LATE GESTATION MATERNAL IMMUNE FUNCTION AND CONCURRENT *ESCHERICHIA COLI* INTRAMAMMARY INFECTION DYNAMICS

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Cows are susceptible to new *Escherichia coli* intramammary infections (IMI) in the non-lactating period of late gestation (known as the 'dry period'). These IMI often persist up until parturition without inducing highly inflammatory responses and increase the risk of postpartum mastitis in the subsequent lactation. The bovine maternal immune system is hypothesized to be regulated during late gestation to prevent highly inflammatory, cell-mediated responses however the mechanisms in generating such tolerance have not been fully elucidated. In other mammalian species mononuclear phagocytes play a primary role in generating maternal immune tolerance, and although beneficial for the fetus, often negatively impacts immune responses to invading pathogens. The work presented here investigated changes in maternal immunity and its relationship to dry period *E. coli* IMI dynamics through the use of experimental and mathematical approaches. The objectives were to 1) characterize changes in blood monocyte composition and monocyte-derived dendritic cell (moDC) function over pregnancy to identify unique changes in late gestation, 2) investigate pregnancy-associated factors that regulate these cells, 3) investigate the relationship between maternal immune regulation and dry period *E. coli* IMI dynamics and risk of postpartum mastitis through mathematical models, and 4) investigate the effect of intramammary immunization with UV-killed *E. coli* on IMI dynamics and host response. Pregnancy was accompanied with a decrease in inflammatory monocytes and impaired moDC maturation following *E. coli* stimulation. Aspects of hindered moDC maturation could be induced by *in vitro* treatment of late gestation levels of progesterone and estradiol. Deviations in

prepartum monocyte composition related to risk of postpartum disease. Mathematical model results indicated these shifts in cytokine production alone were not able to recapitulate IMI dynamics in the dry period, but rather it involved an interaction between maternal immune regulation and physiological and immunological changes to the mammary gland that accompany the dry period. Intramammary immunization at dry-off generated protective responses against *E. coli* challenge later in the dry period. Overall, shifts in maternal immunity both in the periphery and in the mammary gland during the dry period relate to persistent *E. coli* IMI and postpartum mastitis, and local immunity can be manipulated to generate protection against IMI in the dry period.

BIOGRAPHICAL SKETCH

Brianna Pomeroy's passion for dairy cattle health was cultivated early in life through experiences working on her grandfather and uncles' dairy farm in her small hometown in southern New Hampshire. She combined this passion for dairy farming with her fervor for math and science. She transferred from her initial start in the Chemical & Biomedical Engineering bachelors program at the Johns Hopkins University to the Animal Science program at Cornell University in January 2010. Brianna received her Bachelor of Science with a distinction in research from the Department of Animal Science at Cornell University in May 2012. Upon finishing her bachelor degree she continued her studies at Cornell University as a doctoral student in the field of Comparative Biomedical Sciences in the Biological and Biomedical Sciences program housed at the College of Veterinary Medicine in August 2012. Following the completion of laboratory rotations in the labs of Dr. Ynte Schukken, Dr. David Russell, and Dr. Avery August she ultimately joined the lab of Dr. Ynte Schukken to pursue her research interests in the epidemiological and immunological aspects of bovine infectious disease. In February 2014 Brianna successfully completed her candidacy exam. In the first years of her doctorate she was awarded a financial gift from Zoetis and USDA AFRI NIFA Pre-doctoral Fellowship both of which enabled her to complete her doctoral research. Throughout her years at Cornell University she has gained invaluable teaching experiences through teaching assistantships and project leadership roles. She has had the opportunity to present her work at multiple workshops and conferences. The majority of her dissertation work has been published in peer-reviewed journals. In the final stages of her doctoral degree she worked as a visiting scholar at Wageningen University in the Netherlands where she was able to expand both her network and knowledge on dairy farming within the Dutch system. In addition to her graduate work, Brianna had the

opportunity to explore options outside of academia. She participated in Zoetis' summer internship program in research and development working on novel adjuvants and immumodulator research. She will pursue a career in industry so she may apply the knowledge and skills developed from her academic training to create useable products to improve animal health and the lives of dairy farmers.

POSITIONS AND HONORS

2010-2012	Research Assistant, Cornell University. Labs of Dr. Thomas Overton, Dr. Michael Van Amburgh, & Dr. Daryl Nydam
2011	Teacher Assistant, Introductory Microbiology Laboratory, Cornell University
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2012 (May)	Distinction in Honors Research, Magnum Cum Laude, B.S. in Animal Science, Cornell University, Ithaca, NY
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PUBLICATIONS

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And last, but certainly not least, I would like to thank my friends and family who provided me with support during the moments of celebration and the many times of stress which accompany graduate school. I would like to thank my parents, Linda and Kirk Pomeroy, and brother, Bret Pomeroy, for their continual support and encouragement, I could not have made this accomplishment without you. I would like to thank my father, my uncles, Kevin Pomeroy and Greg Pomeroy, and my grandfather, Keith Pomeroy, for introducing me to dairy farming in our home town of Mont Vernon, New Hampshire; this is where I found my passion for dairy farming, animal health, and science. I would like to thank my friends I've made over the years from New Hampshire to Baltimore to Ithaca to Kalamazoo to Wageningen and back for the encouragement, the strength, the laughter, the patience, the love, the kindness, and for creating space to let go and reflect. Thank you all.

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CHAPTER 1:

INTRODUCTION

1. Mastitis

1.1. Definition, Epidemiology, Etiology, and Risk Factors

Mastitis is one of the most prevalent and costly diseases afflicting dairy farms worldwide (Seegers et al., 2003, Bannerman, 2009). Financial losses associated with mastitis are attributed to decreased milk production and quality, increased culling rates, discarded milk, treatment costs, and adverse effects on fertility and early pregnancy (Hortet and Seegers, 1998, Seegers et al., 2003, Hertl et al., 2014a). The greatest incidence of mastitis occurs within the first weeks following parturition that overlaps with the transition period, a time of negative energy balance following the onset of lactation and immune dysfunction (Barkema et al., 1998, Green et al., 2002). Clinical signs of mastitis include painful swelling of the afflicted mammary gland, increased somatic cell counts (SCC), decreased milk production and abnormal secretions, and, in extreme cases where bacteremia occurs, systemic signs such as rumen stasis, shock, or even death (Burvenich et al., 2003, Schukken et al., 2011). Bovine mastitis is most commonly caused by bacterial intramammary infections (IMI) (Bannerman, 2009). *Escherichia coli* is the most prevalent of the gram-negative pathogenic agents responsible for IMI and cases of clinical mastitis in dairy cattle and that are typically of greater severity and mortality than other mastitis-causing pathogens (Barkema et al., 1998, Bannerman, 2009, Rinaldi et al., 2010, Quesnell et al., 2012, Oliveira et al., 2013). Common environmental sources of *E. coli* include contaminated bedding, manure solids and unhygienic housing conditions. *E. coli* IMI are often self-limiting and short in duration with high bacterial counts, although some strains are known to cause mild, chronic forms of mastitis and have the ability to form intracellular reservoirs in mammary

epithelial cells (MEC) (Dopfer et al., 2000, Dogan et al., 2006, White et al., 2010, Schukken et al., 2011, Dogan et al., 2012).

1.2. Immune Response Associated with *E. coli* IMI

E. coli express an array of virulence factors, most notability the endotoxin lipopolysaccharide (LPS) which is central to pathogenesis in IMI (Wenz et al., 2006, Lippolis et al., 2014). Factors other than LPS also contribute to the pathogenesis of *E. coli*; some strains express adhesins, invasins, and secreted factors related to serum resistance that enable them to persist in the gland (Wenz et al., 2006, Suojala et al., 2011, Dogan et al., 2012, Lippolis et al., 2014). The clinical severity of *E. coli* mastitis is thought to be dependent largely on cow-specific factors and host response to these virulence factors (Burvenich et al., 2003, Schukken et al., 2011). Specifically, stage of lactation, parity, metabolic status, and immune function are associated with clinical severity of mastitis (Burvenich et al., 2003, Burvenich et al., 2007). In addition, initial sensing and strength of the innate immune response to invading *E. coli* is thought to be dependent on bacterial load (Vangroenweghe et al., 2004, Mehrzad et al., 2008, Bannerman, 2009, Schukken et al., 2011). Both Vangroenweghe et al. (2004) and Mehrzad et al. (2008) found that increasing the *E. coli* inoculum in primiparous cows induced a more rapid inflammatory cytokine response and increased CD8+ T cell trafficking to the gland, but this immune response did not correspond to a more rapid clearance of the bacteria. These findings suggest that the ability to clear *E. coli* IMI efficiently involves more complex elements than strong, early initiation of the inflammatory cascade (Vangroenweghe et al., 2004, Mehrzad et al., 2008).

The mammary gland innate immune defense is comprised of physical barriers such as the teat sphincter and keratin, soluble factors in mammary gland secretions including lactoferrin,

complement, and defensins, and cellular defenses at the mucosa including tissue-resident cells and recruited leukocytes i.e. MEC, monocytes (Mo), dendritic cells (DC), macrophages (Mφ), lymphocytes, neutrophils (PMN), mast cells, natural killer (NK) cells, etc. (Riollet et al., 2000, Burvenich et al., 2003, Hodgkinson et al., 2009, Maxymiv et al., 2012). Inflammation induced by *E. coli* IMI is initiated by toll-like receptor (TLR) signaling, primarily through TLR2 and TLR4 expressed on the surface of MEC, mast cells, and myeloid cells (Mo, DC, Mφ) which recognize pathogen-associated molecular patterns (PAMP) such as LPS. Lipopolysaccharide-induced signal transduction via TLR2/4 with co-receptors CD14 and MD-2 leads to recruitment of adaptor proteins (MyD88, TRIF, TIRAP/MAL, TRAM, SARM) and ultimately results in translocation of transcription factor NF-κB into the nucleus to induce pro-inflammatory cytokine production (IL-1β, IL-6, IL-8, TNFα) (Akira et al., 2006, Schukken et al., 2011, Piras and Selvarajoo, 2014). The inflammatory cascade leads to the recruitment of leukocytes from the blood to the infected mammary gland and eventual clearance of *E. coli*, however, changes in immune function i.e. PMN diapedesis, ROS production, phagocytosis, cytokine production at different stages of lactation, parity, age, etc. influence severity of clinical mastitis and outcome of IMI (Vangroenweghe et al., 2005, Burvenich et al., 2007, Bannerman et al., 2008, Detilleux, 2009, Wenz et al., 2010, Quesnell et al., 2012). Antigen presenting cells, including Mo, Mφ, and DC are present in the mammary gland; these tissue-resident cells orchestrate innate and adaptive immunity imperative to efficient bacterial clearance and mucosal vaccine efficacy (Sordillo and Streicher, 2002, Bharathan and Mullarky, 2011, Maxymiv et al., 2012). Following parturition cows experience a state of immune dysfunction leading to uncontrolled inflammation and increased incidence and severity of infectious diseases. Increased nutritional demands of lactation in addition to dramatic changes in hormone profiles following calving are thought to

regulate aspects of the immune response (Burvenich et al., 2007, Sordillo, 2016). Periparturient cows have reduced circulating levels of T cells, decreased IFN-γ secretion, and impaired PMN oxidative function, all of which are likely to contribute to heightened risk of clinical mastitis (Hoeben et al., 2000, Vangroenweghe et al., 2005, Rinaldi et al., 2008). However, the specific factors and mechanisms that regulate immune cell function and ultimately lead to changes in IMI dynamics in dairy cattle are not fully elucidated (Vangroenweghe et al., 2005).

1.3. Vaccination against *E. coli* mastitis

In efforts to prevent this highly prevalent and costly disease, farms employ management practices to improve milking protocols and milking hygiene, housing conditions and cow hygiene and nutrition. Some farms may also choose implement vaccination programs against *E. coli* mastitis. The commercially available systemic *E. coli* J5 bacterin mastitis vaccine is commonly administered in the non-lactating period of late gestation known as the 'dry period'; J5 vaccination is meant to aid in the reduction of clinical severity in postpartum *E. coli* mastitis through enhanced J5-specific IgM, IgG1, and IgG2 serum levels (Dosogne et al., 2002, Wilson et al., 2009). However, these commercially available J5 vaccines are not labeled for the prevention of *E. coli* IMI rather they labeled to aid in clinical signs associated with *E. coli* mastitis. There are numerous challenges associated with generating mucosal immunity and eliciting strong, appropriate immune response to vaccination; the route of vaccination and immune status of the animal may impact vaccine efficacy (Bharathan and Mullarky, 2011). Preventing new infections in the dry period prior to the transition period, a period of known immune dysfunction, would greatly reduce the incidence of postpartum mastitis. Parameters related to immune status of the cow influence both susceptibility to disease, and infection dynamics, and response to vaccination is not fully elucidated. Identifying the key parameters that substantially impact the outcome of

IMI and vaccination are necessary to optimize management strategies and aid in efforts of generating effective and appropriate bio-therapeutics, vaccines, and schedule for prophylaxis administration to prevent cases of clinical mastitis postpartum.

2. Dry period and late gestation

2.1. Definition of dry period

The non-lactating period prior to calving in primiparous and multiparous cows, known as the 'dry period', is a time for turnover of MEC meant to maximize milk production in the coming lactation, and coincides with a time of considerable fetal growth (Esposito et al., 2014). Typically the dry period ranges between 40-60 days prior to calving, a period in which the mammary gland goes through involution characterized by apoptosis and subsequent cell regeneration (De Vries et al., 2010). Concurrent to changes in mammary gland physiology, there are also numerous alterations to immunity within the mammary gland.

2.2. Intramammary infections in the dry period

The dry period is a known time of heightened susceptibility to new IMI, specifically around dry-off and colostrogenesis when milk leakage is likely to occur due to the increased pressure within the mammary gland; conjointly, the risk of IMI increases with possible delays in keratin plug formation following milk cessation in high producing cows (Smith et al., 1985a, Burvenich et al., 2007). In addition to milk leakage, there is a delay in leukocyte recruitment to the mammary gland by approximately a week following the onset of dry-off until protective levels of leukocytes are reached; additionally, upon reaching the gland these cells have decreased phagocytic function from ingestion of milk fat and cell debris in the early dry period (Burvenich et al., 2007). The lowest incidence of new IMI occurs in the mid-dry period when the gland remains in steady-state involution, the teat ends are sealed, conditions are unfavorable for

bacterial growth, and there is a high concentration of phagocytes, lactoferrin, acute phase proteins, and other soluble mediators in the gland and secretions (Ziv and Gordin, 1973, Jensen and Eberhart, 1981, Todhunter et al., 1990). The majority of cases of clinical mastitis occurring in the first 60 days post-calving are caused by IMI acquired in the dry period (Fig. 1); among the mastitis-causing pathogens, *E. coli* is the most common isolate from the dry period associated with clinical mastitis in the subsequent lactation (Smith et al., 1985a, Smith et al., 1985b, Barkema et al., 1998, Green et al., 2002). Interestingly, signs of inflammation typically observed with *E. coli* mastitis in lactation do not accompany IMI acquired in the dry period; often these IMI persist in the dry period without signs of inflammation up until parturition (Quesnell et al., 2012).

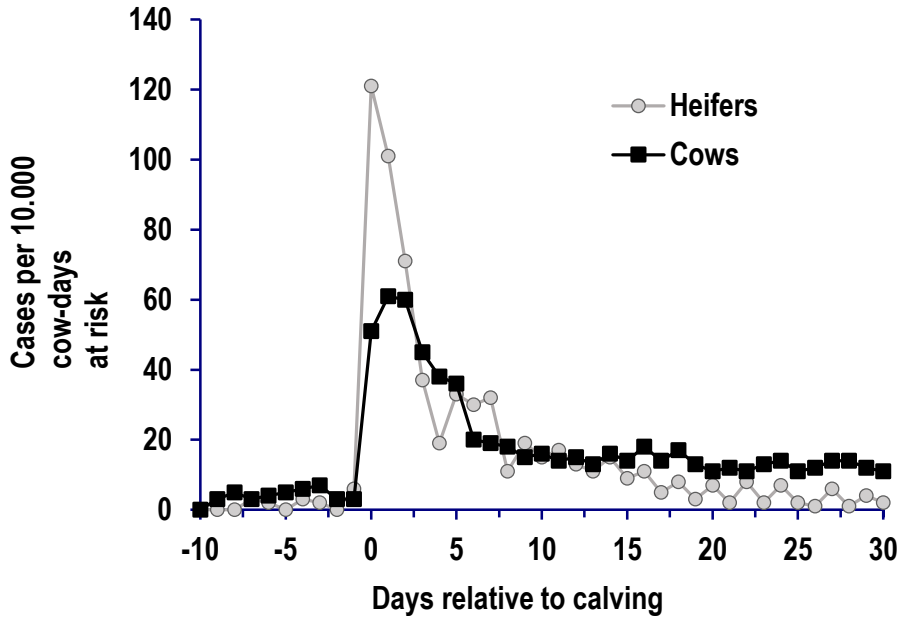

Figure 1. Distribution of incidence rate of clinical mastitis of weeks following calving. Adapted from Barkema et al. (1998).

Previous work by Quesnell et al. (2012) and Gurjar et al. (2013) showed that when multiparous dairy cows were given an experimental intramammary challenge of *E. coli* ECC-Z

in the dry period the infection persisted until parturition without clinical signs or upregulation of pro-inflammatory cytokines (TNFα, IL-1β, IFNγ); following calving cows developed clinical mastitis and upregulated these pro-inflammatory cytokines hallmark of *E. coli* mastitis in challenged quarters. Sipka et al. (2013) found when mid-lactation multiparous cows were given similar intramammary challenges with *E. coli* ECC-Z, animals developed clinical mastitis and upregulated pro-inflammatory cytokines within 24hr following challenge. As mentioned previously, the severity of *E. coli* mastitis and response to infection are thought to be largely dependent on cow factors. The specific factors associated with late gestation and dry period which impact IMI dynamics and allow for persistence of infection without clinical signs in the dry period are poorly understood.

3. Maternal Immune Regulation in Pregnancy

3.1. Maternal Immune Tolerance

During pregnancy the maternal immune system is presented with the challenge of needing to concurrently tolerate the growth of a semi-allogenic fetus and respond to invading pathogens. Highly inflammatory, cell-mediated T helper 1 (Th1) and T helper 17 (Th17)-type responses in pregnancy can be detrimental to the health of the fetus, thus, the maternal immune system must be carefully regulated (Rosbottom et al., 2008, Betz, 2012, Krishnan et al., 2013, Lash and Ernerudh, 2015, Regal et al., 2015). Though highly inflammatory responses appear to negatively impact pregnancy success, the original dogma that the maternal immune system is suppressed and biased towards Th2/Treg-type responses throughout pregnancy in order to tolerate the conceptus is an oversimplification of maternal immune tolerance. The manner in which the maternal immune system is regulated varies depending on the needs of particular stages of pregnancy, i.e. fertilization, implantation, maintenance, preparation for labor and

parturition (Oliveira et al., 2012, Bauersachs and Wolf, 2013, Gomez-Lopez et al., 2014, Sandra et al., 2015, Nair et al., 2016). These changes to the maternal immune system occur locally at the tissues apart of the fetal-maternal interface and also systemically in peripheral blood and other mucosal sites in the body (Bauersachs and Wolf, 2013, Schumacher et al., 2014). However, as observed in human and murine pregnancy, the dam's immune system still maintains aspects of immune competency, and it is also possible for maternal immune tolerance to be disrupted; the immune response elicited to invading pathogens is differentially regulated by the stage of pregnancy (Entrican, 2002, Innes, 2007, Krishnan et al., 2013). Maternal immune regulation in pregnancy is extensively studied in both human and mice, but less is known about changes made to the maternal immune system in dairy cows. However, given the wide biological differences and energy demands of a production animal, the findings in humans and mice may not be directly transferable to a bovine model.

