Brigham Young University
BYU ScholarsArchive

All Theses and Dissertations

2017-06-01

Comparison of Cytokine Expression and Bacterial Growth During Periparturient and Mid Lactation Mastitis in a Mouse Model

Rhonda Nicole Chronis
Brigham Young University

Follow this and additional works at: https://scholarsarchive.byu.edu/etd

 Part of the Microbiology Commons

BYU ScholarsArchive Citation

Chronis, Rhonda Nicole, "Comparison of Cytokine Expression and Bacterial Growth During Periparturient and Mid Lactation Mastitis in a Mouse Model" (2017). *All Theses and Dissertations*. 6847.
https://scholarsarchive.byu.edu/etd/6847

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.

Comparison of Cytokine Expression and Bacterial Growth During Periparturient and Mid-Lactation Mastitis in a Mouse Model

Rhonda Nicole Chronis

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Eric Wilson, Chair
David Erickson
Scott Weber

Department of Microbiology and Molecular Biology

Brigham Young University

Copyright © 2017 Rhonda Nicole Chronis

All Rights Reserved

ABSTRACT

Comparison of Cytokine Expression and Bacterial Growth During Periparturient and Mid-Lactation Mastitis in a Mouse Model

Rhonda Nicole Chronis
Department of Microbiology and Molecular Biology, BYU
Master of Science

Clinical cases of bovine mastitis are most severe in the early stages of lactation. The causes of this increased propensity for severe mastitis during early lactation, compared to mid and late lactation are unclear. In order to better understand the early lactation immune response to mastitis, a murine model of mastitis was employed. Intramammary inoculation of a mastitis causing *Escherichia coli* strain was performed in lactating mice at various stages of lactation to model the immune response seen in cows during lactation. In our experiments, mice in the early stages of lactation exhibited altered mRNA expression of cytokines IL-1β, IL-6, IL-10, and TNFα over the course of infection when compared to mice at mid-lactation. Additionally, increased bacterial growth was observed in the mammary gland of mice infected during early lactation compared to late lactation. These results are consistent with the immune response observed in cows at early lactation. These results suggest that the mouse may provide a useful model to study differences in the immune response seen during different times in lactation.

Keywords: mastitis, periparturient, mouse model

ACKNOWLEDGMENTS

I am so grateful for the opportunity I have had to attend graduate school at Brigham Young University. The faculty and atmosphere has made this a truly enjoyable experience. I would sincerely like to thank my graduate committee for their guidance and support. I'd like to thank Dr. Wilson for allowing me to pursue a project that excited me and for being encouraging and understanding every step of the way. (And for all the chocolate donuts!) Dr. Erickson and Dr. Weber have been instrumental in helping me focus my project and understand what is important and see the big picture. I am so grateful for all of their feedback and in pushing me to be a better researcher and writer.

My fellow graduate students have been a great support system through all the ups and downs during my graduate program. Lauren Johnson, Jared Hoffman and Michael Olson have not only shared in my triumphs, but have also lent an understanding and listening ear when needed. I'd especially like to thank Sara Mason for her unselfish help and guidance throughout my project; this would not have been possible without her.

Lastly, but certainly not least, I'd like to thank my family and friends who have supported me throughout this process. I have been truly blessed to have such an amazing support system. I could not have done this without their love, support, patience and prayers. Thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Mastitis

Inflammation of the mammary gland, known as mastitis, is a common disease of mammals. Mastitis is the result of an inflammatory response in the mammary gland due to trauma, metabolic and physiologic changes, or most commonly as the result of an infectious pathogen (1). Between 10-30% of lactating women are affected by mastitis (2). Additionally, mastitis is the most common and costly infectious disease of the dairy industry, resulting in a multibillion dollar economic loss each year (3, 4). The costs associated with bovine mastitis are the result of treatment, decreased milk production, culling, and death (5), with mastitis being the leading cause of death in adult dairy cows (6). Over the past century, dairy cattle have been genetically selected to maximize milk production. However, this singular focus on maximum milk production has had a negative effect on the cow's ability to resist mastitis (7). The increased incidence of mastitis in commercial dairy herds has contributed to the overuse of antibiotics which has in turn contributed to the emergence of antibiotic resistant bacteria, which are a public health concern (8).

Mammary gland infections in cows are caused by a wide variety of organisms; these include bacteria, mycoplasma, yeasts, viruses and even algae, with bacteria being the most common (9). Infectious agents that cause mastitis are typically classified as contagious or environmental. Contagious pathogens are those that are adapted to survive within the host, establish subclinical infections, and spread from cow to cow (often via contaminated milking equipment). The most common contagious pathogens include: *Acholeplasma laidlawii, Mycoplasma agalactiae, Mycolplasma bovigenitalium, Mycoplasma bovirhinis, Mycoplasma*

mycoides, Pasteuralla haemolytica, Streptococcus agalactiae and *Staphylococcus aureus,* with *S. aureus* being the predominant mastitis causing pathogen in this group (9). In contrast, environmental pathogens are opportunistic organisms that are often not well adapted to survive long term within the mammary gland. These pathogens commonly cause acute disease, and are then rapidly eliminated by the host immune system (5). The past several decades has seen a dramatic reduction in incidence of mastitis caused by contagious pathogens such as *S. aureus*. This has indirectly led to an increased percentage of clinical mastitis cases caused by opportunistic pathogens such as *Campylobacter coli, Pseudomonas aeruginosa, Streptococcus uberis,* and *Escherichia coli* (9). Currently, infections from *Escherichia coli* (*E. coli*) are the leading cause of acute mastitis in dairy cattle (9, 10). Infections from *E. coli* can induce a severe clinical case of mastitis that can cause extensive tissue damage to the mammary gland and in some cases death (11). Due to the environmental prevalence of *E. coli*, this pathogen remains a persistent threat to the dairy industry (12).

In the past, differences in disease severity of mastitis infections has often been attributed to cow factors rather than differences in bacterial virulence (13). Although differences in the immune response seen in individual cows certainly plays a role in disease progression and severity, recent research strongly indicates that mammary pathogenic *E. coli* (MPEC) likely express a different complement of genes compared to other *E. coli* pathotypes. Potential MPEC virulence factors include type six secretion systems, long polar fimbriae and a ferric dicitrate iron acquisition system (14-16). These virulence genes may allow bacteria to thrive in specific niches within the mammary gland, such as the gland cistern, milk tubules and alveolar epithelium (17). While mastitis caused by *E. coli* has been subject to extensive epidemiological studies, the genetic analysis of MPEC virulence remains limited.

Immune System

The immune system is a tightly regulated host defense system. Physical barriers, such as skin, prevent pathogens from invading the host. Once the invading organism breaches these barriers they are met by an array of innate immune defenses. The innate immune response is a rapid non-specific response that does not result in long lasting immune protection (18). However, components of the innate immune system interact with the adaptive immune system, presenting antigens that initiate immunological memory. Immunological memory provides for a stronger and faster immune response when a pathogen is encountered a second time. The activation and duration of both the innate and adaptive immune system is also regulated by increases and decreases in production of pro- and anti-inflammatory cytokines. Dysregulation of the immune system results in unrestricted growth of microbes and accompanying host pathology.

Mammary Gland Immunology

There are several aspects of physiology and immunity that play a role in prevention and recovery from a mastitis infection. Before an infectious agent can cause disease, it must first gain access to the lumen of the mammary gland via the teat canal. The teat canal is sealed between milkings in an effort to prevent bacterial colonization of the mammary gland (19). After milking, it takes about two hours for the teat end to reseal and close the canal, leaving the mammary gland vulnerable to microbial invasion (20). Once in the mammary gland, bacteria must overcome the antimicrobial defenses of the gland to establish disease. If cattle are unable to eliminate the infectious agents, bacterial will grow in the mammary gland, ultimately resulting in damage to the mammary epithelium. Effective immune protection of the mammary gland is highly dependent on the innate immune system (1, 5, 10, 19-72). Conserved features found on invading pathogens, known as pathogen-associated molecular patterns (PAMPS), stimulate this innate

immune response. These features include lipopolysaccharide (LPS), peptidoglycan, methylated DNA, and the negative charge found on the outer membrane of bacteria. Recognition of PAMPS by the immune system leads to inflammation and the recruitment/stimulation of immune cells and production/activation of soluble effector molecules (21).

Cellular Defenses

Cellular defenses of the mammary gland play a vital role in limiting the severity and duration of an infection. The resident and recruited leukocytes of the mammary gland include neutrophils, macrophages and lymphocytes. In a healthy lactating mammary gland, macrophages are the dominant cell type in milk and tissues (1, 3, 5, 10, 11, 22). During a bacterial infection, macrophages phagocytize invading bacteria and kill them with proteases and reactive oxygen species (ROS). Macrophage numbers during an infection remain relatively constant in the mammary gland. However, they play a vital role in secreting soluble effectors that enhance migration and antibacterial activity of neutrophils (22). Macrophages also function as a component of the adaptive immune system, by presenting phagocytized bacterial antigens on MHC II receptors to stimulate the $CD4^+$ Th1 response (24, 25).

Neutrophils are thought to be the most important cell type of the innate immune response in the mammary gland (26). During the early stage of an infection, neutrophils are recruited to the mammary gland by a variety of inflammatory mediators such as cytokines, chemokines and complement, expressed by resident immune cells. During an infection, greater than 90% of the leukocyte population found in mammary tissue and milk are neutrophils (26). The importance of neutrophil mediated protection of the lactating mammary gland is illustrated by a study indicating that the magnitude of an *E. coli* mastitis infection is directly related to the rate of bacterial growth *in vivo* prior to the influx of neutrophils (27). Experimental mastitis studies have

shown neutrophils are recruited to tissues infected with *E. coli* approximately 1-2 hours following exposure to infectious agent (22).

Neutrophils kill bacteria in multiple ways, including through phagocytosis and intracellular killing, as well as through the release of bactericidal proteins. Phagocytosis of bacteria by neutrophils is facilitated by opsonizing agents such as antibodies, complement and cytokines. The bactericidal effect of neutrophils is mediated through proteases and respiratory bursts that produce ROS (28). Interestingly, milk neutrophils have been shown to have lower ROS activity than blood neutrophils (29). However, excessive inflammation attributed to neutrophil activity in the mammary gland often results in host tissue damage and decreased milk production (34).

Soluble Defenses

Soluble factors are an important line of defense in the mammary gland. The innate soluble factors present in the mammary gland play a critical role in combating invading pathogens. Many of these soluble defenses are classified as antimicrobial peptides, these proteins directly inhibit growth or cause death of invading microbes. Antimicrobial peptides known to be important in immune protection of the lactating mammary gland include lactoferrin and lysozyme. Antimicrobial proteins function in a variety of ways, including iron sequestration and inhibiting cell wall biosynthesis (30, 35).

Cytokines are produced by many different cell types in the mammary gland and have been shown to have overlapping activities as regulators of the proliferation and differentiation of immune cells (18, 31). Cytokines present in the milk and mammary gland are critical in orchestrating the immune response needed to clear a microbial infection (31). There are four major groups of cytokines: interleukins (IL), interferons (IFN), colony-stimulating factors (CSF),

and tumor necrosis factor (TNF). Table I provides a brief overview of the cytokines known to be expressed during a mammary inflammatory response and their observed effects. These cytokines are critical for an efficient and effective immune response and in the clearance of invading pathogens. Proinflammatory cytokines expressed during a mammary gland infection and known to be critical during an immune response include IL-1β, TNF-α, IL-6, IFN-γ, IL-2, and G-CSF (18).

Previous research has shown that a number of proinflammatory cytokines are critical to effective mammary gland immune responses (36). During an *E. coli* infection, there is a sharp increase of IL-1β levels, which is associated with the influx of neutrophils (36). Pro-IL-1β is secreted by activated macrophages, resulting in increased body temperature and circulating acute-phase proteins (37). Additionally, IL-1β receptor binding activates signal transduction of various mediators, which promote kinase activation, resulting in the activation of NF-κB and MAP kinase dependent nuclear factors (37).

IL-6 is secreted by T and B lymphocytes, monocytes, fibroblasts and activated macrophages (38). IL-6 bound to its receptor activates the JAK/STAT pathway (37). Stimulation of bovine mammary gland epithelial lines with LPS induces IL-6 (39), and in mammary glands infected with *E. coli*, IL-6 expression has been detected as early as eight hours post infection (40). It has been suggested that IL-6 facilitates the inflammatory process in transitioning from the influx of neutrophils to monocytes, which is essential for the appropriate immune response to decrease the potentially harmful effect of neutrophils (41).

TNF-α expression is significantly increased during coliform mastitis, and in LPS infused mammary glands (42, 43). TNF-α plays a critical role in the immunological response of the mammary gland to coliform mastitis by recruiting and activating neutrophils and elevating levels

of nitrite and nitrate, which aid in the killing of invading pathogens (44). Macrophages secrete TNF-α upon stimulation. TNF-α inhibits expression of TLR4 on monocytes in conjunction with an IL-6 increase and enhances neutrophil superoxide production (37, 45, 46).

Following a robust inflammatory response and clearance of invading pathogens, a new set of regulatory cytokines are expressed. These cytokines are anti-inflammatory and act to repress the immune response and prevent tissue damage. IL-10 is a strong anti-inflammatory cytokine, which mediates decreased proinflammatory cytokine response. IL-10 is secreted by activated T cells, monocytes/macrophages, mast cells and keratinocytes (37).

The appropriately timed expression of these cytokines is key to an effective immune response and previous research has shown that the acute symptoms of a mastitis infection are often the result of rapid growth by the invading organism combined with an inappropriate pro inflammatory response (18). Controlling the delicate balance of a strong proinflammatory response to kill invading pathogens, that is also limited in duration, is key to an effective and safe immune response.

