingly, a QTL for clinical mastitis has been identified in Norwegian Red cattle within 1 Mb of a cluster of genes encoding CXC chemokines, including CXCL5 [187], providing further evidence that that variation in this gene could contribute to mastitis resistance.

Two genes involved in neutrophil recruitment, *GRO1* and *CXCL8*, were upregulated following LPS challenge in mastitis-resistant bMECs only. This suggests that

bMECs from mastitis-resistant cattle may have an enhanced ability over bMECs from mastitis-susceptible cows to rapidly recruit neutrophils to the mammary gland following exposure to mastitis pathogens. *In vivo*, the intensity and rapidity of neutrophil recruitment varies between individual cows, and can contribute to the speed of bacterial clearance and recovery from mastitis [30].

Among the genes downregulated in mastitis-resistant bMECs following LPS challenge, downregulation of tumor necrosis factor (TNF) is particularly noteworthy. Tumor necrosis factor is a multifunctional, pro-inflammatory cytokine with well-known roles in the pathogenesis of mastitis, and is upregulated by mammary gland macrophages and bMECs during mastitis [188]. Similarly, TNF is upregulated in primary bMECs following challenge by *E. coli* or LPS, with most studies reporting increased expression by 6 hours after challenge and more marked increases after 12-24 hours [39,118,170]. In this study, TNF expression was not significantly different following a 6-hour LPS challenge in mastitis-susceptible bMECs, but was significantly different between unchallenged and LPS-challenged, mastitis-resistant bMECs. As discussed above, the time required for mastitis-susceptible bMEC to respond to LPS may exceed the 6-hour time period used in this study, providing a possible explanation for the lack of an observed change in TNF expression.

More striking is the *pattern* of TNF expression difference noted among mastitis-resistant bMECs. Tumor necrosis factor expression was significantly downregulated, rather than upregulated, by nearly ten-fold following LPS challenge. Tumor necrosis factor is known to influence the severity of clinical signs of mastitis [24]. High levels of

TNF induce both local and systemic effects contributing to the morbidity and mortality in *E. coli* mastitis, including promoting the development of endotoxic shock during coliform mastitis [189]. High milk and plasma TNF concentrations as a result of LPS-induced mastitis have been noted conjunction with excessive milk concentrations of nitric oxide and severe systemic clinical signs [190]. Conversely, inhibition of TNF expression during mastitis appears to have a protective effect on the mammary gland. For example, decreased severity of histopathologic changes in the mammary gland in a mouse model of mastitis [191] and the severity of *E.coli* mastitis-induced local and systemic signs in cattle [189] were noted in conjunction with administration of treatments that decreased TNF expression. Given the known contributions of TNF to mastitis severity, downregulation could be an important mechanism for mastitis resistance. In fact, a parity-dependent association was identified between, and polymorphism within, the TNF gene and clinical mastitis variables in Holstein-Friesian dairy cattle [192], further supporting a potential role of this gene in mastitis resistance.

**Conclusions**

Bovine MECs from mastitis-resistant and mastitis-susceptible cattle display differences in the expression of genes relevant to inflammation in response to LPS challenge. Upregulation of *CXCL5,* a gene involved in neutrophil recruitment and activation, occurs in both groups, while upregulation of two additional genes involved in neutrophil recruitment was observed in mastitis-resistant but not mastitis-susceptible bMECs. Additionally, 40 immune genes were downregulated in mastitis-resistant

bMECs, including *TNF*, which may be beneficial in minimizing the clinical severity of mastitis. These results suggest that a possible mechanism for mastitis resistance provided by bMECs may be an enhanced capacity by mastitis-resistant bMECs as compared with mastitis-susceptible bMECs to respond rapidly to exposure to mastitis pathogens. Differences in gene expression were not observed between mastitis-resistant and mastitis-susceptible bMECs under unchallenged conditions, indicating that immune responses rather than constitutive immune gene expression are more important in the contribution of bMECs to mastitis resistance.

Table 5: Significant ($p < 0.05$) inflammatory gene expression fold-changes (≥2) in LPS-challenged relative to unchallenged bMECs from mastitis-resistant and mastitis-susceptible cows. P-values are shown.

| Gene Symbol | Gene | Fold-change | P-value |
|---|---|---|---|
| **Mastitis-resistant bMECs** | | | |
| **Upregulated** | | | |
| CXCL5 | Chemokine (C-X-C motif) ligand 5 | 9.1 | 0.004001 |
| GRO1 | Chemokine (C-X-C motif) ligand 2 | 5.84 | 0.001459 |
| CXCL8 | Interleukin 8 | 3.76 | 0.008736 |
| **Downregulated** | | | |
| C5 | Complemet component 5 | -18.93 | 0.009573 |
| CCR5 | Chemokine (C-C motif) receptor 5 | -18.89 | 0.009591 |
| IL13 | Interleukin 13 | -18.74 | 0.012933 |
| PF4 | Platelet factor 4 | -17.81 | 0.009447 |
| CCL11 | Chemokine (C-C motif) ligand 11 | -17.56 | 0.009998 |
| LOC510185 | Interleukin 2 receptor, beta | -17.34 | 0.010016 |
| CCR2 | Chemokine (C-C motif) receptor 2 | -17.34 | 0.010016 |
| CXCL10 | Chemokine (C-X-C motif) ligand 10 | -17.3 | 0.010019 |
| CD40LG | CD40 ligand | -16.29 | 0.010186 |
| CCR1 | Chemokine (C-C motif) receptor 1 | -16.22 | 0.010134 |
| IL7 | Interleukin 7 | -15.74 | 0.010203 |
| CXCR1 | Chemokine (C-X3-C motif) receptor 1 | -15.68 | 0.010319 |
| CCR4 | Chemokine (C-C motif) receptor 4 | -15.47 | 0.010258 |
| IL27 | Interleukin 27 | -15.45 | 0.010259 |
| IL3 | Interleukin 3 | -14.8 | 0.010412 |
| CXCR3 | Chemokine (C-X-C motif) receptor 3 | -14.68 | 0.010452 |
| IL16 | Interleukin 16 | -14.38 | 0.010472 |
| CCR8 | Chemokine (C-C motif) receptor 8 | -14.35 | 0.010453 |
| CX3CR1 | Chemokine (C-X3-C motif) receptor 1 | -14.23 | 0.008799 |
| CXCL9 | Chemokine (C-X-C motif) ligand 9 | -13.91 | 0.010708 |
| TNFSF14 | Tumor necrosis factor (ligand) superfamily, member 14 | -13.51 | 0.009462 |
| CSF3 | Colony stimulating factor 3 (granulocyte) | -13.17 | 0.010952 |
| IL17B | Interleukin 17B | -13.04 | 0.010758 |
| LTB | Lymphotoxin beta | -12.49 | 0.011005 |
| TNFRSF11B | Tumor necrosis factor receptor superfamily, member 11b | -11.28 | 0.010725 |
| CCL4 | Chemokine (C-C motif) ligand 4 | -11.22 | 0.012553 |

| | | | |
|---|---|---|---|
| IL9R | Interleukin 9 receptor | -10.95 | 0.009601 |
| OSM | Oncostatin M | -10.34 | 0.011304 |
| IL2RG | Interleukin 2 receptor, gamma | -10.12 | 0.011248 |
| TNF | Tumor necrosis factor | -9.96 | 0.013403 |
| IL4 | Interleukin 4 | -9.11 | 0.013379 |
| CXCR5 | Chemokine (C-X-C motif) receptor 5 | -7.9 | 0.012634 |
| IL15 | Interleukin 15 | -6.82 | 0.003445 |
| LTA | Lymphotoxin alpha | -4.92 | 0.013147 |
| CXCL12 | Chemokine (C-X-C motif) ligand 12 | -4.46 | 0.04327 |
| TNFSF13B | Tumor necrosis factor (ligand) superfamily, member 13b | -4.16 | 0.040672 |
| IL10RA | Interleukin 10 receptor, alpha | -3.71 | 0.016316 |
| CCL17 | Chemokine (C-C motif) ligand 17 | -3.61 | 0.030804 |
| IL6R | Interleukin 6 receptor | -2.5 | 0.009601 |
| TNFSF4 | Tumor necrosis factor (ligand) superfamily, member 4 | -2.16 | 0.024155 |
| **<u>Mastitis-susceptible bMECs</u>** | | | |
| **Upregulated** | | | |
| CXCL5 | Chemokine (C-X-C motif) ligand 5 | 8.25 | 0.001102 |
| **Downregulated** | | | |
| None | | | |

Table 6: Fold expression differences (≥2) in unchallenged bMECs from mastitis-resistant relative to mastitis-susceptible cattle. P-values are shown; none are significant ($p < 0.05$).

| Gene Symbol | Gene | Fold-difference | P-value |
|---|---|---|---|
| **Higher expression** | | | |
| CXCR1 | Chemokine (C-X-C motif) receptor 1 | 2.01 | 0.282959 |
| FASLG | Fas ligand | 40.67 | 0.207079 |
| IL15 | Interleukin 15 | 2.4 | 0.283571 |
| IL33 | Interleukin 33 | 4.44 | 0.291716 |
| **Lower expression** | | | |
| BMP2 | Bone morphogenetic protein 2 | -4.31 | 0.166194 |
| CCL2 | Chemokine (C-C motif) ligand 2 | -3.84 | 0.242192 |
| CCL5 | Chemokine (C-C motif) ligand 5 | -4.4 | 0.277475 |
| CXCL5 | Chemokine (C-X-C motif) ligand 5 | -3.49 | 0.067625 |
| CXCL8 | Interleukin 8 | -3.06 | 0.25974 |
| GRO1 | Chemokine (C-X-C motif) ligand 2 | -4.09 | 0.112732 |
| HPRT1 | Hypoxanthine phosphoribosyltransferase 1 | -2 | 0.116871 |
| IL10RB | Interleukin 10 receptor, beta | -2.78 | 0.255559 |
| IL1A | Interleukin 1, alpha | -2.36 | 0.269131 |
| IL1R1 | Interleukin 1 receptor, type 1 | -2.08 | 0.276355 |
| IL1RN | Interleukin 1 receptor antagonist | -3.04 | 0.276084 |
| IL6R | Interleukin 6 receptor | -2.23 | 0.276033 |
| TBP | TATA box binding protein | -2.65 | 0.252476 |
| TNFSF10 | Tumor necrosis factor (ligand) superfamily, member 10 | -2.42 | 0.279517 |
| TNFSF13 | Tumor necrosis factor (ligand) superfamily, member 13 | -2.5 | 0.2726 |
| TNFSF4 | Tumor necrosis factor (ligand) superfamily, member 4 | -2.08 | 0.457644 |
| VEGFA | Vascular endothelial growth factor A | -2.73 | 0.063398 |

# CHAPTER 4

# PRIMARY BOVINE DERMAL FIBROBLASTS DISPLAY SIMILAR INTERLEUKIN 1β EXPRESSION CHANGES TO PRIMARY BOVINE MAMMARY EPITHELIAL CELLS IN RESPONSE TO LIPOPOLYSACCHARIDE


## Abstract

Mastitis is an important disease among dairy cattle worldwide. Primary bovine mammary epithelial cells (bMECs) provide an *in vitro* model for studies of the bovine mammary gland, including investigations of the cellular mechanisms involved in mastitis. Bovine milk provides a source of viable bMECs and establishment of bMECs from this source is a non-invasive, repeatable method that is more practical than explant methods. However, establishment of primary bMECs from bovine milk is challenging due to the low concentration of bMECs, generally requiring a large volume of milk. Bovine fibroblasts (bFs) are a second resident cell type involved in immune responses to intramammary infection, and are readily established and maintained in culture. In order to systematically investigate the common or differential innate immune responses to mastitis-causing pathogens between bMECs and bFs, we established primary bMEC and bF lines from seven lactating Holstein cattle and profiled the inflammatory responses of bMECs and bFs to lipopolysaccharide (LPS) challenge using the Fluidigm Biomark HD system. Additionally, we describe a method for establishment of primary bMECs from bovine milk with reduced milk volume and FBS requirements. Under static (unchallenged) conditions, bFs demonstrate significantly different expression from

bMECs of interleukin 1α (IL1A), IL-1 receptor antagonist (IL1RN), C-C motif chemokine ligand 20 (CCL20), C-X-C motif chemokine ligand 5 (CXCL5), IL-1 receptor 1 (IL1R1), and IL-6. In response to LPS challenge, both bMECs and bFs downregulate IL-1β (IL1B), and bMECs additionally downregulate CCL20. Therefore, bFs could potentially be used as a model for bMEC IL1B responses but may not provide a universal model for bMEC inflammatory gene responses.

**Background**

Mastitis is the most prevalent production-related disease among dairy cattle and is associated with substantial economic impacts [193]. Decreased milk yield accounts for much of the cost associated with mastitis [194] and is a result of temporary or permanent functional compromise of the mammary gland by direct pathogen effects or inflammatory-mediated damage [55].

The inflammatory response to mastitis pathogens is due to contributions from multiple cell types. Bovine mammary epithelial cells (bMECs), lining the teat canal, ductal system, and alveoli, are extensively involved in innate defenses against invading pathogens. In addition to providing a physical barrier against bacterial entry into mammary tissue via tight junctions, these cells have the capacity to initiate components of the inflammatory response through the production of cytokines, chemokines, and other secreted factors. Secreted factors with bacteriostatic, bactericidal, and/or modulatory effects on inflammation include lactoferrin, citrate, xanthine oxidase, arachidonic acid metabolites, and host defense peptides [30]. Mammary epithelial cells are capable of

recognizing antigen from invading organisms through expression of pattern recognition receptors, including Toll-like receptors (TLR) 2 and 4, facilitating generation of pathogen-specific cytokine/chemokine profiles. Intramammary infection by *Escherichia coli*, an important mastitis pathogen, initiates the release of cytokines by bMECs, including interleukins (ILs), chemokines, and tumor necrosis factor (TNF) [30,195].

Because of the importance of bMECs in mastitis, bMEC immune responses have been examined in numerous *in vitro* and *in vivo* studies. *In vitro* studies provide a practical approach whereby immune responses of bMECs can be isolated from other mammary gland and systemic components within a highly controlled environment. Primary bMECs retain characteristics of MECs *in vivo*, including epithelial morphology, desmosomes, abundant tonofilaments, and formation of acinar structures [177,196], and gene expression of cytokeratins, casein, and whey proteins [197,198]. Additionally, primary bMECs are capable of eliciting pathogen-specific inflammatory responses by differential expression of multiple inflammatory components, including lactoferrin, IL-1β, IL-8, TNF-α, serum amyloid A, and β-defensin [38,170]. Furthermore, the effects of factors such as energy balance in the donor animal on bMEC responses can be studied [171]. Variation in immune responses of primary bMECs from donor animals with differing levels of susceptibility to mastitis have been demonstrated. For example, primary bMECs from animals classified as  exhibiting high and low susceptibility to mastitis based on genotype at a quantitative trait locus for udder health traits on BTA 18 showed differential expression of several key innate immune genes in response to challenge by two mastitis pathogens [118]. Additionally, a faster and stronger immune

response was initiated in primary bMECs from less susceptible animals [157]. These and other studies support the usefulness of primary bMECs in studies to elucidate specific mechanisms underlying mastitis pathogenesis, including mastitis resistance.

Primary bMECs may be established from explant culture of mammary gland tissue or from isolation from bovine milk. Explant culture requires postmortem or biopsy tissue, the acquisition of which requires euthanasia or an invasive procedure. As stated in its policy on the use of animals in research, the American Veterinary Medical Association recommends the use of non-animal methods and "refinement of experimental methods to eliminate animal pain and distress" [199], which can be achieved with the use of *in vitro* models attained by minimally-invasive methods. Bovine milk provides a source of viable bMECs for primary culture and presents a non-invasive, repeatable, and practical alternative to explant methods. Previously reported methods of bMEC establishment from milk require collection of a large volume of milk, which may be impractical under some circumstances. Here, we describe a method by which bMECs can be successfully established from a reduced volume of milk.

In addition to bMECs, bovine fibroblasts (bFs) are a resident component of the mammary gland and contribute to inflammatory responses during mastitis [200]. Differences in inflammatory responses of primary bFs between individual animals have been demonstrated to be reflective of mammary gland immune responses *in vivo* [201]. Like bMECs, bFs should be considered as potential contributors to mastitis susceptibility.

*In vitro* models based on primary cell culture are vital in differentiating the contributions of specific cell types to mastitis under highly controlled conditions. As the

inflammatory responses of both bMECs and bFs may contribute to mastitis susceptibility, defining the responses of both to inflammatory stimuli is expected to yield a more complete picture of the mechanisms of mastitis resistance than would the study of one cell type alone. Direct comparisons between the responses of these two cell types from the same individual cattle are needed to better define the mechanisms of mastitis resistance. In order to compare inflammatory responses, we isolated bMECs and bFs from seven lactating Holstein dairy cows and examined differences between the two cell types in the expression of 13 pro-inflammatory genes under unchallenged as well as lipopolysaccharide (LPS)-challenged conditions.

**Methods**

Bovine MEC collection, isolation, and establishment

Seven primary bMEC lines were established from bovine milk using methods modified from those previously reported [39,177]. Milk samples for bMEC isolation were collected from lactating Holstein cows in the milking parlor prior to attachment of the milking cluster. Prior to milk collection, teat ends were cleaned and disinfected with a 7.5% povidone-iodine solution applied with a spray bottle. Disinfectant was allowed approximately ten seconds contact time before being removed using a clean paper towel. Four hundred ml of milk was then hand-stripped from one or more mammary gland quarters into sterile, 1 liter-capacity plastic bottles containing an equal volume of collection media. Collection media consisted of Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) supplemented with penicillin (60 μg/ml), streptomycin (200

μg/ml), gentamycin (120 μg/ml), and nystatin (50 mg/L). Samples were kept on ice within an insulated container for transport to the laboratory and short-term storage (up to four hours) prior to cell isolation.