3.2. Cell Types Involved in Generating Maternal Immune Tolerance

Myeloid and lymphoid cell populations such as Mo, Mφ, DC, NK cells, T cells, and B cells, are directly and indirectly regulated by various pregnancy-associated factors including hCG (in the case of humans), progesterone (PG), estradiol (E2), pregnancy-associated glycoproteins, etc. to promote tolerance of the fetus (Schumacher et al., 2014). Myeloid cells (Mo, Mφ, DC), in particular DC, are known to play a critical role in the generation and maintenance of maternal immune tolerance and are regulated both locally at the feto-maternal interphase and in the periphery (Bachy et al., 2008, Negishi et al., 2012, Leno-Duran et al., 2014, Schumacher et al., 2014). Dendritic cells are potent antigen presenting cells that both initiate immune responses and induce peripheral tolerance; the type of response elicited depends heavily on DC maturation status and cytokine production (Hivroz et al., 2012). In human and murine

pregnancy, the composition of DC subsets changes and regulatory subsets such as plasmacytoid and regulatory myeloid DC increase, which promote Treg expansion and are pertinent to fetal survival (Miyazaki et al., 2003, Ban et al., 2008, Fang et al., 2016). The regulation of conventional DC and monocyte derived DC (moDC) maturation in humans and rodents is a key mechanism in the generation of immune tolerance through the inhibition of antigen presentation (MHC II), dampened expression of co-stimulatory molecules (CD80, CD86, CD40), and generation of antigen-specific, cell-mediated responses; maternal DC regulation varies by species, stage of pregnancy, and subset of DC (Lutz and Schuler, 2002, Bachy et al., 2008). Tolerance is often generated by immature and semi-mature DC due to the weak and insufficient receptor and cytokine signaling provided to naïve T cells thereby hindering antigen-specific immunity and other innate immune cell populations including neutrophils and NK cells (Lutz and Schuler, 2002, Doz et al., 2013). Previous work in humans and mice indicates DC maturation is impaired during mammalian pregnancy due tothe influence of circulating pregnancy-associated factors in order to debilitate strong cellular, inflammatory responses detrimental to the fetus and favor Th2/regulatory immunity (Uemura et al., 2008, Segerer et al., 2009, Della Bella et al., 2011, Xu et al., 2011, Cordeau et al., 2012, Schumacher et al., 2013, Schumacher et al., 2014).

Monocyte and Mφ populations are also modulated in pregnancy. Monocytes are classified by CD14 and CD16 expression into classical (cM), intermediate (intM), and nonclassical (ncM) monocytes with unique functions, however these subsets have only recently been defined in cattle and subset functional characteristics appear to deviate from other species (Hussen et al. 2013). In humans and rats, high levels of intM relative to cM during pregnancy is associated with the inflammatory disease pre-eclampsia (Faas et al., 2014). Monocytes may also

differentiate into Mφ and DC following tissue infiltration. Macrophages are thought to play essential roles in spiral artery formation during placentation in humans and predominantly maintain an anti-inflammatory, M2 phenotype over M1 (Faas et al., 2014).

In bovine pregnancy very little is known about the mechanisms involved in maternal immune regulation including which cell types are subject to regulation and which factors regulate them. Manjari et al. (2016) observed dampening of pro-inflammatory responses through changes in gene expression profiles in PMN and plasma cytokine levels as early as implantation. Changes in T cell populations are observed throughout stages of bovine pregnancy (Asai et al., 1998, Oliveira and Hansen, 2008, Maeda et al., 2013). Cattle experience an increase in peripheral $CD4^+CD25^+$ T cells in response to pregnancy, and aspects of T cell function are regulated in pregnancy; PG inhibits the mRNA expression of Th1 and Th17 cytokines IFN-γ and IL-17 and transcription factors T-bet and RORC, and enhanced the mRNA expression of IL-4 in cells from pregnant cows (Oliveira and Hansen, 2008, Maeda et al., 2013). Asai et al. (1998) found an increase in CD4+/CD8+ T cell ratio and IL-2 and IL-4 production from cells in mammary gland secretions in the dry period relative to lactating stages of non-pregnancy and pregnancy. Work on early pregnancy in cattle suggests the important role of myeloid cells at the feto-maternal interphase Specifically Mansouri-Attia et al. (2012) found there was a substantial increase in the number of $CD14^+$ cells and $CD172a^+CD11c^+$ cells (Mo, Mφ, DC) in the endometrium during very early stages of pregnancy. Kamat et al. (2016) observed comparable changes in pregnant heifers with increases in numbers of CD14+CD11c+ cells and tolerance-inducing indoleamine 2,3-dioxygenase (IDO) expression in both blood and endometrium in early pregnancy. Other studies from later stages of pregnancy showed accumulation of Mφ in the mammary gland during involution within the early dry period and in the endometrium approaching calving

(Sordillo and Streicher, 2002, Oliveira and Hansen, 2008, Oliveira and Hansen, 2009). Despite their central role in human and murine pregnancy little is known about functional changes to various myeloid cell populations during bovine pregnancy, mechanisms that regulate these cells, and their specific roles in maternal immune tolerance across pregnancy.

3.3. Endocrine factors involved in maternal immune tolerance

Multiple factors in pregnancy regulate various maternal immune cell populations; these cells may be regulated differently depending on the stage of pregnancy in order to meet the specific demands for successful implantation, maintenance of pregnancy, and preparation for parturition (Bauersachs and Wolf, 2013, Gomez-Lopez et al., 2014, Robertson and Moldenhauer, 2014). Immune-endocrine crosstalk is essential for pregnancy maintenance. In human and murine pregnancies gonadotropin and steroid hormones suppress detrimental maternal responses to fetal antigens by generating and maintaining maternal immune tolerance. Specifically, pregnancy associated hormones may reduce the antigen-presenting capacity of DC, Mo, and Mφ as well as regulate NK cells, T and B cells i.e. support the proliferation of uterine NK cells and induction of regulatory T and B (B1) cells (Laskarin et al., 2007, De Vries et al., 2010, Muzzio et al., 2014, Schumacher et al., 2014, Nair et al., 2016). In humans, pregnancy recognition following fertilization relies heavily on conceptus produced luteotropic hormone human chorionic gonadotropin (hCG) that directly acts upon the corpus luteum (CL) to sustain progesterone (PG) production. In bovine pregnancy the initial cross-talk between the conceptus and the dam is from locally produced, conceptus-derived, antiluteolytic IFNτ which blocks luteolysis through paracrine actions on the uterine endometrium (Bazer et al., 2010). These pregnancy-recognition, conceptus-derived molecules also act on the maternal immune system to generate tolerance in the early pregnancy. For example, in regards to human pregnancy, hCG

modulates multiple cell populations to initiate maternal immune tolerance towards the conceptus. Specifically, hCG induces Treg cell formation and recruitment to the fetal maternal interface, inhibits IL-2 production by peripheral mononuclear cells (PBMC) leading to a reduction in T cell activation, and generates tolerogenic DC (Bansal et al., 2012, Polese et al., 2014). In ruminants, IFNτ primarily acts locally within the uterine tissue, but also has systemic effects analogous to the effects of hCG in humans that promote PG secretion from luteal cells through systemic activation and recruitment of neutrophils by IFNτ (Shirasuna et al., 2015).

Estradiol (E2) and PG are known to regulate aspects of maternal immune tolerance and are related to the maintenance of pregnancy (Schumacher et al., 2014). Estradiol and PG are relatively high in bovine gestation, and in the case of E2, this hormone drastically increases as cows approach calving in the third trimester of pregnancy (Hunter et al., 1970, Echternkamp and Hansel, 1973, Smith et al., 1973). Maeda et al. (2013) showed that *in vitro* treatments with PG influenced the expression of T cell transcription factors to promote T regulatory cell formation in bovine PMBC. Lamote et al. (2004), (2006) found E2 and PG regulated the function and phenotype of bovine neutrophils. Previous work on human and murine pregnancy found E2 and PG to have immunosuppressive effects on DC differentiation and maturation i.e. reduction in CD80 and MHC II, enhanced anti-inflammatory cytokine production with concurrent suppressed pro-inflammatory cytokine responses, and T cell stimulatory capacity (Hughes et al., 2008, Segerer et al., 2009, Jones et al., 2010, Xu et al., 2011, Lasarte et al., 2013). Estradiol and PG in conjunction with other pregnancy-associated hormones such as cortisol, relaxin, and prostaglandin play a role in preparation for labor both directly on the tissues, and indirectly through influence of local immune cell populations (Shynlova et al., 2013). For example, in non-bovine species, IL-1β and IL-10 are key cytokines involved in priming the uterus for labor and

preventing preterm labor in late gestation, respectively, and E2 and P4 are known to influence the expression of these cytokines and related receptors (Sato et al., 2001, Schaefer et al., 2005, Pioli et al., 2006, Robertson et al., 2006, Robertson et al., 2007, Boro et al., 2014). Despite the importance of both immune endocrine crosstalk and myeloid cells in maternal immune regulation across other species, we lack an understanding on the impact of hormones on myeloid cell populations.

3.4. Immunity to Infection in Late Gestation

Pregnancy poses the unique challenge for the maternal immune system; it must be capable of simultaneously promoting maternal immune tolerance to the semi-allogenic fetus and responding to invading pathogens. Maternal immune regulation in mammalian pregnancy is dynamic, and dairy cattle show varying responses to infectious agents a different stages of pregnancy (Innes, 2007, Rosbottom et al., 2008, Quesnell et al., 2012, Sipka et al., 2013). In human and murine pregnancy, women and mice are more susceptible to intracellular pathogens like *L. monocytogenes*, *Salmonella enterica Typhimurium*, influenza, etc. which rely on Th1/Th17, cell-mediated responses for effective clearance (Krishnan et al., 2013). Previous work investigating the interplay between maternal immune regulation and infectious disease within pregnancy has primarily focused on abortion-causing pathogens. However, as mentioned previously both Quesnell et al. (2012) and Gurjar et al. (2013) have shown there are alterations to bovine IMI immune response in the dry period which coincides with late gestation. Namely, there are hindered pro-inflammatory responses and clearance of *E. coli* in the mammary gland throughout the dry period (Quesnell et al., 2012, Gurjar et al., 2013).

Similar to IMI dynamics in the dry period, previous work on *Neospora caninum* pathogenesis has shown gestational stage-dependent changes in infection dynamics with notable

differences in late gestation relative to earlier stages of pregnancy (Innes, 2007, Rosbottom et al., 2008). *N. caninum* is a major abortion-causing parasite in cattle which is transmitted via ingestion of oocyst-contaminated feed or water and leads to transplacental transmission to the fetus resulting in abortion or full-term congenitally infected fetuses (Innes, 2007). Previous work has shown that when cattle are experimentally infected with *N. caninum* in early pregnancy during the first trimester (less than 100 days carrying calf) fetal death is most likely to occur, whereas the risk of transmission from dam to fetus is highest in the last trimester (Williams et al., 2000, Maley et al., 2003, Macaldowie et al., 2004, Maley et al., 2006, Gibney et al., 2008, Rosbottom et al., 2008). These differences in *N. caninum* infection outcome are largely thought to be due to the changes in maternal immune system which vary by stage of pregnancy. Early induction of cell-mediated responses including early production of Th1-type cytokines IFN-γ and IL-12, NK cell lysis of infected cells, and antigen-specific T cell responses are necessary to eliminate this obligate intracellular pathogen; these responses are most severe in early pregnancy when fetal death is greatest and mildest in late pregnancy when infections are most likely to persist (Gazzinelli et al., 1996, Marks et al., 1998, Staska et al., 2003, Boysen et al., 2006, Maley et al., 2006, Innes, 2007, Rosbottom et al., 2008, Canton et al., 2014). These studies indicate is the greatest regulation of cell-mediated immune responses occurs in late gestation and hinders effective responses to infection; the specific role maternal immune regulation plays in IMI dynamics in late gestation and the dry period is not fully known. Our limited understanding of maternal immune regulation hinder our ability to elucidate complexities of host-pathogen interactions at periods of heightened susceptibility and thus, hinder the development of optimal preventative interventions in the dry period to eliminate IMI.

4. Objectives and hypotheses

The overall objective of the work presented in this dissertation was to evaluate differences in late gestation immune response as it relates to the outcome of *E. coli* IMI in the dry period compared to other stages of lactation. The known roles of myeloid cells in other mammalian species indicate that they are key drivers in the immune response to infection, the antigen-specific responses to immunization, and in the maternal immune tolerance. Thus, we chose to investigate changes to Mo and moDC in bovine late gestation as a parameter related to *E. coli* IMI dynamics in the dry period and subsequent postpartum disease. The studies presented here investigated the specific hypothesis that bovine myeloid cells, including Mo and DC populations, have reduced capacity to promote cell-mediated, inflammatory and antigen-specific responses in late gestation which contribute to a suboptimal defense against *E. coli* IMI and pathogen clearance. The following experiments used *E. coli* ECC-Z strain which is an isolate from a case of clinical bovine mastitis (Dogan et al., 2006) and has been used in a number of intramammary challenge studies (Quesnell et al., 2012; Gurjar et al., 2013; Sipka et al., 2013). The first objective of this dissertation was to compare the ability of moDC from late gestation dairy cows to mature in response to UV irradiated *E. coli* ECC-Z stimulation to those from non-pregnant dairy cows in a cross-sectional study. The second objective captured the dynamics of monocyte subsets, and the phenotypic maturation and cytokine production induced by *E. coli* ECC-Z of respective moDC populations through a prospective longitudinal study in cows from mid-gestation through calving into the subsequent lactation to elucidate stage-specific changes. The third objective was to characterize influence of steroid hormones on moDC using an *in vitro* model of moDC co-cultured with pregnancy-associated steroid hormones, in order to further understand what factors may regulate changes observed in late gestation.

To investigate the role of maternal immune regulation in intramammary infection dynamics in the dry period of late gestation and subsequent postpartum disease, the remaining objectives of this dissertation investigate the relationship between prepartum immune function and postpartum disease. The fourth objective investigated maternal immune function and postpartum disease by elucidating relationship between prepartum monocyte composition and postpartum disease using logistic regression models to analyze prospective data. The fifth objective was an expansion of a pre-existing mathematical model on dynamics of *E. coli* IMI to account for the altered immunity in late gestation using data from other objectives. The sixth and final objective investigated the impact of intramammary immunization with UV irradiated *E. coli* ECC-Z in the dry period on intramammary challenge with the same strain on IMI and postpartum mastitis.

The long term goal of the studies proposed here is to characterize the influence of gestation on bovine immune function to permit the development of optimal dry cow management, vaccination schemes, and potential immune-modulators to prevent IMI in the dry period thereby reducing risk of clinical mastitis in early lactation.

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CHAPTER 2:
MONOCYTE-DERIVED DENDRITIC CELLS FROM LATE GESTATION COWS HAVE AN IMPAIRED ABILTY TO MATURE IN RESPONSE TO *E. COLI* STIMULATION IN A RECEPTOR AND CYTOKINE-MEDIATED FASHION

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ABSTRACT

During late gestation the bovine immune system is less capable of eliciting inflammatory responses and eliminating invading pathogens. The maternal immune system is directed towards tolerance in order to prevent fetal rejection due to recognition of paternal antigens. In humans and mice, dendritic cell (DC) populations maintain a tolerogenic phenotype essential in the generation and preservation of maternal immune tolerance throughout pregnancy. However, the primary mechanisms which facilitate maternal immune tolerance involved in bovine gestation remain poorly understood. In order to determine if DC phenotype and function were regulated towards tolerance during bovine gestation, we compared *in vitro* generated monocyte-derived DC (moDC) from monocytes isolated from cows in late gestation (LG) to those from non-pregnant (NP) cows in their ability to mature following stimulation with UV irradiated *E. coli*. Our results show moDC from LG cows have an impaired ability to mature in response to *E. coli* stimulation in a receptor and cytokine-mediated fashion in comparison to those from NP cows. Specifically, moDC from LG cows were unable to upregulate MHC II and maintained high expression of CD14, both indicative of an immature phenotype following *E. coli*-stimulation. Only moDC from LG showed significant increase in IL-10 production and had a significantly lower ratio of production of the Th1-polarizing cytokine IL-12 to regulatory cytokine IL-10 following *E. coli* stimulation compared to moDC from NP cows. Our findings demonstrate moDC from LG cows have a stifled capacity to develop a mature phenotype and drive pro-inflammatory Th1-type responses to *E. coli* stimulation. Results from this study provide insight into DC immune modulation in bovine pregnancy and elucidate host factors which may contribute to the heightened susceptibility to infection in late gestation.

1. Introduction

Successful maintenance of a pregnancy requires careful regulation of the maternal immune system in order to tolerate the growth of a semi-allogeneic fetus (Betz, 2012, Zenclussen, 2013). Highly inflammatory, Th1-type responses must be mitigated during pregnancy to prevent fetal rejection. However, this may result in deleterious effects on appropriate responses required for pathogen elimination (Krishnan et al., 2013). The ability to mount Th1-type responses is compromised in late gestating dairy cattle, and the susceptibility to new intramammary infections greatly increases during this stage of pregnancy (Sordillo and Streicher, 2002, Green et al., 2005). Intramammary infections acquired during the non-lactating period of late gestation (also known as the 'dry period') greatly increase the risk of early lactation clinical mastitis (Barkema et al., 1998, Green et al., 2002). Quesnell et al. (2012) and Gurjar et al. (2013) demonstrated that dairy cattle in the dry period failed to elicit pro-inflammatory, Th1-type immunity in response to experimental *E. coli* intramammary infections, and were unable to clear the infecting pathogen throughout the remainder of gestation leading to subsequent mastitis post-calving. However, Sipka et al. (2013) found mid-lactation dairy cows challenged with the same dose of the same *E. coli* strain developed clinical mastitis and were fully capable of eliciting an inflammatory response.

Previous work on leukocyte populations in periparturient cows suggests that the bovine immune system experiences an anti-inflammatory, Th2/regulatory bias in late gestation analogous to maternal immune regulation that has been described in other mammals. Previous studies observed a decrease in the proportion of circulating CD4, CD8, and IL-2 expressing lymphocytes in late gestation dairy cattle which gradually increased following parturition (Kimura et al., 1999, Meglia et al., 2005). More recent work found circulating lymphocytes in

late gestation cattle had suppressed Th17 and Th1-related gene expression and were regulated by epigenetic changes at the IL-4 and IFNγ promoters to sustain a Th2/regulatory bias by hindering IFNγ production and promoting IL-4 production (Maeda et al., 2013, Paibomesai et al., 2013). Other immune cell populations responsible for maintaining maternal immune tolerance and providing signals to regulate circulating lymphocytes remain poorly characterized in dairy cattle.

Dendritic cells (DC) are known to play a critical role in the generation and maintenance of maternal immune tolerance in murine and human pregnancy (Bachy et al., 2008, Negishi et al., 2012, Leno-Duran et al., 2014). Dendritic cells are potent antigen presenting cells which both initiate immune responses and induce peripheral tolerance depending heavily on DC maturation status and cytokine production (Hivroz et al., 2012). The suppression of DC maturation is a key mechanism in the generation of immune tolerance (Lutz and Schuler, 2002). Immature DC have low expression of co-stimulatory molecules (CD40, CD86, CD80) and MHC molecules, and are not capable of producing high levels of pro-inflammatory cytokines (IL-12, TNFα, IL-1β, IL-6). Tolerance is often generated by immature and semi-mature DC due to the weak and insufficient receptor and cytokine signaling provided to naïve T cells and other innate immune cell populations including neutrophils and NK cells (Lutz and Schuler, 2002, Doz et al., 2013). Dendritic cell maturation is impaired during pregnancy by the influence of circulating pregnancy-associated factors in order to debilitate strong cellular, inflammatory responses detrimental to the fetus and favor Th2/regulatory immunity (Uemura et al., 2008, Segerer et al., 2009, Della Bella et al., 2011, Xu et al., 2011, Cordeau et al., 2012, Schumacher et al., 2013, Schumacher et al., 2014).