Tissue Damage

Mammary gland tissue damage occurs as the bacterial load increases, toxins accumulate and excessive inflammation occurs. The mammary gland alveoli begin to lose their structural integrity and visible changes to milk and mammary gland occur (35). Mammary epithelial cells are damaged during mastitis by a variety of mechanisms, including the release of cellular and extracellular products from the invading bacterial pathogens, ROS and lysosomal enzymes released by phagocytes, and by the release of proteases and cytokines during the proinflammatory immune response (47). In mild and moderate cases of *E. coli* mastitis there is minimal alveolar tissue damage. However, in severe *E. coli* mastitis there is a significant amount

of necrosis of the mammary epithelium (48). Unrestricted neutrophil activity promotes tissue damage within the mammary gland by ROS production and degranulation (26). Direct damage to epithelial cells, as a result of neutrophil activity, has been shown by Mehrzad when mastitic milk was co-incubated with healthy mammary tissue resulting in tissue degradation (49). Additionally, proinflammatory cytokines such as TNF-α and IL-1 can induce apoptosis in bovine and human mammary endothelial cells (50).

Apoptosis and necrosis of tissues occurs during an infection in response to a variety of inducers, such as cytokines and bacterial toxins (51). The apoptosis of cells is tightly regulated by a cascade of signaling events that involve both pro-apoptotic and anti-apoptotic factors. These regulators include pro-apoptotic factors Bax, Bak, Bad, caspase-1 and anti-apoptotic factor Bcl-2. Pro-apoptotic proteins promote permeabilization of the mitochondrial membrane and release of ROS (52). Bcl-2 is localized on the mitochondria membrane and functions by inhibiting ROS species activity. Increases in expression of Bax and caspase-1 and a decrease of expression of Bcl-2 have been demonstrated in bovine mammary gland infections with *E. coli* (53).

Early and Late Lactation

It has been well established in dairy cattle that the efficiency of the mammary gland immune response is directly related to the stage of lactation (56-72). Studies have shown over 50% of clinical mastitis cases occur around the time of parturition, and of those that are severe, 25% of will result in culling or death (56). A similar pattern of severe mastitis occurring shortly after birth and less severe mastitis occurring latter in lactation is also seen in other mammals such as goats and humans. (2, 56).

Clinical coliform mastitis is rare during mid to late lactation and is most severe during the first few weeks of lactation (11). Previous studies have shown that during mid-lactation *E. coli*

were promptly eliminated without causing damage to the mammary tissues. However, *E. coli* infections during the periparturient time resulted in severe infections (60). This increased susceptibility to clinical coliform mastitis has been hypothesized to be related to the changes in composition, magnitude and efficiency of the mammary gland immune response. There has been an overwhelming amount of evidence of the immunological dysfunction of neutrophils and lymphocytes in periparturient cattle (22, 61-69). Additionally, previous research has shown that during the periparturient period $CD4^+$ T cells produce less proinflammatory cytokines and more anti-inflammatory cytokines than $CD4^+$ cells during the later stages of lactation (70).

Cytokine production at different stages of lactation has been widely studied. Monocytes during the periparturient period produce higher levels of TNF-α than monocytes isolated at mid to late lactation (71). The higher levels of this potent mediator at early lactation may contribute to the severe acute inflammatory response in the mammary gland during coliform mastitis. IL-2 produced by T-lymphocytes is responsible for the clonal expansion of the T-lymphocytes during the initial stages of infection. Studies have shown that around parturition, IL-2 activity was low, correlating to the decreased cell function and increased susceptibility to mastitis during this period (72).

Although the differences in mastitis severity between cows infected early or late in the lactation cycle has been well documented, the causes of this are not well understood. The cost of large animal models as well as the lack of immunology reagents and knockout bovine animal lines has limited the understanding of the immunological differences occurring at early and mid-lactation. To further our understanding of the differences in immune response at varying stages of lactation a small animal model is required.

Mouse model

In the 1970s, Chandler developed and characterized an experimental mouse model of mastitis (73). This model is comparatively inexpensive and uses standard laboratory animal facilities. The mouse mammary gland provides the unique environment for the bacteria to grow in milk and interact with the host cells and immune components. Bacterial counts, as well as neutrophil numbers and histological changes are similar in bovine and murine mastitis (9, 73).

The mouse possesses five pairs of mammary glands that are functionally and anatomically independent from each other. Each gland has only one teat opening and one ductal gland. The glands are identified from head to tail by a letter and number depicting their relative position. Glands L4 and R4 (fourth on the left and fourth on the right respectively) are typically used for intramammary infections because they are the largest and easiest to observe and harvest (74).

In addition to the similarities listed above, there are some differences between the mammary glands of cows and mice. Milk composition between the species differs in terms of protein, fat and carbohydrates and the bovine udder contains more resident phagocytic cells than the murine mammary gland (75). Milk collection is also a difficult procedure in mice and assessing the intensity of inflammation is done indirectly by observing tissue damage.

Results obtained from experiments with mice are not directly applicable to cattle and require final assessment in cows. However, studies in mice are highly indicative of outcomes in cattle. The accessibility of large numbers of experimental animals allows the screening of a variety of potential antimicrobial compounds in mice where similar experiments in cows would be expensive and time-consuming (9). Statistical significance is also achieved with a smaller number of mice due to the use of genetically identical animals.

The capability of the murine mastitis model to replicate bovine mastitis observed during mid-late lactation has been well-documented (76-83). However, to our knowledge, the mouse model has not been used to compare potential differences in the immune response of mice infected during early and mid-lactation.

Experimental Approach

The purpose of this study was to determine if a mouse could be used to model the immunological differences observed in cows infected with *E. coli* at early and mid-lactation. The mouse lactation cycle is typically three weeks, in this study I have chosen to use 24-36 hours post-partum as an early lactation time point and 10-11 days post-partum as the mid-lactation time point. I hypothesized that a mouse model of early and mid-lactation mastitis, would accurately represent the immunological differences seen in cows during early and mid-lactation.

MATERIALS AND METHODS

Bacterial Strains

Twenty *E. coli* strains, isolated from cows exhibiting clinical cases of mastitis, were obtained from Cornell University. Preliminary work in wax moth larvae showed an *E. coli* strain (designated M12) exhibited high virulence and was chosen to be used in these experiments. All bacteria were grown at 37˚C in Luria Broth (LB) overnight and subcultured in fresh media to an O.D. of 1 using an absorbance of 600nm. This culture was then diluted in sterile PBS until the desired concentration of $4x10^3$ CFU/mL was reached. The number of bacteria used in each inoculum was confirmed by plating on LB agar and counting colony-forming units (CFU) following overnight incubation. To ensure a uniform dose in each injection, a stock of *E. coli*,

strain M12, was frozen in phosphate buffered saline (PBS) and used in all mammary gland injections. The number of bacteria recovered from mammary glands, post infection was determined by serial dilution of mammary homogenates followed by plating on LB agar.

Intramammary Inoculation

Lactating BALB/c mice between 9-12 weeks of age were used in all experiments. To replicate the early lactation time observed in other mammals, lactating mothers 24-36 hours post-partum were used. Lactating mothers 10-11 days post-partum were chosen for the mid-lactation stage. Mice were infected based on the method described by Brouillette (74) with slight modifications. Briefly, mice were anesthetized using 75µl-100µl of a ketamine/xylazine solution. A 50µl volume of bacteria (~200 CFU) in PBS was then injected directly into the lumen of the L4 and R4 mammary glands. Injections were performed using a 33G needle with a beveled end. Control mice were inoculated with 50µl of sterile PBS in both L4 and R4 glands. All protocols were approved by IUCAC, Brigham Young University (protocol number 140303).

RNA Isolation

Mice were sacrificed at various times post infection and dissected to expose the mammary glands. Lymph nodes in L4 and R4 glands were excised and mammary glands were removed and homogenized in 1mL sterile PBS. 100µl of homogenate was then used for bacterial counts by serial dilution and plated on LB agar. The remaining homogenate was centrifuged at 3,800 rpm for five minutes. Supernatant fluid was then discarded and the tissue pellet was resuspended in 1mL RLT buffer (Qiagen RNeasy Kit) and again homogenized. RNA isolation was then performed according to manufacturer's instructions and frozen for cytokine analysis.

RT-qPCR

RNA levels were quantified using the absorbance at 260nm as determined by a Nanodrop spectrophotometer. Working solutions were prepared by dilution in sterile ddH_2O to 100ng/µl. qPCR primers were purchased from Integrated DNA Technologies and Applied Biosystems. Catalog numbers for each primer probe set are found in Table II. ROX qPCR Mastermix was purchased from Thermo Scientific. Primers were diluted to 30X. Multiplex qPCR reactions were performed in a 20µl volume, 0.5µl of GAPDH and 1µl of each target gene primer were used in each reaction. GAPDH was used as endogenous control.

Cycling conditions for RT-qPCR were as follows: 15 minutes at 60°C, followed by 15 minutes at 95°C, finished with 40 cycles of: 15 seconds at 95°C and 15 seconds at 60°C. All reactions were performed on a StepOne Real-Time PCR System and analyzed using StepOne Software v2.1.

Statistical Analysis

For cytokine analysis there were four control samples for both early and late lactation groups. Each control sample was used as a reference sample to obtain RQ values. (For example: Mouse #1 at 4 hours post infection was measured against each reference sample, resulting in four separate RQ values.) All RQ values were then combined for each time point and averaged and outliers removed from data set. Standard errors of the mean were calculated. Data is expressed as the mean and standard error of the mean. Significance was calculated using *t*-test with a p value <0.05 being considered statistically significant.

RESULTS

E. coli proliferation in the mastitic mouse mammary gland

In these experiments, we sought to determine if a uniform inoculum of mastitis causing *E. coli* injected into the mammary gland of mice at early vs mid-lactation would result in different numbers of bacteria being recovered from the infected glands. In these short-term experiments, pups were removed from lactating females immediately before infection and remained separated for the duration of experiment. Lactating mice were sacrificed at 4, 8, 12, 16, and 24 hours post infection and bacterial counts performed on mammary gland homogenates.

Bacterial growth in the early lactation and mid-lactation groups were similar at four and eight hours post infection with an average increase of 3.2x10^5 CFU and 2.6x10^5 CFU respectively, as seen in Figure 1. The number of bacteria recovered at 12 hours post infection showed a slightly higher number of bacteria in early lactation mice compared to mid-lactation mice, with a concentration of 1.3x10^8CFU and 1x10^7CFU/gland respectively. (These differences; however, are not statistically significant.) At 24 hours post infection, early lactation mice and mid-lactation mice once again had similar levels of bacterial growth in infected mammary glands.

Pro-inflammatory cytokine expression in mammary gland of mastitic mice

The effectiveness of an immune response is highly dependent on appropriate cytokine expression. Cytokines IL-1β, IL-6 and TNF-α are important components of the pro-inflammatory immune response within the mammary gland during a mastitis infection (31). In these experiments, relative changes in cytokine gene expression were quantified by RT-qPCR at 4, 8, 12, 16, and 24 hours post infection. Gene expression was determined by comparison of

infected tissues to mammary gland tissue from non-infected mice collected at the same stage in the lactation cycle. Gene expression in the calibrator sample was set to a value of one and gene expression in experimental groups is represented as fold change, compared to the calibrator sample. RT-qPCR analysis of mRNA isolated from mammary tissue showed that non-infected control gland cytokine expression between early and mid-lactation mice were uniformly low (data not shown). Cytokine mRNA levels from mice four hours post infection were also relatively unchanged in both early and mid-lactation mice. At eight hours post infection, pro-inflammatory cytokine mRNA expression increased in both the early and mid-lactation groups. Early lactation mice expressed 1.41x more IL-1β in response to infection, whereas mid-lactation mice showed a 3.7 fold increase after infection. The differences between these two groups (early vs mid-lactation was statistically significant ($p=0.002$). IL-6 had a similar difference of expression between early and mid-lactation mice with a higher increase of expression in mid-lactation mice, $p= 0.0005$. Additionally, at this time point TNF-α mRNA expression increased in both early and mid-lactation groups with a fold increase of 15.87 and 39.04 respectively $p= 0.002$ (Figure 2). The observed delay in pro-inflammatory cytokine response in mice infected during early lactation compared to levels observed in mice at mid-lactation coincides with the pattern of pro-inflammatory cytokine expression previously observed in mastitic cows (40, 68, 72).

At 12 hours post infection, IL-1β expression in early lactation mice was elevated to 101.54 compared to a smaller fold increase in mid-lactation mice of 6.08, $p= 0.0000004$. Similar increases in expression of IL-6 and TNF-α in early lactation mice was also observed at 12 hours post infection, as seen in Figure 2. Conversely, 16 hours post infection exhibited similar levels of expression of IL-1β and IL-6 in both early and mid-lactation mice. TNF-α expression at 16 hours

post infection was higher in mid-lactation mice compared to early lactation mice, p= 0.03. Twenty-four hours post infection experiments showed early lactation mice exhibited higher mRNA expression of pro-inflammatory cytokines than at mice in mid-lactation (Figure 2).

Nitric oxide synthase expression in mammary gland of mastitis mice

Levels of iNOS (NOS2) mRNA were measured in an effort to measure nitric oxide synthase which is indicative of neutrophil activity (28). TNFα is a known activator of iNOS expression (44). Results indicated that levels of iNOS in mid-lactation mice mimicked the gene expression pattern of TNF-α in this group, with sharp increases of expression at eight and 16 hours post infection as seen in Figure 3. However, mice in early lactation had a delay in the initial expression of iNOS, with increases observed latter in infection (12 and 24 hours post infection). Twenty-four hours post infection results showed a significant drop in expression of iNOS in mid-lactation mice and a rise in expression of iNOS in early lactation mice. These fluctuations in iNOS gene expression coincide with increases and decreases of the pro-inflammatory cytokine TNF-α, as seen in Figure 2.