In all subsequent steps where sample containers were open, samples were handled within a cell culture hood to maintain asepsis. Milk samples were divided into two 500-ml capacity conical centrifuge bottles and centrifuged for ten minutes at 3000 rpm at room temperature. The supernatant was discarded, and the cell pellets were resuspended in 12 ml of fresh collection media and transferred into a 50 ml sterile conical tube. Resuspended cell pellets from the same individual animal were combined into the same 50 ml tube. Samples were centrifuged for ten minutes at 3000 rpm at room temperature. The supernatant was discarded, and the remaining cell pellet resuspended in 24 ml of fresh collection media and transferred to a new 50 ml conical tube through a 100 μm cell strainer. Samples were centrifuged again at 3000 rpm for ten minutes at room temperature, and the supernatant discarded. The cell pellet was resuspended in 5 ml of growth media with 10% FBS, seeded into a T-25 culture flask, and incubated at 38.5°C with 5% $CO_2$. Growth media consisted of HuMEC Ready Medium (Life Technologies, Grand Island, NY) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), and nystatin (50 μg/ml). After 12-18 hours, media was exchanged for growth media without FBS. Media was changed every 2-3 days. After large, coalescing colonies formed or the culture had reached confluence, cells were passaged using trypsin 0.05%/EDTA 0.02% (Sigma-Aldrich, St. Louis, MO). Cells were maintained to the second, at which point LPS challenge was carried out.

Bovine fibroblast collection, isolation, and establishment

Bovine fibroblast primary cultures were established as previously described [202], with some modifications, from dermal ear notch samples from the cows for which bMEC lines were established. Ear notches were rinsed in a 7.5% povidone-iodine solution followed by 70% ethanol, and transferred into collection media as described above for transport to the laboratory on ice. Under a cell culture hood, ear notches were minced to 2 $mm^3$ pieces, with exclusion of the epidermis and hair. Tissue pieces were rinsed twice with collection media and transferred to cell culture wells containing 0.25% trypsin. After a one-hour incubation at 37˚C with 5% $CO_2$, trypsin was exchanged for growth media as described above with 10% FBS. Media was exchanged every 2-3 days thereafter. Once adherent monolayer cells morphologically consistent with fibroblasts had formed around explant tissue pieces, tissue was removed. Cells were passaged when confluence was reached as described for bMECs. Cells were maintained to the second passage, at which point LPS challenge was carried out.

Cell lineage verification

Verification of epithelial lineage of representative bMECs was achieved using observation of cell morphology and cytokeratin 8 (KER8) expression as determined by polymerase chain reaction (PCR). Observations of cell morphology were made at $P_0$ and all subsequent passages using phase-contrast microscopy. Primers for KER8 were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and based on NCBI reference sequence NM_001033610.1 and were as follows: forward

primer AATCAAGTATGAGGAGCTGC; reverse primer CATCCTTAACAGCCATCTCA. Polymerase chain reaction conditions consisted of initial denaturation at 98˚C for 30 seconds followed by 32 cycles of 98˚C for 10 seconds, 57 ˚C for 15 seconds, and 72˚C for 30 seconds. Gel electrophoresis was used to confirm the presence of PCR product. Sequencing of PCR product to confirm KER8 amplification was carried out by the Center for Integrated Biotechnology, Utah State University (Logan, UT). Uniformity of cytokeratin expression was also confirmed by immunocytochemistry using a rabbit anti-cytokeratin, wide spectrum screening primary antibody (Z0622; Agilent Technologies, Santa Clara, CA), carried out by the Utah Veterinary Diagnostic Laboratory (Logan, UT).

LPS challenge and RNA isolation

Bovine MECs and bFs were exposed to LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) to mimic exposure to a mastitis-causing pathogen. Lipopolysaccharide challenge methods were similar to those described previously [173]. Briefly, bMECs and bFs were split after the second passage into two T-25 culture flasks. Once 50-100% confluence was reached, media was exchanged for growth media with or without 50 µg/ml LPS. Cells were incubated at culture conditions for 6 hours, after which media was removed.

Following LPS challenge, bMECs and bFs were lysed and total RNA was isolated and purified using the Purelink RNA Mini Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Quality of the RNA (RNA integrity number

> 8.0) was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Complementary DNA was generated using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, with reverse transcription PCR conditions as follows: primer annealing at 25˚C for 10 minutes, reverse transcription at 42˚C for 1 hour, and enzyme inactivation at 85˚C for 5 minutes.

Quantitative PCR for gene expression

Relative expression of 13 pro-inflammatory genes was assessed via quantitative PCR (qPCR). Genes were selected based on known roles in inflammation and/or bovine mastitis and included Toll-like receptor 4 (TLR4), interleukin-6 (IL6), IL33, IL1α, IL1β, IL2 receptor subunit gamma (IL2RG), IL1 receptor antagonist (IL1RN), IL1 receptor type 1 (IL1R1), C-X-C motif chemokine ligand 5 (CXCL5), interleukin 8 (CXCL8), C-C motif chemokine receptor 2 (CCR2), C-C motif chemokine ligand 20 (CCL20), and chemokine (C-X-C motif) ligand 1 (GRO1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitously expressed transcript protein (UXT) were used as reference genes. Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and are shown in Table 7. Efficiency of all primers was between 80-110%.

Quantitative PCR was performed by the Utah State University Center for Integrated Technology (Logan, UT) using BioMark technology (Fluidigm) and EvaGreen (Bio-Rad) detection chemistry. The ΔΔCt normalization method was used for relative

quantification as implemented by Fluidigm Real-Time PCR Analysis software (Fluidigm). Results were analyzed in a mixed model approach implemented in SAS® statistical software, Version 9.4 [203], using the "proc mixed" command, with a significance level set at $p < 0.05$. Comparisons in expression of the 13 genes were made between bMECs and bFs under unchallenged conditions. Additionally, expression of these genes was compared between unchallenged and LPS-challenged bMECs and between unchallenged and LPS-challenged bFs.

## Results

### Improved bMEC isolation methods

Methods described for bMEC establishment include explant culture from mammary gland biopsies or postmortem tissue and isolation from bovine milk. Establishment of primary bMECs from postmortem tissue carries the disadvantage that further cultures at a later time cannot be obtained from the same cow. Additionally, slaughter of cattle solely to obtain such tissue may be economically impractical as well as ethically questionable. Mammary gland biopsies can overcome these problems, but are time-consuming and may not be practical under some circumstances, particularly if repeated establishment of cell lines from the same animals is required. With both these methods, other cell types such as bFs are isolated along with bMECs and steps must be taken to remove these contaminating cell types [170,176]. Establishment of primary bMECs from bovine milk is a non-invasive method that minimizes contamination of bMEC lines by bFs and other cell types relative to explant-based methods [177,204].

We successfully established bMEC primary cultures from bovine milk from a reduced volume of milk (400 ml) as compared with previously-reported methods. Moreover, use of hand-milking for milk collection, use of collection media supplemented by antimicrobials, and centrifugation speed of 3000 rpm during isolation steps were important factors. Additionally, use of FBS in bMEC culture media can be minimized, as FBS was eliminated from the culture media from the first media change onward.

All established bMEC lines demonstrated cell and colony morphology uniformly consistent with their respective expected cell types. Among bMEC lines, rare to moderate numbers of individual, adherent cells with polygonal morphology, oval nuclei, and elongated cytoplasmic processes were present by 12 hours after seeding. By four days after seeding, colonies of 2-5 cells were observed, demonstrating the cobblestone arrangement and polygonal individual cell morphology consistent with MECs grown on monolayer [205–207]. Cultures had formed large, coalescing colonies by 21-28 days after seeding and were initially passaged at this time. Cellular senescence was identified by prominent flattening, enlargement, and nuclear vacuolation of cells in addition to a cessation of cell division. Senescence among a majority of cells, with no further growth of colonies, was observed in occasional cell lines at initial passage ($P_0$) and $P_1$ and these cultures were excluded from the LPS challenge study. However, most cell lines did not exhibit senescence among a majority of cells by confluence at $P_2$. All bMEC lines expressed KER8. Immunocytochemistry demonstrated cytokeratin expression in the majority of cells in a representative bMEC line.

Primary bFs formed small colonies of spindle-shaped cells bordering explant tissue pieces and reached confluence within 7-10 days. All bF lines demonstrated uniform sheets and individual cell morphology consistent with primary fibroblasts on monolayer.

Our results demonstrate that primary bMEC lines can be established from 400 ml of bovine milk. The use of a small volume of milk allows for a reduction in the time required for sample collection, improving the utility of this method.

Constitutive expression of inflammatory genes differs between bMECs and bFs

To investigate differences in constitutive expression of pro-inflammatory genes between bFs and bMECs, we evaluated the expression of 13 pro-inflammatory genes by qRT-PCR in unchallenged cell lines. Four genes, IL6, ILR1, CXCL5, and CCL20, were significantly ($p < 0.05$) more highly expressed in bMECs than bFs under unchallenged conditions, while two genes, IL1RN and IL1A, were more highly expressed in bFs (Table 8). Results show that constitutive expression of a number of pro-inflammatory genes varies between bFs and bMECs. This finding highlights the importance of examining the two cell types separately when conclusions are drawn regarding possible influences of mammary gland constitutive gene expression in mastitis.

Expression of pro-inflammatory genes between bMECs and bFs upon LPS challenge

Changes in the expression of 13 pro-inflammatory genes between the two cell types as a result of LPS challenge were compared.

Lipopolysaccharide challenge resulted in differential expression of two pro-inflammatory genes among bMECs and one pro-inflammatory gene among bFs. Among bMECs, both IL1B and CCL20 were significantly downregulated. Among bFs, IL1B was significantly downregulated (Table 9). The expression of the remaining 11 genes examined was not significantly affected by LPS challenge in either cell type. These findings demonstrate that in response to LPS, both cell types respond similarly with regard to changes in expression of IL1B, an important cytokine in bovine mastitis. The pattern of expression of CCL20, however, differs between the two cell types in response to LPS. Therefore, bFs could potentially be used as a model for bMEC IL1B responses but may not provide a good universal model for bMEC inflammatory gene responses.

**Discussion**

Primary bMEC cultures can be established from a small volume of bovine milk with minimal use of FBS

Our results reinforce that bovine milk provides a source of viable bMECs that can be collected non-invasively and repeatedly. Challenges encountered in bMEC primary culture from bovine milk include recovery of an adequate number of viable bMECs and avoidance of culture contamination by bacterial and fungal organisms. In our experience, obtaining milk through hand-milking, use of collection media and antimicrobials, and a

centrifugation speed of 3000 rpm during isolation steps contributed to the success of establishing bMEC primary cultures from a 400 ml volume of milk.

The concentration of viable bMECs detected within milk from dairy cattle tends to be low [204,208]. As such, previously-reported methods for bMEC isolation from milk require relatively large volumes of milk, at 950 ml or greater [39,171,177], which may be impractical in some settings. For example, as we experienced, sampling of cows on a commercial farm within the milking parlor may unduly disrupt the milking schedule due to the time required to collect a large volume of milk. Reducing the volume of milk needed allows disruption to be minimized.

In previously reported methods of bMEC establishment from milk [39,171,177], the technique of milk collection is unspecified. In our experience, collection of milk by mechanical milking equipment resulted in a lower rate of successful bMEC establishment, as well as a higher number of cultures discarded as a result of bacterial contamination, than did manual collection by hand-milking. We speculate that manual collection may result in less cellular damage from shearing stress to bMECs, and therefore a higher success rate, than does milking by mechanical means. In the setting of a commercial dairy farm, as was used in our study, hand milking additionally lends a higher degree of control over maintaining asepsis, thereby reducing bacterial and fungal contamination. We observed a lower frequency of bacterial contamination of bMEC cultures obtained through manual sampling than those obtained through mechanical milking, in agreement with a previous study [209].

During the initial centrifugation step in bMEC isolation, cells are separated from the majority of the liquid and fat components of the milk. A variable amount of fat separates from the liquid component and forms a layer on the surface of the milk during centrifugation, while cells, debris, and some fat form a pellet beneath the liquid layer. However, in agreement with a previous study [210], we suspect that some cells may become trapped in the upper fat layer of the milk during centrifugation. Dilution of the sample with collection media may help to minimize cell trapping by reducing the viscosity of the milk and the concentration of lipids, allowing cells to more easily pellet during centrifugation.

We experimented with low (2000 rpm) to high (4000 rpm) centrifugation speeds during bMEC isolation attempts, and found that centrifugation at 3000 rpm was optimal for establishment of bMEC lines. We believe that low-speed centrifugation may cause less damage to cells, preserving viability, but may leave many cells remaining in suspension. High-speed centrifugation may result in a larger number of cells recovered from suspension, but may cause cellular damage and decreased viability (Gertrude C. Beuhring, personal communication).

Antimicrobials in collection media and growth media were used to prevent bacterial and fungal contamination of bMEC and bF cultures. Prior to the use of antimicrobial-supplemented collection media, we observed a higher rate of contamination of bMEC and bF cultures at initial establishment or early passages. Although milk samples for bMEC establishment were collected with asepsis in mind, some level of contamination by environmental debris nevertheless occurred, evident as grossly or

microscopically visible acellular debris embedded within the cell pellet following centrifugation. We expect that the use of antimicrobials helped to control bacterial and fungal proliferation in milk and ear notch samples prior to seeding of isolated cell pellets or explant tissue, respectively, into antimicrobial-supplemented growth media. Overall, evidence of bacterial contamination was uncommon when milk was collected manually. However, fungal contamination of cultures at early passages was common in both bMEC and bF lines when no collection media was used or when amphotericin B was used as an antifungal. As a result, we substituted nystatin for amphotericin B in both collection and growth media. Concurrently, the source of the iodine disinfectant used to clean teats prior to sampling was switched from the on-farm pre-dip solution to a solution prepared aseptically in the laboratory. The use of this iodine disinfectant was also added to the bF isolation protocol, wherein ear notches were rinsed with the iodine solution prior to ethanol rinsing. The implementation of these changes successfully prevented fungal contamination in bMEC and most bF lines.

In previously reported methods of bMEC isolation and culture from milk, FBS or fetal calf serum (FCS) is used in the culture media at concentrations of 5-10% in growth media in order to facilitate cell-substrate attachment and achieve sustained growth of bMECs [38,39,170,177]. However, the use of serum in cell culture media has a number of disadvantages. The components of serum, as well as their concentrations, are incompletely defined, and may exhibit large lot-to-lot variation. Serum is additionally a potential source of hemoglobin, endotoxin, and microbial contaminants, all of which have the potential to influence cellular responses and experimental results [211]. Serum can

inhibit the growth of some epithelial cell types, including human mammary epithelial cells [206,212]; a similar effect on bMECs is possible. Furthermore, the presence of serum in media may influence inflammatory cytokine secretion or bioactivity by bMECs [213]. These considerations, in addition to the expense and ethical concerns regarding the collection of serum from bovine fetuses, justify development of cell culture methods that minimize or eliminate the use of serum in cell culture [211,214]. Human MECs have been cultured successfully in serum-free media, and may in fact be more easily cultured using alternatives to FBS [206]. In our studies, we found that FBS is required for initial attachment of bMECs, as reported previously [177]. However, using a specialized serum-free media developed for human MEC culture, we were able to eliminate FBS from the growth media from the first media change onward.

Isolation of bMECs from bovine milk has some disadvantages, such as a low number of cells relative to what can be achieved through explant culture [177]. However, in studies that do not require a large number of cells, isolation from milk provides a number of advantages over explant culture. The modifications described here to previously-reported milk isolation methods improve the versatility of bMEC isolation from milk by reducing the volume of milk needed and minimizing the requirement for serum.

The use of bFs in mastitis studies

Fibroblasts are a component of the bovine mammary gland and respond to intramammary infection by secretion of cytokines such as TNF-α and IL-6 [215]. *In vitro,*

mammary gland-derived bFs from donor animals with and without intramammary infection display differential expression of genes involved in cell junction and adhesion, immune response, and biosynthesis/metabolism cellular pathways, and additionally affect proliferation and secretion of TNF-α and IL-8 by co-cultured bMEC [215]. Dermal bFs exposed *in vitro* to LPS display increased expression of IL-8, IL-6, and some matrix metalloproteinases [201,216,217].

Similar to bMECs, individual animal variation has been observed in immune responses of primary bFs. Variability in IL-6, IL-8, and TLR-4 expression between dermal bF lines from different animals was noted upon *in vitro* exposure of these cells to LPS or IL-1β [201,216,218]. Intriguingly, responses of dermal bFs to LPS *in vitro* were predictive of the rate of mastitis resolution and return to mammary gland productive capacity upon intramammary infusion of *Escherichia coli* in lactating Holstein cows [201]. These findings indicate that primary bFs, like bMECs, have the potential to provide a suitable model for certain mastitis studies. We demonstrate that bFs and bMECs respond similarly to LPS challenge in their expression of IL1B, but demonstrate differences in CCL20 expression.

IL1B is downregulated by both bMECs and bFs following LPS challenge

The role of IL1B in bovine mastitis is well recognized. Interleukin 1β has been detected in both healthy and infected bovine mammary glands [219] and is produced by monocytes/macrophages, bMECs, and possibly mammary gland endothelial cells [213,220]. Functions during mastitis include neutrophil chemotaxis and adhesion

molecule expression by endothelial cells. The relative importance of IL1B responses in mastitis varies by pathogen [220], but increased expression in the mammary gland is observed in the acute stages of mastitis caused by several important pathogens, including *Streptococcus uberis* [221], *Escherichia coli* [195,222], and *Staphylococcus aureus* [222]. Measurement of serum IL1B and calculation of the serum IL1RN:IL1B ratio has been suggested as a predictive assay for mastitis severity in cattle [219]. Our finding that IL1B was downregulated following LPS exposure is in contrast to many studies that demonstrate upregulation of IL1B in response to inflammatory stimuli, although downregulation of IL1B in bovine mammary tissue with mastitis has been reported [223].

The finding that bFs show similar IL1B responses to LPS to bMECs suggests that bFs could be used in place of bMECs in understanding some aspects of the immune responses of mastitis. Changes in IL1B expression by bFs could potentially be used as a tool for predicting bMEC immune responsiveness to various conditions. For example, bFs could be used in the initial investigation of compounds theorized to dampen bMEC responses and thereby reduce inflammatory-mediated damage to the mammary gland during mastitis. The responsiveness of bFs in place of bMECs to LPS in the presence of these compounds could be evaluated. Because bFs grow more readily and rapidly in culture than bMECs, bF-based models have the potential to improve the efficiency of such initial investigations, the findings of which could be used as guidance for the direction of more targeted investigations using bMECs or *in vivo* models.