Dendritic cells present in the bovine mammary gland are hypothesized to play an important role in defense against invading pathogens (Maxymiv et al., 2012). Impaired DC

maturation in late gestation could negatively impact appropriate inflammatory responses necessary for clearance of intramammary infections caused by *E. coli*. Bovine DC function has not been characterized in late gestation despite their important role in both orchestrating innate and adaptive immune activation and inducing tolerance.

The objective of this study was to compare the ability of monocyte-derived DC (moDC) from late gestation (LG) dairy cows to mature in response to UV irradiated *E. coli* ECC-Z stimulation to those from non-pregnant (NP) dairy cows. For this study we selected non-pregnant dairy cows earlier in lactation as opposed to mid-gestation because highest incidence of clinical mastitis caused by natural infection occurs early in lactation following calving, and this also excluded other pregnancy factors present in mid-gestation (Barkema et al., 1998, Green et al., 2002). The *E. coli* ECC-Z strain is an isolate from a case of clinical bovine mastitis (Dogan et al., 2006) and has been used in a number of intramammary challenge studies (Quesnell et al., 2012, Gurjar et al., 2013, Sipka et al., 2013). This study characterized moDC maturation by measuring changes in surface marker expression (CD14, CD80, MHC II) and cytokine production (IL-10, IL-12) 24 hours following *E. coli* ECC-Z stimulation. The selection of parameters was based on findings in current literature in rodent and human models which indicated both CD80 and HLA-DR/MHC II expression and IL-10 and IL-12 production were altered during pregnancy and/or affected by pregnancy-associated hormones (Bachy et al. 2008, Della Bella et al. 2011, Xu et al. 2011). This study provides evidence for maternal immune regulation of bovine moDC populations in late gestation.

2. Materials and methods

2.1. Animals

Fourteen Holstein-Friesian cows, 7 non-lactating cows in the third trimester of pregnancy within 45-60 days prior to expected calving date, to be called 'late gestation' (LG) and 7 non-pregnant, lactating cows past the periparturient period to be called 'non-pregnant cows' (NP) were selected from the Cornell University Veterinary College Teaching Dairy Barn. All animals enrolled were matched by lactation, and the same number of cows in 2nd and 3rd lactation was sampled for each group. The mean age of cows in the pregnant group at time of sampling was 3.81 years old with a standard deviation of 0.80 years and the mean age of cows in the non-pregnant group at time of sampling was 3.62 years old with a standard deviation of 0.42 years. No disease (i.e. metabolic, reproductive, mastitis) was recorded within 30 days prior to the time of sampling for all enrolled animals. Peripheral blood and serum were collected by jugular venipuncture from enrolled animals into bottles with vacuum containing EDTA and 10ml vacutainer glass serum tubes with no additive (Becton Dickinson, Franklin Lakes, NJ). Blood samples were put on ice and transported immediately to the laboratory for further processing. All procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number: 2007-0110).

2.2. Generation of peripheral blood monocyte-derived dendritic cells (moDC)

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood diluted 1:1 in PBS by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO). Mononuclear cells were isolated from the interphase and pooled. Isolated PBMC were washed twice with PBS. Any remaining red blood cells were lysed with hyposmotic NaCl solution and washed once more in PBS following erythrolysis. Cells were resuspended in MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2mM EDTA) and passed over a 0.44µm^2 filter to remove any clumps. Mononuclear cells were counted, pelleted by centrifugation, and

resuspended in 80µl of cold MACS buffer per 10^7 cells. Monocytes were isolated using magnetic-activated cell sorting (MACS): PBMC were incubated with anti-human CD14 antibodies conjugated with paramagnetic microbeads (Miltenyi Biotech, Inc., Auburn, CA) at 50µl bead solution per 10^8 cells for 20 minutes, washed twice in MACS buffer, pelleted, resuspended in MACS buffer, and transferred to MACS LS magnetic separation column (Miltenyi Biotech, Inc., Auburn, CA). After the column was washed repeatedly with MACS buffer, magnetically labeled cells were collected. Purity was determined by flow cytometry, using mouse anti-bovine CD14 (Kingfisher Biotech, Inc., Saint Paul, MN) and Alexafluor488-conjugated goat anti-mouse IgG1 secondary antibody to detect $CD14^+$ cells, and purity was shown to be >90%. Monocytes were resuspended in differentiation media (phenol-free complete RPMI with L-glutamine, supplement with 10% autologous serum, 20ng/ml recombinant bovine IL-4and , 20ng/ml recombinant bovine GM-CSF, both Kingfisher Biotech, Inc., Saint Paul, MN) then plated in 6-well tissue culture plates at 1.3-1.8x10^6 cells in 3ml differentiation media per well. Cells were cultured for 5 days (37°C, 5% CO_2) in presence of recombinant cytokines to generate immature moDC. One third of the media was replaced after three days in culture.

2.3. Inactivation of *E. coli* ECC-Z

Frozen stock (-80°C) of the previously characterized *E. coli* ECC-Z strain known to cause mild persistent clinical mastitis (Dogan et al., 2006, Lippolis et al., 2014) was inoculated into LB liquid broth, and grown to log phase (37°C, agitation). Bacteria were pelleted and diluted in cold PBS. Bacterial suspension was plated on agar plates prior to inactivation to determine concentration of stock solution. The bacterial suspension was inactivated using UV irradiation in the laboratory of Dr. Randy W. Worobo (Department of Food Science and Technology, Cornell University). *E. coli* were inactivated using UV irradiation in order to

preserve the bacterial structure while preventing bacterial growth during the experiment. Bacterial suspension inactivated using the described UV irradiation failed to produce colonies on LB agar plates (37°C). Aliquots of UV irradiated *E. coli* were resuspended in sterile 10% glycerol/PBS were stored at -80°C.

2.4. Maturation of bovine moDC with UV irradiated *E. coli*

Maturation of moDC was induced by bacterial stimulation through the addition of UV irradiated *E. coli* to immature moDC cultures in an MOI (multiplicity of infection) of 10 (Cavatorta et al., 2009). Cultures of moDC without bacterial stimulation served as media controls. Following 24 hours of culture with UV irradiated *E. coli*- stimulated moDC were considered mature moDC. At 24 hours in culture with UV irradiated *E. coli*, moDC were harvested from all cultures for phenotypic analysis by flow cytometry. Cell culture supernatants were collected and stored at -80°C until further cytokine analysis by ELISA.

2.5. Flow cytometry

Expression of surface markers on moDC was measured with flow cytometry. Cells were washed in FACS buffer (PBS containing 0.5% BSA and 0.05% sodium azide), spun at 400x*g* for 3 minutes to pellet cells, and resuspended and blocked with 10% normal goat serum (Life Technologies, Grand Island, NY) for 15 minutes at 4°C. Blocked cells were incubated with primary, unlabeled mouse mAb: anti-bovine MHC II DR orthologue (TH4B; Kingfisher Biotech), CD14 (MM61A; Kingfisher Biotech), and CD80 (ILA159; Kingfisher Biotech) diluted in FACS buffer at 1:50 for anti-bovine MHC II DR and CD14 mAb and 1:100 for anti-bovine CD80 for 30 minutes at 4°C. Following incubation with primary mAb, cells were washed twice in FACS buffer and incubated with Alexa Fluor® 488 conjugated goat anti-mouse IgG_1 or $IgG_{2\alpha}$ mAb (Life Technologies, Grand Island, NY) diluted at 1:100 for 20 minutes at 4°C in the dark.

Secondary Alexa Fluor® 488 conjugated mAb controls were incubated with the same concentration as used for cells labeled with primary mAb. No differences were found in baseline autofluoresence as determined by consistent MFI of secondary controls between cows (data not shown). Labeled cells were washed in FACS buffer and then stained with LIVE/DEAD® Fixable Red (Life Technologies, Grand Island, NY) according to manufacturer instructions to exclude dead cells during analysis. Labeled cells were then fixed with 4% paraformaldehyde for 15 minutes at 4°C in the dark. Following fixation, cells were washed twice in FACS buffer and measured with a FACSCalibur (Becton Dickinson). A minimum of 10,000 events gated by morphology of viable cells were recorded for each sample. Flow cytometry data were analyzed using FlowJo (TreeStar Inc., Ashland, OR).

2.6. Measurement of IL-10 and IL-12 by ELISA

Supernatant levels of bovine IL-10 and IL-12 were measured using ELISA. Thermo Cliniplate EB flat-bottom 96-well plates (ThermoFisher, Vantaa, Finland) were coated with 100µl of capture antibody diluted in 0.05M of carbonate buffer with a pH of 9.6: mouse anti-bovine IL-10 (clone CC320; AbDSerotec, Raleigh, NC) at 1µg/ml for 1.5 hours at room temperature, and IL-12 (clone CC301; AbDSerotec) at 2µg/ml overnight at 4°C. Plates were washed three times with wash buffer (PBS containing 0.05% Tween-20) then blocked with 2% fish skin gelatin (Sea Block buffer, ThermoFisher) in PBS for 1 hour at room temperature. Blocked plates were washed four times with wash buffer and 100µl of supernatant diluted in PBS at 1:5 for IL-10 and 1:20 for IL-12 was added to each well. Known concentrations of recombinant bovine IL-10 (Kingfisher Biotech Inc., St. Paul, MN) and IL-12 (Kingfisher Biotech Inc., St. Paul, MN) were assayed simultaneously with the respective ELISA to generate a standard curve from which sample concentrations were determined. Plates were incubated for 1.5

hours at room temperature. Following sample and standard incubation, plates were washed four times with wash buffer, and 100µl of respective biotinylated detection antibodies: mouse anti-bovine IL-10 (clone CC320; AbD Serotec) at 1µg/ml and IL-12 (clone CC301; AbD Serotec) at 5µg/ml were added to each well. All samples and standard dilutions of known recombinant protein concentration were assayed in triplicate. Samples were corrected for their respective dilution factor to determine the actual concentrations of the cytokine.

2.7. Statistical Analysis

Data were analyzed with a paired, two-tailed, non-parametric Wilcoxon signed rank test and unpaired, two-tailed, Mann Whitney test. Data were analyzed using a SAS statistical analysis program, version 9.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Increased CD14 expression in moDC from LG cows

After 5 days of culturing CD14+ monocytes with recombinant GM-CSF and IL-4, LG and NP cultures had non-adherent and loosely adherent cells with typical DC morphology of cells with dendrite-like projections. Following the 5 day differentiation period, maturation was induced by 24 hour stimulation with UV irradiated *E. coli* ECC-Z. Monocyte-derived DC from NP and LG cows decreased in relative levels of CD14 expression following UV *E. coli* ECC-Z maturation ($p=0.047$ and $p=0.026$, respectively; Fig. 2 A-C). However, *E. coli* ECC-Z-stimulated moDC from LG cows had higher levels of expression of CD14 compared to moDC from NP cows ($p= 0.007$; Fig. 2 C). Though numerically different, difference in levels of CD14 expression between immature moDC of NP and LG cows were not statistically significant ($p=0.064$; Fig. 2 C). Only moDC from NP cows had a significant decrease in the percentage of

CD14+ moDC from a median of 95.1% CD14+ to 75.6% CD14+ following *E. coli* ECC-Z stimulation (p=0.026; Fig. 2 D).

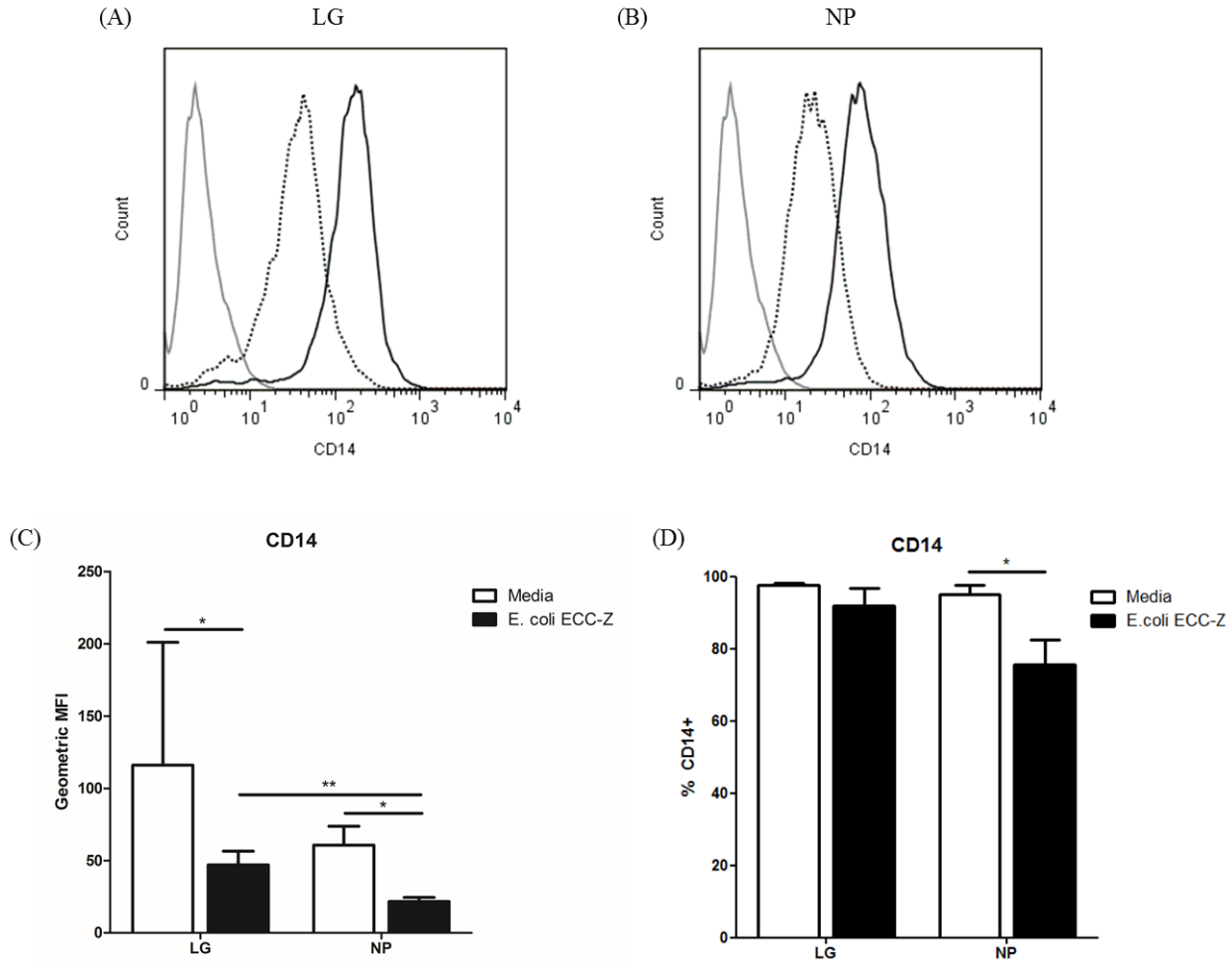

Figure 2. CD14 expression in moDC from late gestation cows (LG) and non-pregnant cows (NP) following 24hr stimulation with UV irradiated *E. coli* ECC-Z. Representative histograms of CD14 expression measured by flow cytometry from LG (A) and NP (B) moDC cultures. Solid black lines represent media control cultures, dotted black lines represent *E. coli* ECC-Z stimulated cultures, and solid grey lines represent secondary antibody staining control. Relative level of CD14 expression as determined by geometric mean fluorescence intensity (MFI) (C), and proportion of moDC expressing surface marker CD14 (D) following *E. coli* ECC-Z stimulation. White bars represent moDC media controls, and black bars represent *E. coli* ECC-Z-stimulated moDC. Bars represent median (n=7) with error bars representing interquartile range. Significant differences determined by Wilcoxon signed rank test when comparing media controls to *E. coli* ECC-Z-stimulated moDC, and Mann Whitney-U test when comparing between LG and NP of same treatment group.*p<0.05, **p<0.01

3.2. Impaired upregulation of MHC II expression in *E. coli*-activated moDC from LG cows

Activation of naïve T cells requires expression of co-stimulatory signals and antigen presentation in DC. Stimulation with UV irradiated *E. coli* ECC-Z induced a significant up-regulation in the level of expression and percentage of cells expressing co-stimulatory molecule CD80 in moDC from both LG and NP cows (p=0.016, p=0.016, p=0.031, and p=0.016, respectively Fig. 3 A-D). No differences were found in CD80 expression levels or percentage of cells expressing CD80 between *E. coli*-stimulated moDC from LG cows and NP cows (p=0.908 and p=0.749, respectively, Fig. 3 C&D).