IL-10 levels are increased in early lactation compared to mid-lactation.

IL-10 is an anti-inflammatory cytokine that functions in part by downregulating the secretion of pro-inflammatory cytokines such as IFN-γ and TNF-α (84). Appropriate IL-10 expression has been shown to prevent excessive damage to host tissues during an inflammatory response. However, increased expression of IL-10 can also result in an inappropriate immune response against invading pathogens, as reviewed by Cyktor (85). Gene expression of IL-10 within the mammary gland was measured by qPCR at 4, 8, 12, 16 and 24 hours post infection as seen in Figure 4. Interestingly, at 4 hours post infection there was a significant upregulation of IL-10 mRNA expression seen in mice at early lactation, but not mice mid-lactation. Analysis

eight hours post infection revealed similar levels of IL-10 mRNA expression in both groups of mice. However, at 12 hours post infection, mice in early lactation had elevated levels of IL-10 when compared to mice in mid-lactation with a fold increase of 44.60 and 5.43 respectively. Levels of IL-10 mRNA expression at 16 and 24 hours post infection in both groups of mice exhibited similar increases (figure 4).

Pro-apoptosis gene expression

The regulation of apoptosis during an infection is accomplished by the expression of pro and anti-apoptotic genes. Caspase-1 and Bax induce apoptosis of epithelial cells during a mammary gland infection (53). Importantly, levels of pro apoptotic genes are also increased following weaning of the nursing young, as extensive cell death occurs during involution and accompanying remodeling of the lactating mammary gland (86-88). In these experiments, relative levels of Caspase-1 and Bax gene expression were measured at 4, 8, 12, 16 and 24 hours post infection. Importantly, in these experiments, mice were infected with bacteria and separated from their pups for the remainder of the experiment. Results show that in as little as four hours post infection significant differences were observed in the levels of both Bax as well as Caspase with mice in mid-lactation consistently expressing higher mRNA levels when compared to mice early in lactation Figure 5.

Anti-apoptosis gene expression

Bcl-2 promotes cellular survival and inhibits actions of pro-apoptotic proteins. In these experiments gene expression of Bcl-2 was measured by qPCR at 4, 8, 12, 16 and 24 hours post infection and compared to control glands (uninfected). Bcl-2 gene expression in the infected mammary gland showed significant differences in early, compared to mid-lactation mice, as

shown in Figure 5. Throughout the course of infection, mice in mid-lactation had higher levels of Bcl-2 gene expression in harvested tissues than the mice in early lactation.

Bacterial growth and cytokine expression in mice infected at early and mid-lactation during normal nursing.

As discussed above, expression of inflammatory cytokines in the lactating mammary gland appear to be altered in the infected mammary glands between the two groups of mice. However, in the experiments described above, lactating mice were separated from their pups following mammary gland infection. In the following experiment, mice were infected with a standard inoculum of *E. coli* at both early and mid-lactation time points as described above. However, in these experiments pups were allowed to nurse normally following infection. Results of these experiments show that bacterial numbers as well as apoptotic gene regulation differed in mice infected during early and mid-lactation. Mammary glands of mice infected at early lactation time points had increased numbers of bacteria compared to mice infected at mid-lactation at 24 hours post infection (Figure 6).

In these experiments all pro and anti-inflammatory cytokines were elevated in infected glands when compared to control glands following a 24 hour infection, as seen in Figure 7. Mice in early lactation also exhibited higher gene expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α than in mid-lactation. Levels of iNOS gene expression was higher in mice in mid-lactation than in mice in early lactation. Anti-inflammatory cytokine IL-10 exhibited significantly higher levels of gene expression in early lactation mice with a fold increase of expression of 691 while mice in mid-lactation showed a fold increase of 90. Of the apoptosis regulators, Bcl-2 was the only gene expressed at a significantly different level between early lactation and mid-lactation mice as seen in Figure 7.

DISCUSSION

Mastitis is the most economically costly disease of the dairy industry (3, 4). Most mastitis infections (both in terms of severity and frequency) occur during the periparturient period (33, 56, 58, 61, 89, 90). Previous research in cows has shown impaired immune responses during this time. Periparturient immune responses are characterized by altered cytokine production, impaired neutrophil activity, and result in increased bacterial growth (23, 59, 62, 65, 69, 91). Better understanding of the factors contributing to this increased risk of mastitis are needed to design effective therapies and preventive strategies. A mouse model has not previously been utilized to study the immune response during the periparturient period. In this study, intramammary infusions with *E. coli* were performed in mice in "early lactation" (24-36 hours post-partum) and in mice in "mid-lactation" (10-11 days post-partum). The purpose of this research was to gain a better understanding of potential differences in the cytokine response of mastitic animals during early and mid-lactation.

Bacterial Growth

A delay in neutrophil recruitment and rapid/uncontrolled bacterial growth in the mammary gland is commonly associated with the periparturient period (92-97). Previous research has determined that even a one-hour delay in neutrophil recruitment to the mammary gland could result in up to an 8-fold higher bacterial load and accompanying higher endotoxin concentration (97, 98). In our experiments, bacterial load (CFUs) in the mammary gland of mice infected early in lactation (when pups were nursing) was higher than the bacterial load in mice at

mid-lactation following a 24 hour infection (Figure 7). This increase in bacterial growth in mice during early lactation is consistent with results seen in cows during early lactation and supports the idea that differences in cytokine expression in early vs. mid-lactation result in varied bacterial growth and disease pathology (56, 60).

In experiments in which mothers were separated from their pups following infection, bacterial growth between the two groups of mice was not significantly different at any point over the course of infection (Figure 1 and 7). The observed differences in bacterial growth at 24 hours post infection in animals which were nursing pups vs mothers not nursing could be associated with the lack of milk removal via nursing. Milk stasis in the lactating mammary gland may have provided bacteria an improved environment to grow and possibly evade the immune components present in the mammary gland, resulting in differences in bacterial growth in these experiments.

E. coli strain M12 was used in all experiments. This strain of *E. coli* is highly virulent, compared to other strains. Future work in which less virulent strains of bacteria are used may allow us to detect more distinct differences in bacterial growth between these two groups of mice.

Proinflammatory Cytokines

The cytokines IL-1β, IL-6 and TNF-α have been shown to be critical in a bovine mammary gland immune response and expression patterns of these cytokine have been shown to differ in early vs. mid-lactation. (56, 99). Elevated levels of these proinflammatory cytokines have been shown to be contributors to tissue damage and severe mastitis (68, 72). In experiments with pups reunited with nursing dams following infection, we observed significantly increased expression of IL-1β, IL-6, and TNF-α in the mammary tissues of mice in early lactation compared to expression levels seen in mice at mid-lactation 24hours post infection (Figure 7).

This increase in proinflammatory cytokine expression during early lactation is consistent with the cytokine profile observed in bovine mastitis during early lactation (18, 40).

The immune response to bovine mastitis at the periparturient time has not been well characterized. However, results from previous experiments using a bovine model of mastitis suggest there may be altered expression of cytokines in periparturient cows during the initial inflammatory response compared to cows later in lactation (40, 68, 100). The direct comparison of cow and mouse models at each time point is difficult, due to the lack of side-by-side experiments in cows during early and mid-lactation.

To investigate the potential of altered expression of cytokines over the course of infection, in this study cytokine expression was measured by RT-qPCR at 4, 8, 12, 16 and 24 hours post inoculation. These mouse experiments were done with pups removed from the nursing dams for the duration of the infection in an effort to reduce variability and ensure total retention of the inoculum. In these experiments the proinflammatory cytokines IL-1β, IL-6, and TNF-α were measured over the course of infection. Results indicate that mice in mid-lactation exhibited a significant increase in IL-1β, IL-6 and TNF-α eight hours post infection compared to control tissues. Conversely, mice in early lactation had a delay in proinflammatory cytokine expression, which peaked at 12 hours post infection (Figure 2). In addition to exacerbated disease pathology caused by elevated levels of proinflammatory cytokines, the observed delay in cytokine expression in mice infected early in lactation may be a contributing factor in the delay of neutrophil recruitment and increased severity of disease at the periparturient time.

TNF-α has been the most extensively researched proinflammatory cytokine in experimental bovine mastitis and has been shown to be critical for an effective immune response during a mammary gland infection (56, 65, 68, 71, 101). Monocytes isolated from cows early in

lactation have been shown to produce higher levels of TNF-α compared to monocytes isolated from cows in mid-late lactation (56, 71). It has also been suggested that the over-production of TNF-α during the early stages of lactation may contribute to immunopathological changes in host tissues, resulting in elevated bacterial growth and in some cases animal death (65, 68, 71, 72). Research in cows has shown TNF-α expression peaked at eight hours post infection in mammary gland tissues (40, 101). Additionally, cows tested during early lactation had a delay of TNF-α expression with peaks occurring 10-16 hours post infection (56, 72). In our experiments, the expression pattern of TNF-α in mice at early lactation and mice at mid-lactation followed the expression patterns previously observed in cows, with peaks of TNF-α occurring at 12 and eight hours post infection, respectively (Figure 2). These results further confirm the validity of the mouse model as a valuable resource to study the cytokine response during a mastitis infection at the different stages of lactation.

The early lactation mouse model allows the unique ability to advance our understanding of these critical cytokines at a molecular level. The mouse could provide a valuable model to study the effect of modulating cytokine production. This could be accomplished through blocking activity of cytokines in the mammary gland through the use of antibodies. Likewise, enhancing levels of cytokines by the addition of exogenous cytokine in the mammary gland is possible using the mouse model. Increasing our understanding of the role of specific pro inflammatory cytokine responses at the periparturient time will aid in the development of therapeutic strategies specifically targeting the high-risk early lactation time.

iNOS

Delayed neutrophil recruitment to infected mammary glands at the periparturient time is thought to contribute to systemic disease and death in severe cases of mastitis. Correspondingly,

cows during the periparturient period generally have minimal influx of neutrophils to the site of infection (60). Additionally, proteomic data on cows in early lactation showed a down regulation of histone H2A.1 in neutrophil membranes, correlating with a decrease in neutrophil function and an increase in disease susceptibility (102). Previously published studies also indicate there is an increase in the percentage of apoptotic neutrophils in the blood, and an increase in necrotic neutrophils in milk in early lactating cows in comparison to mid lactating cows. These factors are thought to increase the risk of severe mastitis during this time (103).

In these studies iNOS levels were measured in mammary tissues in an effort to determine the level of nitric oxide synthase; a protein which is indicative of neutrophil activity (28). In our experiments, we observed lower levels of iNOS, in the first eight hours post infection in mice early in lactation compared to mice in mid-lactation. This reduced level of iNOS expression may reflect decreased neutrophil recruitment and/or increased neutrophil death in mice infected in early lactation.

Neutrophil recruitment and activation within the mammary gland is accomplished by an assortment of chemokines and cytokines, one of which is TNF-α. Cows at mid-lactation with mastitis displayed peaks in expression of TNF-α coinciding with peaks of expression of NO (44, 101). We also found this to be true of mice in mid-lactation with expression of TNF-α and iNOS being tightly correlated over the course of infection (Figure 2 and 4). Mice in the early stages of lactation did not exhibit this correlation in expression, further supporting our hypothesis of immune impairment in the periparturient mouse (59, 102). Future experiments to more specifically study neutrophil differences during early vs. mid-lactation include isolating neutrophils from mice at these time points and testing neutrophil function. These experiments

could confirm the results of iNOS measurements we observed and support our hypothesis of neutrophil impairment in mice during the early stages of lactation.

IL-10

IL-10 is an anti-inflammatory cytokine that is essential in the regulation of the inflammatory response. IL-10 production early in infection is thought to be produced primarily by activated macrophages (104, 105). Autocrine and paracrine activation of macrophages through IL-10 results in initiation of the JAK/STAT signaling pathway, resulting in down-regulation of inflammatory gene expression (106-109). IL-10 functions by blocking or reducing proinflammatory cytokine expression at different stages of the immune response (104, 110).

Resident macrophages in the mammary gland have been shown to be an important line of defense against invading pathogens (22). Macrophages have the ability to modulate the immune response via expression of various cytokines such as IL-1β, TNF-α and IL-10. This is thought to occur due to the wide variety of innate immunological receptors on these cells (111, 112). Macrophages that recognize live bacteria have been shown to adapt the electron transport chain (ETC) of their mitochondria to increase the generation of ROS and subsequently increase the expression of proinflammatory cytokines. Conversely, macrophages when exposed to dead bacteria stimulated a different ETC of the mitochondria, resulting in an increase in expression of anti-inflammatory cytokines such as IL-10 (113). These results suggest the response of the innate immune system is dependent on activation of specific PRR and the consequent activation of specific mitochondrial ETC that result in increased expression of pro or anti-inflammatory cytokines.

Macrophages play a vital role in secreting soluble effectors and stimulating the T_H1 response (22, 23). IL-10 has been shown to inhibit the development of T_H1 response via activation of macrophages (104). Previous research has shown that around the time of parturition there is a shift from a T_H1 response to a T_H2 response in cows (58). This shift results in a predominantly anti-inflammatory response if an animal is infected during the periparturient time. In agreement with this previously published observation an increase in IL-10 expression was seen in our experiments in mice when infected early in lactation (Figure 7).