CCL20 is downregulated in bMECs but not in bFs following LPS challenge

CCL20 is a pro-inflammatory chemokine involved in recruitment of leukocytes [224]. Upregulation of CCL20 by the mammary gland and primary bMECs in response to mastitis pathogens has been demonstrated [225].

Although IL1B responses of bFs and bMECs were similar, the two cell types displayed distinct CCL20 responses following LPS challenge, with differential expression noted only in bMECs. This finding demonstrates that bFs cannot be assumed to universally mimic bMECs and as such cannot be used in place of bMECs for some mastitis studies.

## Conclusions

Constitutive expression of IL6, ILR1, CXCL5, CCL20, IL1RN, and IL1A differ between primary bFs and bMECs under unchallenged conditions, indicating a need to examine each cell type separately in studies of constitutive mammary gland gene expression. Following LPS challenge, both cell types respond by downregulating IL1B, while CCL20 is downregulated in bMECs only. Bovine fibroblasts may therefore be of use as a model for bMEC IL1B expression responses during mastitis but may not be appropriate when the expression of some other inflammatory genes are examined. Primary bMEC cultures can be established from 400 ml of bovine milk and with reduced FBS use by the methods described here.

Table 7: Primers used for qPCR.

| Symbol | Gene | Primer | Sequence | NCBI sequence |
|---|---|---|---|---|
| CCL20 | C-C motif chemokine ligand 20 | F | TTGATGTCAGTGCTATTGCT | NM_174263.2 |
| | | R | ACCCACTTCTTCTTTGGATC | |
| CCR2 | C-C motif chemokine receptor 2 | F | GTGCCCCTTATTTTCCACTA | NM_001194959.1 |
| | | R | GAGCCCAGAAGAGAAAGTAG | |
| CXCL5 | C-X-C motif chemokine ligand 5 | F | TGTGTTTAACCACCACACC | NM_174300.2 |
| | | R | TTGTTCTTTCCACTGTCCA | |
| CXCL8 | Interleukin 8 | F | AAACACATTCCACACCTTTC | NM_173925.2 |
| | | R | TCTTCACAAATACCTGCACA | |
| GRO1 | chemokine (C-X-C motif) ligand 1 | F | ATTCACCTCAAGAACATCCA | NM_175700.1 |
| | | R | GCACTAGCCTTGTTTAGCAT | |
| IL1A | Interleukin 1 alpha | F | GAAGAGGATTCTCAGCTTCC | NM_174092.1 |
| | | R | GATGGGCAACTGATTTGAAG | |
| IL1B | Interleukin 1 beta | F | CTTGGGTATCAAGGACAAGA | NM_174093.1 |
| | | R | TGAGAAGTGCTGATGTACCA | |
| IL1R1 | Interleukin 1 receptor type 1 | F | GAGACAATGGAAGTGGTCTT | NM_001206735.1 |
| | | R | GAAATATTAAGCCGTGCGAG | |
| IL1RN | Interleukin 1 receptor antagonist | F | CACTGACTTGAACCAGAACA | AB005148.1 |
| | | R | GCTGGAAGTAGAACTTGGTG | |
| IL2RG | Interleukin 2 receptor subunit gamma | F | AATTCCAGCTAGAACTGAGC | NM_174359.1 |
| | | R | TTCCGCAAAGTGGGTTATAA | |
| IL33 | Interleukin 33 | F | CAACCAAGAGAAAGACAAGG | NM_001075297.1 |
| | | R | CTCCACAGAGTGCTCCTTAC | |
| IL6 | Interleukin 6 | F | ACTGCTGGTCTTCTGGAGTA | NM_173923.2 |
| | | R | CTTTACCCACTCGTTTGAAG | |
| TLR4 | Toll-like receptor 4 | F | GCATGGAGCTGAATCTCTAC | NM_174198.6 |
| | | R | ATAGGGTTTCCCGTCAGTAT | |

Table 8: Genes differentially expressed between bFs and bMECs under unchallenged conditions.

| Symbol | Gene | P-value | SE |
|---|---|---|---|
| **More highly expressed in bFs** | | | |
| IL1A | Interleukin 1 alpha | 0.0003 | 1.1784 |
| IL1RN | Interleukin 1 receptor antagonist | <0.0001 | 1.1848 |
| **More highly expressed in bMECs** | | | |
| CCL20 | C-C motif chemokine ligand 20 | 0.0003 | 1.36 |
| CXCL5 | C-X-C motif chemokine ligand 5 | 0.0415 | 0.8957 |
| IL1R1 | Interleukin 1 receptor type 1 | 0.0071 | 1.4813 |
| IL6 | Interleukin 6 | 0.0026 | 2.1505 |

SE: Standard Error

Table 9: Genes downregulated following LPS challenge in bFs and bMECs.

| Symbol | Gene | P-value | SE |
|---|---|---|---|
| **Downregulated in bFs** | | | |
| IL1B | Interleukin 1 beta | 0.0322 | 1.2603 |
| **Downregulated in bMECs** | | | |
| IL1B | Interleukin 1 beta | 0.0009 | 1.2603 |
| CCL20 | C-C motif chemokine ligand 20 | <0.0001 | 1.36 |

# CHAPTER 5

# EXOGENOUS PHOSPHOLIPASE A2 AFFECTS EXPRESSION OF INTERLEUKIN-8 AND CHEMOKINE (C-X-C MOTIF) LIGAND 1 BY PRIMARY BOVINE MAMMARY EPITHELIAL CELLS IN RESPONSE TO LIPOPOLYSACCHARIDE


## Abstract

Bovine mastitis causes substantial economic losses to the dairy industry. Phospholipase A2 (PLA2) is an endogenous enzyme involved in phospholipid metabolism in all mammalian tissues and has both pro-inflammatory and anti-inflammatory functions as well as bactericidal characteristics. Modulation of PLA2 levels locally within tissue has been proposed as a therapeutic approach to various diseases, and evidence exists for an anti-inflammatory effect when administered as an intramammary treatment during murine mastitis. Little is known about the effects of exogenous PLA2 on the bovine mammary gland and its effects on bovine mastitis. We used an *in vitro* model to investigate the effects of exogenous PLA2 on primary bovine mammary epithelial cells (bMECs). We established bMEC lines from 12 lactating Holstein dairy cows and compared the expression of 13 pro-inflammatory genes under unchallenged and lipopolysaccharide (LPS)-challenged conditions with and without concurrent treatment with bovine pancreatic PLA2G1B. No differences in the expression of these genes were noted between PLA2-treated and untreated bMECs under unchallenged conditions. However, following LPS challenge, untreated bMECs exhibited significant ($p < 0.05$) downregulation of interleukin-8 (IL8), interleukin-1β (IL1B), C-C motif chemokine

ligand 20 (CCL20), and chemokine (C-X-C motif) ligand 1 (GRO1). In contrast, PLA2-treated bMECs exhibited significant downregulation of IL1B and CCL20 only, indicating that exogenous PLA2 affects the expression of some pro-inflammatory factors in immune-stimulated bMECs. Further studies are required to determine whether PLA2 affects the expression of other inflammatory genes by bMECs or other mammary gland cell types, or if a dose-dependent effect exists that was not apparent in this study.

**Background**

Bovine mastitis has a major impact on the dairy industry due to the substantial impacts of decreased milk yield, altered milk production, and the costs of diagnostic, therapeutic, and preventative measures [3,193]. Currently, the majority of dairy cattle with mastitis are treated with antibiotics, despite evidence indicating that bacteriological cure rates may not be improved with antibiotic treatment over spontaneous cure rates [226,227].

Changes in the production capacity of the mammary gland as well as alterations in milk composition are a result of damage to bovine mammary epithelial cells (bMECs), the milk-producing compartment. Damage can result from direct effects of mastitis pathogens on bMECs [228,229]. A substantial portion of damage, however, is a consequence of the host immune response itself, wherein host defense mechanisms such as neutrophil degranulation cause bystander-effect cellular injury [47]. Minimizing the damaging effects of host inflammatory responses should be considered an important factor in the management of inflammatory diseases such as mastitis. With the use of

antibiotics alone in cases of bovine mastitis, minimizing inflammation-induced host tissue damage relies solely on elimination of the infecting organism. Alternative therapeutic approaches to bovine mastitis that include strategies to minimize host-induced damage to mammary gland tissue are needed.

One proposed therapeutic approach in the treatment of a number of inflammatory diseases in various species involves modulation of phospholipase A2 [230]. The phospholipase $A_2$ superfamily of enzymes are involved in several cellular processes, including influencing inflammatory processes, predominantly via generation of lipid mediators. Inhibition of PLA2 activity has therefore been proposed in the treatment of inflammatory diseases [231]. However, because of the involvement of PLA2 in other cellular processes such as normal phospholipid metabolism, universal PLA2 inhibition has potentially detrimental effects, including impairment of cell viability [230]. Therefore, selective modulation of specific PLA2 subtypes, such as secreted PLA2 (sPLA2), may be a more optimal approach [230].

Pro-inflammatory actions of sPLA2 are mediated through intrinsic enzymatic and bactericidal activity as well as via interaction with the PLA2 receptor expressed on various mammalian cell types [232,233]. Secreted PLA2 enzymatic activity results in cell membrane and extracellular phospholipid hydrolysis and the consequent release of arachidonic acid, which is subsequently converted into eicosanoids to potentiate inflammation [232,234]. Binding of sPLA2 to transmembrane and soluble forms of the PLA2 receptor (*PLA2R1*) evokes additional cell type-specific biological responses, including initiating receptor-mediated activation of eicosanoid formation independent of

sPLA2 enzymatic activity [232]. For some sPLA2 subtypes, such as sPLA2-1B, this receptor-mediated activity is particularly important, as intrinsic hydrolyzing activity toward intact cell membranes of sPLA2-1B is relatively weak [235]. Additional receptor-mediated pro-inflammatory effects include influences on plasma pro-inflammatory cytokine levels during endotoxic shock [232]. Evidence exists for sPLA2 subtype-specific effects upon receptor binding [232]. Interestingly, in addition to potentiating PLA2 function, *PLA2R1*-sPLA2 binding also has negative regulatory effects on PLA2 activity. The intrinsic enzymatic activity of sPLA2 is abolished upon receptor binding [232], and receptor-mediated endocytosis of sPLA2 facilitates clearance of these enzymes [235]. Binding of sPLA2 to a circulating, soluble form of PLA2 receptor upregulated during endotoxic shock additionally blocks the biological functions of some sPLA2 subtypes [235].

Little information is available on the role of PLA2 in the pathogenesis of bovine mastitis. It is unknown whether endogenous PLA2 activity in the bovine mammary gland changes as a result of mastitis [236]. Effects of PLA2 noted in other species and tissues may be extrapolated to the bovine mammary gland only tentatively, as variation in PLA2-receptor-binding activity is noted among different mammalian species. For example, PLA2 receptor binding affinity of sPLA2 subtype sPLA2-1B is high in rodents but low in humans, and binding of sPLA2-IIA to the PLA2 receptor does not occur in humans [235]. Although PLA2 activity contributes to inflammation-induced damage in some diseases, as discussed above, there is some evidence for an *anti*-inflammatory effect of sPLA2 during mastitis. Intramammary administration of bovine PLA2G1B in mice with

experimental, LPS-induced mastitis reduced inflammation despite displaying no bactericidal activity [233]. Additionally, the effects of murine PLA2G2D are known to include the production of anti-inflammatory lipid mediators [237]. Information on the effects of PLA2 on inflammation in bovine mammary tissue is lacking. Whether PLA2 in the bovine mammary gland during mastitis has beneficial or detrimental effects has yet to be described.

Investigation of the effects of PLA2 on bovine mammary gland tissue will indicate whether further studies of PLA2 or PLA2 inhibitors as potential therapeutic agents are warranted. The objective of this study was to examine the effects of exogenously administered PLA2 on inflammatory responses of primary bMECs. To achieve this, we established primary bMEC lines from 12 lactating Holstein cows and compared the expression of 13 pro-inflammatory genes in PLA2-treated and untreated cell lines at unchallenged as well as LPS-challenged conditions.

## Methods

### Bovine MEC collection, isolation, and establishment

Milk-derived primary bMEC lines were established from adult lactating Holstein dairy cows. Four hundred milliliters of hand-stripped milk were aseptically collected into an equal volume of collection media consisting of Hank's Balanced Salt Solution (Sigma) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), and nystatin (50 mg/L).

Bovine MEC isolation was carried out as previously reported [39,177] with some modifications. Briefly, milk samples were subjected to a series of wash and centrifugation steps (3000 rpm for 10 minutes each) followed by passage of the resuspended cell pellet through a 100 μm pore size cell strainer to separate cell pellets from debris and other milk components. Following a final centrifugation step, the cell pellet was resuspended in 5 ml of growth media consisting of HuMEC Ready Medium (Life Technologies, Grand Island, NY) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), nystatin (50 μg/ml), and 10% fetal bovine serum, and seeded into a T-25 culture flask. Cells were incubated at 38.5°C with 5% $CO_2$. After 12-18 hours, media was exchanged for FBS-free growth media. Media was changed every 2-3 days thereafter.

Cells were passaged when confluency was reached using trypsin 0.05%/EDTA 0.02% (Sigma-Aldrich, St. Louis, MO). Cells were maintained to the third passage, at which point LPS challenge was carried out.

Cell lineage verification

Verification of epithelial and mesenchymal lineage of bMEC was achieved using observation of cell morphology and expression of keratin 8 (KER8). Observations of cell morphology were made at $P_0$ and subsequent passages using phase-contrast microscopy. Colonies that demonstrated a cobblestone pattern comprised of polygonal cells with round to oval nuclei typical of epithelial cells were considered likely to be of bMEC origin. Expression of KER 8, a product of both basal and luminal MECs [178], was

determined via polymerase chain reaction (PCR). Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on NCBI reference sequence NM_001033610.1 and were as follows: forward primer AATCAAGTATGAGGAGCTGC; reverse primer CATCCTTAACAGCCATCTCA. Polymerase chain reactions were: initial denaturation at 98˚C for 30 seconds followed by 32 cycles of 98˚C for 10 seconds, 57 ˚C for 15 seconds, and 72˚C for 30 seconds. Gel electrophoresis was used to confirm the presence of PCR product. Sequencing of PCR product to confirm KER8 amplification was carried out by the Center for Integrated Biotechnology, Utah State University (Logan, UT).

PLA2 treatment, LPS challenge, and RNA isolation

Each cell line was split into four treatment groups at the second to third passage: unchallenged, LPS-challenged, unchallenged with PLA2 treatment, and LPS-challenged with PLA2 treatment. Third-passage bMEC were exposed to LPS from *Escherichia coli* to mimic exposure to a mastitis-causing pathogen. Lipopolysaccharide challenge methods were similar to those described previously [173], with some modifications. Briefly, once 50-100% confluency was reached, media was exchanged for growth media with or without 50 µg/ml LPS from *Escherichia coli* (Sigma) and/or 50 µg/ml PLA2G1B from bovine pancreas (product P8913, Sigma-Aldrich, St. Louis, MO). Cells were incubated at culture conditions for 6 hours, after which media was removed. Cells were immediately lysed, and total RNA was isolated and purified using the Purelink RNA isolation kit (Life Technologies) according to the manufacturer's instructions. Quality of RNA was

determined using the BioAnalyzer (Agilent Technologies, Santa Clara, CA). Complimentary DNA was generated using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, with RT-PCR conditions as follows: primer annealing at 25˚C for 10 minutes, reverse transcription at 42˚C for 1 hour, and enzyme inactivation at 85˚C for 5 minutes.

Quantitative PCR for gene expression

Relative expression of 13 genes related to inflammation were assessed via quantitative PCR (qPCR). Genes were selected based on known roles in inflammation and/or bovine mastitis and included Toll-like receptor 4 (TLR4), Interleukin-6 (IL6), IL33, IL8, IL1α, IL1β, IL2 receptor subunit gamma (IL2RG), IL1 receptor antagonist (IL1RN), IL1 receptor type 1 (IL1R1), C-X-C motif chemokine ligand 5 (CXCL5), CXCL8, C-C motif chemokine receptor 2 (CCR2), C-C motif chemokine ligand 20 (CCL20), and chemokine (C-X-C motif) ligand 1 (GRO1). GAPDH was used as a reference gene. Primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and are shown in Table 10. Efficiency of all primers was between 80-110%.

Quantitative PCR was performed by the Utah State University Center for Integrated Technology (Logan, UT) using Fluidigm Biomark technology and EvaGreen detection chemistry. The ΔΔCt normalization method was used for relative quantification as implemented by Fluidigm Real-Time PCR Analysis software, using GAPDH as a reference gene. Results were analyzed in a mixed model approach implemented in SAS®

statistical software, Version 9.4, using the "proc mixed" command. Comparisons in expression of the 13 genes were made between bMEC from the four treatment groups in each of the 12 cell lines. A significance level was set at $p < 0.05$.

## Results

### Cell lines

Primary bMEC lines were established from 12 cattle. The cellular morphology of all cell lines was consistent with epithelial cells on monolayer culture, and all cell lines expressed KER8 as determined by PCR. Quality of RNA for all cell lines had an RIN >7.0.

### Effects of PLA2 on proinflammatory gene expression by bMECs

In order to investigate the effects of endogenously administered PLA2 on bMEC inflammatory responses, we compared the expression of 13 pro-inflammatory genes by bMECs with and without treatment with PLA2 under unchallenged conditions and following LPS challenge. These results are shown in Table 11.

None of the 14 genes were significantly differentially expressed between unchallenged, PLA2-treated bMECs versus unchallenged, untreated bMECs. Four of the 13 genes were significantly differentially expressed between unchallenged and LPS-challenged cell lines with no PLA2 treatment. These were CCL20, IL8, IL1B, and GRO1. All four were downregulated in LPS-challenged versus unchallenged bMEC. Two of

these genes, CCL20 and IL1B, were also downregulated in PLA2 treated, LPS-challenged bMEC compared with PLA2 treated, unchallenged bMEC.

Based on these findings, exogenous PLA2 treatment does not affect constitutive expression of pro-inflammatory genes by bMECs. However, PLA2 has a dampening effect on the responses of bMECs to LPS, as indicated by a lack of differential IL8 and GRO1 expression responses by PLA2-treated bMECs in contrast to untreated bMECs.