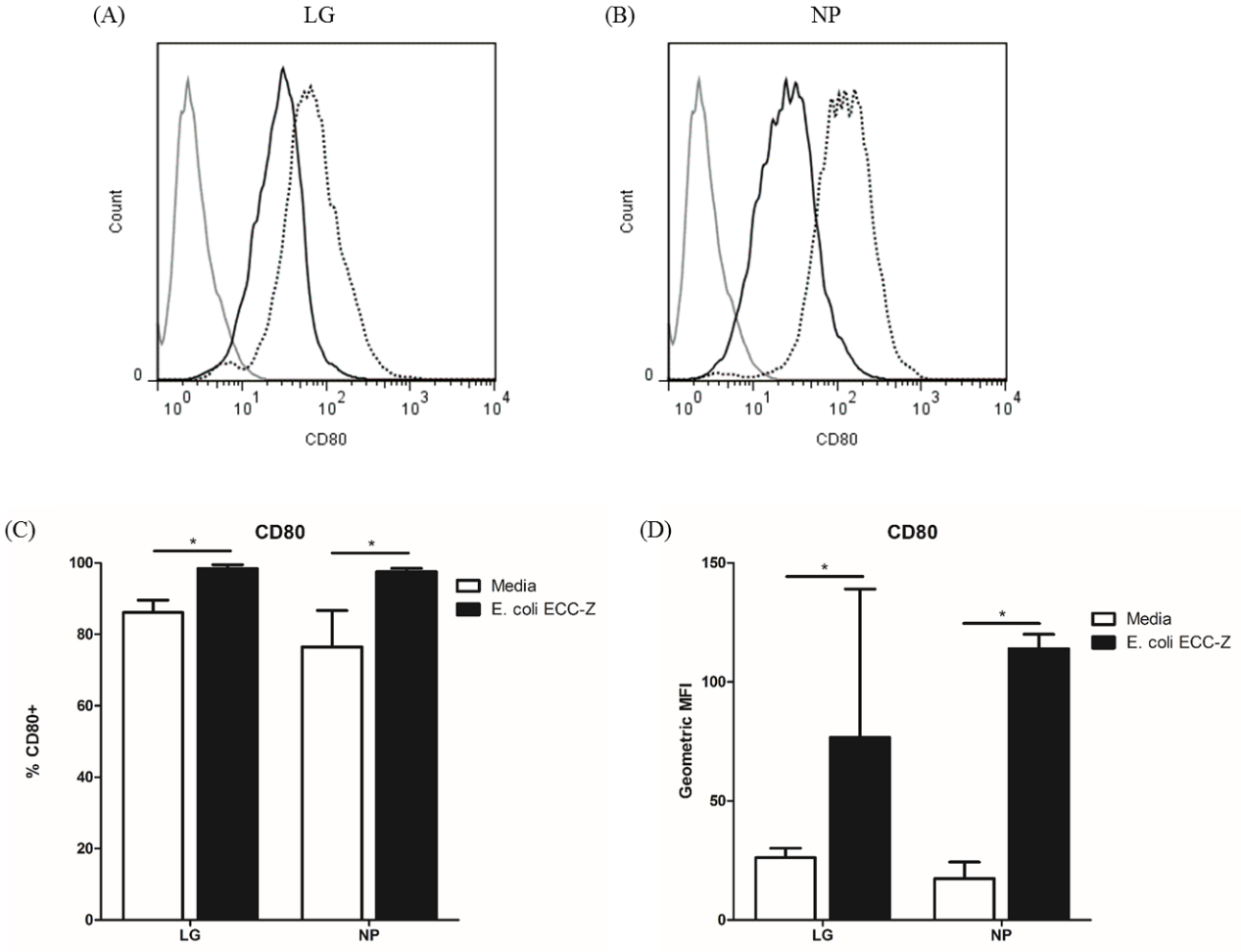

Figure 3. CD80 expression in moDC from late gestation cows (LG) and non-pregnant cows (NP) following 24hr stimulation with UV irradiated *E. coli* ECC-Z. Representative histograms of CD80 expression measured by flow cytometry from LG (a) and NP (b) moDC

cultures. Solid black lines represent media control cultures, dotted black lines represent *E. coli* ECC-Z stimulated cultures, and solid grey lines represent secondary antibody staining control. Proportion of moDC expressing surface marker CD80 (c), and relative level of CD80 expression as determined by geometric mean fluorescence intensity (MFI) (d) following *E. coli* ECC-Z stimulation. White bars represent moDC media controls, and black bars represent *E. coli* ECC-Z-stimulated moDC. Bars represent median (n=7) with error bars representing interquartile range. Significant differences determined by Wilcoxon signed rank test when comparing media controls to *E. coli* ECC-Z-stimulated moDC, and Mann Whitney-U test when comparing between LG and NP of same treatment group. *$p<0.05$, **$p<0.01$

Both, un-stimulated media controls and *E. coli*-stimulated moDC from LG and NP cows did not differ in the percentage of MHC II-expressing moDC, and all cultures had a median expression of ~98% MHC II+ ($p=0.701$ and $p=0.5645$, respectively; Fig. 4 A). Expression levels of MHC II were comparable between moDC un-stimulated, media controls in both LG and NP cows. However, only moDC from NP cows were able to significantly upregulate their level of MHC II expression following *E. coli* stimulation ($p=0.031$; Fig. 4 C), while MHC II expression in stimulated moDC from LG cows remained unchanged ($p=0.578$; Fig. 4 C). Overall NP cows

showed significantly higher MHC II expression with less variation compared to those from LG **cows (p=0.001; Fig 4 C).**

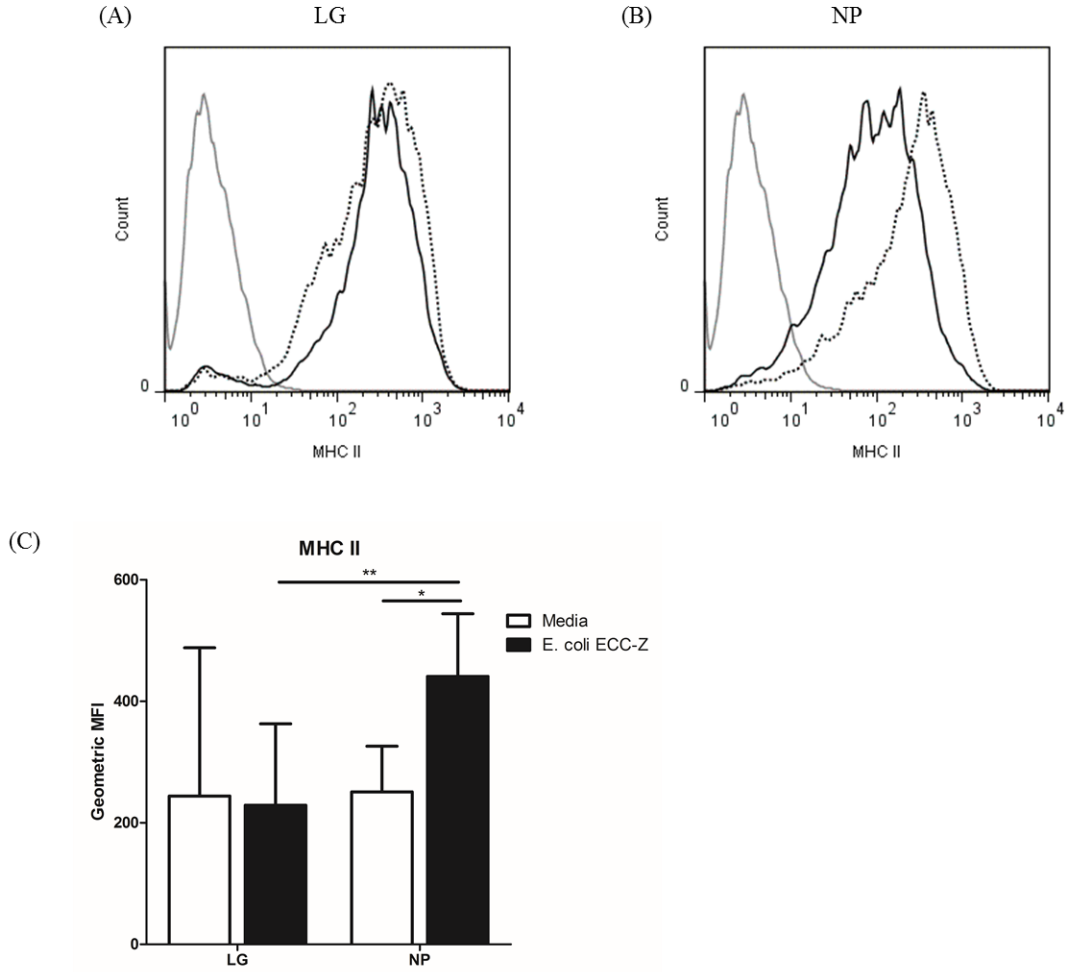

Figure 4. MHC II expression in moDC from late gestation cows (LG) and non-pregnant cows (NP) following 24hr stimulation with UV irradiated *E. coli* ECC-Z. Representative histograms of MHC II expression measured by flow cytometry from LG (A) and NP (B) moDC cultures. Solid black lines represent media control cultures, dotted black lines represent *E. coli* ECC-Z stimulated cultures, and solid grey lines represent secondary antibody staining control. Relative level of MHC II expression as determined by geometric mean fluorescence intensity (MFI) (C) following *E. coli* ECC-Z stimulation. White bars represent moDC media controls, and black bars represent *E. coli* ECC-Z-stimulated moDC. Bars represent median (n=7) with error bars representing interquartile range. Significant differences determined by Wilcoxon signed rank test when comparing media controls to *E. coli* ECC-Z -stimulated moDC, and Mann Whitney-U test when comparing between LG and NP of same treatment group. *$p<0.05$, **$p<0.01$

3.3. Suppressed IL-12 production and elevated IL-10 production by *E. coli*-stimulated moDC from LG cows

Culture supernatants from un-stimulated media controls and *E. coli*-stimulated moDC cultures were harvested after 24 hours to measure levels of the pro-inflammatory Th1 cytokine, IL-12 and the anti-inflammatory cytokine IL-10. Only moDC from LG cows showed a significant increase in IL-10 production following *E. coli*-stimulation (p=0.031; Fig. 5 A). Both, moDC cultures from NP cows and LG cows significantly upregulated level of IL-12 production following *E. coli*-stimulation (p=0.031 and p=0.031, respectively; Fig. 5 B). Upregulation of IL-12 was numerically higher in moDC from NP relative to moDC from LG. Ratios of pro-inflammatory IL-12 to anti-inflammatory IL-10 cytokine production were used to compare cytokine profiles of *E. coli*-stimulated moDC from LG cows to NP cows. Though both groups were able to produce IL-12 in response to *E. coli* stimulation, the ratio of IL-12 to IL-10 produced by *E. coli*-stimulated moDC was significantly lower in cultures from LG cows compared to NP cows (p=0.015; Fig. 5 C).

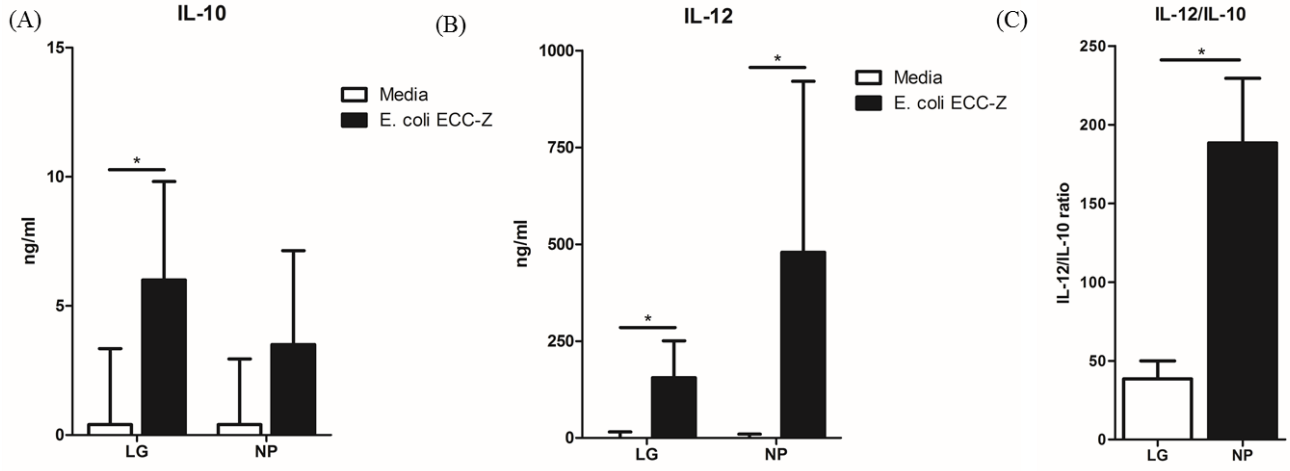

Figure 5. IL-10 and IL-12 production by moDC from late gestation cows (LG) and non-pregnant cows (NP) following 24hr stimulation with UV irradiated *E. coli* ECC-Z. IL-10 concentration (ng/ml) in cell-free supernatant harvested 24hr following UV irradiated *E. coli* ECC-Z stimulation as determined by sandwich ELISA (A). IL-12 concentration (ng/ml) in cell-free supernatant harvested 24hr following UV irradiated *E. coli* ECC-Z stimulation as determined by sandwich ELISA (B). Ratio of IL-12 to IL-10 levels in cell-free supernatant from

E. coli ECC-Z-stimulated moDC cultures from LG and NP cows (C). White bars represent moDC media controls, and black bars represent *E. coli* ECC-Z-stimulated moDC (A, B). Bars represent median (n=6) with error bars representing interquartile range. Significant differences determined by Wilcoxon signed rank test when comparing media controls to *E. coli*-C1-stimulated moDC, and Mann Whitney-U test when comparing between LG and NP. *$p<0.05$, **$p<0.01$

4. Discussion

During gestation the maternal immune system is hypothesized to be directed towards tolerance in order to prevent rejection of the fetus (Dietert and Piepenbrink, 2008). Little is known about the underlying mechanisms involved in generation and maintenance of maternal immune tolerance in dairy cattle. Dendritic cells are known to play a crucial role in the generation of maternal immune tolerance in other mammalian species (Lutz and Schuler, 2002). Our study showed that moDC from LG dairy cows had a biased and impaired ability to fully mature in response to stimulation with UV irradiated *E. coli* both phenotypically and functionally when compared NP cows.

Upon antigen uptake and appropriate microenvironment, DC become activated and subsequently express surface markers that distinguish them from immature DC. Fully differentiated, mature DC are characterized by low levels of CD14 expression (Lei and Hostetter, 2007, Merant et al., 2009). We found *E. coli*-stimulated moDC from LG cows expressed higher levels of CD14 compared to moDC from NP cows (Fig. 2). Only moDC from NP cows showed a decrease in percent of CD14+ moDC. High levels of CD14 expression following *E. coli* stimulation indicate LG cows have impaired moDC maturation relative to NP cows. Zanoni et al. (2009) demonstrated a novel role of CD14-dependent signaling following LPS-stimulation within the DC life cycle as a mechanism of inducing apoptosis of terminally differentiated DC through CD14-mediated CA^{2+} influx and NFAT (nuclear factor of activated T cells) activation. Dendritic cell apoptosis is another mechanism of generating and maintaining immune tolerance

by reducing prolonged T cell priming. However, the importance of CD14-dependent DC regulation during gestation has not yet been described in bovine DC and needs further investigation.

Co-stimulatory molecules including CD80 and MHC-antigen complexes are upregulated during DC activation, and provide necessary signals to activate naïve lymphocytes and generate antigen-specific responses (Cools et al., 2007). Both moDC from LG and NP cows were able to upregulate CD80 in response to *E. coli* stimulation, and CD80 expression between groups were at comparable levels (Fig 3). However, only moDC from NP animals were able to upregulate MHC II following *E. coli*-stimulation (Fig. 4). Della Bella et al. (2011) found a similar semi-mature DC phenotype in the context of human pregnancy in which human moDC conditioned in plasma from healthy pregnant women were able to upregulate co-stimulatory molecule CD80 but were unable to upregulate MHC II. Up-regulation of MHC II-antigen complexes is necessary to generate antigen-specific T cell responses. Impaired MHC II-antigen presentation results in weak T cell receptor signaling and subsequent bias for Th2 differentiation due to reduced activation of ERK (Extracellular Signal-regulated Kinase) (Yamane and Paul, 2013). The inability of moDC from LG cows to upregulate MHC II molecules in response to *E. coli*-stimulation indicates DC in late gestation are unable to undergo complete activation, which may elucidate one mechanism utilized to maintain maternal immune tolerance.

E. coli-stimulated moDC from LG cows had reduced pro-inflammatory cytokine IL-12 production and increased regulatory cytokine IL-10 production, which resulted in a significantly lower IL-12/IL-10 ratio compared to those from NP cows (Fig. 5). The balance of these two cytokines greatly influences the production of IFN-γ and polarization of the immune response; Collins et al. (1999) found IL-12-induced IFN-γ production in bovine PBMC was greatly

impaired in presence of IL-10. Interleukin 12 induces IFN-γ production in NK cells, T cells, and macrophages, and provides the necessary signal to direct Th1 differentiation following T cell activation (Schroder et al., 2004, O'Garra and Murphy, 2009). Interleukin 10 is a potent regulatory cytokine which inhibits Th1-type and other pro-inflammatory responses. High levels of Th1 cytokines often in conjunction with danger signals such as LPS are associated with pregnancy loss in mammalian species (Nahum et al., 2004, Hadfield et al., 2011). The suppression of Th1-type cytokine production appears to be specific to late gestation in dairy cows; Rosbottom et al. (2008) found cattle infected with *Neospora caninum* in the early pregnancy had a strong increase in Th1 cytokine mRNA expression and subsequent fetal loss, but cattle infected during late gestation had minimal increase in Th1 cytokine mRNA expression and experienced no fetal loss. Negishi et al. (2012) demonstrated substantial pregnancy loss either by the depletion of tolerogenic DC populations or addition of exogenous IL-12 in pregnant mice. This demonstrates the crucial role of DC and DC cytokines in maintaining tolerance through Th2/Th1 balance. Our study indicates that bovine DC in late gestation have reduced Th1-promoting cytokine production relative to regulatory cytokine production coinciding with previous work (Bachy et al., 2008, Della Bella et al., 2011, Negishi et al., 2012). The low IL-12/IL-10 ratio produced by *E. coli*-stimulated moDC from LG cows would contribute to the hampered pro-inflammatory responses to *E. coli* infections characteristic of the dry period by self-impaired Th1 cytokine production and inability to stimulate Th1-type responses in other leukocyte populations. Though dampened Th1 cytokine production is favorable to fetal survival, this could enable pathogens to persist in the dry period due to ineffective immune responses. Similar to bovine dendritic cells in mammary gland and afferent lymph, and conventional DC in the blood, bovine moDC express high levels of MHC II, low levels of CD14, and are CD11c+

(Maxymiv et al., 2012). However, Maxymiv et al. (2012) observed different subpopulations of DC in tissue based on CD11a expression, but bovine moDC generated *in vitro* using recombinant GM-CSF and IL-4 have been described as singularly $CD11a^{lo}$ (Werling et al., 1999, Denis and Buddle, 2008). Thus, moDC represent only one of multiple DC subsets found in the tissue, and we must take precaution when making inferences about DC populations found *in vivo*.

Maternal immune tolerance and tolerogenic DC may be generated and maintained by interfering with DC maturation. Dendritic cell function has been shown to be influenced by endocrine factors including estradiol, progesterone, human chorionic gonadotropin, and glycoprotein hormone activin-A to generate a tolerogenic DC phenotype comparable to that observed during pregnancy (Uemura et al., 2008, Della Bella et al., 2011, Schumacher et al., 2014). Our findings indicate moDC generated from LG dairy cows have impaired ability to mature and a biased pro-inflammatory cytokine production in response to *E. coli* stimulation. Although impaired DC maturation is potentially beneficial for maintenance of pregnancy and maternal immune tolerance in dairy cattle, this may impede optimal immune responses to invading pathogens. This is the first study providing evidence for the potential role of DC in the maintenance of maternal immune regulation in dairy cattle through the impairment and bias in DC activation. Precise mechanisms involved in the regulation of DC activation in bovine late gestation still require further investigation.

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CHAPTER 3:
LONGITUDINAL CHARACTERIZATION OF BOVINE MONOCYTE-DERIVED DENDRITIC CELLS FROM MID-GESTATION INTO SUBSEQUENT LACTATION REVEALS NADIR IN PHENOTYPIC MATURATION AND MACROPHAGE-LIKE CYTOKINE PROFILE IN LATE GESTATION

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KEYWORDS: Bovine; pregnancy; monocyte-derived dendritic cells; monocytes

ABBREVIATIONS**:** cM (classical monocyte), DC (dendritic cell), DIM (days in milk), ED (early dry period), EL (early lactation), intM (intermediate monocytes), LD (late dry period), Mφ (macrophage), MG (mid-gestation), moDC (monocyte-derived dendritic cells), MOI (multiplicity of infection), ncM (nonclassical monocyte), PBMC (peripheral blood mononuclear cells), PC (post-calving), T helper 1 (Th1), T helper 17 (Th17)

ABSTRACT

Changes in monocyte and dendritic cell populations during bovine pregnancy and lactation remain poorly described despite the key roles these cells play in immune tolerance and activation. Using a prospective longitudinal study, we characterized CD14+ monocyte-derived dendritic cell (moDC) differentiation and maturation and captured monocyte composition dynamics from mid-gestation through calving and into the subsequent lactation in dairy cows (n=7). First, we measured absolute counts of classical (CD14+CD16-, cM), intermediate (CD14+CD16+, intM), and nonclassical (CD14-CD16+, ncM) monocytes in the blood and determined proportions of individual subsets within the total monocyte population. We found the proportion of cM decreased and intM increased significantly by early lactation, whereas there was a nadir in the proportion of ncM in late gestation, two weeks prepartum. Monocyte composition appears to be regulated in pregnancy, possibly to limit the proportion of highly inflammatory monocytes i.e. intM. Ultimately, we found that moDC differentiated from CD14+ monocytes isolated in the early dry period of late gestation had impaired *E. coli*-induced maturation, with nadirs in upregulation of CD80 and MHC II, and downregulation of CD14. The moDC from late gestation also had altered cytokine profiles with greatest production of pro-inflammatory IL-1β and anti-inflammatory IL-10. These data suggest monocytes in late gestation, in contrast to other stages of pregnancy and lactation, differentiate and maturate into moDC less capable of eliciting strong T cell activation, and have macrophage-like cytokine profiles. These results provide insight into maternal immune modulation and elucidate potential immune changes necessary to facilitate bovine pregnancy.