It has been hypothesized that the production of T_H2 associated cytokines may serve a protective role during disease by down-regulating the production of proinflammatory cytokines. In the current study, a significant increase in expression of IL-10 after four hours of infection was observed in mice in early lactation. The elevated levels of IL-10 in the initial stages of lactation is likely not due to the predominantly immunosuppressive nature of the T helper cells at the time of birth. Rather, this gene expression is most likely a product of resident macrophages and epithelial cells in the mammary gland (37, 84, 114, 115). These data are supported by studies showing macrophages from IL-$10^{-/-}$ mice produce high amounts of TNF-α in response to LPS stimulation. Additionally, in IL-10 deficient macrophages stimulated with IL-10, down-regulation of TNF-α was observed as early as one hour after IL-10 addition (109). To our knowledge, IL-10 gene expression during the initial stages of a mastitis infection in periparturient cows has not been studied. Results from the current study suggest that elevated IL-10 gene expression in the initial stages of infection may be a contributing factor in the delay of pro-inflammatory cytokine expression observed during the initial immune response of cows infected early in lactation.

As mentioned above, macrophages have the ability to transiently increase mitochondrial ROS activity and scale the immune response based on the invading bacteria's pathogenicity (111, 112). Mouse macrophages challenged with dead bacteria decrease their production of IL-1β and increase production of IL-10 (113). This might explain the increase in IL-10 production seen in mice during mid-lactation without nursing pups 24hours post infection, as seen in Figure 4. Regular milk removal from the mammary gland likely results in the continual removal of dead bacteria in the mammary gland. However, in experiments with weaned pups, there would be no removal of milk (and dead bacteria) throughout the infection. I theorize that macrophages in the gland may be sensing both viable and dead bacteria, resulting in expression of both proinflammatory and anti-inflammatory cytokines, explaining the elevated levels of IL-10 in the mice in mid-lactation without nursing pups.

Previous studies in mice have shown forced weaning of the young induces mammary gland involution as early as 12 hours post weaning (115). This involution of non-infected mammary glands resulted in elevated expression of IL-10 in this tissue (115). The differences observed in IL-10 gene expression in mice during mid-lactation, between the nursing and weaned experiments, may be influenced by cytokine expression changes, which occur normally in mammary tissues during normal mammary gland involution following weaning (86-88).

Future experiments blocking the activity of IL-10 during the early stages of infection may prevent the delay of proinflammatory cytokine expression observed in the mice in early lactation experiments. These experiments would contribute to a better understanding of the neutrophil influx and increased disease severity seen in previous experiments. Additionally, enhancing the availability of IL-10 in the mice at various stages of lactation could further our understanding of its role and importance in the immune response during an *E. coli* mammary gland infection.

Bcl-2 and Inflammasomes

Bcl-2 is an anti-apoptotic protein, critical in the regulation of the inflammatory response. This protein acts by suppressing oxidative stress signals in the cell and prevents activation of NLRP1 and NLRP3 inflammasomes (116-119). The activation of the pro-inflammatory cytokine IL-1β has been shown to be directly dependent on NLRP3 inflammasomes (117). The importance of inflammasome activation is well illustrated by experimental results showing that treatment with an experimental drug pDAG, which activates inflammasomes, results in a decrease in the somatic cell count in milk from mastitic cows (120).

Studies in cows during mid-lactation have shown Bcl-2 expression in the mammary gland changes over the course of an infection. Cows in mid-lactation exhibited a decrease in Bcl-2 expression 24 hours post infection, and at 72hours post infection Bcl-2 increased to levels seen in control glands, coinciding with decreases in SCC and inflammation (53). Bcl-2 expression in infected mammary tissues of cows during the early stages of lactation has not been well characterized. However, studies have shown healthy mammary glands in cows in the early stages of lactation exhibited a steady state of low Bcl-2 expression (121).

In the current study, Bcl-2 levels were measured over the course of a mastitic infection, using mice during early and mid-lactation. Results from these experiments indicate that Bcl-2 expression is unchanged over the first 24 hours of an infection during early lactation. Conversely, Bcl-2 expression in an infection during mid-lactation increased significantly (Figure 5). Mice in mid-lactation exhibited a peak in Bcl-2 expression at eight hours post infection, and a sharp decrease in expression (returning to near base line levels) at 12 hours post infection. This drop in Bcl-2 gene expression preceded an increase in IL-1β gene expression at 16 hours post infection.

The inverse correlation between IL-1β and Bcl-2 expression seen during mid-lactation suggests that elevated levels of IL-1β in mice during mid-lactation may be related to the decrease of Bcl-2 expression in infected mammary tissues at 12 hours post infection. The regulation of inflammatory cytokines, such as IL-1β, is a complex process with many positive and negative regulators of expression. The fact that low expression of Bcl-2 in uninfected mice and mice during early lactation does not lead to expression of IL-1β may be due to the combinatorial effect of changes in expression of multiple cytokines, including Bcl-2. Conversely, this inverse association between Bcl-2 expression and IL-1β expression may be coincidental. Future experiments using siRNA knockdown of Bcl-2 expression in cell lines could shed insight into the potential role of Bcl-2 in IL-1β regulation.

The initiation of the innate immune response to an invading pathogen occurs when PRR are bound by pathogen-associated molecular patterns (PAMPS) resulting in the expression of proinflammatory cytokines (122). IL-1β is a potent pro-inflammatory cytokine whose maturation to its active form is tightly regulated by the catalytic activity of caspase-1. Active caspase-1 requires signal-dependent auto-activation within multi-protein complexes known as inflammasomes (123). The NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome has been shown to activate caspase-1 in response to ATP, pore-forming toxins, crystalline substances, nucleic acids, bacterial or viral pathogens and damage-associated molecular patterns (DAMPS) (124).

TLR4 activation of the NLRP3 inflammasome in myeloid cells is essential in the innate immune response to gram-negative bacteria (Schroder 2010). As reviewed by Guo et al (124), binding of TLR4 with extracellular LPS promotes activation of NLRP3 proteins, and the upregulation of pro-IL-1B and NLRP3 mRNA expression via NF-κB signaling. Activation of

NLRP3 leads to oligomerization of other NLRP3 receptors. This clustering of NLRP3 recruits apoptosis-associated speck-like protein containing CARD (ASC) proteins. The binding of ASC proteins to NLRP3 recruits pro-caspase-1. Aggregation of pro-caspase-1 to the inflammasome leads to the auto-cleavage of pro-caspase-1 to it's the active form which ultimately cleaves pro-IL-1B to its active form of IL-1β (124). Formation of the inflammasome is also influenced by potassium efflux, mitochondrial ROS generation and increased intracellular calcium levels (125-128).

The activation of inflammasomes is tightly regulated to prevent inappropriate inflammatory responses. Scavengers and inhibitors of ROS inhibit priming of NLRP3 inflammasomes and block the positive regulation of ROS on pro-inflammatory cytokine expression (129, 133, 134). The anti-apoptotic protein Bcl-2 counterbalances oxidative damage and up-regulates the production of anti-oxidant enzymes (130-132). Thus Bcl-2 expression can lead to prevention of inflammasome activation and a decrease in pro-inflammatory cytokine gene expression (133, 134). Research on the function of Bcl-2 in mastitis typically focuses on its role as an apoptotic regulator, and not on the influence of inhibiting proinflammatory cytokine expression through anti-oxidant abilities (47, 53, 135, 136). Inflammasome activity during a mammary gland infection has not been well characterized, particularly during the periparturient time.

Our experiments suggest the absence of Bcl-2 expression in mice in early lactation during mastitis may contribute to the over-expression of potent pro-inflammatory cytokines observed in cows and mice at the periparturient time. This lack in innate immune response regulation at the early stages of lactation may contribute to the increased severity of disease and tissue damage, characteristic of mastitis infections during the early stages of lactation (40, 68, 84).

The period before parturition is characterized by extensive remodeling of the mammary alveolar glands. Lipoproteins have been hypothesized to inhibit LPS from binding to TLR4 (137-139). This may result in a delayed upregulation of IL-1β and NLRP3 mRNA via the NF-κB signaling pathway. It is possible the lipoproteins present in the colostrum can mask LPS from binding TLR4 and initiating the innate immune response (141). Caseins, whey proteins and lactose synthesis enzymes are highest at early lactation compared to later stages of lactation (142). In our mouse mastitis model, initial IL-1β gene expression was delayed in mice in the early stages of lactation. As mentioned previously, a delay in pro-inflammatory cytokine expression in cows at early lactation may be present in the initial stages of infection. Cows at mid-lactation have been shown to significantly upregulate TLR4 expression 24 hours post infusion with *E. coli* (140). It has been hypothesized the TLR4 signaling cascade initializing expression of IL-1β may be dysfunctional or depressed around parturition (133). It is clear multiple checkpoints are necessary in the regulation of IL-1β production. In the current study, mice in early lactation exhibited an initial delay in IL-1β expression at 8hours post infection, despite low Bcl-2 expression. I hypothesize this delay in expression may be attributed to either IL-10 over-expression at 4 hours post infection (Figure 3), or to a dysfunctional TLR4 signaling cascade during the initial stages of infection.

Conclusions

Results from our early lactation murine mastitis model support the hypothesis that animals infected at different times in the lactation cycle respond with different cytokine responses. Animals infected early in lactation were observed to express higher levels of IL-1β, IL-6, TNF-α and IL-10 at 24 hours post infection, and low expression of Bcl-2 over the course of the infection. Conversely, animals infected later in the lactation cycle expressed higher levels of

iNOS, an indicator of neutrophil activity. The first eight hours of infection resulted in an increase of anti-inflammatory cytokine expression and a delay in proinflammatory cytokine expression in mice infected early in lactation when compared to mice infected later in the lactation cycle.

The use of small animal models has allowed substantial advances in our understanding of diseases that plague humans as well as animals. Continually adapting these models to be more efficient and accurate is necessary for not only furthering our understanding of the immune response, but also for finding better and more efficient methods to combat these important diseases. To our knowledge, expression of IL-10 and Bcl-2 in periparturient cows over the course of infection has not been previously studied. Our results, from a mouse model of mastitis, suggest that immune dysregulation, resulting in rapid IL-10 expression and impaired Bcl-2 modulation in periparturient animals may contribute to increased disease frequency and severity.

Table 1: Overview of the cytokines expressed during a mammary inflammatory response and their observed effects.

Cytokine	Observation
IL-1β	• Increases neutrophil number and enhances phagocytosis and bactericidal activity • Influences acute phase inflammatory response • Recruits neutrophil migration into infected mammary gland
IL-2	• Promotes mononuclear cell proliferation in mammary gland • Enhances bactericidal activities of lymphocytes • Increase plasma cell numbers • Activate NK cells
IL-6	• Neutrophil chemoattractant • Mediates switch from neutrophil to monocyte recruitment as an immune suppressor • Promotes B and T cell differentiation • Promote Th2 and T17 response
IL-8	• Strong chemoattractant • Induces inflammation
IL-10	• Down-regulate proinflammatory cytokines, MHC II expression, and macrophage co-stimulatory molecules • Anti-inflammatory cytokine
G-CSF	• Increase neutrophil numbers in blood and milk • Increase milk somatic cell count • Enhances bactericidal activity
TNF-α	• Enhance acute phase inflammatory response • Enhance neutrophil activity • Promotes expression of endothelial adhesion molecules
IFN-γ	• Enhance neutrophil activity • Activate macrophages • Upregulates MHC II expression • Mediates differentiation of naive CD4 T cells into Th1 effectors

As reviewed by Sordillo (34)

Table 2: qPCR pre-designed primers were ordered from Integrated DNA Technologies. Housekeeping gene GAPDH was ordered from Applied Biosystems.

Gene	Catalog Number
IL-1β	Mm.PT.58.41616450
IL-6	Mm.PT.58.13354106
IL-10	Mm.PT.58.23504055
Bax	Mm.PT.58.14012210
Bcl-2	Mm.PT.58.4362966
Caspase-1	Mm.PT.58.13005595
iNOS	Mm.PT.58.43705194
TNF-α	Mm.PT.58.5812575861
GAPDH	1416074

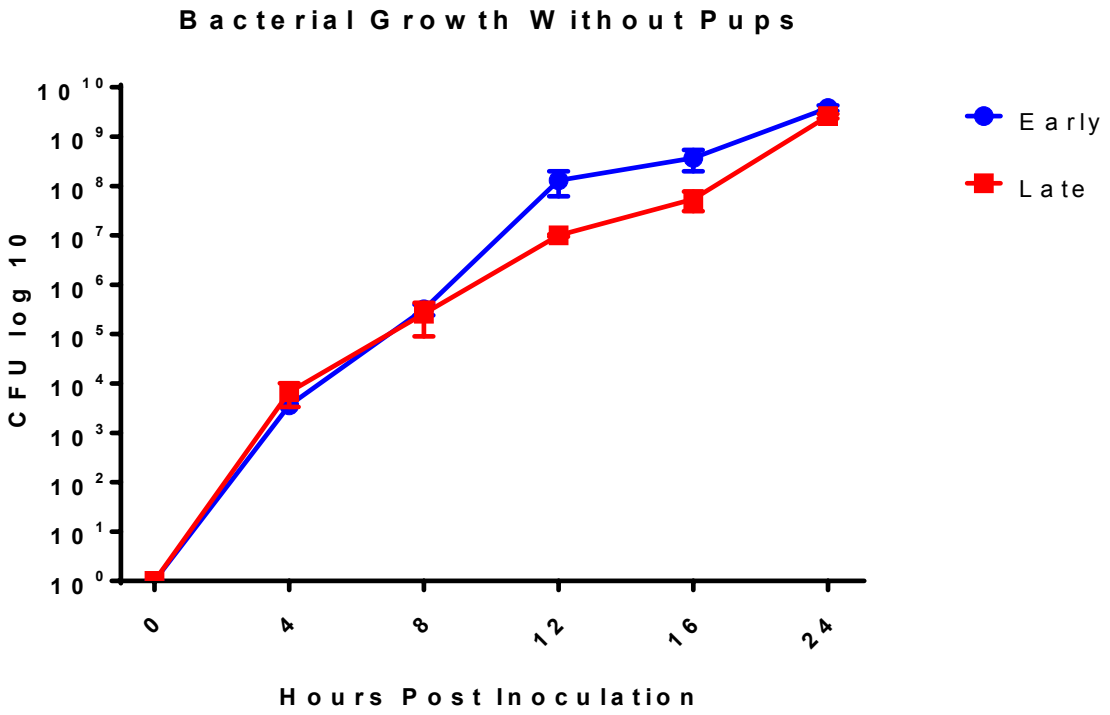

Figure 1: Intramammary growth of *E. coli* following experimental challenge in early and mid-lactating mice with pups removed. Following intramammary infusions with 200 CFU of M12 in L4 and R4 mammary glands. Whole mammary glands were collected at various times, homogenized and plated following serial dilutions. Data are presented as the mean ± S.E.M. n=3-5.