**Discussion**

The actions of sPLA2 in inflammation are complex, comprising both stimulatory and inhibitory effects on other inflammatory factors, and vary by species as well as tissue. We demonstrate an effect of exogenous PLA2 on pro-inflammatory gene expression by primary bMECs following LPS challenge but not on constitutive expression of these genes. Unexpectedly, several pro-inflammatory cytokines were downregulated in primary bMECs as a result of LPS challenge. Both IL1B and CCL20 were downregulated regardless of PLA2 treatment. Two additional genes, IL8 and GRO1, were downregulated only in bMECs that were not treated with PLA2.

The chemokine ligand CCL20, also known as macrophage inflammatory protein 3-alpha, is involved in leukocyte migration and formation of ectopic lymphoid tissue during inflammation [238] as well as possessing antimicrobial activity [239]. The presence of CCL20 has been demonstrated in human milk early in lactation [240], indicating a potential role in mammary gland defenses. In cattle, upregulation of CCL20 by the liver occurs in conjunction with leukocytosis following intramammary LPS

challenge [241]. Although little information is available on the expression of CCL20 in the bovine mammary gland, our study demonstrates basal expression of CCL20 by primary bMECs as well as its downregulation by LPS challenge, regardless of the presence of exogenous PLA2.

Interleukin 1B is a pro-inflammatory cytokine present in the bovine mammary gland during the acute stages of mastitis caused by a number of mastitis pathogens [195,221,222] and is involved in neutrophil chemotaxis and adhesion [220]. Our findings indicate that the expression of IL1B by bMECs is not affected by exogenous PLA2.

Downregulation of IL8 did not occur following LPS challenge in PLA2-treated bMECs, in contrast to untreated bMECs. Interleukin-8 is a pro-inflammatory chemokine involved in neutrophil chemotaxis and activation [181]. During acute bovine mastitis, neutrophils are the major effector leukocyte [242]. Interleukin-8 is upregulated in the mammary gland during mastitis [195,243] and in bMECs *in vitro* in response to mastitis pathogens [157,244]. Correlations between PLA2 and IL8 levels may exist in some tissues during inflammatory diseases. For example, suppression of PLA2-II during pancreatitis in rats resulted in decreased serum levels of IL8 and other pro-inflammatory cytokines [245]. Studies investigating a potential similar correlation are lacking in bovine mammary tissue. Our results show that exposure to increased levels of PLA2 via exogenous administration may affect IL8 expression by bMECs following LPS challenge. However, PLA2 treatment alone, in the absence of LPS, was not sufficient to alter IL8 expression in these cells.

Similarly, downregulation of GRO1 in responses to LPS challenge was prevented by treatment with PLA2. Chemokine (C-X-C motif) ligand 1 is a neutrophil-specific chemoattractant [246]. Upregulation of GRO1 has been demonstrated in the bovine mammary gland during mastitis [247] and in a murine model of bovine mastitis following LPS challenge [248]. Similar to IL8, GRO1 appears to be an important factor in in neutrophil chemotaxis during bovine mastitis and its expression by primary bMECs following LPS challenge is influenced by treatment with exogenous PLA2.

Our findings show that exogenous PLA2 affects the expression of two neutrophil chemotactic factors, IL8 and GRO1, by primary bMECs. Further studies are needed in order to demonstrate whether exogenous PLA2 has an overall pro- or anti-inflammatory effect on bMECs and other bovine mammary gland cell types and the bovine mammary gland *in vivo*.

**Conclusions**

This study provides preliminary evidence that exogenously-administered PLA2 may affect the inflammatory responses of the bovine mammary gland. We demonstrated downregulation of four pro-inflammatory genes following LPS challenge in primary bMECs. In contrast, bMECs that were concurrently treated with PLA2 demonstrated downregulation of only two of these genes, CCL20 and IL1B. Future studies are needed to investigate the effects of exogenous PLA2 on the expression of additional inflammatory genes by bMEC, other bovine mammary gland cell types, and *in vivo* effects.

Table 10: Primers used for qPCR.

| Symbol | Gene | Primer | Sequence | NCBI sequence |
|---|---|---|---|---|
| CCL20 | C-C motif chemokine ligand 20 | F | TTGATGTCAGTGCTATTGCT | NM_174263.2 |
| | | R | ACCCACTTCTTCTTTGGATC | |
| CCR2 | C-C motif chemokine receptor 2 | F | GTGCCCCTTATTTTCCACTA | NM_001194959.1 |
| | | R | GAGCCCAGAAGAGAAAGTAG | |
| CXCL5 | C-X-C motif chemokine ligand 5 | F | TGTGTTTAACCACCACACC | NM_174300.2 |
| | | R | TTGTTCTTTCCACTGTCCA | |
| CXCL8 | Interleukin 8 | F | AAACACATTCCACACCTTTC | NM_173925.2 |
| | | R | TCTTCACAAATACCTGCACA | |
| GRO1 | chemokine (C-X-C motif) ligand 1 | F | ATTCACCTCAAGAACATCCA | NM_175700.1 |
| | | R | GCACTAGCCTTGTTTAGCAT | |
| IL1A | Interleukin 1 alpha | F | GAAGAGGATTCTCAGCTTCC | NM_174092.1 |
| | | R | GATGGGCAACTGATTTGAAG | |
| IL1B | Interleukin 1 beta | F | CTTGGGTATCAAGGACAAGA | NM_174093.1 |
| | | R | TGAGAAGTGCTGATGTACCA | |
| IL1R1 | Interleukin 1 receptor type 1 | F | GAGACAATGGAAGTGGTCTT | NM_001206735.1 |
| | | R | GAAATATTAAGCCGTGCGAG | |
| IL1RN | Interleukin 1 receptor antagonist | F | CACTGACTTGAACCAGAACA | AB005148.1 |
| | | R | GCTGGAAGTAGAACTTGGTG | |
| IL2RG | Interleukin 2 receptor subunit gamma | F | AATTCCAGCTAGAACTGAGC | NM_174359.1 |
| | | R | TTCCGCAAAGTGGGTTATAA | |
| IL33 | Interleukin 33 | F | CAACCAAGAGAAAGACAAGG | NM_001075297.1 |
| | | R | CTCCACAGAGTGCTCCTTAC | |
| IL6 | Interleukin 6 | F | ACTGCTGGTCTTCTGGAGTA | NM_173923.2 |
| | | R | CTTTACCCACTCGTTTGAAG | |
| TLR4 | Toll-like receptor 4 | F | GCATGGAGCTGAATCTCTAC | NM_174198.6 |
| | | R | ATAGGGTTTCCCGTCAGTAT | |

Table 11: Genes downregulated following LPS challenge in untreated and PLA2-treated bMECs. P-values are shown.

| Symbol | Gene | P-value | SE |
|---|---|---|---|
| **Untreated bMECs** | | | |
| IL1B | Interleukin-1 beta | <0.0001 | 0.7311 |
| IL8 | Interleukin-8 | 0.0272 | 1.0043 |
| CCL20 | C-C motif chemokine ligand 20 | <0.0001 | 1.0126 |
| GRO1 | chemokine (C-X-C motif) ligand 1 | 0.0084 | 0.8879 |
| **PLA2-treated bMECs** | | | |
| IL1B | Interleukin-1 beta | <0.0001 | 0.7311 |
| CCL20 | C-C motif chemokine ligand 20 | <0.0001 | 1.0126 |

# CHAPTER 6

# ESTABLISHMENT OF A MILK-DERIVED BOVINE MAMMARY EPITHELIAL CELL LINE WITH EXTENDED GROWTH CAPABILITIES BY TRANSFECTION WITH A SIMIAN VIRUS 40 LARGE T ANTIGEN-CONTAINING PLASMID CONSTRUCT


## Abstract

Primary bovine mammary epithelial cells (bMECs) have been used as models of bovine mammary gland immunity and milk production. However, challenges associated with the establishment and maintenance of primary bMECs from bovine milk may limit their use in some studies that require long-term cell line maintenance or large numbers of cells. Immortalization can facilitate long-term growth of primary bMECs and thereby improve their versatility. In order to create an immortalized bMEC line, we established milk-derived primary bMECs and transfected them with a plasmid containing Simian Virus large T antigen (SV40 L Tag), a protein known to facilitate cell cycle progression and reduce apoptosis and a well-established method of primary cell immortalization. The transfected cell line was grown through passage 27 ($P_{27}$), at which time it was cryopreserved, with morphologic characteristics of cellular senescence not evident in any cells after $P_9$. In contrast, all cells in a non-transfected bMEC line established from the same initial isolation exhibited morphologic characteristics of cellular senescence and complete cessation of division by $P_{11}$. Cellular morphologic characteristics and cytokeratin expression suggest preservation of basic bMEC characteristics. This bMEC

line could provide a valuable model of the bovine mammary gland with the advantage over primary bMECs of continued growth. Our establishment of an immortalized bMEC line derived from bovine milk is unique, and the methods described here could be used in studies that require the use of continuous cell lines from multiple cows.

**Background**

Bovine mammary epithelial cells (bMECs) are the milk-producing cells of the mammary gland, and are also important contributors to innate immunity. Primary bMECs provide an alternative to animal-based studies within a controlled environment, and have been used as a model for milk synthesis and secretion [249],the response of the mammary gland to mastitis-causing pathogens [250], and factors influencing mastitis susceptibility [204].

Primary bMECs may be established from explant culture or bovine milk. Establishment of bMECs from bovine milk provides several advantages over explant culture, including a decreased risk of contamination by other cell types, facilitation of bMEC line establishment from large numbers of animals, and decreased cost [177]. An additional advantage of this method that it is non-invasive, minimizing animal pain and distress as compared with biopsy explant methods and eliminating the need for euthanasia as required by explant methods using postmortem tissue. These principles are in keeping with the American Veterinary Medical Association's policy on the use of animals in research [199].

However, a major limitation of milk-derived bMEC establishment is the low number of cells that can be obtained as compared with explant methods, limiting the use of this technique in studies that require large numbers of cells [177]. In our experience, many milk-derived bMEC lines rapidly undergo cellular senescence and a cessation of division at a low passage number (unpublished data), allowing for only a low number of population doublings before growth has ceased. Immortalization of primary cell lines provides a tool by which the division potential of cell lines can be extended. As this immortalization using SV40 L Tag has been proven in explant-derived bMECs [196,251], we chose to use this method for immortalization of milk-derived bMECs.

In nature, the SV40 L Tag protein, produced by a simian polyomavirus, promotes survival and replication of the virus via increasing proliferative potential and decreasing apoptosis of infected host cells. Host cell expression of SV40 L Tag can result in tumorigenesis *in vivo*, and cell line immortalization *in vivo* [252] through alteration of the function of tumor suppressor and cell cycle regulatory proteins and suppression of apoptosis [253].

SV40 large T antigen affects the function of retinoblastoma proteins pRB, p107, and p130. In the normal cell, pRB proteins serve as a block to progression from the G1 to S phase of the cell cycle by binding in their hypophosphorylated state to members of the E2F transcription factor family, preventing E2F-mediated transcription. This block is lifted in the presence of cyclins E/cdk2 and D1/cdk4, which induce hyperphosphorylation of pRB proteins and concurrent release of E2F. The SV40 large T antigen induces release of the normal block on E2F-mediated transcription by binding pRB proteins in their

hypophosphorylated state, thereby sequestering the proteins so they are unavailable for binding to E2F. Additionally, SV40 large T antigen promotes dissociation of p130 from E2F-4 as well as degradation of p130 [6,252].

The SV40 large T antigen additionally affects p53, a tumor suppressor protein. In the normal cell, p53 levels increase in response to stimuli indicative of cellular damage, such as when DNA damage occurs. As a transcription factor, p53 promotes transcription of a number of genes involved in cell cycle arrest and apoptosis, thus preventing damaged cells from replicating. Important genes involved include p21, bax, cyclin G1, and mdm2. The SV40 large T antigen, however, is hypothesized to interfere with p53-dependent transcription via binding to p53, which results in an inability of p53 to bind DNA as well as preventing its degradation via the ubiquitin pathway [253].

Another effect of the SV40 large T antigen is through its effects on p300 and CREB-binding protein (CBP). In the normal cell, these proteins interact with p53, mdm2, and the NFκB p65 subunit, contributing to a number of effects including p53-dependent transcription and degradation of p53. Additionally, CPB/p300 is known to interact with the E2F-1 transcription factor, increasing its transcriptional activity of factors promoting cell cycle progression. The SV40 large T antigen binds CPB and p300, possibly in association with p53, which may facilitate E2F-mediated transcription as well as inhibition of p53 degradation. SV40 large T antigen has also been shown to bind p400, a protein similar in structure to p300 and CPB, although the exact function of this protein and the effects of its interaction with SV40 large T antigen has yet to be defined [252,253].

Finally, SV40 large T antigen has anti-apoptotic effects due to a partial structural homology with the bcl-2 family's BH1 domain. The BH1 domain is integral to the anti-apoptotic activity of the bcl-2 proteins mainly through interaction with pro-apoptotic proteins Bax and Bak [254]. Additional mechanisms of SV40 large T antigen-induced transformation and immortalization remain to be fully defined.

Here, we describe the method by which we created an immortalized bMEC line by transfecting an SV40 L Tag-containing plasmid into first-passage bMECs isolated from the milk of a Holstein dairy cow. This cell line was grown through passage 27 ($P_{27}$) before cryopreservation, with no morphologic evidence of cellular senescence. Although morphologic features of this cell line were consistent with bMECs and cytokeratin expression was demonstrated, further investigation is needed to characterize this cell line for retention of specific bMEC characteristics. This bMEC line could potentially provide a useful model for bovine mammary gland studies.

**Methods and Results**

Bovine MEC collection, isolation, and establishment

Bovine MECs were isolated from bovine milk using methods modified from those previously reported [39,177]. Briefly, 400 ml of bovine milk was collected into an equal volume of Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), and nystatin (50 mg/L). Milk was subjected to a series of wash and centrifugation steps similar to those described using a centrifugation speed of 3000 rpm. Bovine MECs

isolated by this method were seeded into a T-25 culture flask in growth media consisting of HuMEC Ready Medium (Life Technologies, Grand Island, NY) supplemented with penicillin (60 μg/ml), streptomycin (200 μg/ml), gentamycin (120 μg/ml), and nystatin (50 μg/ml), with 10% fetal bovine serum (FBS). Cells were cultured at 38.5°C with 5% $CO_2$. Growth media was changed after 12 hours and every 2-3 days thereafter.

Cells were passaged after large, coalescing colonies had formed using trypsin 0.05%/EDTA 0.02% (Sigma-Aldrich, St. Louis, MO). Growth media was removed and cells rinsed with one ml of wash media. One ml of trypsin solution was added and the culture flask returned to the incubator. Every five minutes, cell lines were observed by phase-contrast light microscopy for cell detachment. After the majority of cells had detached, the trypsin solution with suspended cells was removed, added to an equal volume of growth media, and centrifuged at 2000 rpm for three minutes. The supernatant was discarded and the cell pellet re-suspended in one ml of growth media, split, and seeded into 2 T-25 flasks. After reaching confluency, bMECs were trypsinized as described, with one flask used for transfection (transfected line) as described below and the other seeded directly into a new T-25 flask (non-transfected line). Cultures were passaged once 70-100% confluency was reached in all subsequent passages. For all passages in non-transfected bMECs and up to $P_{16}$ in transfected bMECs, trypsinized cells were seeded at a 1:2 or 1:3 dilution. At $P_{16}$, transfected bMECs were seeded at a 1:6 dilution, and thereafter at a 1:10 dilution until final cryopreservation at $P_{27}$.

Transfection of bMECs with SV40 L Tag

Bovine MECs were immortalized by transfection with a plasmid construct containing the SV40 L Tag amino acids 109-708 (SV40 1:pBSSVD2005, Addgene plasmid # 21826, David Ron) and an ampicillin resistance gene, provided within transformed bacteria in an agar stab preparation. Bacteria were plated onto lysogeny broth (LB) agar containing ampicillin. After incubation at 37°C for 12 hours, a single bacterial colony was transferred to vial containing 50 ml of LB and incubated at 37°C for a further 12 hours. Bacteria were pelleted by centrifugation at 4.4 rpm for 6 minutes and the supernatant discarded. Plasmids were isolated from bacteria using the QIAGEN Plasmid Plus Midi Prep kit (QIAGEN, Hilden, Germany).

First-passage bMECs were transfected with the SV40 1:pBSSVD2005 plasmid using the Amaxa 4D-Nucleofector kit (Lonza) and 4D-Nucleofector X Unit (Lonza) according to the Amaxa 4D-Nucleofector protocol for transfection of human mammary epithelial cells in suspension. Following trypsinization, approximately 5,000,000 cells were used for transfection with 5 µg of plasmid. Following transfection, cells were re-suspended in growth media and returned to culture conditions.

Cryopreservation of $P_{27}$ transfected bMECs

Following detachment of bMECs and centrifugation during trypsinization as described, cell pellets were re-suspended in one ml of growth media with 10% dimethyl sulfoxide and transferred to two 2-ml capacity cryopreservation tubes. The cryopreservation tubes were cooled at a rate of approximately -1°C per minute until

reaching -80°C. After 24 hours, cryopreservation tubes were transferred to a liquid nitrogen storage unit for long-term storage.

Cell characterization and growth

Verification of epithelial origin of the cultured cells was achieved through observations of cellular and colony morphology and cytokeratin expression.

Cytokeratin expression was verified by immunocytochemistry in the transfected ($P_{21}$) and non-transfected ($P_2$) cell lines using a monoclonal mouse anti-human cytokeratin antibody (cytokeratin AE1/AE3, product M3515, Dako, Glostrup, Denmark) and an anti-mouse secondary antibody (Mouse-On-Farma AP-Polymer, product BRR4010, Biocare Medical, Concord, CA), with IP Warp Red chromogen (product IPK5024, Biocare Medical) and IP FLX hematoxylin counterstain (IPCS5006, Biocare Medical). Immunocytochemistry was carried out by the Utah Veterinary Diagnostic Laboratory (Logan, UT). Both cell lines exhibited cytokeratin expression in the majority of cells (Figure 2).