1. Introduction

During pregnancy the maternal immune system is presented with the challenge of needing to concurrently tolerate the growth of a semi-allogenic fetus and respond to invading pathogens. Highly inflammatory, T helper 1 (Th1) and T helper 17 (Th17)-type responses in pregnancy have been shown to be detrimental to the health of the fetus, thus, the maternal immune system must be carefully regulated (Rosbottom et al., 2008, Betz, 2012, Krishnan et al., 2013). Maternal immune regulation is dynamic; dairy cattle show varying responses to infectious agents depending upon their stage of pregnancy and lactation (Innes, 2007, Rosbottom et al., 2008, Quesnell et al., 2012, Sipka et al., 2013). Previous work found cows in the third trimester of pregnancy, particularly during the non-lactating period of late gestation (known as the 'dry period'), had a heightened susceptibility to persistent infections caused by mastitis- and abortion-causing pathogens including *E. coli* and *N. caninum* with hindered pro-inflammatory, Th1-type responses relative to the postpartum period and earlier stages of gestation (Williams et al., 2000, Green et al., 2002, Anderson, 2007, Rosbottom et al., 2008, Quesnell et al., 2012). Monocytes and monocyte derived cells play a major role in maternal immune regulation throughout pregnancy across multiple species; these cells are involved at all stages of pregnancy including implantation, maintenance of pregnancy, and parturition and show dynamic regulation by stage of gestation (Oliveira et al., 2012, Gomez-Lopez et al., 2014, Leno-Duran et al., 2014, Schumacher et al., 2014). Though previous work on dairy cattle indicated that certain immune cell populations have altered function in pregnancy, this area of research remains largely underdeveloped (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013, Pomeroy et al., 2015). We lack an understanding of underlying mechanisms in immune tolerance, gestational

stage-dependent changes in maternal immune function, and how this relates to overall health of the dam and fetus.

Bovine monocytes have only recently been classified into three major subsets, classical (cM), intermediate (intM), and nonclassical (ncM), based on CD14 and CD16 expression analogous to their human counterparts (Hussen et al., 2013, Hussen et al., 2014, Corripio-Miyar et al., 2015, Hussen et al., 2016). Unlike CD14- ncM, both CD14+ subsets, cM and intM, have been shown to be highly responsive to LPS stimulation (Hussen et al., 2013, Hussen et al., 2014). Changes in monocyte subset composition have been shown to influence disease susceptibility and pregnancy outcome in species such as mouse and human, but these changes have not been characterized in cattle (Al-ofi et al., 2012, Melgert et al., 2012, Devevre et al., 2015, Tang et al., 2015b). These monocyte populations circulating in the periphery migrate into tissues either during inflammation or steady-state conditions where they may differentiate into macrophages (Mφ) or dendritic cells (DC).

Across multiple species, DC are shown to be regulated in pregnancy and play a crucial role in generating maternal immune tolerance toward fetal antigens (Bachy et al., 2008, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Pomeroy et al., 2015). Recently we found key differences in monocyte derived DC (moDC) phenotype and cytokine production in a cross-sectional study comparing moDC from late gestation cows to moDC from non-pregnant cows in early lactation. We found moDC had limited Th1-type responses and were likely to have a poor ability to activate T cells during late gestation (Pomeroy et al., 2015).

Here we aim to expand upon our previous work and capture the dynamics of monocyte subsets, and the function and phenotype of respective moDC populations through a prospective longitudinal study in cows from mid-gestation through calving into the subsequent lactation. The

objective of this study was to analyze differentiation and maturation with UV-killed *E. coli*-stimulation of moDC, derived from blood CD14+ across pregnancy and into early lactation. We investigated phenotype and function analyzing surface marker expression (CD14, CD40, CD80, MHC II) and cytokine production (IL-1β, TNFα, IL-10) following *E. coli* stimulation. Furthermore we measured monocyte subset composition (cM, intM, ncM) in blood at the designated time points to describe the composition of monocyte subsets in blood as a baseline and further understand the implications of starting monocyte population on moDC differentiation and maturation.

2. Materials and methods

2.1. Animals

Seven Holstein–Friesian cows, in the second trimester of pregnancy between 90-110 days carrying calf ('mid-gestation', MG, 90-110dcc/P-190-170d) as determined from insemination date were selected from the Cornell University Veterinary College Teaching Dairy Barn. No disease *(i.e.* metabolic, reproductive, mastitis) was recorded within 30 days prior to the time of first sampling for all enrolled animals. Peripheral blood was collected by jugular venipuncture from enrolled animals into 250ml vacuum bottles containing EDTA and 10 ml vacutainer glass serum tubes with no additive (Becton Dickinson, Franklin Lakes, NJ). Each enrolled animal was sampled repeatedly throughout the remainder of pregnancy and into the subsequent lactation as depicted in figure 6. Blood was collected at MG, early dry period within the first week since dry-off ~60-53 days prior to expected calving date ('early dry', ED, 1-7d dry/P-60-53d), late dry period ~2 weeks prior to expected calving date ('late dry', LD, 43d dry/P-14d), 4-6 days following parturition ('post-calving', PC, P+4-6d), and the final collection ~45-55 days following parturition before breeding ('early lactation', EL, P+45-55d) (Fig. 6). Disease was

monitored daily and recorded in DairyComp® by farm management; metritis cases were diagnosed within the first two weeks postpartum by abnormal vaginal discharge and foul odor of the discharge. All animal procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number: 2007-0110).

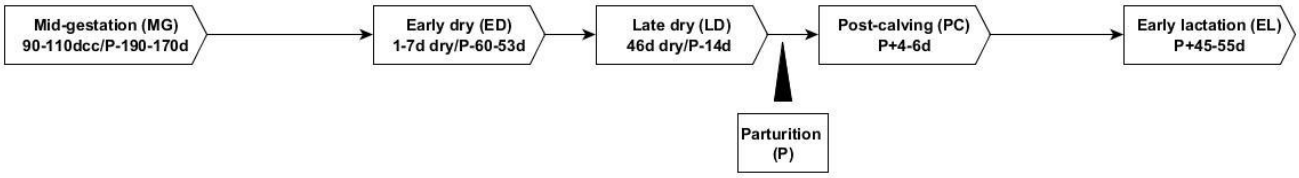

Figure 6. Blood sampling timeframe over pregnancy and lactation. Sample time points starting in mid-gestation (MG) at 90-110d carrying calf (dcc)/approximately 190-170 d prior to parturition (P) estimated by insemination date and final blood samples taken in subsequent lactation in early lactation (EL) at 45-55d after parturition. The dry period begins at approximately 60d prior to the expected calving date. The '-' signifies prior to, and '+' signifies after parturition. Abbreviations: parturition (P), days carrying calf (dcc), days dry (d dry), mid-gestation (MG), early dry (ED), late dry (LD), post-calving (PC), early lactation (EL).

2.2. Generation of peripheral blood CD14+ moDC

Monocyte derived DC were generated from CD14+ monocytes as described previously by Pomeroy et al. (2015); CD14+ positive selection was preformed based on the use of UV irradiated *E. coli* ECC-Z stimulation to induce moDC maturation in conjunction with previous work from Hussen et al. (2013) and (2014) indicating CD14+ cM and intM are the monocyte subsets responsive to LPS stimulation. In brief, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. The PBMC were incubated with anti-human CD14 antibodies conjugated with paramagnetic microbeads (Miltenyi Biotech, Inc., Auburn, CA) in MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), transferred to MACS LS magnetic separation column, and after repeatedly washing the column with MACS buffer the magnetically labeled cells were collected (Miltenyi Biotech, Inc.). Purity was determined by flow cytometry and was shown to be >90%. Monocytes were re-suspended in

differentiation media (phenol-free complete RPMI with l-glutamine, supplemented with 10% autologous serum, 20 ng/ml recombinant bovine IL-4 and, 20 ng/ml recombinant bovine GM-CSF both from Kingfisher Biotech), plated in 6-well tissue culture plates at 1.5×10^6 cells in 3 ml differentiation media per well, and cultured for 5 days (37 °C, 5% CO_2) in presence of recombinant cytokines to generate immature moDC; at 2-3 d cultures were given 1ml/well of fresh differentiation media.

2.3. Inactivation of *E. coli* ECC-Z

E. coli ECC-Z was inactivated as described by Pomeroy et al. (2015). In brief, frozen stock of the previously characterized *E. coli* ECC-Z strain known to cause mild persistent clinical mastitis (Dogan et al., 2006, Lippolis et al., 2014) was grown to log phase in LB liquid. Bacteria were pelleted and diluted in PBS. Bacterial suspension was plated on agar plates prior to inactivation to determine concentration of stock solution. The bacterial suspension was inactivated using UV irradiation in the laboratory of Dr. Randy W. Worobo (Department of Food Science and Technology, Cornell University). The UV irradiated *E. coli* suspension failed to produce colonies on LB agar plates. Aliquots of UV irradiated *E. coli* were resuspended in sterile 10% glycerol/PBS and stored at −80 °C.

2.4. Maturation of moDC with UV irradiated *E. coli*

Maturation of moDC was induced by bacterial stimulation through the addition of UV irradiated *E. coli* to immature moDC cultures in an MOI (multiplicity of infection) of 10 for 24 h based on previous work (Cavatorta et al., 2009, Pomeroy et al., 2015). Cultures of moDC without bacterial stimulation served as media controls. Cells were harvested after 24 h *E. coli* stimulation for phenotypic analysis by flow cytometry. Cell culture supernatants were collected at this time and stored at −80 °C until further cytokine analysis by ELISA.

2.5. Flow cytometry

2.5.1. Monocyte subset staining

Composition of monocyte subsets in PBMC was determined using a similar staining protocol as described by Hussen et al. (2013). To identify monocyte subsets isolated PBMC were analyzed using directly labeled mouse monoclonal antibodies against human CD16:FITC (MCA5665F, AbD Serotec, Raleigh, NC), mouse anti-human CD14:RPE (MCA1568PE, AbD Serotec), and CD172a (Kingfisher Biotech) conjugated with Alexa Fluor® 647 dye using monoclonal antibody labeling kit (Life Technologies). Discrimination between live and dead cells was done by adding propidium iodide (PI, 1 µg/ml, Sigma Aldrich). A minimum of 75,000 events gated on viable cells were recorded for each sample. Due to issues with reagent availability, PBMC from MG were isolated and stored in freezing media (8mM HEPES, 9% DMSO, 45% HI FBS, antibiotic-antimitotic in phenol-free RPMI, +L-glut), slowly frozen at -80 ^{o}C, and transferred to liquid nitrogen until monocyte subset staining could be conducted. No differences were found in monocyte populations between frozen and fresh samples (data not shown). Absolute counts of monocyte subsets were estimated based on PBMC counts isolated per volume of blood.

2.5.2. Monocyte-derived dendritic cell staining

Expression of surface markers on moDC was measured with flow cytometry as described previously by Pomeroy et al. (2015). In brief, moDC were blocked with 10% normal goat serum (Life Technologies), subsequently incubated with primary unlabeled monoclonal mouse antibodies against bovine MHC II DR orthologue (TH4B), CD14 (MM61A), CD40 (ILA158A), and CD80 (ILA159) (Kingfisher Biotech), and then indirectly labeled with secondary Alexa Fluor® 488 conjugated monoclonal goat antibodies against mouse IgG_1 or $IgG_{2\alpha}$ (Life

Technologies). Secondary controls were of moDC stained only with Alexa Fluor® 488 conjugated secondary antibodies. No differences were found in baseline autofluoresence as determined by consistent MFI of secondary controls across samples (data not shown). Labeled cells were stained with LIVE/DEAD® Fixable Red (Life Technologies) to exclude dead cells during analyses, and then fixed with 4% paraformaldehyde. A minimum of 10,000 events gated on viable cells were recorded for each sample. All measurements were taken with a FACSCalibur (Becton Dickinson). All flow cytometry data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

2.6. Measurement of IL-10, TNFα, and IL-1β by ELISA

Supernatant levels of bovine IL-10 were measured using ELISA as described in Pomeroy et al. (2015). In brief, Thermo Cliniplate EB flat-bottom 96-well plates (ThermoFisher, Vantaa, Finland) were coated with capture monoclonal mouse antibody against bovine IL-10 (1 μg/ml; CC320; AbDSerotec) in 0.05 M carbonate buffer (pH 9.6). Plates were washed then blocked with 2% fish skin gelatin (Sea Block buffer, ThermoFisher) in PBS. Following blocking supernatant samples were added. Known concentrations of recombinant bovine IL-10 (Kingfisher Biotech) were assayed simultaneously to generate a standard curve from which sample concentrations were determined. Following sample and standard incubation, plates were washed, and biotinylated detection monoclonal mouse antibody against bovine IL-10 (1 μg/ml, CC320, AbD Serotec) was added. All samples and standards were assayed in duplicate.

Supernatant levels of TNFα and IL-1β were measured according to using commercially available kits according to the manufacturer (bovine TNFα DuoSet® ELISA, DY2279, R&D Systems, Minneapolis, MN; bovine IL-1β ELISA, Thermo Scientific, Rockford IL).

2.7. Statistical analysis

All data were analyzed using the SAS v 9.4 statistical analysis program (SAS Institute Inc., Cary, NC). Initially, data were graphed and evaluated for outliers. Outliers were inspected and reanalyzed where necessary. Significant changes in monocyte subset composition in blood, moDC surface marker expression, and moDC cytokine production over time were determined using polynomial regression (SAS, PROC MIXED); linear models adjusted for repeated measures within cow and accounted for between subject factors. Regression coefficients were evaluated for statistical significance. If data did not fit a linear polynomial model, data were analyzed using multiple comparison tests with repeated measures ANOVA; a Tukey for post hoc tests was used. For all analyses a type I error of $\leq$0.05 was used.

3. Results

All cows completed all samplings and carried their pregnancies to full term without any recorded or observed clinical disease or other complications during pregnancy. After calving, 2 cows developed metritis.

3.1. Monocyte population dynamics show increasing intM and ncM, and decreasing cM composition postpartum relative to late gestation

Greatest proportion (mean=31.7%, SD=13.1%) and absolute count (mean=$7.591x10^5$ cells/ml, SD=$2.671x10^5$ cells/ml) of total monocyte population occurred PC (Fig. 7 A, Table 1). Monocyte subset composition is shown in figure 7 B. The proportion of cM had a significant time-dependent quadratic relationship in which lowest values occurred in EL, with a plateau occurring in late gestation; cM proportions over pregnancy (MG, ED, LD) and PC were relatively consistent (Fig. 7 C). There was no significant difference detected in absolute counts of cM across pregnancy or subsequent lactation (Table 1). The proportion of intM had a significant time-dependent linear relationship in which lowest values occurred in MG, slightly increasing as

pregnancy progressed into the subsequent lactation and reached highest proportions at EL (Fig. 7 D). There was a significant increase in absolute counts of intM from LD to PC (p=0.05); cows which acquired metritis (n=2) were outliers with low intM counts at PC ($0.44591x10^5$ cells/ml, $0.44639x10^5$ cells/ml) (Table 1). The absolute counts of cM and intM used in moDC culture were estimated based on proportions measured in PBMC; cultures from EL had highest intM counts and lowest cM counts compared to all time points (Fig. 8). The proportion of ncM had a significant time-dependent quadratic relationship in which it decreased from MG until a nadir occurred in LD and then subsequently increased into EL (Fig. 7 E). In conjunction, there was a significant increase in absolute counts of ncM at PC (ED v. PC (p=0.05) LD v. PC (p=0.008), EL v PC (p=0.03); cows which acquired metritis (n=2) were outliers with low ncM counts at PC ($0.29045x10^5$ cells/ml, $0.19966x10^5$ cells/ml) (Table 1).

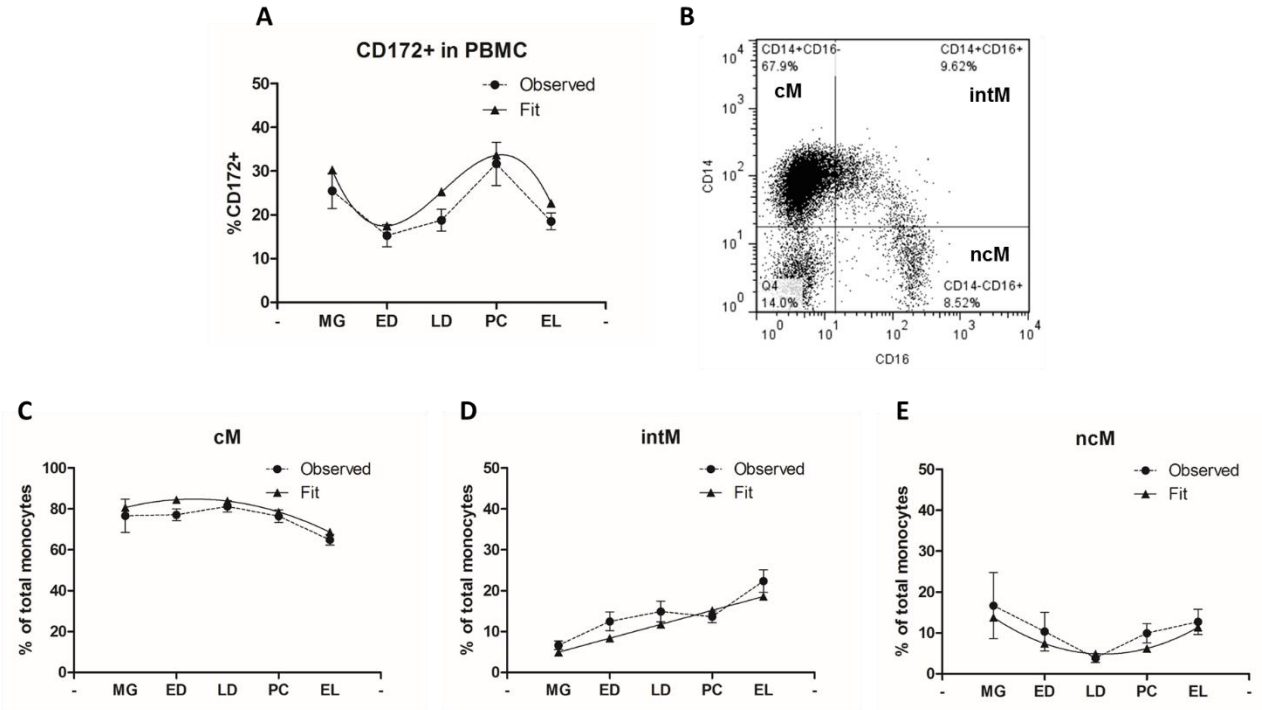

Figure 7. Changes in monocyte subset proportions from peripheral blood over pregnancy and lactation. The mean of the proportion (A) of CD172a+ cells in peripheral blood mononuclear cells (PBMC). Gating strategy on CD172a+ population used to determine relative proportion and absolute counts of individual subsets (B). The mean of the proportion of classical monocytes (cM) (C), intermediate monocytes (intM) (D), and nonclassical monocytes (ncM) (E)

within total monocyte population (cM+intM+ncM). (n=7, missing data at MG (n=5) and ED (n=6)). Error bars represent SEM. Significant changes (minimum and maximums) were determined using polynomial regression; only significant fits as indicated with triangles and solid black lines were are depicted. Terms of the regression models were considered significant if $p<0.05$ when tested against the null hypothesis that the coefficients of the terms were zero. All regression models correct for repeated measures.