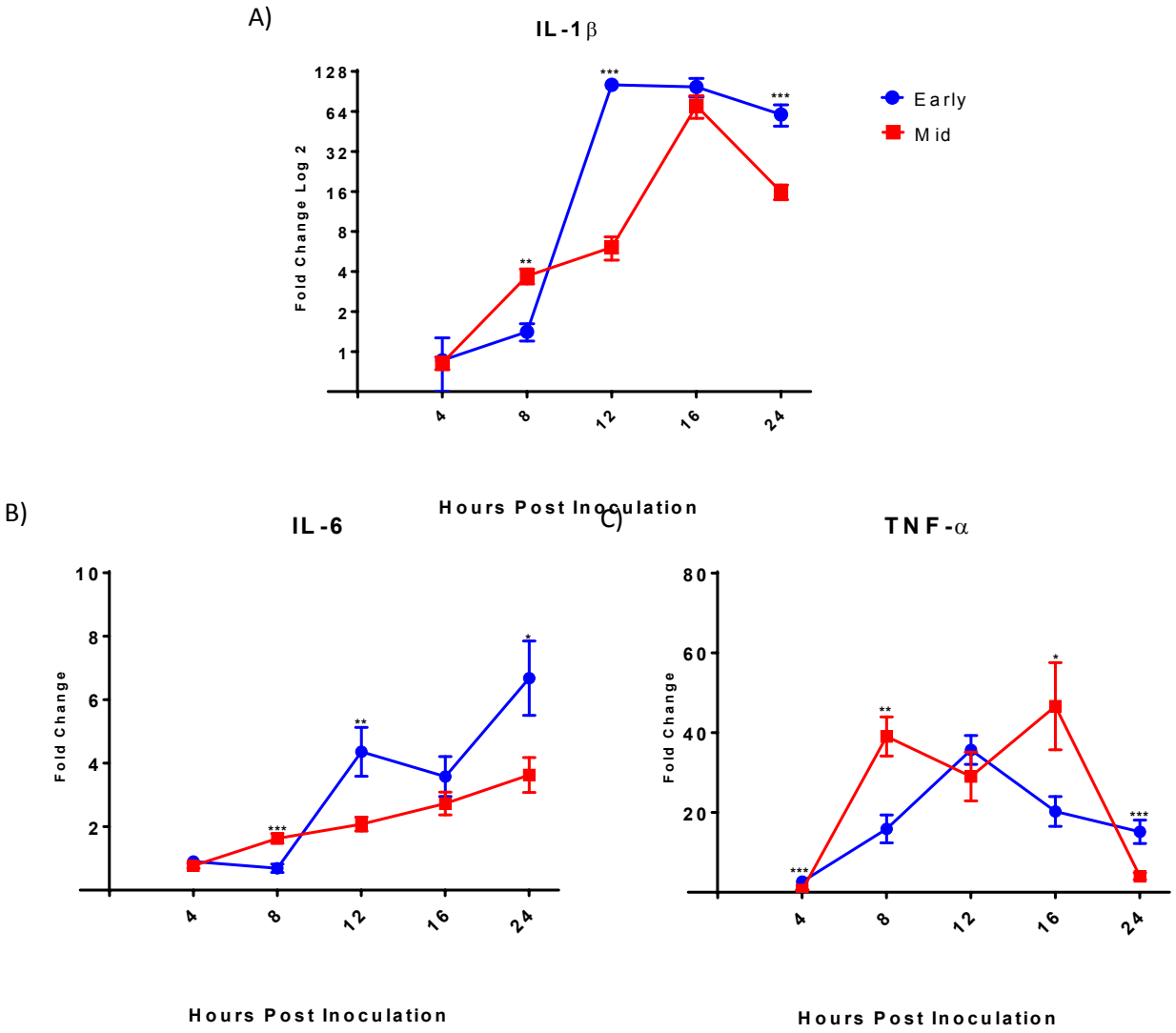

Figure 2: Effect of intramammary challenge with *E. coli* on mRNA expression of pro-inflammatory cytokines. The expression of (A) IL-1β, (B) IL-6, and (C) TNF-α was determined by measuring the expression of each target gene and using GAPDH as an internal reference and converted to relative fold induction compared to non-infected control glands. Data are presented as the mean ± S.E.M. n=3-5 at each time point in each group. P-value: *<0.05, **<0.001, ***<0.0001

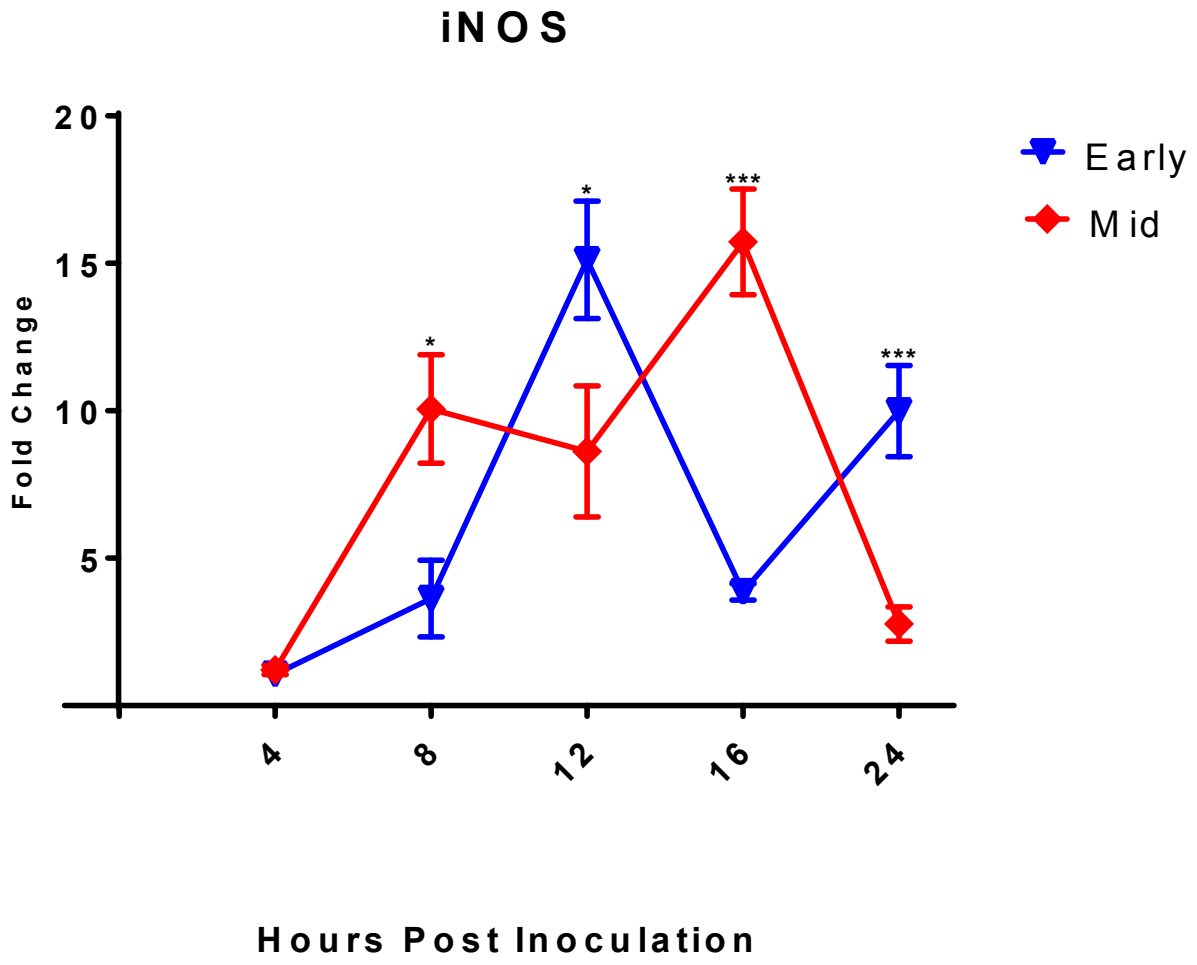

Figure 3: Effect of Intramammary challenge with *E. coli* on mRNA expression of iNOS. The expression iNOS was determined at each time point. Relative fold induction of gene expression was determined by comparing experimental to control tissues. Data are presented as the mean ± S.E.M. n=3-5 at each time point in each group. P-value *<0.05 and ***<0.0001.

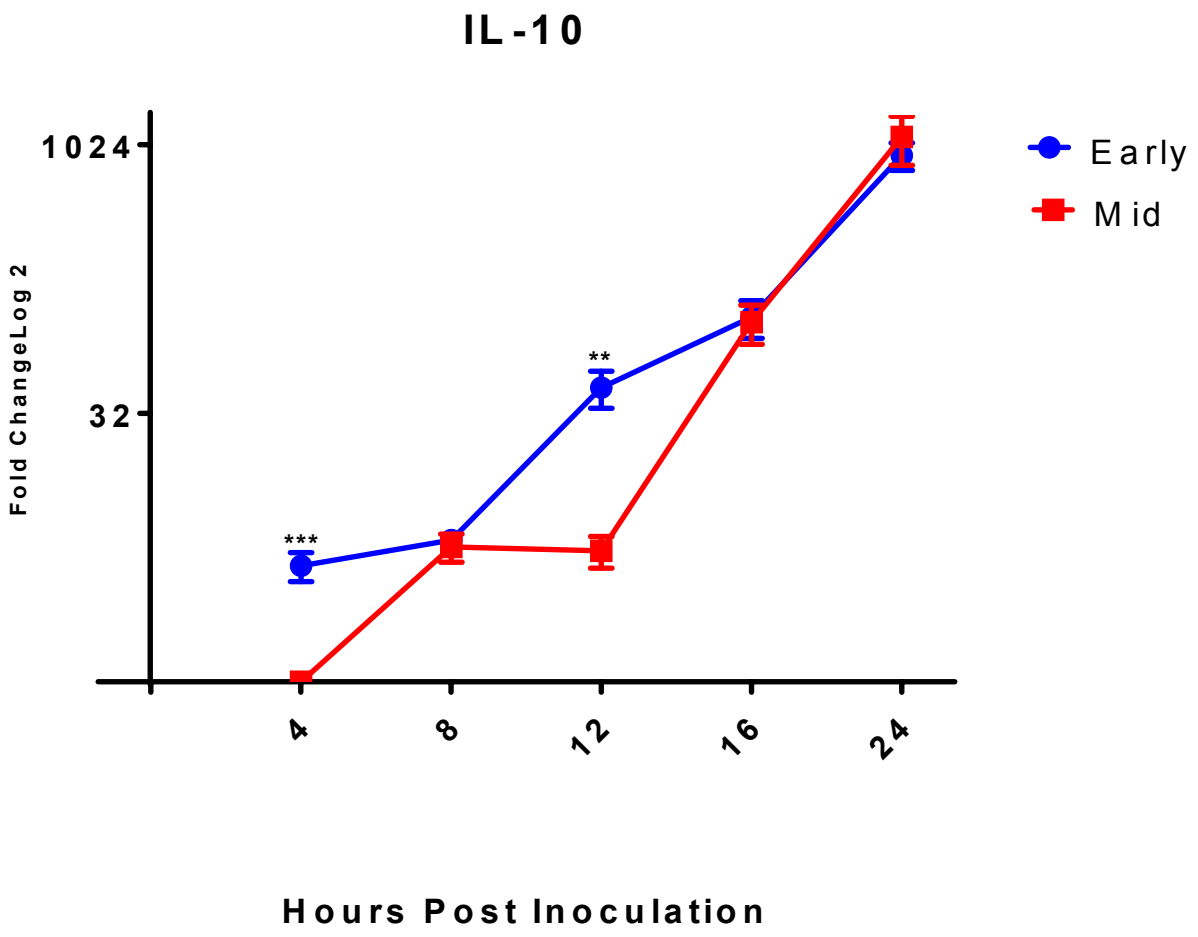

Figure 4: Effect if Intramammary challenge with *E. coli* on mRNA expression of anti-inflammatory cytokine IL-10. The expression of IL-10 was determined at each time point. Relative fold induction of gene expression was determined by comparing experimental to control tissues. Data are presented as the mean ± S.E.M. n=3-5 at each time point in each group. P-value: **<0.001 and ***<0.0001.