Observations of cell colony morphology were made at $P_0$ and subsequent passages using phase-contrast microscopy. At all passages, both cell lines demonstrated colony and individual cell morphology consistent with MECs on monolayer [207]. Two types of cells were recognized, consistent with morphology of milk-derived bMECs described previously [177]. The first type was characterized by small to moderate amounts of cytoplasm and formation of colonies. Individualized, squamous-like cells with large amounts of variably vacuolated cytoplasm comprised the second type.

Characteristics of the second cell type were considered indicative of cellular senescence, as these cells exhibited no further division. Small numbers of senescent cells were observed at $P_0$ to $P_9$ in transfected bMECs but not thereafter. Small to moderate numbers of senescent cells were observed in non-transfected bMECs at $P_0$ to $P_7$. In subsequent passages, senescent cells predominated, and comprised all cells at $P_{11}$. After 30 days at $P_{11}$ with no growth observed, this cell line was terminated. The continued growth of transfected cells with a lack of senescent cells after $P_9$ suggests that immortalization was successful in removing blocks to cell cycle progression, in contrast with non-transfected cells that all underwent senescence by $P_{11}$ (Figure 3). Our laboratory has established numerous additional non-transfected bMEC lines by the methods described here, all of which underwent senescence at $P_{10}$ or earlier (unpublished data).

No steps were undertaken to verify SV40 L Tag expression in the transfected cell line to verify this mechanism as responsible for the continued growth observed. Non-transfected bMECs were not subjected to sham transfection; therefore, we cannot rule out the possibility that continued growth was the result of the transfection process itself rather than SV40 L Tag expression. Nevertheless, promotion of cell cycle progression through expression of SV40 L Tag is considered most likely.

**Conclusions**

We established a milk-derived bMEC line with extended growth by transfection with an SV40 L Tag-containing plasmid. This cell line was successfully grown to confluence at $P_{27}$, with cellular morphology consistent with bMECs throughout all

passages and cytokeratin expression demonstrated at $P_{21}$. In contrast, a non-transfected bMEC line established from the same cell line split after $P_1$ ceased further growth and exhibited cellular morphology consistent with cellular senescence at $P_{11}$. Further characterization is needed on this cell line to determine whether characteristics of bMECs besides cytokeratin expression are preserved, to verify the expression of SV40 L Tag, and to determine whether growth is continuous after $P_{27}$. With further characterization, this cell line could provide a valuable tool as a model for the bovine mammary gland.

Figure 2: Cytokeratin expression among non-immortalized (A) and immortalized (B) bMECs. In both bMEC lines, the majority of cells are cytokeratin-positive. Monoclonal mouse anti-human cytokeratin AE1/AE3 primary antibody; 100x magnification.

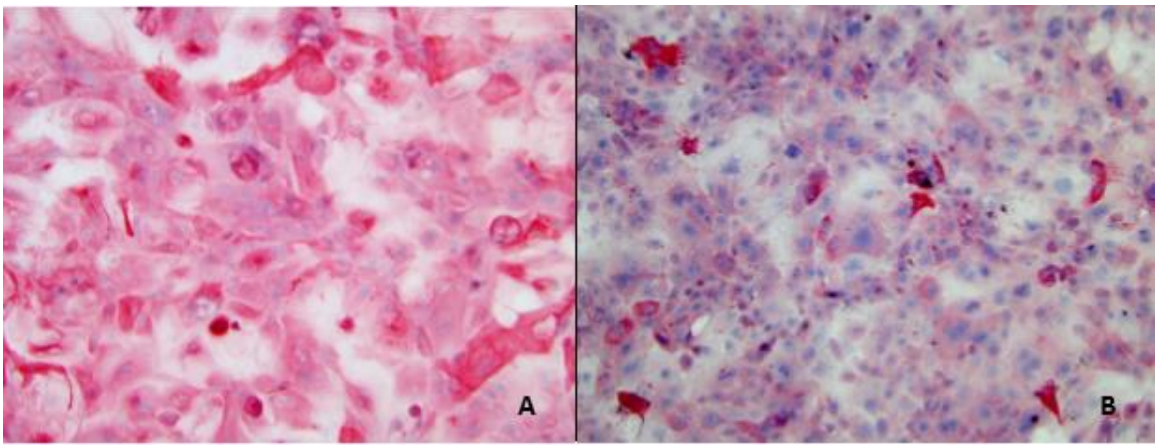
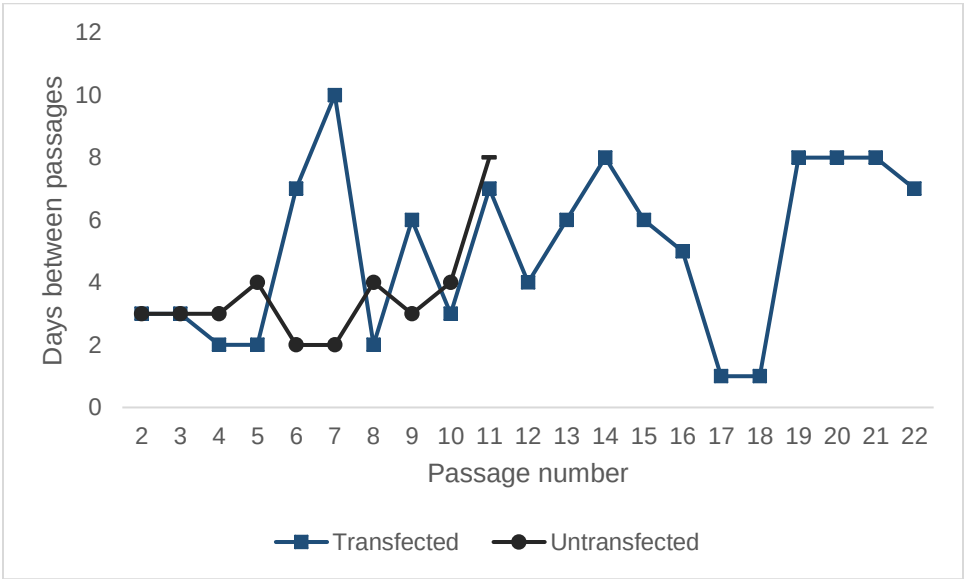



Figure 3: Growth rates of transfected and non-transfected bMEC lines from $P_2$ to $P_{22}$. Transfection with the SV40 L Tag-containing plasmid in the immortalized line occurred at $P_1$. The non-immortalized cell line ceased growth at $P_{11}$.



# CHAPTER 7
# CONCLUSIONS

Bovine mastitis results in substantial economic losses to the dairy industry worldwide and presents considerable animal welfare issues. As such, the development of strategies to decrease the prevalence of mastitis among dairy cattle is a major focus in the field of dairy science. Additionally, novel therapeutic approaches are needed in order to overcome problems associated with reliance on antibiotics as a treatment for mastitis. A genetic basis for mastitis resistance has previously been demonstrated. Despite relatively low heritability of some individual mastitis traits, genomic-based approaches to decreasing mastitis incidence among dairy cattle have proven effective and valuable. The application of genetics to guide mastitis prevention strategies relies on the identification of robust genetic markers of mastitis resistance as well as an understanding of the mechanisms underlying the relevant genetic factors.

My dissertation research identified single nucleotide polymorphism (SNP) genetic factors and quantitative trait loci of mastitis resistance in Holstein dairy cattle. In addition, I have demonstrated that bovine mammary epithelial cells (bMECs), the milk-producing cells of the mammary gland, may contribute to mastitis resistance, display distinct responses from primary bovine fibroblasts (bFs), and respond to exogenously-administered phospholipase A2 (PLA2) through differential expression of pro-inflammatory genes. Additionally, I described a method for establishment of milk-

derived primary bMECs with extended division potential through transfection with a viral protein.

Through a genome-wide association study (GWAS) using a selective genotyping approach and a single-locus mixed model (SLMM), I identified 117 SNPs suggestive of genome-wide significance for mastitis resistance. From these 117 SNPs, I identified 27 quantitative trait loci (QTLs) potentially associated with mastitis resistance, including ten QTLs that have not been reported previously. The remaining QTLs overlap with previously identified QTLs of mastitis or udder conformation traits, including several QTLS for teat length, suggesting that this trait is potentially important in mastitis resistance. Additionally, I identified a candidate gene, RAS guanyl releasing Protein 1, which may be involved in bovine mastitis and is overlapped by a SNP suggestive of genome-wide association in this study. The cows used in this study were rigorously phenotypically characterized using multiple measures of intramammary infection status over an eight-month period. Identification of the most mastitis-resistant and mastitis-susceptible animals within the sample population for use in a selective genotyping approach GWAS may have facilitated identification of the ten novel QTLs.

A subset of the cattle genotyped in the GWAS were selected for establishment of milk-derived primary bMECs. I used a polymerase chain reaction (PCR) array covering 84 bovine genes relevant to inflammation to examine inflammatory responses of bMEC from mastitis-resistant and mastitis-susceptible cattle. The constitutive expression of these 84 genes did not differ between bMECs from mastitis-resistant and mastitis-susceptible cattle. However, following a six-hour lipopolysaccharide (LPS) challenge,

mastitis-resistant bMECs demonstrated differential regulation of 43 genes, while mastitis-susceptible bMECs demonstrated differential regulation of one gene only. This gene, chemokine (C-X-C motif) ligand 5, was upregulated in both groups and is involved in neutrophil chemotaxis and activation. The differences observed between mastitis-resistant and mastitis-susceptible bMECs in this study suggest that rapid differential expression of inflammatory genes by bMECs in response to inflammatory stimuli may comprise one mechanism of mastitis resistance in cattle.

To compare the expression of bMEC and bFs, two cell types present in the mammary gland, I examined the expression of 13 pro-inflammatory genes by primary bMECs and bFs isolated from the same cattle. These two cell types display differences in the constitutive expression of six of these genes. As a result of LPS challenge, both cell types upregulated interleukin-1β, while only bMECs upregulated C-C motif chemokine ligand 20. Thus, both similarities and differences exist in the responses of these two cell types to inflammatory stimuli. Because challenges exist in the establishment and maintenance of primary bMECs, the use of primary bFs could be considered for some but not all types of bovine mastitis studies.

Phospholipase A2 is an enzyme involved in generation of inflammatory mediators in mammalian tissues. I demonstrated that PLA2 influences the expression of pro-inflammatory genes by primary bMECs, reducing their responsiveness to LPS. Whereas four pro-inflammatory genes out of 13 examined were differentially regulated following LPS challenge in untreated cells, only two of these genes were differentially regulated following LPS challenge in bMECs treated concurrently with exogenous bovine PLA2.

This finding highlights PLA2 as a candidate for future studies to investigate its potential effects on mastitis severity in cattle.

Cessation of primary bMEC division at early passage numbers presents a major limitation to their use in some studies requiring large numbers of cells or long-term maintenance. I describe the establishment of a milk-derived bMEC line with extended division potential through transfection with simian virus large T antigen (SV40 L Tag), a protein known to influence progression of the cell cycle and apoptosis. This bMEC line demonstrated continued division that far exceeded that of a non-transfected line derived from the same animal, with continued division and an absence of morphologic evidence of cellular senescence through passage 27 as compared with complete cellular senescence by passage 11 in the non-transfected line. The methods used to establish this bMEC line, as well as the cell line itself, could facilitate future studies using the bMEC model.

Collectively, the findings of this dissertation research contribute to knowledge of genetic factors of mastitis resistance in dairy cattle, the underlying molecular mechanisms that may be involved, and the use and versatility of a primary bMEC-based model for bovine mammary gland studies.

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# APPENDICES

# APPENDIX A

Table 12: Single nucleotide polymorphisms suggestive of genome-wide significance for bovine mastitis resistance. The allele associated with mastitis resistance is shown.

| Marker | Chr | Position | -log10(P-Value) | Protective Allele |
|---|---|---|---|---|
| rs43358044 | 3 | 91840020 | 3.996653467 | G |
| rs137266495 | 3 | 91852910 | 3.996653467 | A |
| rs109319807 | 4 | 101537884 | 3.980910125 | T |
| rs136808611 | 27 | 42783896 | 3.97700887 | A |
| rs136937812 | X | 57802477 | 3.975335219 | A |
| rs110284739 | 10 | 27951935 | 3.974500075 | A |
| rs109526058 | 12 | 84803111 | 3.964518728 | C |
| rs134217424 | 15 | 51068247 | 3.952684594 | G |
| rs111020787 | 10 | 15230978 | 3.939244754 | T |
| rs109587178 | 10 | 34260761 | 3.936591969 | C |
| rs135295512 | 9 | 53003499 | 3.929227883 | C |
| rs110055960 | 15 | 3927915 | 3.9101623 | C |
| rs109308232 | 10 | 34184801 | 3.903017995 | G |
| rs43506128 | 7 | 32204119 | 3.894230637 | C |
| rs134337435 | 18 | 43565043 | 3.869328826 | T |
| rs135850795 | 18 | 43566644 | 3.869328826 | C |
| rs41879780 | 18 | 43568721 | 3.869328826 | G |
| rs42748473 | 27 | 151528 | 3.866788625 | C |
| rs135442750 | 17 | 41733436 | 3.843632627 | A |
| rs110555626 | 17 | 41739893 | 3.843632627 | G |
| rs109816246 | 17 | 41753937 | 3.843632627 | T |
| rs42781654 | 17 | 41758421 | 3.843632627 | T |
| rs43616389 | 10 | 14261766 | 3.839667402 | T |
| rs110785064 | 14 | 40832903 | 3.835638804 | C |
| rs133822572 | 14 | 40834978 | 3.835638804 | G |
| rs109701184 | 2 | 135044225 | 3.826885025 | A |
| rs134262073 | 2 | 135061232 | 3.826885025 | G |
| rs110866096 | 10 | 34482573 | 3.826134783 | T |
| rs41604830 | 26 | 28196945 | 3.815561566 | G |
| rs43354976 | 3 | 92983189 | 3.802056534 | A |
| rs135734418 | 10 | 29032168 | 3.77771954 | T |
| rs43700325 | 11 | 29205354 | 3.763347317 | G |
| rs43701535 | 11 | 29206310 | 3.763347317 | A |
| rs109119871 | 11 | 29207614 | 3.763347317 | G |
| rs43701552 | 11 | 29208446 | 3.763347317 | G |
| rs43048540 | 11 | 29228506 | 3.763347317 | A |
| rs42306995 | 17 | 41702459 | 3.761873315 | G |
| rs135443516 | 9 | 65859392 | 3.753918619 | A |
| rs133087401 | 9 | 65860153 | 3.753918619 | C |
| rs134332052 | 9 | 65861243 | 3.753918619 | C |
| rs135270243 | 9 | 65879171 | 3.753918619 | G |
| rs133800930 | 9 | 65889358 | 3.753918619 | T |
| rs137234024 | 9 | 65890250 | 3.753918619 | C |

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|---|---|---|---|---|
| rs136293798 | 9 | 65891235 | 3.753918619 | T |
| rs136979275 | 9 | 65895727 | 3.753918619 | G |
| rs109202959 | 19 | 7514783 | 3.746281982 | A |
| rs134340673 | 10 | 15144718 | 3.7416025 | C |
| rs109711228 | 15 | 50978021 | 3.719140182 | T |
| rs135987526 | 28 | 18520683 | 3.714851977 | G |
| rs136467850 | 15 | 54649868 | 3.704686149 | T |
| rs43618989 | 10 | 11496248 | 3.701449371 | G |
| rs111023847 | 16 | 20608750 | 3.694447987 | C |
| rs110918390 | 16 | 20609335 | 3.694447987 | G |
| rs110001905 | 16 | 20610171 | 3.694447987 | A |
| rs109403663 | 16 | 20612167 | 3.694447987 | A |
| rs108942580 | 16 | 20614885 | 3.694447987 | A |
| rs109726329 | 16 | 20615451 | 3.694447987 | C |
| rs110132689 | 16 | 20616455 | 3.694447987 | A |
| rs109513395 | 16 | 20617139 | 3.694447987 | A |
| rs110663077 | 10 | 39854351 | 3.693917704 | C |
| rs42435974 | 26 | 28877559 | 3.677934056 | T |
| rs137733108 | 12 | 12976126 | 3.675972311 | G |
| rs42422111 | 3 | 90823349 | 3.675190282 | A |
| rs42422098 | 3 | 90825605 | 3.675190282 | C |
| rs42422096 | 3 | 90826586 | 3.675190282 | C |
| rs110502897 | 6 | 45848609 | 3.668025217 | C |
| rs109139712 | 10 | 11478732 | 3.666290057 | T |
| rs135391817 | 10 | 11482238 | 3.666290057 | T |
| rs43488809 | 6 | 109419395 | 3.661360545 | C |
| rs109048873 | 17 | 37988898 | 3.653290211 | T |
| rs109752781 | 17 | 37999567 | 3.653290211 | T |
| rs135575219 | 17 | 41741046 | 3.651960319 | T |
| rs41611251 | 17 | 41742069 | 3.651960319 | T |
| rs109407328 | 17 | 41742777 | 3.651960319 | T |
| rs110264878 | 17 | 41746116 | 3.651960319 | T |
| rs109199653 | 17 | 41747600 | 3.651960319 | G |
| rs137323892 | 19 | 11786116 | 3.64943487 | T |
| rs136457662 | 29 | 28578533 | 3.648974591 | A |
| rs41856186 | 18 | 5214466 | 3.648636063 | A |
| rs109442613 | 18 | 5225895 | 3.648636063 | A |
| rs110805371 | 10 | 34407598 | 3.64764349 | T |
| rs134256203 | 29 | 46072112 | 3.640522943 | A |
| rs42230220 | 10 | 29751247 | 3.638958744 | G |
| rs110803408 | 9 | 103674333 | 3.637892378 | C |
| rs41708297 | 13 | 61537602 | 3.631269978 | G |
| rs109484288 | 6 | 108468990 | 3.628915132 | C |
| rs109133788 | 18 | 49684020 | 3.627804715 | T |
| rs136768665 | 3 | 91342794 | 3.623859956 | T |
| rs109699387 | 11 | 42600063 | 3.622735086 | G |
| rs43617435 | 10 | 14229935 | 3.617247186 | T |
| rs43616344 | 10 | 14279631 | 3.617247186 | T |
| rs42179869 | 7 | 89788219 | 3.604189492 | A |
| rs110465605 | 7 | 89800236 | 3.604189492 | T |