Table 1. Changes in absolute counts of monocyte subsets from peripheral blood over pregnancy and lactation. Mean with standard deviation of all enrolled animals (n=7, missing data at MG (n=5) and ED (n=6)). Bold indicates time points that had one or more significant differences from other time points ($p<0.05$).

	Mid-gestation	Early dry	Late dry	Post-calving	Early lactation
Total monocyte population (CD127a+) 10^5 cells/ml	4.97534 (±1.06231)	5.15939 (±1.34377)	4.76751 (±1.33409)	**7.5881 (±2.67147)**	4.99155 (±1.79158)
Classical monocytes (CD172a+CD14+CD16-) 10^5 cells/ml	2.79034 (±0.99817)	3.63731 (±0.90306)	3.646 (±0.68536)	3.6753 (±3.22558)	3.09059 (±1.08718)
Intermediate monocytes (CD172a+CD14+CD16+) 10^5 cells/ml	1.04582 (±1.62374)	0.57665 (±0.26718)	0.74694 (±0.59223)	**2.58210 (±2.24307)**	1.06926 (±0.51890)
Nonclassical monocytes (CD172a+CD14-CD16+) 10^5 cells/ml	0.83274 (±1.14193)	0.61384 (±0.68549)	0.17373 (±0.13838)	**2.8976 (±2.71907)**	0.60789 (±0.58731)

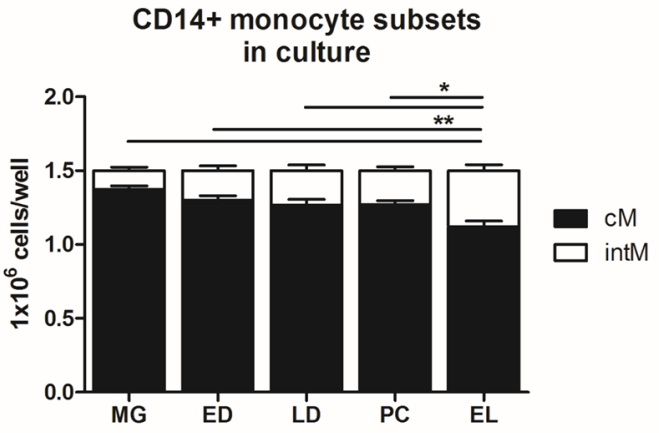

Figure 8. Changes in CD14+ monocyte subset composition in culture isolated from peripheral blood over pregnancy and lactation. Black bars represent the means of cM counts estimated in culture and white bars represent the means of the intM counts estimated in culture.

Error bases represent SEM. Counts of individual subsets were estimated by using proportion of intM and cM in blood and total number of monocytes plated. Significant differences indicated by *p<0.05, **p<0.01

3.2. *E. coli*-stimulated moDC have hindered MHC II and CD80 upregulation, and high CD14 expression in early dry period of late gestation

As shown in figure 9, moDC had greater phenotypic maturation following calving (PC, EL) compared to stages of late gestation (ED, LD). The expression of CD14 as determined by MFI had a significant time-dependent cubic relationship in which maximum expression occurred in ED, and minimum expression at PC, with higher levels of CD14 expression across pregnancy (MG, ED, LD) relative to non-pregnant stages (PC, EL) (Fig. 9 A&B). The MFI of CD14 expression in *E. coli*-stimulated moDC shows least variation during PC and EL (Fig. 9 A&B). All moDC upregulated co-stimulatory molecules CD40 and CD80 following *E. coli* stimulation, however, percent CD80+ *E. coli*-stimulated moDC had a significant time-dependent cubic relationship over the sampling period which indicated a nadir in CD80+ cells at ED and a plateau with maximum CD80+ cells at PC into EL (Fig. 9 A&C, Table 1S). There were no significant changes in CD40 expression; this marker was constituently expressed throughout pregnancy into the subsequent lactation at comparable levels (Fig. 9 A, data not shown). We found upregulation of MHC II expression as determined by delta MFI (MFI *E. coli*-stimulated moDC– MFI

unstimulated moDC) had a significant time-dependent quadratic relationship indicating a nadir at ED, maximum in EL (Fig. 9 A&D).

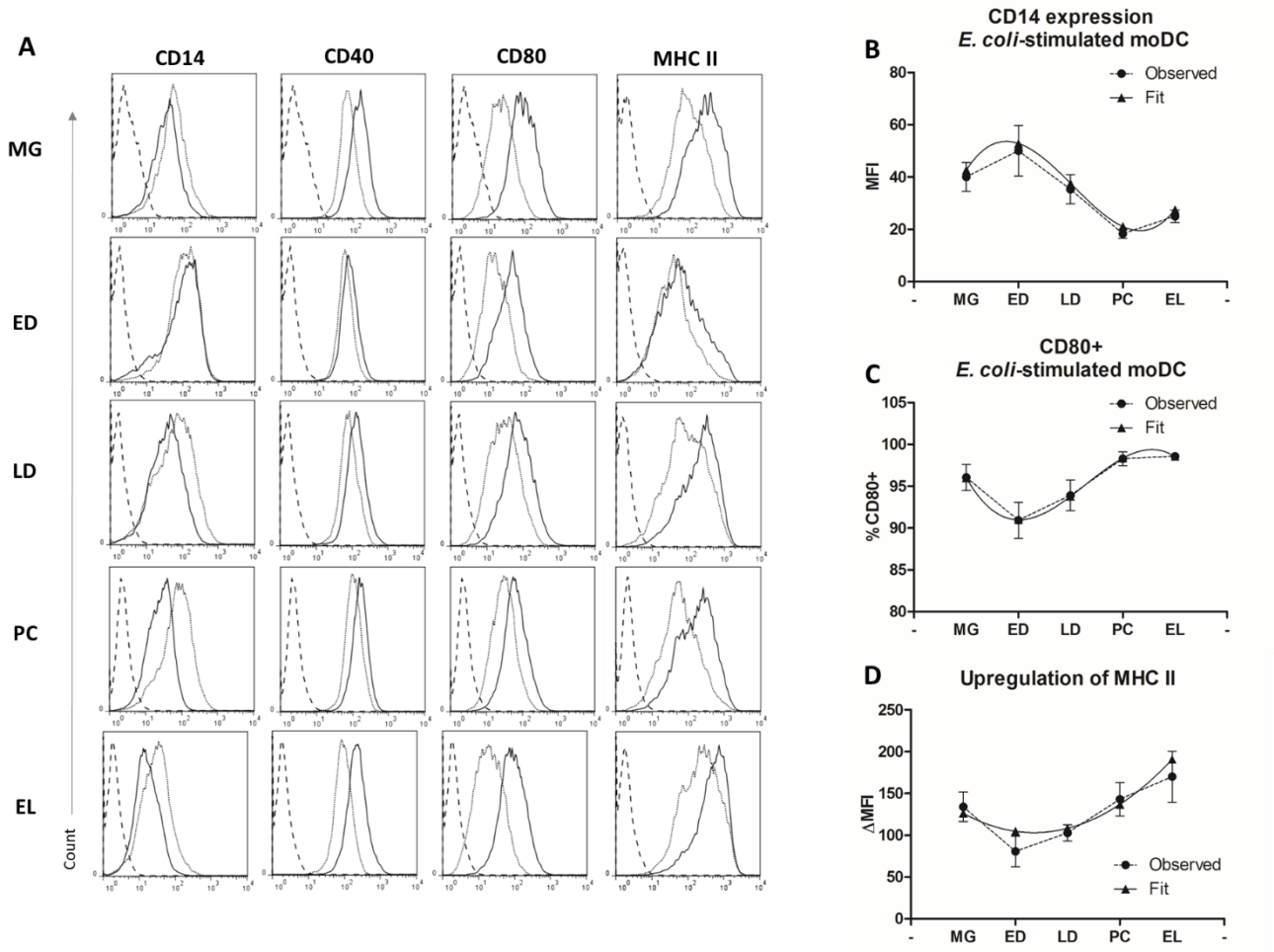

Figure 9. Phenotypic maturation induced by 24hr stimulation with UV irradiated *E. coli* ECC-Z stimulation in moDC generated from CD14+ monocytes over pregnancy into lactation. Representative histograms of moDC expression of CD14, CD40, CD80, and MHC II from one cow from MG to EL; dashed line represents secondary, negative staining control, dotted line represents stained, unstimulated moDC, and solid line represents stained, *E. coli*-stimulated moDC (A) . Dynamics of relative expression as determined by geometric mean fluorescence intensity (MFI) of CD14 (B) in *E. coli*-stimulated, moDC. Dynamics of *E. coli*-stimulated moDC population positive for CD80 (C). Delta MFI of MHC II (D) in moDC to investigate MHC II upregulation in response to *E. coli* stimulation Circles represent the mean (n=7) at each time point (B-D). Error bars represent SEM. Significant changes (minimum and maximums) were determined using polynomial regression; only significant fits as indicated with triangles and solid black lines were are depicted. Terms of the regression models were considered significant if $p<0.05$ when tested against the null hypothesis that the coefficients of these terms were zero. All regression models correct for repeated measures.

3.3. moDC have greatest production of IL-1β and IL-10 in late gestation

Concentrations of IL-1β following *E. coli* stimulation had a significant time-dependent cubic relationship over the sampling period which indicated a transient peak in production at ED and higher levels at LD relative to MG, PC, and EL (Fig. 10 A&D). We found moDC from all stages were capable of upregulating TNFα production in response to *E. coli*,-induced maturation; there was no significant time-dependent relationship (Fig. 10 B&E). No significant production of IL-1β or TNFα was detected in the supernatant from unstimulated moDC. Concentrations of IL-10 following *E. coli* stimulation had a significant time-dependent quadratic relationship which indicated maximum concentrations at LD (Fig. 10 C&F). Interleukin 10 was detected a varying levels in supernatant from unstimulated moDC across the sampling period so delta IL-10 (*E. coli*-stimulated moDC–unstimulated moDC) was used to compare IL-10 upregulation following *E. coli* stimulation (Fig. 10 C&G). Upregulation of IL-10 had a significant time-dependent cubic relationship which indicated maximum upregulation at ED and a nadir at PC (Fig. 10 C&G).

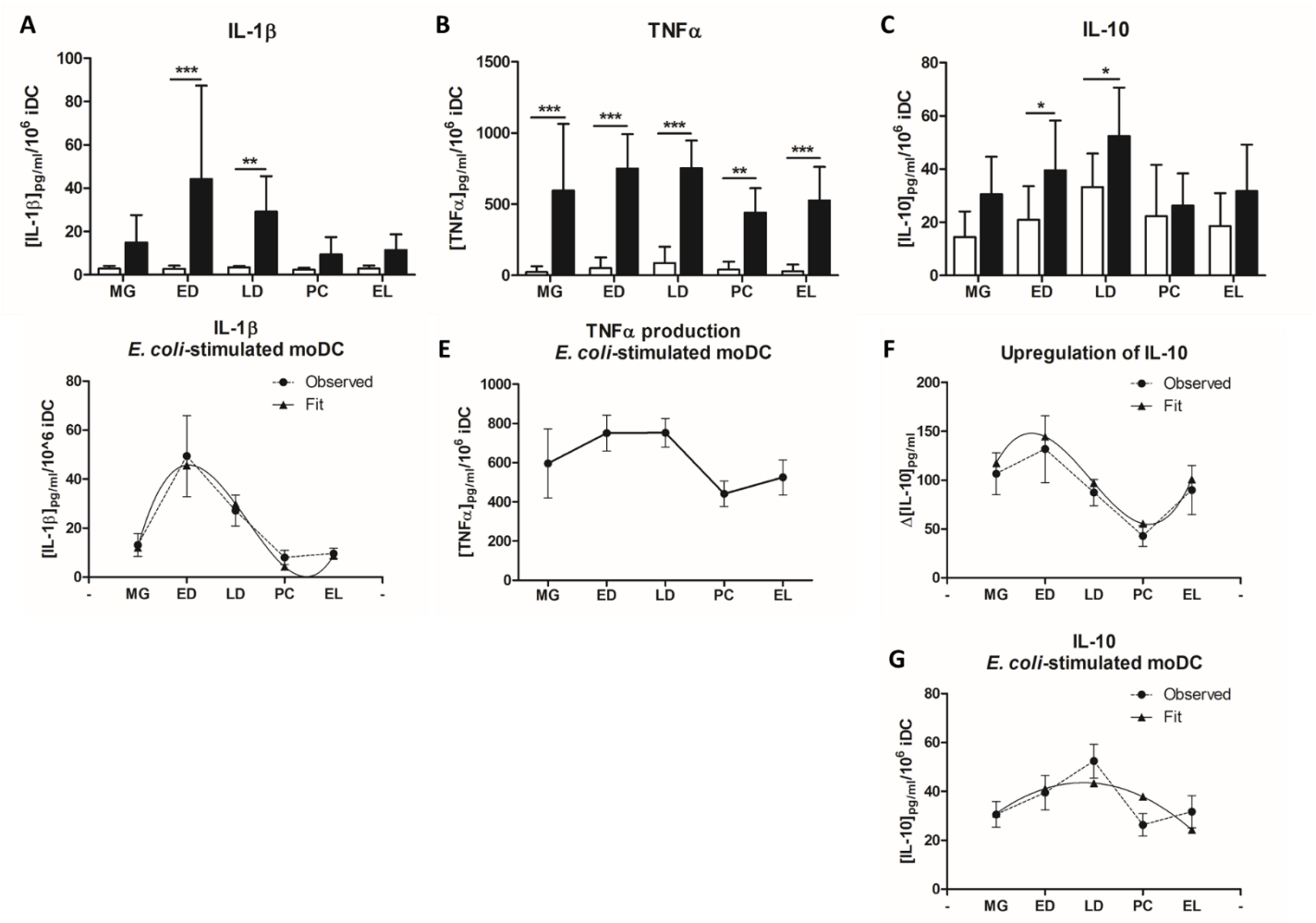

Figure 10. Cytokine production following 24hr stimulation with UV irradiated *E. coli* ECC-Z in moDC generated from CD14+ monocytes over pregnancy into lactation. IL-1β (A,D), TNFα (B,E), and IL-10 (C,F,G) concentration (pg/ml/10^6 unstimulated, iDC) in cell-free supernatant harvested 24hr following UV irradiated *E. coli* ECC-Z stimulation as determined by sandwich ELISA. Upregulation of cytokines determined by comparing unstimulated, iDC to *E. coli* ECC-Z, mDC (A, B, C). White bars represent unstimulated, immature moDC (media control), and black bars represent *E. coli* ECC-Z-stimulated moDC (A, B, C). Bars represent mean (n=7) with error bars representing SD. Significant differences were determined by repeated measures ANOVA and Tukey post-test and are indicated by *p<0.05, **p<0.01, ***p<0.001. Significant changes (minimum and maximums) were determined using polynomial regression for (D-G); only significant fits as indicated with triangles and solid black lines were are depicted. Terms of the regression models were considered significant if p<0.05 when tested against the null hypothesis that the coefficients of the terms were zero. All regression models correct for repeated measures.

4. Discussion

In non-bovine species, monocytes and DC are known to be key drivers of maternal immune tolerance and are differentially regulated to accommodate unique needs at each stage of pregnancy; similar dynamic changes have not been characterized in cattle (Blois et al., 2007, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Schumacher et al., 2014). In this longitudinal study, we identified unique profiles in monocyte composition and moDC maturation at specific stages of gestation and subsequent lactation that suggest greatest alterations to these cell populations occurs over the dry period in late gestation.

Peripheral monocyte composition experienced dynamic changes dependent on the stage of pregnancy and lactation (Fig. 7&8). Within the total monocyte population we observed the greatest proportion of intM and lowest proportion of cM at early lactation 45-55 d following calving where all previous stages had relatively little change (Fig. 7 C&D, Fig. 8). Cows also experienced a nadir in the proportion of ncM 2 wk prior to calving, suggesting monocyte composition may relate to changes associated with preparation for labor in addition to pregnancy maintenance. Both intM and ncM have been associated with a pro-inflammatory response during various inflammatory conditions in humans, including preeclampsia in which affected pregnant women have significantly higher levels of both subsets indicating monocyte populations must be regulated to maintain a healthy pregnancy (Al-ofi et al., 2012, Melgert et al., 2012, Devevre et al., 2015, Tang et al., 2015b). Bovine intM and cM share functional homology to their human counterparts; intM are highly pro-inflammatory and more mature than cM (Hussen et al., 2013, Hussen et al., 2014, Corripio-Miyar et al., 2015, Hussen et al., 2016). Minimizing inflammatory and allogenic responses throughout pregnancy by limiting the available reservoir of intM may be

one possible mechanism in maternal immune tolerance, but without functional data we cannot confirm this.

Like monocyte composition, moDC maturation had dynamic, stage dependent changes over pregnancy and lactation. In line with our previous work, we observed the lowest levels of CD14 expression and greatest upregulation of MHC II expression following *E. coli* stimulation in moDC from non-pregnant stages of lactation compared to the early dry period which had the highest and lowest, respectively (Fig. 3, Table 1S) (Pomeroy et al. 2015). Additionally, there was a significant nadir in the percentage of CD80+ moDC following maturation in the early dry period, with highest levels plateauing following calving carrying into early lactation (Fig. 8 C, Table 1S). High CD14 expression indicates moDC from the early dry period are phenotypically less mature in contrast to those from the post-calving period and early lactation. The nadir in co-stimulatory molecule CD80 and antigen-presentation molecule MHC II upregulation in the early dry period further indicates these moDC had hindered maturation which likely coincides with weaker T cell activation; the maximum upregulation of both these markers was observed in early lactation. Like in other species, bovine moDC differentiation and maturation may be influenced by the initial monocyte composition (Sanchez-Torres et al. 2001; Randolph et al. 2002). The increase in high MHC II expressing intM and decrease in cM at early lactation may relate to the peak in moDC MHC II upregulation observed at this time point (Fig. 8, Fig. 9 D) (Hussen et al 2013). However, regression models of the dynamic, stage-dependent changes in markers of moDC maturation were not identical in shape to those of the starting monocyte composition suggesting other pregnancy factors also play a role in moDC maturation.

In addition to phenotypic changes, moDC cytokine production following maturation was also dependent on stage of pregnancy and lactation. Dendritic cells may secrete and upregulate

of mRNA expression of IL-10 and IL-1β depending upon the stimulant, though typically to a lesser extent when compared to Mφ (Norimatsu et al., 2003, Langelaar et al., 2005, Heller et al., 2012). The greatest upregulation of both pro- and anti-inflammatory cytokines, IL-1β and IL-10, by moDC was observed in the early dry period (Fig. 10). Our findings pertaining to IL-10 production go in line with our previous cross sectional study in which moDC from late gestation dry cows had greater upregulation of IL-10 following *E. coli*-stimulation compared to those from early lactation cows (Pomeroy et al., 2015). As previously mentioned, moDC can be regulated by steroid hormones (Papenfuss et al., 2011, Xu et al., 2011, Lasarte et al., 2013). Poili et al. (2006) found estradiol enhanced production of IL-1β in both human uterine macrophages and blood monocytes. Dairy cattle increasing circulating levels of estradiol in late gestation which greatly surpass levels observed at estrus; autologous serum used to culture moDC containing these circulating factors in addition to the monocytes prior *in vivo* exposure may be related to the rise in IL-1β production we observed in the dry period (Echternkamp and Hansel, 1973, Smith et al., 1973). In non-bovine species, IL-1β and IL-10 are key cytokines involved in priming the uterus for labor and preventing preterm labor in late gestation, respectively (Sato et al., 2001, Robertson et al., 2006, Boro et al., 2014). In conjunction with our previous work (Pomeroy et al., 2015) showing reduced moDC IL-12 production in late gestation cows, these increased levels of IL-1β and IL-10 with unsuppressed TNFα levels are more indicative of Mφ cytokine profile rather than that of DC (Werling et al., 2004, Denis and Buddle, 2008). This preferential *in vitro* differentiation of monocytes into Mφ-like cells in late gestation despite the addition of exogenous GM-CSF and IL-4 to promote DC differentiation may relate to previous work describing the accumulation of Mφ in the mammary gland during involution within the early dry

period and in the endometrium approaching calving (Sordillo and Streicher, 2002, Oliveira and Hansen, 2008, Oliveira and Hansen, 2009).