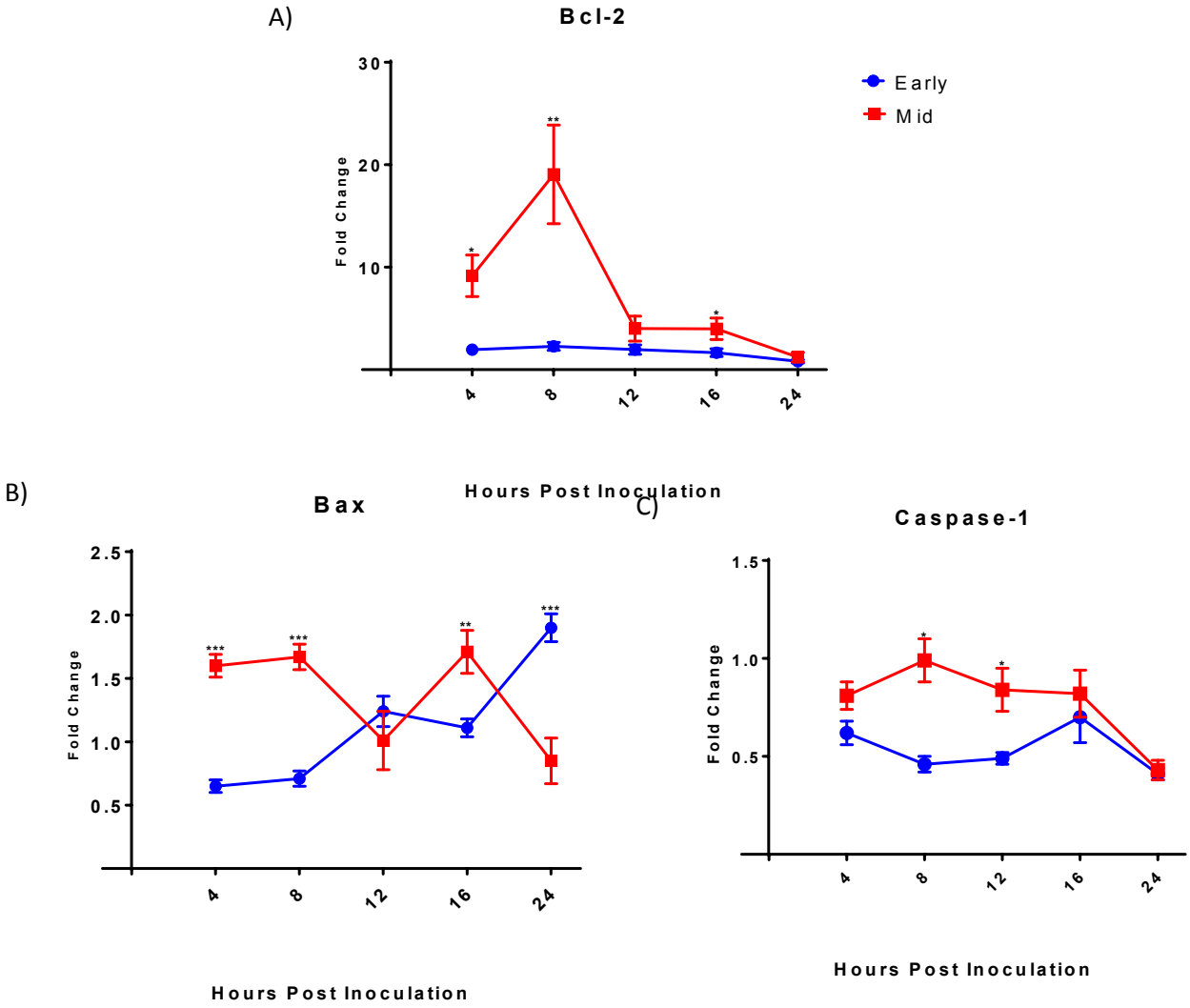

Figure 5: Effects of intramammary challenge with *E. coli* on mRNA expression of pro-apoptotic and anti-apoptotic markers. The expression of anti-apoptotic Bcl-2 (A) and pro-apoptotic Bax and Caspase-1 (B and C) was determined at each time point. Relative fold induction of gene expression was determined by comparing experimental to control tissues. Data are presented as the mean ± S.E.M. n=3-5 at each time point in each group. P-value *<0.05, **<0.001 and ***<0.0001.

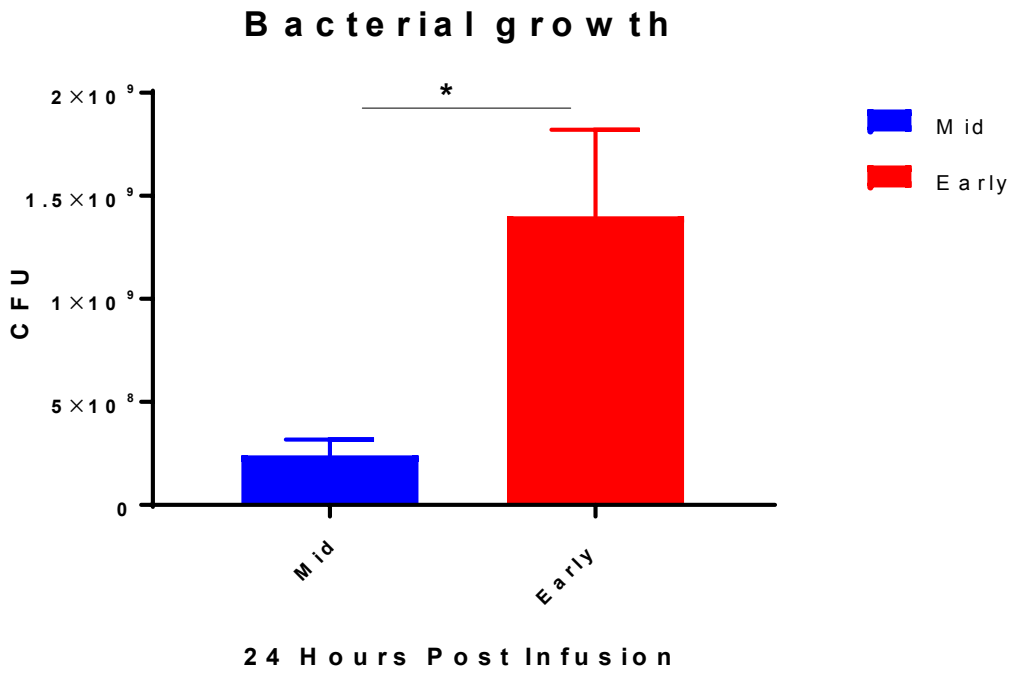

Figure 6: Intramammary growth of *E. coli* following 24 hour experimental challenge with pups nursing. Following intramammary infusions of 200 CFU of M12 into L4 and R4 glands in early lactation mice and mid-lactation mice. Whole mammary glands were collected at 24 hour post infection. Serial dilutions of tissue homogenates were plated and bacterial colonies counted. Data are presented as the mean ± S.E.M. P-value <0.05, n=5-6.

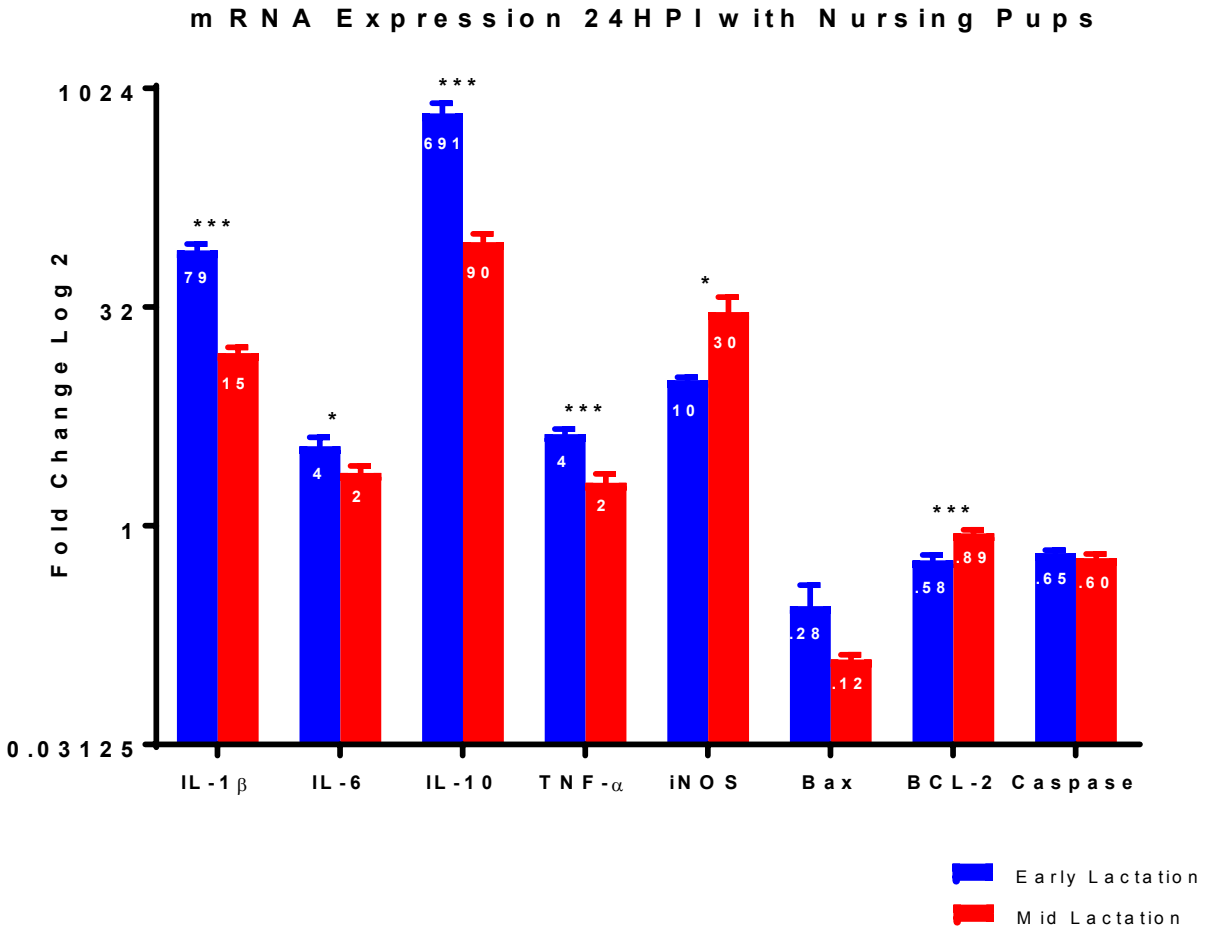

Figure 7: Effect of intramammary challenge with *E. coli* on mRNA expression with nursing pups. The expression of IL-1β, IL-6, IL-10, TNF-α, iNOS, Bax, Bcl-2 and Caspase was determined 24 hours post infection. Relative fold induction of gene expression was determined by comparing tissues of mice infected with pups nursing and with pups weaned to uninfected control tissues. Data are presented by mean ± S.E.M. n=5-6 for each group. P-value: *<0.05 and ***<0.0001

REFERENCES

1. Oviedo-Boyso, J., Valdez-Alarcón, J. J., Cajero-Juárez, M., Ochoa-Zarzosa, A., López-Meza, J. E., Bravo-Patiño, A., & Baizabal-Aguirre, V. M. (2007). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. Journal of Infection, 54(4), 399-409.
2. Foxman, B., D'Arcy, H., Gillespie, B., Bobo, J. K., & Schwartz, K. (2002). Lactation mastitis: Occurrence and medical management among 946 breastfeeding women in the United States. American Journal of Epidemiology, 155(2), 103-114.
3. Wilson, D. J., Gonzalez, R. N., & Das, H. H. (1997). Bovine mastitis pathogens in new york and pennsylvania: Prevalence and effects on somatic cell count and milk production. Journal of Dairy Science, 80(10), 2592-2598.
4. Seegers, H., Fourichon, C., & Beaudeau, F. (2003). Production effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary Research, 34(5), 475-491.
5. Bradley, A. J. (2002). Bovine mastitis: An evolving disease. The Veterinary Journal, 164(2), 116-128.
6. Kossaibati, M., & Esslemont, R. (1997). The costs of production diseases in dairy herds in england. The Veterinary Journal, 154(1), 41-51.
7. Heringstad, B., Rekaya, R., Gianola, D., Klemetsdal, G., & Weigel, K. (2003). Genetic change for clinical mastitis in norwegian cattle: A threshold model analysis. Journal of Dairy Science, 86(1), 369-375.
8. White, L. J., Schukken, Y. H., Lam, T. J., Medley, G. F., & Chappell, M. J. (2001). A multispecies model for the transmission and control of mastitis in dairy cows. Epidemiology and Infection, 127(3), 567-576.
9. Notebaert, S., & Meyer, E. (2006). Mouse models to study the pathogenesis and control of bovine mastitis. A review. Veterinary Quarterly, 28(1), 2-13.
10. Blowey, R., & Edmondson, P. (1995). Mastitis–causes epidemiology and control. Mastitis Control in Dairy Herds (Ed.R Blowey and P Edmondson), 27-45.
11. Eberhart, R. (1977). Coliform mastitis [bacterial diseases, cattle]. Journal American Veterinary Medical Association,
12. Shpigel, N. Y., Elazar, S., & Rosenshine, I. (2008). Mammary pathogenic escherichia coli. Current Opinion in Microbiology, 11(1), 60-65.
13. Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., & Duchateau, L. (2003). Severity of E. coli mastitis is mainly determined by cow factors. *Veterinary research*, *34*(5), 521-564.
14. Fernandes, J. B. C., Zanardo, L. G., Galvão, N. N., Carvalho, I. A., Nero, L. A., & Moreira, M. A. S. (2011). Escherichia coli from clinical mastitis: serotypes and virulence factors. *Journal of Veterinary Diagnostic Investigation*, *23*(6), 1146-1152.
15. Liu, Y., Liu, G., Liu, W., Liu, Y., Ali, T., Chen, W., ... & Han, B. (2014). Phylogenetic group, virulence factors and antimicrobial resistance of Escherichia coli associated with bovine mastitis. *Research in microbiology*, *165*(4), 273-277.
16. Blum SE & Leitner G (2013) Genotyping and virulence factors assessment of bovine mastitis Escherichia coli. *Vet. Microbiol.* 163(3-4):305-312.