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|---|---|---|---|---|
| rs42179853 | 7 | 89808465 | 3.604189492 | T |
| rs42179846 | 7 | 89816912 | 3.604189492 | A |
| rs42179845 | 7 | 89820454 | 3.604189492 | G |
| rs136757535 | 7 | 89821932 | 3.604189492 | T |
| rs42178916 | 7 | 89831709 | 3.604189492 | A |
| rs42178892 | 7 | 89842459 | 3.604189492 | A |
| rs109533174 | 16 | 62901191 | 3.59584457 | G |
| rs110257587 | 12 | 12918128 | 3.583356138 | A |
| rs110745310 | 12 | 12925593 | 3.583356138 | A |
| rs109716027 | 12 | 12935119 | 3.583356138 | A |
| rs135512574 | 12 | 12941960 | 3.583356138 | C |
| rs110541423 | 12 | 12946676 | 3.583356138 | T |
| rs210940686 | 12 | 12950359 | 3.583356138 | C |
| rs110770556 | 12 | 12959938 | 3.583356138 | T |
| rs109138785 | 12 | 12980738 | 3.583356138 | A |
| rs109474774 | 12 | 13002261 | 3.583356138 | A |
| rs110219834 | 12 | 13008208 | 3.583356138 | A |
| rs110094067 | 12 | 13020618 | 3.583356138 | A |
| rs109450381 | 26 | 26296160 | 3.577415756 | C |
| rs43418248 | 26 | 27874379 | 3.575512545 | T |
| rs43418238 | 26 | 27878019 | 3.575512545 | G |
| rs43418237 | 26 | 27879888 | 3.575512545 | T |
| rs109126903 | 11 | 46523735 | 3.575329135 | G |
| rs137303803 | 21 | 21173650 | 3.567662303 | C |
| rs110079373 | 11 | 96756491 | 3.56603655 | G |
| rs110796580 | 11 | 96777054 | 3.56603655 | A |
| rs136932995 | 26 | 25658416 | 3.5653789 | C |
| rs137549287 | 3 | 5770104 | 3.554231467 | G |
| rs132946079 | 17 | 10716265 | 3.552360613 | C |
| rs41573750 | 17 | 10726289 | 3.552360613 | T |
| rs110260876 | 17 | 10727750 | 3.552360613 | G |
| rs42572201 | 4 | 91124601 | 3.547840315 | T |
| rs42572206 | 4 | 91127591 | 3.547840315 | T |
| rs110741324 | 4 | 91130350 | 3.547840315 | A |
| rs109847541 | 4 | 91136670 | 3.547840315 | T |
| rs109527045 | 4 | 91158590 | 3.547840315 | G |
| rs43019584 | 4 | 91161129 | 3.547840315 | T |
| rs43019591 | 4 | 91163203 | 3.547840315 | G |
| rs42957734 | 4 | 91180659 | 3.547840315 | C |
| rs42956722 | 4 | 91181932 | 3.547840315 | T |
| rs42956709 | 4 | 91183368 | 3.547840315 | T |
| rs42573425 | 4 | 91311286 | 3.547840315 | G |
| rs110656903 | 21 | 21893359 | 3.545937526 | A |
| rs109351174 | 3 | 56556783 | 3.544667915 | A |
| rs109747432 | 3 | 56570552 | 3.544667915 | A |
| rs109256407 | 3 | 56585697 | 3.544667915 | C |
| rs133343209 | 29 | 26200863 | 3.539963879 | A |

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|---|---|---|---|---|
| rs135942451 | 4 | 90401943 | 3.53243049 | A |
| rs43061363 | 4 | 90407999 | 3.53243049 | G |
| rs134862481 | 4 | 90414075 | 3.53243049 | C |
| rs42953972 | 4 | 90415012 | 3.53243049 | T |
| rs42954026 | 4 | 90422526 | 3.53243049 | A |
| rs110623899 | 4 | 90425762 | 3.53243049 | G |
| rs135621455 | 4 | 90428930 | 3.53243049 | A |
| rs136015399 | 4 | 90431785 | 3.53243049 | T |
| rs42954434 | 4 | 90456245 | 3.53243049 | A |
| rs42436989 | 4 | 90502505 | 3.53243049 | T |
| rs42954027 | 19 | 11469210 | 3.53243049 | T |
| rs110459211 | 15 | 22583487 | 3.531664681 | A |
| rs132635669 | 10 | 11663811 | 3.527354767 | A |
| rs110383759 | 2 | 135067905 | 3.52190773 | C |
| rs110296238 | 2 | 135069441 | 3.52190773 | T |
| rs137654606 | 11 | 30120909 | 3.519420207 | G |
| rs110578425 | 10 | 34260119 | 3.517448796 | T |
| rs110822305 | 10 | 34269720 | 3.517448796 | G |
| rs110174252 | 10 | 34271375 | 3.517448796 | A |
| rs109718130 | 10 | 34278604 | 3.517448796 | A |
| rs135485607 | 15 | 3936865 | 3.510710974 | G |
| rs110521330 | 27 | 43792463 | 3.509502691 | G |
| rs109247220 | 27 | 43795174 | 3.509502691 | G |
| rs134139377 | 15 | 46032011 | 3.503671841 | T |
| rs136575211 | 27 | 25015766 | 3.502020606 | T |
| rs109202339 | 10 | 27908334 | 3.501847277 | T |
| rs134911710 | 10 | 27913156 | 3.501847277 | T |
| rs109630946 | 10 | 27923467 | 3.501847277 | A |
| rs135108032 | 10 | 27926282 | 3.501847277 | A |
| rs110174162 | 10 | 27948713 | 3.501847277 | A |
| rs137223718 | 10 | 27949701 | 3.501847277 | G |
| rs136175781 | 10 | 27950452 | 3.501847277 | C |
| rs133857484 | 10 | 27951385 | 3.501847277 | A |
| rs109055367 | 10 | 27953197 | 3.501847277 | G |
| rs109712672 | 10 | 27969361 | 3.501847277 | A |
| rs135060899 | 10 | 27971131 | 3.501847277 | G |
| rs109834818 | 10 | 27975781 | 3.501847277 | A |
| rs109929709 | 10 | 27976538 | 3.501847277 | A |
| rs137395442 | 10 | 27978253 | 3.501847277 | C |
| rs110948714 | 10 | 27981920 | 3.501847277 | C |
| rs109276009 | 10 | 27983389 | 3.501847277 | T |
| rs109119406 | 10 | 27987774 | 3.501847277 | T |
| rs109393053 | 10 | 27989220 | 3.501847277 | G |
| rs110741778 | 10 | 27990512 | 3.501847277 | C |
| rs109718041 | 10 | 27991328 | 3.501847277 | A |
| rs110676082 | 10 | 27993659 | 3.501847277 | A |
| rs110933562 | 10 | 27994618 | 3.501847277 | A |

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|---|---|---|---|---|
| rs110968891 | 10 | 27998032 | 3.501847277 | A |
| rs109263205 | 10 | 27998826 | 3.501847277 | C |
| rs110438571 | 10 | 28000036 | 3.501847277 | T |
| rs110061866 | 10 | 28001148 | 3.501847277 | A |
| rs109345446 | 10 | 28001876 | 3.501847277 | G |
| rs109406918 | 10 | 28002566 | 3.501847277 | C |
| rs137404908 | X | 106584624 | 3.491051617 | C |
| rs134000682 | X | 106601943 | 3.491051617 | G |
| rs41641989 | 19 | 7511302 | 3.49066656 | G |
| rs135723042 | 17 | 42173612 | 3.48390421 | T |
| rs109491704 | 17 | 42186164 | 3.48390421 | T |
| rs109795284 | 17 | 42189018 | 3.48390421 | T |
| rs136231792 | 17 | 42190919 | 3.48390421 | T |
| rs110808681 | 10 | 34182291 | 3.47996144 | A |
| rs41623451 | 10 | 34193887 | 3.47996144 | T |
| rs134408007 | 10 | 34201327 | 3.47996144 | G |
| rs110049315 | 12 | 76826267 | 3.479667966 | C |
| rs137547770 | 12 | 76832234 | 3.479667966 | T |
| rs135018781 | 28 | 43884340 | 3.479083948 | G |
| rs109004955 | 10 | 15226746 | 3.477491518 | G |
| rs110644177 | 10 | 15231607 | 3.477491518 | C |
| rs137247805 | 28 | 30866421 | 3.458749724 | A |
| rs136227126 | 28 | 30867142 | 3.458749724 | T |
| rs110234333 | 10 | 34393478 | 3.458190041 | C |
| rs137236292 | 21 | 8053012 | 3.457490709 | T |
| rs41963113 | 21 | 8061425 | 3.457490709 | T |
| rs41963125 | 21 | 8062897 | 3.457490709 | T |
| rs110332445 | 21 | 8065472 | 3.457490709 | G |
| rs41963076 | 21 | 8071325 | 3.457490709 | T |
| rs135664235 | 26 | 25265647 | 3.45634136 | T |
| rs110429039 | 9 | 97571198 | 3.455458218 | C |
| rs109503693 | 9 | 97572172 | 3.455458218 | A |
| rs134754035 | 9 | 97573217 | 3.455458218 | T |
| rs110418388 | 9 | 97574028 | 3.455458218 | C |
| rs137051476 | 3 | 24671921 | 3.452247361 | G |
| rs134675792 | 16 | 80383679 | 3.448342703 | T |
| rs136087996 | 16 | 80385720 | 3.448342703 | A |
| rs134310314 | 15 | 47264048 | 3.448021966 | C |
| rs41766844 | 15 | 47264872 | 3.448021966 | T |
| rs41766852 | 15 | 47266773 | 3.448021966 | T |
| rs41766857 | 15 | 47268306 | 3.448021966 | A |
| rs41631859 | 15 | 47274125 | 3.448021966 | T |
| rs41766870 | 15 | 47274957 | 3.448021966 | A |
| rs41766877 | 15 | 47275830 | 3.448021966 | T |
| rs41766891 | 15 | 47278941 | 3.448021966 | T |
| rs110616111 | 15 | 47279621 | 3.448021966 | A |
| rs110069583 | 15 | 47280330 | 3.448021966 | A |

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|---|---|---|---|---|
| rs135331709 | 15 | 47283531 | 3.448021966 | T |
| rs41764625 | 15 | 47290269 | 3.448021966 | G |
| rs41764630 | 15 | 47290932 | 3.448021966 | G |
| rs41765840 | 15 | 47294915 | 3.448021966 | T |
| rs41765850 | 15 | 47297442 | 3.448021966 | T |
| rs135103940 | 15 | 47298250 | 3.448021966 | A |
| rs41765859 | 15 | 47299091 | 3.448021966 | C |
| rs41765876 | 15 | 47302403 | 3.448021966 | T |
| rs41765898 | 15 | 47304572 | 3.448021966 | A |
| rs41765904 | 15 | 47305423 | 3.448021966 | C |
| rs41765907 | 15 | 47306109 | 3.448021966 | T |
| rs109916280 | 15 | 47307206 | 3.448021966 | G |
| rs41765913 | 15 | 47307914 | 3.448021966 | C |
| rs42669165 | 15 | 47336499 | 3.448021966 | G |
| rs42669195 | 15 | 47357365 | 3.448021966 | C |
| rs42669201 | 15 | 47358546 | 3.448021966 | G |
| rs42669211 | 15 | 47359452 | 3.448021966 | A |
| rs42669215 | 15 | 47360926 | 3.448021966 | A |
| rs110650579 | 15 | 47363154 | 3.448021966 | T |
| rs110477122 | 16 | 20611330 | 3.437492686 | G |
| rs134322109 | 10 | 35133400 | 3.436990559 | A |
| rs135507523 | 10 | 35134070 | 3.436990559 | T |
| rs42976756 | 3 | 5907195 | 3.435443723 | T |
| rs42976790 | 3 | 5909921 | 3.435443723 | T |
| rs137400107 | 2 | 118870999 | 3.430142009 | C |
| rs109940736 | 11 | 102335697 | 3.427253851 | C |
| rs109462487 | 26 | 26358551 | 3.423511281 | T |
| rs43418314 | 26 | 27866959 | 3.421461295 | A |
| rs43418304 | 26 | 27867615 | 3.421461295 | A |
| rs43418296 | 26 | 27868998 | 3.421461295 | A |
| rs43418241 | 26 | 27875295 | 3.421461295 | C |
| rs136871582 | 25 | 29026876 | 3.418423118 | C |
| rs134778236 | 25 | 29028549 | 3.418423118 | G |
| rs109296602 | 25 | 29029462 | 3.418423118 | T |
| rs133195919 | 25 | 29030397 | 3.418423118 | T |
| rs134990112 | 25 | 29032886 | 3.418423118 | A |
| rs29026849 | 25 | 29033600 | 3.418423118 | T |
| rs134505616 | 10 | 14915348 | 3.417097085 | C |
| rs134775008 | 10 | 14992318 | 3.417097085 | G |
| rs135993868 | 11 | 36693585 | 3.41288933 | T |
| rs109550935 | 10 | 14509430 | 3.410021525 | A |
| rs800022227 | 18 | 49707682 | 3.406825082 | C |
| rs110936103 | 18 | 49826315 | 3.406825082 | A |
| rs109752915 | 18 | 49827804 | 3.406825082 | T |
| rs110052628 | 18 | 49829235 | 3.406825082 | G |
| rs109584832 | 18 | 49830124 | 3.406825082 | T |
| rs110511465 | 18 | 49830798 | 3.406825082 | C |

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| rs110721957 | 18 | 49831451 | 3.406825082 | C |
| rs109371909 | 18 | 49832385 | 3.406825082 | A |
| rs136730838 | 5 | 68586107 | 3.4002011 | T |
| rs43615154 | 10 | 14374841 | 3.399155241 | T |
| rs41973031 | 21 | 21202324 | 3.397541583 | T |
| rs41972130 | 21 | 21203119 | 3.397541583 | A |
| rs41972127 | 21 | 21205862 | 3.397541583 | T |
| rs29011369 | 2 | 116210744 | 3.396737757 | A |
| rs43326486 | 2 | 116261117 | 3.396737757 | T |
| rs43326515 | 2 | 116317216 | 3.396737757 | C |
| rs43327275 | 2 | 116368014 | 3.396737757 | T |
| rs43327284 | 2 | 116379355 | 3.396737757 | A |
| rs43328231 | 2 | 116433069 | 3.396737757 | A |
| rs136608836 | 2 | 116443823 | 3.396737757 | T |
| rs110463452 | 2 | 116450940 | 3.396737757 | A |
| rs134877611 | 2 | 116481181 | 3.396737757 | T |
| rs134785162 | 2 | 116502303 | 3.396737757 | C |
| rs110836924 | 2 | 116515561 | 3.396737757 | C |
| rs42350539 | 3 | 91406267 | 3.396283165 | T |
| rs109276053 | 17 | 37366043 | 3.38905385 | C |
| rs42822799 | 4 | 91392323 | 3.388029393 | G |
| rs42822793 | 4 | 91401157 | 3.388029393 | A |
| rs42822797 | 4 | 91410156 | 3.388029393 | G |
| rs110781216 | 11 | 16185893 | 3.38300674 | A |
| rs110700817 | 17 | 39991047 | 3.379336817 | T |
| rs137095327 | 23 | 42900015 | 3.377386947 | T |
| rs132738542 | 11 | 43746272 | 3.371605441 | T |
| rs109954573 | 2 | 97056987 | 3.369612574 | A |
| rs110583355 | 3 | 20726185 | 3.367102737 | A |
| rs110891235 | 2 | 135043582 | 3.362086163 | C |
| rs134075027 | 16 | 80373783 | 3.360130724 | C |
| rs29012884 | 16 | 80375118 | 3.360130724 | T |
| rs137539857 | 16 | 80380895 | 3.360130724 | T |
| rs134130295 | 11 | 96300378 | 3.356836108 | A |
| rs137130502 | 17 | 42379744 | 3.355224205 | G |
| rs110952236 | 28 | 14587431 | 3.352891958 | A |
| rs110529357 | 27 | 3587344 | 3.347190698 | G |
| rs136309760 | 27 | 3603082 | 3.347190698 | C |
| rs136752551 | 27 | 3603744 | 3.347190698 | T |
| rs137721609 | 27 | 3605490 | 3.347190698 | C |
| rs109053110 | 27 | 3606725 | 3.347190698 | G |
| rs133569944 | 27 | 3613160 | 3.347190698 | A |
| rs135112745 | 27 | 3619112 | 3.347190698 | A |
| rs109004577 | 27 | 3621325 | 3.347190698 | C |
| rs133566198 | 27 | 3632970 | 3.347190698 | A |
| rs109288278 | 1 | 9155528 | 3.343575742 | C |
| rs137423771 | 1 | 9172574 | 3.343575742 | G |