5. Conclusion

This study demonstrates there are distinct, dynamic changes in monocytes and respective monocyte derived cells by stage of gestation and lactation in dairy cattle. The data indicate that cows experience a reduction in intM and ncM during pregnancy, and a nadir in phenotypic moDC maturation concurrent with a Mφ-like cytokine profile in the early dry period of late gestation. The reduction in monocytes capable of generating stronger inflammatory responses in conjunction with hindered DC maturation likely promotes and maintains a viable, full term pregnancy by preventing the development of conceptus-derived antigen-specific responses. Future research should address specific roles and function of monocyte subsets and respective derived cells in pregnancy maintenance and preparation for labor as it relates to the dynamic changes we observed.

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CHAPTER 4:
IMPACT OF IN VITRO TREATMENTS OF PHYSIOLOGICAL LEVELS OF ESTRADIOL AND PROGESTERONE OBSERVED IN PREGNANCY ON BOVINE MONOCYTE-DERIVED DENDRITIC CELL DIFFERENTIATION AND MATURATION

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KEYWORDS: Bovine; monocyte-derived dendritic cell; estradiol; progesterone

ABBREVIATIONS: DC (dendritic cell), Dex (dexamethasone), E2 (estradiol), iDC (immature moDC), Mφ (macrophage), mDC (mature moDC), moDC (monocyte-derived dendritic cells),

MOI (multiplicity of infection), PBMC (peripheral blood mononuclear cells), PG (progesterone), T helper 1 (Th1), T helper 17 (Th17)

ABSTRACT

The specific factors which regulate differentiation and maturation of dendritic cells in bovine pregnancy remain unclear. We evaluated the influence of physiologically relevant *in vitro* treatments of progesterone (PG) and estradiol (E2) observed in late pregnancy on the differentiation and maturation of CD14+ monocyte-derived dendritic cell (moDC) from non-pregnant, lactating dairy cows (n=7). We found that moDC differentiated in the presence of both E2 and PG had impaired *E. coli*-induced phenotypic maturation, specifically a significant reduction in CD80 and MHC II expression. Contrary to our previous work characterizing moDC from late gestating dairy cattle, we did not observe an increase in CD14 expression relative to the untreated control; this increase was only observed in the current data in the dexamethasone-treated moDC. The moDC treated with a combination of both E2 and PG had significantly greater upregulation of anti-inflammatory cytokine IL-10 relative to the untreated control, but TNFα production was not suppressed; only dexamethasone-treated moDC showed abrogated TNFα production. These data suggest moDC may be regulated by E2 and PG to hinder phenotypic maturation and regulate inflammatory responses. Pregnancy-associated hormone profiles appear to be involved in the generation of maternal immune tolerance in pregnancy. These hormone-facilitated changes to moDC in pregnancy may also impede optimal immune responses to both invading pathogens and routine vaccinations administered in late gestation through limited antigen presentation and increased anti-inflammatory cytokine production. These results provide insight into maternal immune modulation and elucidate potential immune changes necessary to facilitate bovine pregnancy.

1. Introduction

During pregnancy the maternal immune system is regulated by both maternal and fetal factors to generate tolerance toward the semi-allogenic fetus (Nahum et al., 2004, Schumacher et al., 2014, Fair, 2015). Across multiple species dendritic cells (DC) are known to play a crucial role in generating maternal immune tolerance toward fetal antigens by preventing the development of highly inflammatory, fetal antigen-specific responses (Zenclussen et al., 2005, Kahn and Baltimore, 2010, Petroff, 2011). Our previous work indicated that these cells are regulated in bovine late gestation (Bachy et al., 2008, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Pomeroy et al., 2015). We found previously that relative to non-pregnant cows, monocyte-derived DC (moDC) from cows in late gestation had hindered phenotypic maturation and altered cytokine secretion in response to *E. coli* stimulation and thus were likely to have a poor ability to activate T cells during late gestation. We also showed that these moDC from cows in late gestation showed heightened IL-10 production and hindered MHC II upregulation (Pomeroy et al., 2015). Hormone profiles unique to late gestation may affect the ability of DC to prime and activate T cells during this stage of pregnancy.

Multiple factors in pregnancy have been shown to regulate various maternal immune cell populations; these cells may be regulated differently depending on the stage of pregnancy in order to meet the specific demands for successful implantation, maintenance of pregnancy, and preparation for parturition (Bauersachs and Wolf, 2013, Gomez-Lopez et al., 2014, Robertson and Moldenhauer, 2014). In murine and human models, the ability of DC to differentiate and mature can be altered by pregnancy-associated hormones, including estradiol (E2) and progesterone (PG), both of which are highly upregulated in pregnancy (Lamote et al., 2004, Segerer et al., 2009, Xu et al., 2011, Cordeau et al., 2012, Lasarte et al., 2013, Schumacher et al.,

2014). The relationship of these two hormones, E2 and PG, appears to be complex and dependent on context and concentration. The majority of studies show immunosuppressive effects of both E2 and PG on DC differentiation and maturation i.e. reduction in CD80 and MHC II, enhanced anti-inflammatory cytokine production with concurrent suppressed pro-inflammatory cytokine responses, and T cell stimulatory capacity (Hughes et al., 2008, Segerer et al., 2009, Jones et al., 2010, Xu et al., 2011, Lasarte et al., 2013). Both E2 and PG are relatively high in bovine late gestation relative to other stages of lactation, and in the case of E2, this hormone drastically increases as cows approach calving in the third trimester of pregnancy (Hunter et al., 1970, Echternkamp and Hansel, 1973, Smith et al., 1973). Previous work in dairy cattle showed that *in vitro* treatments of PG had an effect on lymphocyte transcription factors which may promote T regulatory cell formation, and E2 and PG influence the function of neutrophils. However despite the importance of DC in maternal immune regulation across other species, we lack an understanding of the impact of these hormones on DC (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013).

Here we aim to expand upon our previous work and study the effects of two major pregnancy hormones on moDC differentiation and maturation to further understand what factors may regulate changes observed in late gestation. The objective of this study was to analyze differentiation and maturation of bovine moDC, derived from blood CD14+ monocytes with exogenous *in vitro* treatments of physiological levels of E2 and PG found in late gestation. We investigated phenotype and function analyzing surface marker expression (CD14, CD40, CD80, MHC II) and cytokine production (TNFα, IL-10) following *E. coli* stimulation.

2. Materials and methods

2.1. Animals

Seven Holstein–Friesian cows were included in the study. These cows were either in their second or third lactation, were greater than 30 days in milk (DIM) and had not been bred. Cows were selected from the Cornell University Veterinary College Teaching Dairy Barn. No disease *(i.e.* metabolic, reproductive, mastitis) was observed within 30 days prior to the time of first sampling for all enrolled animals. Peripheral blood was collected by jugular venipuncture from enrolled animals into 250ml vacuum bottles containing EDTA. All animal procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number: 2007-0110).

2.2. Generation of peripheral blood CD14+ moDC

Monocyte-derived DC were generated from CD14+ monocytes as described previously by Pomeroy et al. (2015). In brief, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. The PBMC were incubated with anti-human CD14 antibodies conjugated with paramagnetic microbeads (Miltenyi Biotech, Inc., Auburn, CA) in MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), transferred to MACS LS magnetic separation column, and after repeatedly washing the column with MACS buffer the magnetically labeled cells were collected (Miltenyi Biotech, Inc.). Purity was determined by flow cytometry and was shown to be >90%. Monocytes were re-suspended in differentiation media (phenol-free complete RPMI with l-glutamine, supplemented with 10% heat-inactivated charcoal/dextran treated FBS (lot# J13137, Atlanta Biologicals), 20 ng/ml recombinant bovine IL-4 and, 20 ng/ml recombinant bovine GM-CSF both from Kingfisher Biotech), plated in 24-well tissue culture plates at 5-7 × 10^5 cells in 1 ml differentiation media per well containing hormone treatments, and cultured for 5 days (37 °C, 5% CO_2) in presence of recombinant cytokines to generate immature moDC; at 2-3 d cultures were given 0.5ml/well of

fresh differentiation media containing the respective treatments. Monocytes were treated with 65 pg/ml of E2 (E2758, Sigma Aldrich), 10 ng/ml of PG (P8783, Sigma Aldrich), a combination of both E2 and PG, or 50ng/ml of dexamethasone (DEX) (D4902, Sigma Aldrich); an untreated control was cultured in differentiation media with no addition of exogenous hormones. Dexamethasone was used as a positive control to show suppression of moDC differentiation and maturation (van Kooten and Gelderman, 2011). Estradiol and PG treatments were selected based on range of circulating levels measured in plasma and serum documented in the literature and measurements determined by radioimmunoassay (radioimmunoassay preformed by the Endocrinology Laboratory in the Animal Health Diagnostic Center/ New York State Veterinary Diagnostic Laboratory accredited by the American Association of Veterinary Laboratory Diagnosticians) from cows in 60-30 days prior to calving (data not shown) (Hunter et al., 1970, Smith et al., 1973).

2.3. Inactivation of *E. coli* ECC-Z

E. coli ECC-Z was inactivated as described by Pomeroy et al. (2015). In brief, frozen stock of the previously characterized *E. coli* ECC-Z strain known to cause mild persistent clinical mastitis (Dogan et al., 2006, Lippolis et al., 2014) was grown to log phase in LB broth (Merck Millipore, Billerica, MA, USA). Bacteria were pelleted and diluted in PBS. Bacterial suspension was plated on agar plates prior to inactivation to determine concentration of stock solution. The bacterial suspension was inactivated using UV irradiation in the laboratory of Dr. Randy W. Worobo (Department of Food Science and Technology, Cornell University). The UV irradiated *E. coli* suspension failed to produce colonies on LB agar plates. Aliquots of UV irradiated *E. coli* were re-suspended in sterile 10% glycerol/PBS and stored at −80 °C.

2.4. Maturation of moDC with UV irradiated *E. coli*

Maturation of moDC was induced by bacterial stimulation through the addition of UV irradiated *E. coli* to immature moDC cultures in an MOI (multiplicity of infection) of 10 for 24 h (Pomeroy et al., 2015). Cultures of moDC without bacterial stimulation served as media controls. Cell culture supernatants were collected after 24 h *E. coli* stimulation and stored at −80 °C until further cytokine analysis by ELISA. Cells were also harvested at this time for phenotypic analysis by flow cytometry using Accutase™ Solution according to the manufacturer's instructions (Merck Millipore).

2.5. Flow cytometry

Expression of surface markers on moDC was measured with flow cytometry as described previously by Pomeroy et al. (2015). In brief, moDC were blocked with 10% normal goat serum (Life Technologies), subsequently incubated with primary unlabeled monoclonal mouse antibodies against bovine MHC II DR orthologue (TH4B), CD14 (MM61A), CD40 (ILA158A), and CD80 (ILA159) (Kingfisher Biotech), and then indirectly labeled with secondary Alexa Fluor® 488 conjugated monoclonal goat antibodies against mouse $IgG_{2\alpha}$ or Alexa Fluor® 647 conjugated monoclonal goat antibodies against mouse IgG_1 (Life Technologies). Secondary controls were of moDC stained only with conjugated secondary antibodies. No differences were found in baseline autofluoresence as determined by consistent MFI of secondary controls across samples (data not shown). Discrimination between live and dead cells was done by adding propidium iodide (PI, 1 µg/ml, Sigma Aldrich). A minimum of 10,000 events gated on viable cells were recorded for each sample. All measurements were taken with a FACSCalibur (Becton Dickinson). All flow cytometry data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

2.6. Measurement of IL-10 and TNFα by ELISA

Supernatant levels of bovine IL-10 were measured using ELISA as described in Pomeroy et al. (2015). In brief, Thermo Cliniplate EB flat-bottom 96-well plates (ThermoFisher, Vantaa, Finland) were coated with capture monoclonal mouse antibody against bovine IL-10 (1 μg/ml; CC320; AbDSerotec) in 0.05 M carbonate buffer (pH 9.6). Plates were washed then blocked with 2% fish skin gelatin (Sea Block buffer, ThermoFisher) in PBS. Following blocking supernatant samples were added. Known concentrations of recombinant bovine IL-10 (Kingfisher Biotech) were assayed simultaneously to generate a standard curve from which sample concentrations were determined. Following sample and standard incubation, plates were washed, and biotinylated detection monoclonal mouse antibody against bovine IL-10 (1 μg/ml; CC320; AbD Serotec) was added. All samples and standards were assayed in duplicate.

Supernatant levels of TNFα were measured according to using a commercially available kit according to the manufacturer (bovine TNFα DuoSet® ELISA, DY2279, R&D Systems, Minneapolis, MN).

2.7. Statistical analysis

All data were analyzed using the GraphPad Prism 5 statistical analysis program (GraphPad Software, Inc., La Jolla, CA USA). Initially, data were graphed and evaluated for outliers. Outliers were inspected and reanalyzed where necessary. Significant changes in moDC surface marker expression, and moDC cytokine production between untreated control, was determined by testing whether the fold change over untreated control was significantly different from 1 i.e. no fold change from untreated control, using a non-parametric, Wilcoxon-ranked signed test. A type I error of ≤ 0.05 was used.

3. Results

As determined by percent of cells negative for propidium iodide, cell viability was not impacted by hormone treatment; no differences were detected between treatment groups and untreated controls in either unstimulated, immature DC (iDC) or *E. coli*-stimulated mature DC (mDC) cultures (Supplementary data, Fig. 1S).

3.1. *In vitro* E2 and PG treatments hinder MHC II and CD80 expression

Few changes in surface marker expression were detected following differentiation in unstimulated iDC populations. The iDC treated with E2 showed a moderate decrease in CD40 and CD80 following the differentiation period relative to untreated control iDC (median fold change=0.918, p=0.0313; 0.5526, p=0.0156 respectively), however, following maturation with UV-killed *E. coli* CD40 expression increased across treatments and E2 treated mDC reached comparable expression levels as untreated controls. Dexamethasone treatment significantly increased CD14 expression (3.77, p=0.156; 3.1, p=3.13), decreased CD40 expression (0.584, p=0.0156; 0.604, p=0.0156), and decreased MHC II expression (0.157, p=0.0156; 0.0864, p=0.0156) in both iDC and mDC. However, dexamethasone had no significant impact on CD80 expression relative to the untreated control (Fig. 11 a-i). Treatments with E2, PG, and combination of E2 and PG hindered the increase in expression of MHC II following maturation (0.8702, p=0.0156; 0.793 p=0.0156; 0.825, p=0.0156). Similarly, hindered MHC II expression by PG could be observed prior to *E. coli*-stimulation in iDC (0.847, p=0.0313) (Fig. 11 a,e,i). In addition to MHC II expression, unlike dexamethasone, E2 and PG treatment significantly decreased expression of CD80 in mDC at comparative levels (0.8055, p=0.0156; 0.8076, p=0.0313) and the combination of E2 and PG had a similar trend which approached significance (0.8731, p=0.0938). Reduced CD80 expression by E2 treatment could be observed prior to

stimulation in iDC and the combination of E2 and PG had a similar trend that also approached statistical significance (0.5526, p=0.0156; 0.6667, p=0.0781) (Fig. 11 a,d,h).

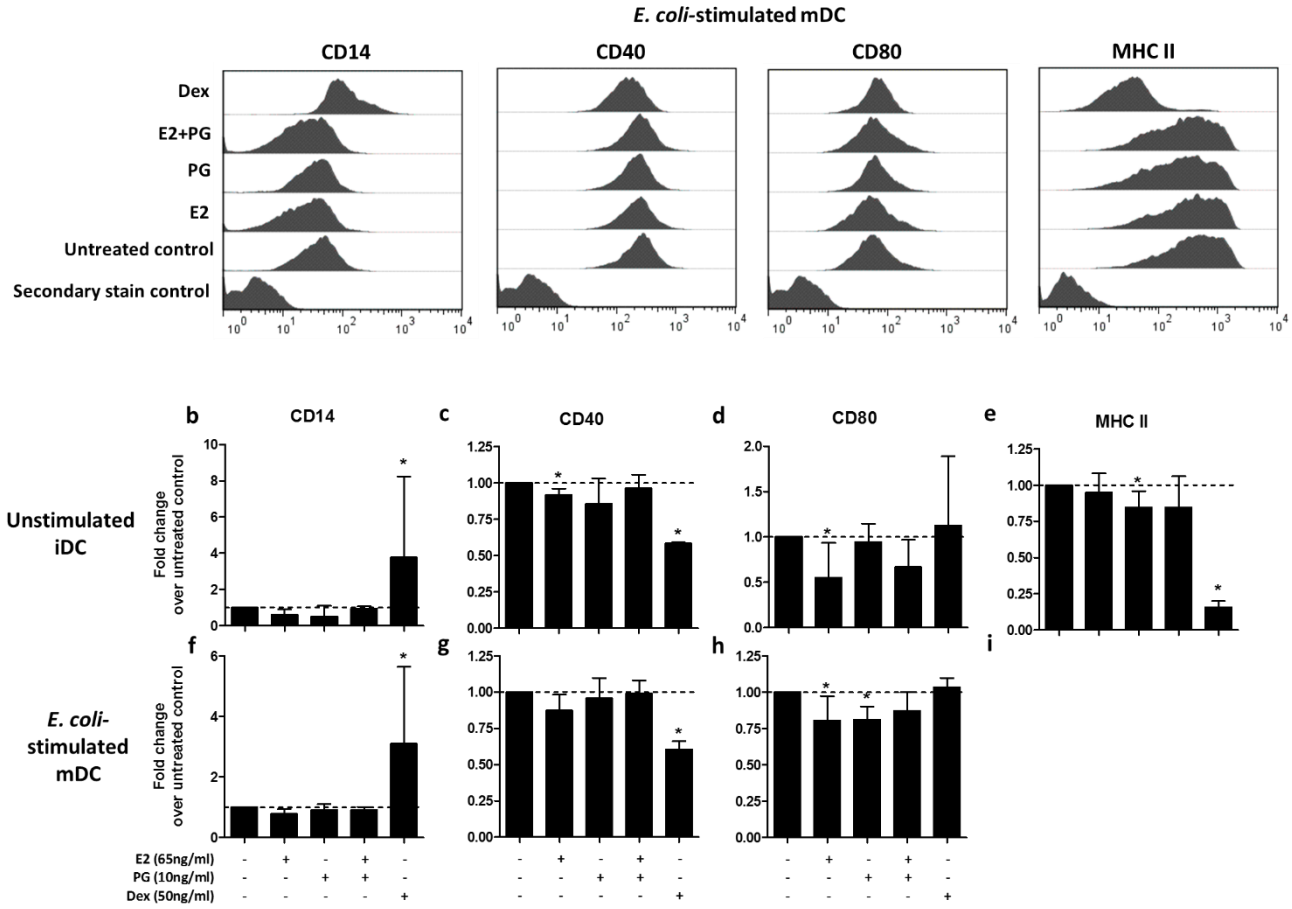

Figure 11. MoDC phenotypic differentiation and maturation induced by 24h stimulation with UV IR *E. coli* ECC-Z. Representative histograms of moDC expression of CD14, CD40, CD80, and MHC II; dashed line represents secondary, negative staining control, dotted line represents stained, unstimulated iDC, and solid line represents stained, *E. coli*-stimulated mDC (a). Fold changes over untreated, control in relative expression of CD14 (b,f), CD40 (c,g), CD80 (d,h), and MHC II (e,i) as determined by geometric mean fluorescence intensity (MFI) in unstimulated, immature moDC (b-e) and UV IR *E. coli*-stimulated moDC (f-i). Solid black bars represent median (n=7). Error bars represent interquartile range. Significant differences were determined using a Wilcoxon-ranked signed test comparing medians to hypothetical value of 1. * indicates significant of $p<0.05$.