17. Kaipainen, T., Pohjanvirta, T., Shpigel, N. Y., Shwimmer, A., Pyörälä, S., & Pelkonen, S. (2002). Virulence factors of Escherichia coli isolated from bovine clinical mastitis. *Veterinary microbiology*, *85*(1), 37-46.
18. Beutler, B. (2004). Innate immunity: An overview. Molecular Immunology, 40(12), 845-859.
19. Rainard, P. (2005). Tackling mastitis in dairy cows. Nature Biotechnology, 23(4), 430-431.
20. Oliver, S., & Mitchell, B. (1983). Susceptibility of bovine mammary gland to infections during the dry Period1. Journal of Dairy Science, 66(5), 1162-1166.
21. Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. Nature Immunology, 11(5), 373-384.
22. Persson-Waller, K., Colditz, I., & Seow, H. (1997). Accumulation of leucocytes and cytokines in the lactating ovine udder during mastitis due to staphylococcus aureus and escherichia coli. Research in Veterinary Science, 62(1), 63-66.
23. Kehrli, M. E., & Goff, J. P. (1989). Periparturient hypocalcemia in cows: Effects on peripheral blood neutrophil and lymphocyte function. Journal of Dairy Science, 72(5), 1188-1196.
24. Fitzpatrick, J., Cripps, P., Hill, A., Bland, P., & Stokes, C. (1992). MHC class II expression in the bovine mammary gland. Veterinary Immunology and Immunopathology, 32(1-2), 13-23.
25. Politis, I., Gorewit, R., Muller, T., & Grosse, R. (1992). Mammary-derived growth inhibitor protein and messenger ribonucleic acid concentrations in different physiological states of the gland. Journal of Dairy Science, 75(6), 1423-1429.
26. Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., & Burvenich, C. (2002). Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. Journal of Mammary Gland Biology and Neoplasia, 7(2), 109-121.
27. Kornalijnslijper, J., Daemen, A., Van Werven, T., Niewold, T., Rutten, V., & Noordhuizen-Stassen, E. (2004). Bacterial growth during the early phase of infection determines the severity of experimental escherichia coli mastitis in dairy cows. Veterinary Microbiology, 101(3), 177-186.
28. Wright, H. L., Moots, R. J., Bucknall, R. C., & Edwards, S. W. (2010). Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford, England), 49(9), 1618-1631. doi:10.1093/rheumatology/keq045 [doi]
29. Prin-Mathieu, C., Le Roux, Y., Faure, G. C., Laurent, F., Bene, M. C., & Moussaoui, F. (2002). Enzymatic activities of bovine peripheral blood leukocytes and milk polymorphonuclear neutrophils during intramammary inflammation caused by lipopolysaccharide. Clinical and Diagnostic Laboratory Immunology, 9(4), 812-817.
30. Wilmes, M., Cammue, B. P., Sahl, H. G., & Thevissen, K. (2011). Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. *Natural product reports*, *28*(8), 1350-1358.
31. Riollet, C., Rainard, P., & Poutrel, B. (2002). Cells and cytokines in inflammatory secretions of bovine mammary gland. Biology of the mammary gland (pp. 247-258) Springer.
32. Schanbacher, F., Goodman, R. E., & Talhouk, R. (1993). Bovine mammary lactoferrin: Implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk Proteins1. Journal of Dairy Science, 76(12), 3812-3831.
33. Shafer-Weaver, K. A., Pighetti, G. M., & Sordillo, L. M. (1996). Diminished mammary gland lymphocyte functions parallel shifts in trafficking patterns during the postpartum period. Proceedings of the Society for Experimental Biology and Medicine, 212(3), 271-279.

34. Sordillo, L. M., & Streicher, K. L. (2002). Mammary gland immunity and mastitis susceptibility. Journal of Mammary Gland Biology and Neoplasia, 7(2), 135-146.
35. Sordillo, L., Shafer-Weaver, K., & DeRosa, D. (1997). Immunobiology of the mammary gland. Journal of Dairy Science, 80(8), 1851-1865.
36. Riollet, C., Rainard, P., & Poutrel, B. (2000). Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with staphylococcus aureus a-toxin. Inflammation Research, 49(9), 486-496.
37. Dembic, Z. (2015). The cytokines of the immune system: The role of cytokines in disease related to immune response Academic Press.
38. Van Snick, J. (1990). Interleukin-6: an overview. *Annual review of immunology*, *8*(1), 253-278.
39. Okada, H., Ohtsuka, H., Satoru, K., Kirisawa, R., Yokomizo, Y., Yoshino, T., & Rosol, Thomas J Thomas J. (1999). Effects of lipopolysaccharide on production of interleukin-1 and interleukin-6 by bovine mammary epithelial cells in vitro. Journal of Veterinary Medical Science, 61(1), 33-35.
40. Lee, J., Bannerman, D. D., Paape, M. J., Huang, M., & Zhao, X. (2006). Characterization of cytokine expression in milk somatic cells during intramammary infections with escherichia coli or staphylococcus aureus by real-time PCR. Veterinary Research, 37(2), 219-229.
41. Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A., & Farnarier, C. (2003). IL-6: A regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends in Immunology, 24(1), 25-29.
42. Hisaeda, K., Hagiwara, K., Eguchi, J., Yamanaka, H., Kirisawa, R., & Hiroshi, I. (2001). Interferon-γ and tumor necrosis factor-α levels in sera and whey of cattle with naturally occurring coliform mastitis. Journal of Veterinary Medical Science, 63(9), 1009-1011.
43. Perkins, K., VandeHaar, M., Burton, J., Liesman, J., Erskine, R., & Elsasser, T. (2002). Clinical responses to intramammary endotoxin infusion in dairy cows subjected to feed restriction. Journal of Dairy Science, 85(7), 1724-1731.
44. Blum, J., Dosogne, H., Hoeben, D., Vangroenweghe, F., Hammon, H., Bruckmaier, R., & Burvenich, C. (2000). Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by escherichia coli infection and endotoxin in dairy cows. Domestic Animal Endocrinology, 19(4), 223-235.
45. Rainard, P., & Riollet, C. (2006). Innate immunity of the bovine mammary gland. Veterinary Research, 37(3), 369-400.
46. Borish, L., Rosenbaum, R., Albury, L., & Clark, S. (1989). Activation of neutrophils by recombinant interleukin 6. *Cellular immunology*, *121*(2), 280-289.
47. Zhao, X., & Lacasse, P. (2008). Mammary tissue damage during bovine mastitis: Causes and control. Journal of Animal Science, 86(13_suppl), 57-65.
48. Frost, A. J., Hill, A. W., & Brooker, B. E. (1980). The early pathogenesis of bovine mastitis due to escherichia coli. Proceedings of the Royal Society of London.Series B, Biological Sciences, 209(1176), 431-439.
49. Mehrzad, J., Desrosiers, C., Lauzon, K., Robitaille, G., Zhao, X., & Lacasse, P. (2005). Proteases involved in mammary tissue damage during endotoxin-induced mastitis in dairy cows. Journal of Dairy Science, 88(1), 211-222.
50. Burow, M. E., Tang, Y., Collins-Burow, B. M., Krajewski, S., Reed, J. C., McLachlan, J. A., & Beckman, B. S. (1999). Effects of environmental estrogens on tumor necrosis factor alpha-mediated apoptosis in MCF-7 cells. Carcinogenesis, 20(11), 2057-2061.

51. Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science, 267(5203), 1456.
52. Tait, S. W., & Green, D. R. (2010). Mitochondria and cell death: Outer membrane permeabilization and beyond. Nature Reviews Molecular Cell Biology, 11(9), 621-632.
53. Long, E., Capuco, A., Wood, D., Sonstegard, T., Tomita, G., Paape, M., & Zhao, X. (2001). Escherichia coli induces apoptosis and proliferation of mammary cells. Cell Death and Differentiation, 8(8), 808.
54. Merto, G. R., Cella, N., & Hynes, N. E. (1997). Apoptosis is accompanied by changes in bcl-2 and bax expression, induced by loss of attachment, and inhibited by specific extracellular matrix proteins in mammary epithelial cells. Cell Growth & Differentiation : The Molecular Biology Journal of the American Association for Cancer Research, 8(2), 251-260.
55. Metcalfe, A. D., Gilmore, A., Klinowska, T., Oliver, J., Valentijn, A. J., Brown, R., . . . Streuli, C. H. (1999). Developmental regulation of bcl-2 family protein expression in the involuting mammary gland. Journal of Cell Science, 112 (Pt 11)(Pt 11), 1771-1783.
56. Burvenich, C., Bannerman, D. D., Lippolis, J., Peelman, L., Nonnecke, B., Kehrli, M., & Paape, M. (2007). Cumulative physiological events influence the inflammatory response of the bovine udder to escherichia coli infections during the transition period. Journal of Dairy Science, 90, E39-E54.
57. Azab, M. E., & Abdel-Maksoud, H. A. (1999). Changes in some hematological and biochemical parameters during prepartum and postpartum periods in female baladi goats. Small Ruminant Research, 34(1), 77-85.
58. Sordillo, L. M. (2005). Factors affecting mammary gland immunity and mastitis susceptibility. Livestock Production Science, 98(1), 89-99.
59. Vangroenweghe, F., Lamote, I., & Burvenich, C. (2005). Physiology of the periparturient period and its relation to severity of clinical mastitis. Domestic Animal Endocrinology, 29(2), 283-293.
60. Hill, A. W. (1981). Factors influencing the outcome of escherichia coli mastitis in the dairy cow. Research in Veterinary Science, 31(1), 107-112.
61. Curtis, C. R., Erb, H. N., Sniffen, C. J., Smith, R. D., & Kronfeld, D. S. (1985). Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in holstein Cows1. Journal of Dairy Science, 68(9), 2347-2360.
62. Stabel, J. R., Kehrli, M. E., Thurston, J. R., Goff, J. P., & Boone, T. C. (1991). Granulocyte colony-stimulating factor effects on lymphocytes and immunoglobulin concentrations in periparturient cows. Journal of Dairy Science, 74(11), 3755-3762.
63. Sordillo, L. M., Afseth, G., Davies, G., & Babiuk, L. A. (1992). Effects of recombinant granulocyte-macrophage colony-stimulating factor on bovine peripheral blood and mammary gland neutrophil function in vitro. Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire, 56(1), 16-21.
64. Cai, T. Q., Weston, P. G., Lund, L. A., Brodie, B., McKenna, D. J., & Wagner, W. C. (1994). Association between neutrophil functions and periparturient disorders in cows. American Journal of Veterinary Research, 55(7), 934-943.
65. Guidry, A. J., Paape, M. J., & Pearson, R. E. (1976). Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. American Journal of Veterinary Research, 37(10), 1195-1200.
66. Burvenich, C., Paape, M., Hill, A., Guidry, A., Miller, R., Heyneman, R., Brand, A. (1994). Role of the neutrophil leucocyte in the local and systemic reactions during experimentally

induced E. coli mastitis in cows immediately after calving. Veterinary Quarterly, 16(1), 45-50.
67. Heyneman, R., Burvenich, C., & Vercauteren, R. (1990). Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced escherichia coli mastitis in cows. Journal of Dairy Science, 73(4), 985-994.
68. Hoeben, D., Burvenich, C., TREVISI, E., BERTONI, G., HAMANN, J., BRUCKMAIER, R. M., & BLUM, J. W. (2000). Role of endotoxin and TNF-α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. Journal of Dairy Research, 67(04), 503-514.
69. Mehrzad, J., Dosogne, H., Meyer, E., Heyneman, R., & Burvenich, C. (2001). Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. Journal of Dairy Research, 68(03), 399-415.
70. Shafer-Weaver, K., Corl, C., & Sordillo, L. (1999). Shifts in bovine CD4 subpopulations increase T-helper-2 compared with T-helper-1 effector cells during the postpartum period. Journal of Dairy Science, 82(8), 1696-1706.
71. Sordillo, L., Pighetti, G., & Davis, M. (1995). Enhanced production of bovine tumor necrosis factor-α during the periparturient period. Veterinary Immunology and Immunopathology, 49(3), 263-270.
72. Sordillo, L. M., Redmond, M. J., Campos, M., Warren, L., & Babiuk, L. A. (1991). Cytokine activity in bovine mammary gland secretions during the periparturient period. Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire, 55(3), 298-301.
73. Chandler, R. (1970). Experimental bacterial mastitis in the mouse. Journal of Medical Microbiology, 3(2), 273-282.
74. Brouillette, E., & Malouin, F. (2005). The pathogenesis and control of staphylococcus aureus-induced mastitis: Study models in the mouse. Microbes and Infection, 7(3), 560-568.
75. Johnson, D., & Otterby, D. (1981). Influence of dry period diet on early postpartum health, feed intake, milk production, and reproductive efficiency of holstein Cows1. Journal of Dairy Science, 64(2), 290-295.
76. Kerr, D., & Wellnitz, O. (2003). Mammary expression of new genes to combat mastitis. Journal of Animal Science, 81(suppl_3), 38-47.
77. Notebaert, S., Demon, D., Berghe, T. V., Vandenabeele, P., & Meyer, E. (2008). Inflammatory mediators in escherichia coli-induced mastitis in mice. Comparative Immunology, Microbiology and Infectious Diseases, 31(6), 551-565.
78. Lee, J., Paape, M. J., & Zhao, X. (2003). Recombinant bovine soluble CD14 reduces severity of experimental escherichia coli mastitis in mice. Veterinary Research, 34(3), 307-316.
79. Trigo, G., Dinis, M., França, Â., Andrade, E. B., da Costa, Rui M Gil, Ferreira, P., & Tavares, D. (2009). Leukocyte populations and cytokine expression in the mammary gland in a mouse model of streptococcus agalactiae mastitis. Journal of Medical Microbiology, 58(7), 951-958.
80. Brouillette, E., Grondin, G., Lefebvre, C., Talbot, B. G., & Malouin, F. (2004). Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of staphylococcus aureus. Veterinary Microbiology, 101(4), 253-262.
81. Demon, D., Ludwig, C., Breyne, K., Guédé, D., Dörner, J., Froyman, R., & Meyer, E. (2012). The intramammary efficacy of first generation cephalosporins against staphylococcus aureus mastitis in mice. Veterinary Microbiology, 160(1), 141-150.