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| rs132926852 | 1 | 9173252 | 3.343575742 | T |
| rs137188372 | 1 | 9174446 | 3.343575742 | G |
| rs109367978 | 1 | 9180914 | 3.343575742 | T |
| rs109114614 | 1 | 9191421 | 3.343575742 | A |
| rs110149968 | 1 | 9196480 | 3.343575742 | A |
| rs134043952 | 9 | 103827095 | 3.338846162 | C |
| rs132692297 | 9 | 96392290 | 3.337454701 | G |
| rs133696224 | 18 | 51793677 | 3.332942754 | T |
| rs110030120 | 18 | 51795302 | 3.332942754 | T |
| rs137178064 | 18 | 51798613 | 3.332942754 | G |
| rs135147402 | 18 | 51800010 | 3.332942754 | T |
| rs41665411 | 9 | 97377573 | 3.331594763 | G |
| rs134859031 | 12 | 76761791 | 3.327441429 | G |
| rs109206774 | 28 | 20301025 | 3.324366482 | G |
| rs137374997 | 28 | 20301881 | 3.324366482 | C |
| rs110611090 | 28 | 20303194 | 3.324366482 | G |
| rs109913025 | 28 | 20307480 | 3.324366482 | T |
| rs109964809 | 28 | 20313646 | 3.324366482 | G |
| rs109577685 | 28 | 20329418 | 3.324366482 | G |
| rs136797470 | 28 | 20330518 | 3.324366482 | T |
| rs110075596 | 28 | 20332119 | 3.324366482 | A |
| rs109137280 | 16 | 20617894 | 3.320623615 | C |
| rs134411715 | 16 | 20619357 | 3.320623615 | A |
| rs110533869 | 16 | 20623978 | 3.320623615 | A |
| rs43306722 | 10 | 39537032 | 3.317268784 | A |
| rs43306706 | 10 | 39546139 | 3.317268784 | T |
| rs109817054 | 10 | 15265576 | 3.316082909 | T |
| rs109344511 | 10 | 15267025 | 3.316082909 | A |
| rs109679240 | 10 | 15269153 | 3.316082909 | A |
| rs109755527 | 10 | 15272047 | 3.316082909 | G |
| rs109793858 | 10 | 15274224 | 3.316082909 | G |
| rs109985287 | 10 | 15291183 | 3.316082909 | A |
| rs133459576 | 10 | 15295267 | 3.316082909 | C |
| rs42620822 | 27 | 42776720 | 3.312793511 | A |
| rs135051801 | 18 | 5113990 | 3.305450691 | A |
| rs109846857 | 11 | 15610039 | 3.30211409 | A |
| rs42743027 | 4 | 91044644 | 3.299742379 | T |
| rs42743021 | 4 | 91045511 | 3.299742379 | G |
| rs135959622 | 4 | 91086150 | 3.299742379 | C |
| rs42741696 | 4 | 91090805 | 3.299742379 | G |
| rs137081039 | 4 | 91095329 | 3.299742379 | C |
| rs41706372 | 13 | 61992391 | 3.299725575 | C |
| rs110493658 | 10 | 34438367 | 3.298523546 | C |
| rs137347983 | 10 | 34447107 | 3.298523546 | A |
| rs136416791 | 10 | 34449394 | 3.298523546 | G |
| rs134051160 | 10 | 34451675 | 3.298523546 | C |
| rs136476283 | 10 | 34454792 | 3.298523546 | C |

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| rs109477546 | 27 | 9014873 | 3.294251483 | G |
| rs134296530 | 15 | 3805680 | 3.286903941 | G |
| rs42188223 | 29 | 46051097 | 3.286597099 | C |
| rs135076653 | 29 | 46069381 | 3.286597099 | T |
| rs137786194 | 29 | 46071340 | 3.286597099 | A |
| rs135523988 | 29 | 46073378 | 3.286597099 | A |
| rs133850293 | 18 | 49844762 | 3.285519188 | A |
| rs137429611 | 18 | 49846906 | 3.285519188 | T |
| rs134644515 | 18 | 49854465 | 3.285519188 | T |
| rs42625901 | 6 | 108569050 | 3.285445958 | A |
| rs136596415 | 18 | 51803425 | 3.283718544 | C |
| rs134480471 | 18 | 51808317 | 3.283718544 | T |
| rs136382907 | 18 | 51810825 | 3.283718544 | C |
| rs110216131 | 27 | 2537760 | 3.283312314 | T |
| rs110326002 | 27 | 2538848 | 3.283312314 | C |
| rs109126345 | X | 120798197 | 3.281319397 | A |
| rs110770536 | 26 | 27897409 | 3.280053399 | A |
| rs135171829 | 4 | 91013071 | 3.272038183 | C |
| rs42743846 | 4 | 91025117 | 3.272038183 | G |
| rs134940524 | 4 | 91029900 | 3.272038183 | T |
| rs42743839 | 4 | 91030631 | 3.272038183 | G |
| rs41571562 | 4 | 91051469 | 3.272038183 | A |
| rs42742989 | 4 | 91053740 | 3.272038183 | G |
| rs109212509 | 4 | 91054564 | 3.272038183 | T |
| rs42742950 | 4 | 91056364 | 3.272038183 | G |
| rs42742955 | 4 | 91057950 | 3.272038183 | A |
| rs42742959 | 4 | 91059678 | 3.272038183 | C |
| rs42742963 | 4 | 91060347 | 3.272038183 | C |
| rs42742969 | 4 | 91060986 | 3.272038183 | C |
| rs42742974 | 4 | 91061839 | 3.272038183 | G |
| rs42742985 | 4 | 91064044 | 3.272038183 | C |
| rs137402389 | 4 | 91074086 | 3.272038183 | C |
| rs133246170 | 4 | 91114783 | 3.272038183 | G |
| rs109558636 | 21 | 5418214 | 3.271931695 | G |
| rs110195286 | 21 | 5421568 | 3.271931695 | C |
| rs109620070 | 21 | 5422909 | 3.271931695 | C |
| rs133390385 | 21 | 5430195 | 3.271931695 | T |
| rs134693433 | 21 | 5432530 | 3.271931695 | A |
| rs137550200 | 21 | 5433346 | 3.271931695 | G |
| rs109326223 | 21 | 5440289 | 3.271931695 | T |
| rs110785020 | 21 | 5442814 | 3.271931695 | T |
| rs133787467 | 21 | 5444628 | 3.271931695 | T |
| rs42307500 | 17 | 41682839 | 3.268182182 | A |
| rs110950041 | 10 | 15240169 | 3.268062731 | G |
| rs134759074 | 10 | 15240747 | 3.268062731 | T |
| rs110608086 | 10 | 15241322 | 3.268062731 | T |
| rs110739273 | 10 | 15244368 | 3.268062731 | G |

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| rs110774021 | 10 | 15245376 | 3.268062731 | A |
| rs110954734 | 10 | 15262140 | 3.268062731 | A |
| rs110353392 | 11 | 102319320 | 3.266517518 | T |
| rs133980943 | 4 | 91528967 | 3.265842726 | T |
| rs136842498 | 6 | 10677303 | 3.261761701 | T |
| rs134993675 | 27 | 43799710 | 3.261477609 | C |
| rs109829168 | 27 | 43801103 | 3.261477609 | T |
| rs42434777 | 9 | 62300403 | 3.260836335 | C |
| rs133369187 | 6 | 108463671 | 3.259525167 | T |
| rs43706008 | 12 | 68755240 | 3.257897754 | C |
| rs136633863 | X | 17647347 | 3.252831054 | C |
| rs137201853 | 28 | 20353894 | 3.249712305 | C |
| rs133190904 | 11 | 102309223 | 3.249568667 | G |
| rs134715529 | 11 | 102310272 | 3.249568667 | T |
| rs136173225 | 17 | 10404541 | 3.246660635 | T |
| rs132676958 | 9 | 65909445 | 3.245590821 | G |
| rs110893833 | 17 | 41778376 | 3.243968586 | G |
| rs110946010 | 9 | 103692309 | 3.242853175 | T |
| rs109631568 | 9 | 103692964 | 3.242853175 | G |
| rs110789880 | 9 | 103697889 | 3.242853175 | A |
| rs132729670 | 9 | 103722885 | 3.242853175 | A |
| rs110277541 | 9 | 103724091 | 3.242853175 | A |
| rs110296542 | 6 | 45833672 | 3.237847779 | G |
| rs110860625 | 7 | 89520670 | 3.233998582 | C |
| rs109391677 | 9 | 98238057 | 3.233200069 | C |
| rs41667147 | 17 | 41720570 | 3.227988184 | A |
| rs41667178 | 17 | 41723453 | 3.227988184 | T |
| rs41679969 | 12 | 76906934 | 3.227322103 | G |
| rs135237762 | 9 | 97532057 | 3.22542096 | T |
| rs132843341 | 9 | 97532971 | 3.22542096 | T |
| rs43354180 | 3 | 92472331 | 3.22451027 | T |
| rs43354182 | 3 | 92484809 | 3.22451027 | T |
| rs43354191 | 3 | 92500456 | 3.22451027 | T |
| rs43354201 | 3 | 92505029 | 3.22451027 | A |
| rs42199706 | 7 | 31224439 | 3.224271457 | G |
| rs42890503 | 29 | 28662664 | 3.218625428 | C |
| rs134551478 | 18 | 49841563 | 3.214677758 | T |
| rs110499373 | 27 | 24803258 | 3.213846196 | A |
| rs41703808 | 13 | 60739302 | 3.209255175 | C |
| rs41703814 | 13 | 60740826 | 3.209255175 | A |
| rs135915757 | 2 | 72801094 | 3.208396431 | A |
| rs135464279 | 12 | 67062990 | 3.207516544 | A |
| rs42094261 | 26 | 26098742 | 3.206601056 | A |
| rs42309434 | 4 | 118211468 | 3.204172924 | T |
| rs109072712 | 27 | 30671561 | 3.201586133 | C |
| rs137813779 | 15 | 6996317 | 3.200806888 | G |
| rs41574223 | 11 | 102329426 | 3.200555935 | T |

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| rs41571435 | 11 | 102331407 | 3.200555935 | T |
| rs136400600 | 11 | 102333185 | 3.200555935 | G |
| rs109104765 | 11 | 102336231 | 3.200555935 | G |
| rs134244722 | 11 | 102347819 | 3.200555935 | C |
| rs110950384 | 10 | 34633682 | 3.199509483 | C |
| rs110976214 | 10 | 34636005 | 3.199509483 | C |
| rs133491211 | 28 | 18509947 | 3.199087297 | A |
| rs133796959 | 28 | 18515950 | 3.199087297 | A |
| rs134041938 | 7 | 98557529 | 3.197304808 | A |
| rs109354718 | 7 | 98566391 | 3.197304808 | C |
| rs110620868 | 14 | 37960177 | 3.195762246 | G |
| rs109588849 | 4 | 9155979 | 3.194702115 | A |
| rs134925464 | 4 | 9157466 | 3.194702115 | T |
| rs110747955 | 4 | 9169373 | 3.194702115 | G |
| rs108978929 | 4 | 9171340 | 3.194702115 | G |
| rs109200993 | 4 | 9175665 | 3.194702115 | A |
| rs109550058 | 4 | 9176248 | 3.194702115 | A |
| rs110782546 | 4 | 9180014 | 3.194702115 | C |
| rs110766969 | 4 | 9181924 | 3.194702115 | G |
| rs110542894 | 4 | 9184443 | 3.194702115 | A |
| rs109872926 | 4 | 9186322 | 3.194702115 | G |
| rs109555021 | 4 | 9187560 | 3.194702115 | T |
| rs133113391 | 4 | 9188973 | 3.194702115 | G |
| rs110008785 | 4 | 9190080 | 3.194702115 | T |
| rs109830573 | 4 | 9192487 | 3.194702115 | A |
| rs136833626 | 4 | 9193149 | 3.194702115 | C |
| rs110227344 | 4 | 9194698 | 3.194702115 | T |
| rs137741495 | 4 | 9195864 | 3.194702115 | C |
| rs109582833 | 4 | 9196384 | 3.194702115 | C |
| rs110996175 | 4 | 9202160 | 3.194702115 | C |
| rs41649756 | 4 | 9203380 | 3.194702115 | T |
| rs109304683 | 4 | 9203946 | 3.194702115 | C |
| rs135642012 | 4 | 90507887 | 3.192964556 | C |
| rs110508072 | 4 | 90510460 | 3.192964556 | C |
| rs135728603 | 4 | 90522341 | 3.192964556 | T |
| rs110807111 | 4 | 90525031 | 3.192964556 | T |
| rs134895773 | 4 | 90525835 | 3.192964556 | C |
| rs110041213 | 4 | 90529431 | 3.192964556 | G |
| rs109847272 | 4 | 90533691 | 3.192964556 | C |
| rs135236859 | 4 | 90535585 | 3.192964556 | G |
| rs133775221 | 4 | 90540601 | 3.192964556 | C |
| rs109172349 | 4 | 90541223 | 3.192964556 | G |
| rs136148020 | 4 | 90545284 | 3.192964556 | G |
| rs110575673 | 11 | 42584714 | 3.191254642 | G |
| rs109447838 | 11 | 42585516 | 3.191254642 | T |
| rs109438861 | 11 | 42593606 | 3.191254642 | C |
| rs109889274 | 11 | 42596497 | 3.191254642 | T |

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| rs42287596 | 7 | 33883766 | 3.189005816 | T |
| rs133957935 | 4 | 19344855 | 3.188761105 | T |
| rs135986439 | 4 | 19346351 | 3.188761105 | T |
| rs132639525 | 4 | 19347907 | 3.188761105 | G |
| rs134542151 | 4 | 19350451 | 3.188761105 | T |
| rs132948098 | 3 | 91375387 | 3.187737698 | G |
| rs136849824 | 11 | 37467110 | 3.183881606 | G |
| rs137255173 | 11 | 37487155 | 3.183881606 | G |
| rs109278103 | 11 | 37493130 | 3.183881606 | A |
| rs110025285 | 11 | 37494839 | 3.183881606 | A |
| rs132976440 | 7 | 31015249 | 3.183524696 | A |
| rs43579588 | 8 | 105240087 | 3.181751899 | A |
| rs110453415 | 24 | 53850563 | 3.181485439 | G |
| rs135719064 | 16 | 20502906 | 3.179219707 | A |
| rs109920725 | 16 | 20509935 | 3.179219707 | A |
| rs110536976 | 16 | 20547179 | 3.179219707 | T |
| rs41255381 | 11 | 102372360 | 3.176418169 | G |
| rs108986684 | 11 | 102384045 | 3.176418169 | G |
| rs109581668 | 24 | 54495215 | 3.176163505 | A |
| rs110134632 | 24 | 54495934 | 3.176163505 | C |
| rs137033644 | X | 90821291 | 3.174601939 | T |
| rs135644841 | 26 | 25671490 | 3.171533024 | G |
| rs42097093 | 26 | 25675473 | 3.171533024 | G |
| rs109399466 | 10 | 28046636 | 3.16912882 | G |
| rs41668105 | 12 | 76851725 | 3.169066788 | C |
| rs41884306 | 18 | 49803763 | 3.166440454 | G |
| rs109818611 | 4 | 17256442 | 3.165984424 | C |
| rs137621015 | 4 | 17265172 | 3.165984424 | G |
| rs110756908 | 4 | 17265949 | 3.165984424 | T |
| rs110291582 | 4 | 17266853 | 3.165984424 | T |
| rs109783538 | 4 | 17267806 | 3.165984424 | A |
| rs133565311 | 4 | 17271497 | 3.165984424 | A |
| rs110884608 | 4 | 17273366 | 3.165984424 | G |
| rs109154308 | 4 | 17274912 | 3.165984424 | G |
| rs109585036 | 19 | 8240893 | 3.161083557 | A |
| rs41666998 | 12 | 68271937 | 3.159651103 | A |
| rs136402214 | 23 | 40834768 | 3.159447603 | T |
| rs133897861 | 23 | 40836579 | 3.159447603 | A |
| rs41569491 | 10 | 13373269 | 3.157691601 | C |
| rs41619924 | 17 | 11367675 | 3.151175936 | A |
| rs41619926 | 17 | 11391752 | 3.151175936 | A |
| rs42880540 | 17 | 11407035 | 3.151175936 | C |
| rs42691544 | 15 | 5058540 | 3.150457207 | T |
| rs42415316 | 10 | 39028282 | 3.150169767 | C |
| rs136359869 | 10 | 39029090 | 3.150169767 | A |
| rs42415320 | 10 | 39029787 | 3.150169767 | T |
| rs42410538 | 10 | 39032213 | 3.150169767 | C |

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| rs109328536 | 10 | 39037912 | 3.150169767 | T |
| rs43618980 | 10 | 11495213 | 3.149188625 | G |
| rs133096287 | 11 | 69539045 | 3.148441969 | G |
| rs109986884 | 11 | 69540272 | 3.148441969 | A |
| rs41604688 | 3 | 90770539 | 3.148305403 | G |
| rs42423304 | 3 | 90765771 | 3.148305403 | A |
| rs42423302 | 3 | 90769435 | 3.148305403 | A |
| rs41836702 | 26 | 28192862 | 3.144206218 | C |
| rs41604826 | 26 | 28193472 | 3.144206218 | A |
| rs41836713 | 26 | 28194285 | 3.144206218 | G |
| rs41837632 | 26 | 28195737 | 3.144206218 | G |
| rs41837646 | 26 | 28197815 | 3.144206218 | A |
| rs41837651 | 26 | 28199991 | 3.144206218 | C |
| rs134942554 | 26 | 28200869 | 3.144206218 | A |
| rs41837664 | 26 | 28202542 | 3.144206218 | G |
| rs41837665 | 26 | 28203265 | 3.144206218 | A |
| rs109135855 | 6 | 45845263 | 3.144023491 | T |
| rs135890677 | 6 | 45853339 | 3.144023491 | G |
| rs110858846 | 6 | 45861086 | 3.144023491 | T |
| rs136076532 | 9 | 97586690 | 3.142161648 | G |
| rs132952962 | 10 | 14658435 | 3.137810362 | G |
| rs109180951 | 28 | 31204958 | 3.136475937 | T |
| rs136456101 | 28 | 31206165 | 3.136475937 | G |
| rs136341552 | X | 92725516 | 3.134137478 | A |
| rs42584654 | 6 | 4888244 | 3.134052041 | G |
| rs110946154 | 5 | 97426617 | 3.129526615 | T |
| rs110729080 | 5 | 97435197 | 3.129526615 | A |
| rs136811150 | 7 | 39875547 | 3.12811969 | G |
| rs109542089 | 11 | 78673403 | 3.128089748 | A |
| rs136068749 | 11 | 78679962 | 3.128089748 | G |
| rs110792012 | 11 | 78687979 | 3.128089748 | A |
| rs109788835 | 11 | 78693214 | 3.128089748 | A |
| rs42718765 | 3 | 93811195 | 3.127079567 | T |
| rs133304868 | 17 | 33892418 | 3.126364031 | C |
| rs137641657 | 27 | 309276 | 3.12223744 | A |
| rs29013678 | 2 | 119390554 | 3.122146871 | C |
| rs110184871 | 2 | 119404166 | 3.122146871 | A |
| rs110397335 | 2 | 119405235 | 3.122146871 | C |
| rs110037371 | 18 | 49703830 | 3.119651106 | C |
| rs134829952 | 26 | 8468758 | 3.117604293 | A |
| rs136971417 | 4 | 20277122 | 3.11717339 | T |
| rs136177868 | 8 | 7488870 | 3.116148286 | T |
| rs42914113 | 10 | 90274472 | 3.113837034 | T |
| rs137408584 | 10 | 90294811 | 3.113837034 | C |
| rs43619617 | 10 | 31072492 | 3.11062073 | C |
| rs43619619 | 10 | 31079958 | 3.11062073 | C |
| rs134753825 | 10 | 31093137 | 3.11062073 | A |

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|---|---|---|---|---|
| rs43769813 | 10 | 31100363 | 3.11062073 | A |
| rs137148762 | 17 | 37971352 | 3.108355235 | T |
| rs135668452 | 17 | 37974917 | 3.108355235 | A |
| rs110458553 | 17 | 37975827 | 3.108355235 | C |
| rs134482616 | 17 | 37976731 | 3.108355235 | G |
| rs133316400 | 17 | 37991827 | 3.108355235 | T |
| rs41567083 | 17 | 38001105 | 3.108355235 | A |
| rs137692466 | 29 | 22208092 | 3.106706804 | A |
| rs133730050 | 10 | 18557936 | 3.105290125 | C |
| rs42307494 | 17 | 41686505 | 3.102338037 | A |
| rs136937470 | 17 | 41687319 | 3.102338037 | T |
| rs42306997 | 17 | 41701959 | 3.102338037 | T |
| rs42306988 | 17 | 41710220 | 3.102338037 | G |
| rs41594602 | 17 | 41710814 | 3.102338037 | G |
| rs42306972 | 17 | 41714001 | 3.102338037 | G |
| rs110262369 | 11 | 80363995 | 3.101961624 | G |
| rs110066674 | 11 | 80365976 | 3.101961624 | C |
| rs109072977 | 7 | 33452602 | 3.099882435 | T |
| rs43618994 | 10 | 11498562 | 3.096714343 | T |
| rs43619004 | 10 | 11502981 | 3.096714343 | A |
| rs108947346 | 10 | 11504567 | 3.096714343 | G |
| rs109320019 | 10 | 11505110 | 3.096714343 | C |
| rs136837752 | 2 | 77402401 | 3.095079099 | A |
| rs134898712 | 2 | 77408791 | 3.095079099 | G |
| rs43114451 | 2 | 77410104 | 3.095079099 | A |
| rs133646028 | 9 | 96369957 | 3.092594656 | A |
| rs137193544 | 20 | 12386335 | 3.089779692 | A |
| rs135048392 | 4 | 9321332 | 3.089742433 | G |
| rs109123301 | 27 | 3808044 | 3.089295934 | C |
| rs110915880 | 27 | 3809006 | 3.089295934 | C |
| rs110727594 | 27 | 3810639 | 3.089295934 | T |
| rs109338247 | 27 | 3820475 | 3.089295934 | T |
| rs41971212 | 21 | 21547822 | 3.087393124 | G |
| rs134446726 | 13 | 61484398 | 3.083348862 | T |
| rs109300977 | 16 | 21376068 | 3.082209646 | T |
| rs137292613 | 15 | 23175841 | 3.08175322 | G |
| rs110258423 | 10 | 29718121 | 3.079582163 | C |
| rs43616376 | 10 | 14258096 | 3.078740179 | T |
| rs109609662 | 10 | 14264457 | 3.078740179 | T |
| rs110956596 | 10 | 14271326 | 3.078740179 | C |
| rs137608138 | 27 | 20504395 | 3.076785852 | T |
| rs133261547 | 27 | 20505934 | 3.076785852 | C |
| rs41598713 | 10 | 11571743 | 3.07580214 | A |
| rs29022854 | 4 | 106321900 | 3.070621877 | A |
| rs136741195 | 4 | 106328752 | 3.070621877 | G |
| rs136442787 | 5 | 41037898 | 3.069245785 | A |
| rs134869709 | 5 | 41048689 | 3.069245785 | C |

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| rs137793969 | 5 | 41051042 | 3.069245785 | G |
| rs41619458 | 17 | 10396137 | 3.067403711 | G |
| rs109460994 | 7 | 32226154 | 3.066312091 | A |
| rs110338491 | 9 | 20446443 | 3.06585316 | C |
| rs137384335 | 9 | 20474683 | 3.06585316 | C |
| rs41566638 | 11 | 94071468 | 3.061720153 | G |
| rs41566639 | 11 | 94095363 | 3.061720153 | G |
| rs41566640 | 11 | 94101466 | 3.061720153 | T |
| rs137848095 | 11 | 94103660 | 3.061720153 | C |
| rs137111321 | 11 | 94125261 | 3.061720153 | A |
| rs135844990 | 11 | 94126990 | 3.061720153 | C |
| rs133488368 | 11 | 94130741 | 3.061720153 | A |
| rs135362767 | 11 | 94137569 | 3.061720153 | G |
| rs133163960 | 11 | 94142045 | 3.061720153 | C |
| rs133228922 | 11 | 94157282 | 3.061720153 | C |
| rs137261893 | 11 | 94161778 | 3.061720153 | A |
| rs41566641 | 11 | 94446742 | 3.061720153 | T |
| rs136735251 | 11 | 94458546 | 3.061720153 | A |
| rs134890269 | 22 | 38209582 | 3.060969843 | G |
| rs110151708 | 24 | 53584633 | 3.059266467 | G |
| rs134469680 | 24 | 53647985 | 3.059266467 | C |
| rs41587428 | 3 | 20727422 | 3.058836777 | A |
| rs133503031 | 17 | 41750810 | 3.055687311 | G |
| rs109685974 | 6 | 1646307 | 3.054860245 | C |
| rs42998566 | 6 | 1712714 | 3.054860245 | G |
| rs42873303 | 6 | 1776509 | 3.054860245 | G |
| rs109101146 | 11 | 37436464 | 3.052703378 | T |
| rs135507951 | 11 | 37499319 | 3.052703378 | T |
| rs134539773 | 1 | 9136608 | 3.051983047 | G |
| rs134476953 | 3 | 90818585 | 3.050303974 | A |
| rs134508681 | 27 | 25171321 | 3.047656763 | T |
| rs42633912 | 26 | 36608784 | 3.046595379 | G |
| rs43120804 | 17 | 11784143 | 3.045144937 | C |
| rs134112858 | 4 | 91304669 | 3.04413363 | G |
| rs110363109 | 4 | 91305332 | 3.04413363 | T |
| rs132996808 | 4 | 91307320 | 3.04413363 | A |
| rs42574539 | 4 | 91308203 | 3.04413363 | G |
| rs135393323 | 4 | 91309151 | 3.04413363 | G |
| rs133214867 | 4 | 91310256 | 3.04413363 | G |
| rs135925889 | 11 | 80423078 | 3.044019522 | A |
| rs135415596 | 11 | 80432266 | 3.044019522 | T |
| rs110562044 | 11 | 80440180 | 3.044019522 | G |
| rs135752409 | 9 | 35835329 | 3.040729485 | A |
| rs41668076 | 12 | 76871379 | 3.038966693 | C |
| rs109250741 | 17 | 41738251 | 3.035858125 | C |
| rs42174547 | 29 | 25876364 | 3.035625358 | A |
| rs42174558 | 29 | 25877577 | 3.035625358 | C |

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|---|---|---|---|---|
| rs132896883 | 29 | 25878907 | 3.035625358 | G |
| rs133952565 | 26 | 25665978 | 3.034792469 | A |
| rs137389486 | X | 56899350 | 3.032715962 | C |
| rs42358070 | 16 | 64382007 | 3.03201204 | G |
| rs135141127 | 5 | 55223818 | 3.031366658 | C |
| rs110079960 | 4 | 14382671 | 3.030198654 | T |
| rs136816057 | 8 | 103151786 | 3.03013748 | C |
| rs109933177 | 18 | 49804652 | 3.030089291 | A |
| rs133858057 | 18 | 49806791 | 3.030089291 | G |
| rs109833294 | 18 | 49808001 | 3.030089291 | T |
| rs110634591 | 18 | 49808532 | 3.030089291 | T |
| rs110700417 | 18 | 49810196 | 3.030089291 | A |
| rs42550173 | 9 | 57878818 | 3.029814845 | C |
| rs109073837 | 9 | 57938963 | 3.029814845 | A |
| rs133836094 | 16 | 52097690 | 3.029701627 | A |
| rs42435944 | 26 | 28854387 | 3.028548934 | A |
| rs109106349 | 4 | 15641166 | 3.02742991 | T |
| rs133633650 | 9 | 85232186 | 3.026904167 | G |
| rs135320876 | 17 | 46212141 | 3.021482544 | T |
| rs132870941 | 17 | 46214774 | 3.021482544 | T |
| rs134152512 | 17 | 46215571 | 3.021482544 | C |
| rs135356708 | 17 | 46217001 | 3.021482544 | A |
| rs133010301 | 17 | 46218318 | 3.021482544 | A |
| rs132770494 | 9 | 87270703 | 3.021111305 | A |
| rs135200117 | X | 142871442 | 3.018211447 | C |
| rs136742797 | 29 | 22180284 | 3.017631735 | T |
| rs137115882 | 3 | 105906013 | 3.016694846 | C |
| rs133218184 | 1 | 13753312 | 3.016631115 | A |
| rs135399735 | 1 | 13879534 | 3.016631115 | G |
| rs109400802 | 1 | 13899013 | 3.016631115 | A |
| rs110695322 | 1 | 13901306 | 3.016631115 | A |
| rs136598771 | 1 | 13997990 | 3.016631115 | G |
| rs41620921 | 7 | 32655349 | 3.015837284 | A |
| rs42065895 | 7 | 32664122 | 3.015837284 | A |
| rs110391851 | 7 | 35805518 | 3.015837284 | A |
| rs109830170 | 7 | 35814180 | 3.015837284 | T |
| rs133228921 | 3 | 90794047 | 3.015756824 | A |
| rs137161699 | X | 105051845 | 3.015376242 | C |
| rs135689626 | 10 | 35112151 | 3.015309983 | A |
| rs110868693 | 17 | 41718080 | 3.014450931 | A |
| rs109973902 | 10 | 15367638 | 3.013635399 | T |
| rs43410902 | 4 | 91099205 | 3.013601801 | C |
| rs42179558 | 29 | 28584509 | 3.01279943 | C |
| rs110521499 | 3 | 88708244 | 3.00966213 | T |
| rs134331064 | 2 | 72885823 | 3.007280514 | T |
| rs110713433 | 2 | 135120564 | 3.006187294 | C |
| rs110367201 | 2 | 135128893 | 3.006187294 | A |

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|---|---|---|---|---|
| rs135870948 | 2 | 135130866 | 3.006187294 | C |
| rs136792452 | 2 | 135131655 | 3.006187294 | T |
| rs134612066 | 2 | 135132299 | 3.006187294 | C |
| rs135740697 | 2 | 135132854 | 3.006187294 | T |
| rs110581623 | 1 | 15591002 | 3.004488715 | T |
| rs133956665 | 24 | 54038094 | 3.004295257 | A |
| rs109346861 | 13 | 564192 | 3.004100587 | T |
| rs109531470 | 13 | 576926 | 3.004100587 | T |
| rs109645110 | 13 | 584808 | 3.004100587 | A |
| rs134953798 | 13 | 599841 | 3.004100587 | T |
| rs110838698 | 3 | 86361340 | 3.00152615 | G |
| rs109496760 | 12 | 79248382 | 3.001163077 | T |

CURRICULUM VITAE

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97 S 100 W
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## SUMMARY

American College of Veterinary Pathologists Board-certified veterinary anatomic pathologist. Currently working as a Clinical Assistant Professor (Temporary) and Extension Veterinary Pathologist for the Utah Veterinary Diagnostic Laboratory, Utah State University. PhD in animal molecular genetics at Utah State University. Veterinary graduate of the University of Edinburgh, Scotland. Supporting BS degree in in animal science, and experience in veterinary research, regulated laboratory procedures, and livestock husbandry. Member of the Royal College of Veterinary Surgeons (United Kingdom), and licensed veterinarian in the state of Utah.

## EDUCATION

Veterinary Anatomic Pathology Residency – 2011 to 2017
Utah Veterinary Diagnostic Laboratory, Utah State University – Logan, UT

PhD, Animal Molecular Genetics – 2012 to 2017
Utah State University – Logan, UT

BVM&S, with Distinction – 2010
University of Edinburgh, Royal (Dick) School of Veterinary Studies – Edinburgh, UK
*AVMA-accredited veterinary degree.*

BS, Animal Science, *magna cum laude* – 2006
Cornell University, College of Agriculture and Life Sciences – Ithaca, NY

## EXPERIENCE

UTAH STATE UNIVERSITY 2017-Present

Clinical Assistant Professor, Temporary, Animal, Dairy, and Veterinary Science

American College of Veterinary Pathologists Board-certified anatomic veterinary pathologist with extension veterinary pathologist (70%), teaching (25%), and departmental service (5%) duties. Extension veterinary pathologist duties include performance, interpretation, and reporting of necropsies, histopathology, and ancillary testing. Teaching responsibilities include assistance of primary instructors for general and systemic veterinary pathology courses in the USU veterinary school, resident training, and organization and teaching of resident seminar series. Departmental service duties encompass service on departmental committees.

UTAH STATE UNIVERSITY 2011-2017

Veterinary Anatomic Pathology Residency

Veterinary residency working toward eligibility for Board certification under the American College of Veterinary Pathologists. Duties involved primary case responsibility for animals submitted for necropsy and samples submitted for surgical pathology, and graduate teaching assistant assignments in Microscopic Anatomy, General Pathology, and Systemic Pathology at the veterinary school level.

UNIVERSITY OF EDINBURGH AND AVIAGEN – Edinburgh, UK 2010

Poultry Medicine Elective Rotation

Veterinary rotation working with the poultry veterinary service at the University of Edinburgh and the global poultry genetics company Aviagen. Duties performed included poultry handling, health and welfare assessment, disease detection and treatment, post mortem examination, egg quality assessment, and flock management.

UNIVERSITY OF EDINBURGH, VETERINARY PATHOLOGY UNIT – Edinburgh, UK 2009

Research Assistant
Member of scientific research team investigating causes of mortality in red squirrels in Scotland, contributing to knowledge vital to wildlife conservation efforts. Lead author on resulting publication.

VETERINARY PRACTICES AND SERVICES – UK and USA 2008-2010

Clinical Veterinary Extramural Work
Placements

Clinical veterinary work external to university schooling included placements in small animal practice (10 weeks), farm animal practice (2 weeks), farm assurance assessment (2 weeks), equine hospital/intensive care (2 weeks), exotics/wildlife hospital (1 week), and abattoir (1 week). Duties included assistance with client consultations, clinical examination, patient monitoring and treatment, disease diagnosis, surgery, post mortem examination, herd health evaluation, and welfare assessment.

ADVION BIOSERVICES – Ithaca, NY 2001-2006

Laboratory Technician Intern

Performed and documented instrument and laboratory maintenance, sample custody, and clinical supply logistics in a regulated bioanalytical laboratory.

CORNELL UNIVERSITY SHEEP RESEARCH & TEACHING UNIT – Hartford, NY 2002-2005

Farm Worker

Responsible for the husbandry of sheep, including lambing ewes and lambs, in a 750-ewe flock.

HEGDALE FARM – Cumbria, UK 2004

Resident Farm Worker

Responsible for the daily husbandry and management of sheep, calves, pigs, and free-range poultry on a small family-run farm.

## PROFESSIONAL AFFILIATIONS

Diplomate, American College of Veterinary Pathologists

Licensed veterinarian, state of Utah

Member of the Royal College of Veterinary Surgeons (United Kingdom)

## PUBLICATIONS

A.J. Van Wettere, J. P. Kurz, A. Wilhelm, and J. D. Ipsen. Opisthotonos and unilateral internal hydrocephalus associated with aberrant migration of *Serratospiculum spp.* in a prairie falcon (*Falco mexicanus*). Journal of Veterinary Diagnostic Investigation, accepted 2017.

D. J. Wilson and J. P. Kurz. (2017) Genetic sequencing using 16S rRNA for pathogen identification in retropharyngeal lymph nodes from wild elk. Human-Wildlife Interactions 11(1), 19-21.

J. P. LaRose, A.L. Meredith, D.J. Everest, C. Fiegna, C.J. McInnes, D.J. Shaw, and E.M. Milne. (2010) Epidemiological and post-mortem findings from 262 red squirrels (*Sciurus vulgaris*) in Scotland (2002-2009). Veterinary Record 167, 297-302.

## AWARDS

2014 College of Agriculture and Applied Sciences Graduate Student Teacher of the Year. Utah State University, Logan, Utah. 18 September 2014.

2013 Utah State University Animal, Dairy, and Veterinary Science Department Graduate Student Symposium, 1st place PhD track oral presentation. Utah State University, Logan, UT. 5 August 2013.

## LECTURES

"Genetic Markers of Bovine Mastitis Resistance." Animal, Dairy, and Veterinary Science/Center for Integrated Biotechnology Seminar Series. Utah State University, Logan, UT. 7 September 2017.

"Lecture Series: Lower GI System: Intestine, Parts I, II, and III." Systemic Pathology, VM 7546. Utah State University, Logan, UT. September 2017, September 2016, and September 2015.

"Lecture series: Ruminant Stomach, Parts I and II." Systemic Pathology, VM 7546. Utah State University, Logan, UT. September 2017, September 2016, September 2015, and September 2014.

"Case Study: Hyperkalemic Periodic Paralysis in a Quarter Horse." Veterinary Physiology I, VM 7519. Utah State University, Logan, UT. October 2015 and October 2014.

"Digestive System: Intestine." Microscopic Anatomy, VM 7510. Utah State University, Logan, UT. October 2012.

## RESEARCH SUPPORT

2014-2015: Molecular characterization of the involvement of phospholipid A2 (PLA2) in the immune response to mastitis pathogens in dairy cattle in vivo and in vitro. PI: Z. Wang. Utah Agriculture Experiment Station Grants Program, $9,004.52 awarded.

2013-2014: Differences in production of IL-1β, IL-6, and TNF-α by cultured bovine mammary epithelial cells from mastitis-resistant and mastitis-susceptible Holstein dairy cows. PI: D.J. Wilson, Co-PI: J. LaRose. Utah Agriculture Experiment Station Grants Program, $10,000 awarded.