82. Glynn, D. J., Hutchinson, M. R., & Ingman, W. V. (2014). Toll-like receptor 4 regulates lipopolysaccharide-induced inflammation and lactation insufficiency in a mouse model of mastitis 1. Biology of Reproduction, 90(5), Article 91, 1-11.
83. Gonen, E., Vallon-Eberhard, A., Elazar, S., Harmelin, A., Brenner, O., Rosenshine, I., Shpigel, N. Y. (2007). Toll-like receptor 4 is needed to restrict the invasion of escherichia coli P4 into mammary gland epithelial cells in a murine model of acute mastitis. Cellular Microbiology, 9(12), 2826-2838.
84. Burton, J., Madsen, S., Yao, J., Sipkovsky, S., & Coussens, P. (2003). An immunogenomics approach to understanding periparturient immunosuppression and mastitis susceptibility in dairy cows. Acta Veterinaria Scandinavica, 44(1), S71.
85. Cyktor, J. C., & Turner, J. (2011). Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. Infection and Immunity, 79(8), 2964-2973. doi:10.1128/IAI.00047-11
86. Atabai, K., Sheppard, D., & Werb, Z. (2007). Roles of the innate immune system in mammary gland remodeling during involution. *Journal of mammary gland biology and neoplasia*, *12*(1), 37-45.
87. Clarkson, R. W., & Watson, C. J. (2003). Microarray analysis of the involution switch. *Journal of mammary gland biology and neoplasia*, *8*(3), 309-319.
88. Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., ... & Gusterson, B. A. (2003). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Research*, *6*(2), R75.
89. Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., & Halbwachs-Mecarelli, L. (2000). Neutrophils: Molecules, functions and pathophysiological aspects. Laboratory Investigation, 80(5), 617.
90. Menzies, P. I., & Ramanoon, S. Z. (2001). Mastitis of sheep and goats. Veterinary Clinics of North America: Food Animal Practice, 17(2), 333-358.
91. Detilleux, J., Kehrli, M., Stabel, J., Freeman, A., & Kelley, D. (1995). Study of immunological dysfunction in periparturient holstein cattle selected for high and average milk production. Veterinary Immunology and Immunopathology, 44(3-4), 251-267
92. Brooker, B. E., Frost, A. J., & Hill, A. W. (1981). At least two toxins are involved inEscherichia coli mastitis. *Cellular and Molecular Life Sciences*, *37*(3), 290-292.
93. Dosogne, H., Burvenich, C., van Werven, T., Roets, E., Noordhuizen-Stassen, E. N., & Goddeeris, B. (1997). Increased surface expression of CD11b receptors on polymorphonuclear leukocytes is not sufficient to sustain phagocytosis during Escherichia coli mastitis in early postpartum dairy cows. *Veterinary immunology and immunopathology*, *60*(1-2), 47-59.
94. Frost, A. J., & Brooker, B. E. (1986). Hyperacute Escherichia coli mastitis of cattle in the immediate post-partum period. *Australian veterinary journal*, *63*(10), 327-331.
95. Shuster, D. E., Lee, E. K., & Kehrli Jr, M. E. (1996). Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. *American Journal of Veterinary Research*, *57*(11), 1569-1575.
96. Van Werven, T., Noordhuizen-Stassen, E. N., Daemen, A. J. J. M., Schukken, Y. H., Brand, A., & Burvenich, C. (1997). Preinfection in vitro chemotaxis, phagocytosis, oxidative burst,

and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with Escherichia coli. *Journal of dairy science*, *80*(1), 67-74.
97. Vandeputte-Van Messom, G., Burvenich, C., Roets, E., Massart-Leën, A. M., Heyneman, R., Kremer, W. D., & Brand, A. (1993). Classification of newly calved cows into moderate and severe responders to experimentally induced Escherichia coli mastitis. *Journal of dairy research*, *60*(01), 19-29.
98. Kehrli, M. E., & Harp, J. A. (2001). Immunity in the mammary gland. *Veterinary clinics of North America: food animal practice*, *17*(3), 495-516
99. Alluwaimi, A. M. (2004). The cytokines of bovine mammary gland: prospects for diagnosis and therapy. *Research in veterinary science*, *77*(3), 211-222. Schmitz, S., Pfaffl, M. W., Meyer, H. H. D., & Bruckmaier, R. M. (2004). Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domestic animal endocrinology*, *26*(2), 111-126.
100. Gunther, J., Esch, K., Poschadel, N., Petzl, W., Zerbe, H., Mitterhuemer, S., Seyfert, H. M. (2011). Comparative kinetics of escherichia coli- and staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha. Infection and Immunity, 79(2), 695-707. doi:10.1128/IAI.01071-10
101. Schmitz, S., Pfaffl, M. W., Meyer, H. H. D., & Bruckmaier, R. M. (2004). Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domestic animal endocrinology*, *26*(2), 111-126.
102. Lippolis, J. D., Peterson-Burch, B. D., & Reinhardt, T. A. (2006). Differential expression analysis of proteins from neutrophils in the periparturient period and neutrophils from dexamethasone-treated dairy cows. *Veterinary immunology and immunopathology*, *111*(3), 149-164.
103. Van Oostveldt, K., Vangroenweghe, F., Dosogne, H., & Burvenich, C. (2001). Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early and midlactating healthy cows. Veterinary Research, 32(6), 617-622.
104. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology*, *19*(1), 683-765.
105. Saraiva, M., & O'garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature reviews immunology*, *10*(3), 170-181.
106. Murray, P. J., Wang, L., Onufryk, C., Tepper, R. I., & Young, R. A. (1997). T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *The Journal of Immunology*, *158*(1), 315-321.
107. Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Förster, I., & Akira, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity*, *10*(1), 39-49.
108. Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., ... & Johnson, E. M. (1998). Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell*, *93*(3), 373-383.
109. Lang, R., Patel, D., Morris, J. J., Rutschman, R. L., & Murray, P. J. (2002). Shaping gene expression in activated and resting primary macrophages by IL-10. *The Journal of Immunology*, *169*(5), 2253-2263.

110. Donnelly, R. P., Dickensheets, H., & Finbloom, D. S. (1999). The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *Journal of interferon & cytokine research*, *19*(6), 563-573.
111. Blander, J. M., & Sander, L. E. (2012). Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nature Reviews Immunology*, *12*(3), 215-225.
112. Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D., & Gordon, S. (2005). Macrophage receptors and immune recognition. *Annu. Rev. Immunol.*, *23*, 901-944.
113. Garaude, J., Acín-Pérez, R., Martínez-Cano, S., Enamorado, M., Ugolini, M., Nistal-Villán, E., ... & Sancho, D. (2016). Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense. *Nature Immunology*, *17*(9), 1037-1045.
114. Agace, W., Hedges, S., Andersson, U., Andersson, J., Ceska, M., Svanborg, C. (1993) Selective cytokine production by epithelial cells following exposure to *Escherichia coli*. *Infect Immun* **61**: 602–609.
115. Sohn, B. H., Hyung-Bae, M. O. O. N., Tae-Yoon, K. I. M., Han-Sung, K. A. N. G., BAE, Y. S., Kyung-Kwang, L. E. E., & KIM, S. J. (2001). Interleukin-10 up-regulates tumour-necrosis-factor-α-related apoptosis-inducing ligand (TRAIL) gene expression in mammary epithelial cells at the involution stage.*Biochemical Journal*, *360*(1), 31-38.
116. Voehringer, D. W., & Meyn, R. E. (2000). Redox aspects of Bcl-2 function. *Antioxidants and Redox Signaling*, *2*(3), 537-550.
117. Bruey, J. M., Bruey-Sedano, N., Luciano, F., Zhai, D., Balpai, R., Xu, C., ... & Matsuzawa, S. I. (2007). Bcl-2 and Bcl-X L regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell*, *129*(1), 45-56.
118. Shimada, K., Crother, T. R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., ... & Rentsendorj, A. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity*, *36*(3), 401-414.
119. Zhou, F., Yang, Y., & Xing, D. (2011). Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS journal*, *278*(3), 403-413.
120. Thacker, J. D., Balin, B. J., Appelt, D. M., Sassi-Gaha, S., Purohit, M., Rest, R. F., & Artlett, C. M. (2012). NLRP3 inflammasome is a target for development of broad-spectrum anti-infective drugs. *Antimicrobial agents and chemotherapy*, *56*(4), 1921-1930.
121. Colitti, M., Wilde, C. J., & Stefanon, B. (2004). Functional expression of bcl-2 protein family and AIF in bovine mammary tissue in early lactation. *Journal of dairy research*, *71*(01), 20-27.
122. Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, *140*(6), 805-820.
123. Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*(6), 821-832.
124. Guo, H., Callaway, J. B., & Ting, J. P. (2015). Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature medicine*, *21*(7), 677-687.
125. Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., ... & Sutterwala, F. S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. *Proceedings of the National Academy of Sciences*, *105*(26), 9035-9040.
126. Cruz, C. M., Rinna, A., Forman, H. J., Ventura, A. L., Persechini, P. M., & Ojcius, D. M. (2007). ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *Journal of Biological Chemistry*, *282*(5), 2871-2879.

127. Perregaux, D., & Gabel, C. A. (1994). Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *Journal of Biological Chemistry*, *269*(21), 15195-15203.
128. Heinen, A., Camara, A. K., Aldakkak, M., Rhodes, S. S., Riess, M. L., & Stowe, D. F. (2007). Mitochondrial Ca2+-induced K+ influx increases respiration and enhances ROS production while maintaining membrane potential. *American Journal of Physiology-Cell Physiology*, *292*(1), C148-C156.
129. Bauernfeind, F., Bartok, E., Rieger, A., Franchi, L., Núñez, G., & Hornung, V. (2011). Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *The Journal of Immunology*, *187*(2), 613-617.
130. Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. *Nature*, *387*(6635), 773.
131. Hockenbery, D. M. (1995). bcl-2, a novel reguator of cell death. *Bioessays*, *17*(7), 631-638.
132. Jang, J. H., & Surh, Y. J. (2003). Potentiation of cellular antioxidant capacity by Bcl-2: implications for its antiapoptotic function. *Biochemical pharmacology*, *66*(8), 1371-1379.
133. Bulua, A. C., Simon, A., Maddipati, R., Pelletier, M., Park, H., Kim, K. Y., ... & Siegel, R. M. (2011). Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *Journal of Experimental Medicine*, jem-20102049.
134. van de Veerdonk, F. L., Smeekens, S. P., Joosten, L. A., Kullberg, B. J., Dinarello, C. A., van der Meer, J. W., & Netea, M. G. (2010). Reactive oxygen species–independent activation of the IL-1β inflammasome in cells from patients with chronic granulomatous disease. *Proceedings of the National Academy of Sciences*, *107*(7), 3030-3033.
135. Boutet, P., Boulanger, D., Gillet, L., Vanderplasschen, A., Closset, R., Bureau, F., & Lekeux, P. (2004). Delayed neutrophil apoptosis in bovine subclinical mastitis. *Journal of dairy science*, *87*(12), 4104-4114.
136. Madsen, S. A., Chang, L. C., Hickey, M. C., Rosa, G. J., Coussens, P. M., & Burton, J. L. (2004). Microarray analysis of gene expression in blood neutrophils of parturient cows. *Physiological Genomics*, *16*(2), 212-221.
137. Strandberg, Y., Gray, C., Vuocolo, T., Donaldson, L., Broadway, M., & Tellam, R. (2005). Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine*, *31*(1), 72-86.
138. Harris, H. W., Grunfeld, C., Feingold, K. R., & Rapp, J. H. (1990). Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. *Journal of Clinical Investigation*, *86*(3), 696.
139. Eichbaum, E. B., Harris, H. W., Kane, J. P., & Rapp, J. H. (1991). Chylomicrons can inhibit endotoxin activity in vitro. *Journal of Surgical Research*, *51*(5), 413-416.
140. Petzl, W., Zerbe, H., Günther, J., Yang, W., Seyfert, H. M., Nürnberg, G., & Schuberth, H. J. (2008). Escherichia coli, but not Staphylococcus aureus triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Veterinary research*, *39*(2), 1-23.
141. De Schepper, S., De Ketelaere, A., Bannerman, D. D., Paape, M. J., Peelman, L., & Burvenich, C. (2008). The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of Escherichia coli mastitis in dairy cattle. *Veterinary research*, *39*(1), 1-23.

142. Wickramasinghe, S., Rincon, G., Islas-Trejo, A., & Medrano, J. F. (2012). Transcriptional profiling of bovine milk using RNA sequencing. *BMC genomics*, *13*(1), 45.