3.2. Combined treatment of E2 and PG enhances IL-10 upregulation following UV-killed *E. coli*-stimulation

Interleukin 10 was detected at varying levels in supernatant from unstimulated moDC. Therefore, delta IL-10 (*E. coli*-stimulated mDC–unstimulated iDC) was used to compare IL-10 upregulation following *E. coli* stimulation (Fig. 12 a). Upregulation of IL-10 following UV-killed *E. coli*-stimulation was enhanced in moDC treated with a combination of both E2 and PG relative to untreated controls (1.258, p=0.0156). However individual treatments of either E2 or PG alone did not enhance IL-10 upregulation (Fig. 12 a). Interestingly, dexamethasone treatment did not enhance IL-10 production (Fig. 12 a).

No TNFα was detected in the supernatant from unstimulated iDC across treatments thus only concentrations of TNFα produced from *E. coli*-stimulated mDC were compared to untreated control mDC. We found mDC treated with E2 or PG or the combination of E2 and PG were capable of upregulating TNFα and there was no significant increase or decrease relative to the untreated control. There was a trend in moDC treated with both E2 and PG towards an increase in TNFα production (1.363, p=0.0781) (Fig. 12 b). Dexamethasone treated moDC had abrogated TNFα production, and the majority of samples were below limits of detection resulting in a drastic decrease relative to untreated control mDC (0, p=0.0147) (Fig. 12 b).

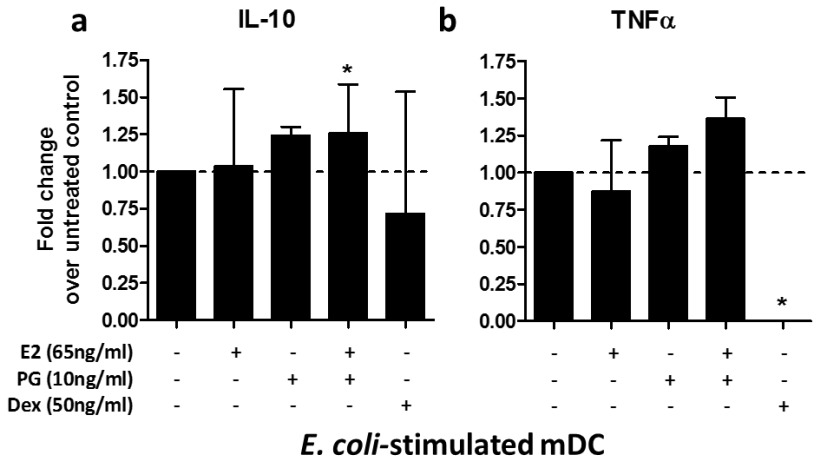

Figure 12. MoDC IL-10 and TNFα production following maturation induced by 24h stimulation with UV IR *E. coli* ECC-Z. Fold changes over untreated, control in upregulation of IL-10 (a); upregulation of IL-10 i.e. ΔIL-10 was defined as the difference in concentration of IL-10 in cell-free, cell culture supernatant between UV IR *E. coli*-stimulated moDC and unstimulated, immature moDC. Fold change over untreated, control in TNFα production in cell culture supernatant from UV IR *E. coli*-stimulated moDC (b). Solid black bars represent median (n=7). Error bars represent interquartile range. Significant differences were determined using a Wilcoxon-ranked signed test comparing medians to hypothetical value of 1. * indicates significant of $p<0.05$.

4. Discussion

Dendritic cells are known to be key drivers of maternal immune tolerance and are regulated to accommodate unique needs at each stage of pregnancy by multiple pregnancy-associated factors; factors which regulate these cells in bovine pregnancy have yet to be determined (Blois et al., 2007, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Schumacher et al., 2014, Pomeroy et al., 2015). In this study, we analyzed the effect of *in vitro* exposure to physiological levels of E2 and PG found in late gestation on aspects of moDC phenotype and cytokine production from non-pregnant dairy cows. Our findings suggest levels of E2 and PG in late gestation regulate DC to promote tolerance through inhibition of phenotypic maturation and increase in the production of anti-inflammatory cytokine IL-10.

We were able to recapitulate some aspects of hindered phenotypic maturation observed in late gestation in moDC from non-pregnant lactating cows using *in vitro* treatments of E2 and PG at levels typically found in circulation during late gestation. We also observed some differences following the differentiation period, in unstimulated iDC in E2 or PG treated cultures relative to untreated controls where treated cells had a reduced expression of co-stimulatory markers (CD40, CD80) or MHC II, respectively (Fig. 11 a-e). Following maturation induced by UV-killed *E. coli* stimulation, the moDC treated with these pregnancy-steroid hormones had reduced expression of CD80 and MHC II (Fig. 11 a, f-i). These results agree with our previous work characterizing moDC from cows in late gestation without addition of exogenous hormones; specifically we had observed hindered MHC II expression following *E. coli* stimulation in moDC from non-lactating cows in late gestation (Pomeroy et al. 2015). Though dexamethasone suppressed MHC II like E2 and PG it did so to a much greater extent. Interestingly, unlike treatments of E2 and PG, dexamethasone treatment did not suppress CD80 expression, but rather it suppressed CD40 expression and further greatly enhanced CD14 expression even following maturation. This difference in pregnancy steroid hormones and dexamethasone effect suggests that pathways which hinder maturation in moDC likely differ between these hormones (Fig. 11) (Yoshimura et al., 2001, Franchimont, 2004). The decrease in co-stimulatory molecule CD80 and antigen-presentation molecule MHC II upregulation in E2 and PG treated moDC indicates that pregnancy steroid hormones may play a role in modulating the immune response specifically in DC and may relate to weaker T cell activation in late gestation.

In addition to phenotypic changes, moDC cytokine production was influenced by *in vitro* hormone treatment. The upregulation of anti-inflammatory cytokine, IL-10 following maturation was relatively higher in moDC treated with both E2 and PG however numerically the data

suggest PG is likely the main driver in these changes (Fig. 12 a). Our findings pertaining to IL-10 production go in line with our previous work in which moDC from late gestation dry cows had greater upregulation of IL-10 following *E. coli*-stimulation compared to those from early lactation cows (Pomeroy et al., 2015). In non-bovine species, IL-10 is a key cytokine involved in preventing preterm labor in late gestation, and maintaining tolerances (Sato et al., 2001, Robertson et al., 2006, Boro et al., 2014). Pro-inflammatory cytokine, TNFα, was not significantly altered with E2 or PG treatment, though there was a trend with treatments including PG suggesting it may elevate production of TNFα. In contrast to E2 and PG, dexamethasone treatment abrogated TNFα production (Fig. 12 b). These cytokine data suggests again that the pathway in which dexamethasone suppresses moDC maturation is likely different from E2 and PG because dexamethasone suppressed pro-inflammatory TNFα while no significant changes were found with regards to IL-10 production, but the combination of E2 and PG enhanced IL-10 production but did not suppress TNFα production (Yoshimura et al., 2001, Franchimont, 2004). The observed upregulation of TNFα may be necessary at various stages of pregnancy, and argument that is similar for certain inflammatory cytokines and what is observed in other mammalian species, (Monzon-Bordonaba et al., 2002, Okuda and Sakumoto, 2003, Fair, 2015). It may also be possible that this pro-inflammatory cytokine is regulated by other factors in pregnancy and not by E2 or PG. Candidates for regulation of pro-inflammatory cytokines were suggested to be metabolic metabolites such as adiponectin (Kabara et al., 2014).

This study demonstrates the effects of pregnancy-associated factors, E2 and PG present at elevated levels simulating bovine late gestation, on moDC and reveals that these steroid hormones may be modulators of maternal immune response in late pregnancy. Previous work in other species generally found these hormones to hinder maturation and alter cytokine profile of

DC generally minimizing T cell activation and highly inflammatory responses; for example, Xu et al. (2011) found *in vitro* treatments of PG diminishes the expression of maturation markers CD80 and MHC II, and elevated IL-10 while concurrently reducing IL-12 production, (Butts et al., 2008, Hughes et al., 2008, Jones et al., 2010, Xu et al., 2011). In non-bovine species, these hormones have been shown to inhibit the NF-κB pathway in multiple ways including, the suppression of translocation via Ikbkg or interfere with NF-κB transcriptional activity. Suppression of the NF-κB pathway negatively impacts effective antigen presentation by DC (Yoshimura et al., 2001, Davies et al., 2004, Lasarte et al., 2013). Diminished DC maturation generated by E2 and PG likely promotes and maintains a viable, full term pregnancy by preventing the development of conceptus-derived antigen-specific responses (Arck et al., 2007). In addition to the effects on DC, both E2 and PG have been shown to regulate other bovine immune cells including neutrophil phenotype and function and T cell transcription factors indicating these hormones play a larger role in maternal immune regulation and work in concert to generate tolerance (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013). Future research should investigate further functional aspects modulated by these hormones, determine if this relates to changes observed *in vivo*, and understand the pathways which are regulated by E2 and PR signaling. In addition to a more in-depth analysis of the effects of E2 and PG on bovine moDC, other pregnancy-associated factors should also be addressed; unlike humans and rodents, dairy cattle experience drastic metabolic changes over the course of pregnancy and lactation that are likely to impact moDC phenotype and function (Sordillo et al., 2009, Rulle et al., 2012, Sim et al., 2016).

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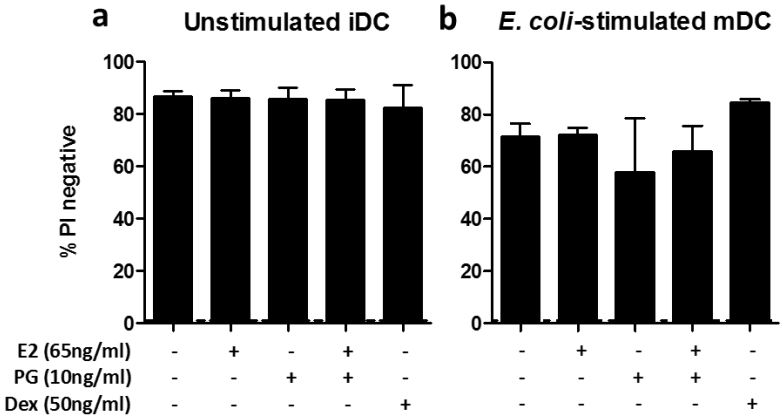

Figure 1S. MoDC viability following differentiation and maturation induced by 24hr stimulation with UV IR *E. coli* ECC-Z. Viability determined by percentage of cells negative for propidium iodide for unstimulated, iDC (a) and *E. coli*-stimulated mDC (b). Solid black bars represent median (n=7). Error bars represent interquartile range. Significant differences were determined using a Wilcoxon-ranked signed test comparing medians to hypothetical value of 1. * indicates significant of $p<0.05$. No significant differences were detected across hormone treatments.

CHAPTER 5:
COUNTS OF BOVINE MONOCYTE SUBSETS PRIOR TO CALVING ARE PREDICTIVE FOR POSTPARTUM OCCURRENCE OF MASTITIS AND METRITIS

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KEYWORDS: monocyte; bovine; disease

ABSTRACT

The heightened susceptibility to infectious diseases in postpartum dairy cows is often attributed to immune dysfunction associated with the transition period. However, the cell populations involved in this immune dysfunction and the dynamics between those populations are not well defined. Monocytes play a crucial role in governing initial immune response in bacterial infections. Bovine monocytes are subdivided in classical ($CD14^+/CD16^-$), intermediate ($CD14^+/CD16^+$) and non-classical monocytes ($CD14^-/CD16^+$) with distinct phenotypic and functional differences. This study investigated the relationship of monocyte subsets counts in blood at 42 and 14 days prior to expected calving date to occurrence of metritis and mastitis within 2 weeks postpartum. In the enrolled prospective cohort of 27 German Holstein cows, housed at the Institute of Animal Nutrition of the Friedrich-Loeffler-Institute Braunschweig, Germany, n=13 developed metritis and/or mastitis postpartum. A multivariable logistic regression was used to analyze the relationship between prepartum cell counts of monocyte subsets and neutrophils with postpartum disease. Our model revealed that higher counts of the two $CD14^+$ monocyte subsets were predictive of disease. In contrast, higher numbers of the CD14- monocyte subset were negatively associated with disease. Interestingly, the neutrophil count, a common hallmark for inflammatory response, was not associated with the outcome variable at either time point. The results indicate that the number and composition of monocyte subsets before calving are related to the susceptibility to infectious disease within 2 weeks postpartum. Furthermore the oppositional effect of $CD14^+$ and $CD14^-$ subsets strengthens the hypothesis that these subsets have different functional roles in the inflammatory response in dairy cows.

1. Introduction

Dairy cows have an increased susceptibility to disease during the first 3 weeks postpartum (LeBlanc, 2010). Clinical mastitis and metritis are frequently observed in this period, and both of these diseases remain an animal welfare concern and a source of major costs for the dairy industry worldwide (Mallard et al., 1998, Bradford et al., 2015). The higher incidence of these diseases is commonly associated with an dysregulated inflammatory response in the animal, which often goes along with an overexpression of pro-inflammatory mediators like TNF-α and IL-1β and increased disease severity (Sordillo and Raphael, 2013). The innate immune system represents the first line of defense against the initial stages of infection and is the key determinant in the outcome of mastitis and metritis (Aitken et al., 2011, Pinedo et al., 2013). Monocytes and monocyte-derived cells play an essential role in orchestrating the main features of the innate immune response (Hume et al., 2002, Auffray et al., 2007). Recently it has become evident that bovine monocytes are a heterogeneous population and can be divided based on the expression of CD14 and CD16 into classical (cM, $CD14^+/CD16^-$), intermediate (intM, $CD14^+/CD16^+$) and non-classical monocytes (ncM, $CD14^-/CD16^+$), with the majority of blood monocytes being CD14 positive (Hussen et al., 2013). Distinct functional features were reported for the different subsets in response to LPS and in the presence of chemokines. Hussen et al. (Hussen et al., 2013) found short term LPS stimulation (3h) induced stronger pro-inflammatory cytokine response and inflammasome activation in $CD14^+$ subsets (cM and intM) compared to the $CD14^-$ ncM; however, Corripio-Miyar et al. (Corripio-Miyar et al., 2015) found that ncM showed an overall stronger pro-inflammatory response after long term LPS (18h) stimulation. In conjunction with inflammatory responses to LPS, cM also are able to produce relatively strong anti-inflammatory responses including high arginase I gene expression and IL-10 production.

Only the $CD14^+$ subsets, cM and intM, are attracted by both the monocyte chemokine CCL5 and neutrophil degranulation products *in vitro* and to respond with significant Ca^{2+} influx (Hussen et al., 2014, Hussen et al., 2016). Though there remains some discrepancies in function in regards to CD14- monocytes it is evident these subsets have unique properties and CD14+ consistently show strong inflammatory responses.

Although the role and dynamics of the different bovine monocyte subsets *in vivo* remain unclear, cows that develop infectious disease postpartum also have an altered monocyte response. Periparturient cows had an overall higher frequency of monocytes in supra mammary lymphnodes and higher TNF-α production in tissue monocytes after stimulation with LPS but significantly lower response in blood monocytes (Sordillo et al., 1995). Monocytes from cows which developed metritis within the first week postpartum have elevated baseline expression of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β at 1 to 12 hours post calving, but showed a less pronounced increase of these mediators in response to bacterial challenge *in vitro* (Galvao et al., 2012). Despite an enhanced baseline pro-inflammatory cytokine production the immune response from those cows failed to prevent the establishment of infection with rapid clearance of the invading pathogen, and the increased inflammatory properties of these cells likely promoted disease. Another study showed that monocyte phagocytic capacity was significantly decreased in cows that developed endometritis within the first 20 days postpartum (Brodzki et al., 2014). It is unclear from these studies if susceptible cows also show changes in monocyte subset composition or an altered function of individual monocyte subsets. In humans however, there are associations between inflammatory conditions and monocyte subset composition. In tuberculosis patients an expansion of $CD16^+$ subsets was reported while *in vitro* $CD16^-$ monocyte subsets were more prone to migrate in response to mycobacteria derived

gradients (Balboa et al., 2015). In an endotoxin tolerance model with human monocytes it was furthermore shown that prior exposure to LPS increased the frequency of CD14+ monocyte subsets after LPS restimulation (Dominguez-Nieto et al., 2015). Additional studies showed that human CD14- ncM were the most inflammatory of the subsets, and an increase in ncM was associated with autoimmune disorders and promotion of Th1-type responses, whereas CD14+ cM and intM attenuated T cell responses and were associated with certain diseases in humans such as sepsis and certain cancers (Wong et al., 2012, Mukherjee et al., 2015). Research pertaining to relationships between disease and monocyte subsets is underdeveloped in bovine immunology, yet the substantial evidence connecting disease and monocyte composition found in other species in conjunction with known species to species differences indicates this area should be further investigated within a bovine model.

Therefore, the objective of this study was to investigate if there were a relationship between prepartum blood monocyte subset composition and postpartum infectious disease in dairy cows. Specifically, we investigated if disease susceptibility was correlated with changes in either $CD14^{+}$ or $CD14^{-}$ subsets. We used logistic regression models with longitudinal prospective data to identify significant relationships between postpartum disease and counts of monocyte subsets.

2. Materials and methods

2.1. Animals and blood sampling

The data was collected from cows enrolled in a previously published study (Eger et al., 2016). Briefly, 27 German Holstein cows housed at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute in Braunschweig, Germany were enrolled in the study. Body condition score (BCS) was determined prior to the experiment and cows were

allotted to a BCS low (BCS 2.77 ± 0.10, mean ± SEM) and a BCS high group (BCS 3.73 ± 0.12) with consideration of milk yield, body weight and lactation number (Table 2). Throughout the experiment the animals were housed in a free-stall barn with individual feeding stations. After calving cows were milked twice per day. Pre-partum, BCS low cows received a diet of 80% roughage, 50% grass silage and 50% corn silage based on dry matter content, and 20% concentrate according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The BCS high group received 40% of the same roughage and 60% concentrate to induce energy oversupply. After calving the concentrate proportion in the diet was raised from 30% to 50% in 2 or 3 weeks for the BCS low and the BCS high group, respectively, to promote negative energy balance in the BCS high group. Half of the enrolled animals were vaccinated against BVD (Bovilis BVD®-MD, MSD) at 42 and 14 days prior to expected calving date; half of the low BCS was vaccination and approximately half of the high BCS was vaccinated. The number of cows with a parity of 2 and cows with a parity of greater than 2 were approximately the same between the groups i.e. four groups based on BCS and vaccination status. Health status was monitored throughout the trial. Cows with purulent uterine discharge detected in the vagina were diagnosed with clinical metritis based on the definition provided by Sheldon et al. (2006). Mastitis cases were identified by clinical signs i.e. clots in milk, elevated somatic cell count, swelling/sensitivity of the mammary gland. Bacteriological cultures were not preformed to determine the etiology of clinical disease; metritis and mastitis were defined by clinical signs. For the purpose of this paper animals that developed metritis and/or mastitis within two weeks postpartum were combined into a single category of postpartum disease; therefore a binary outcome variable of postpartum disease yes or no was used in the subsequent data analyses (Table 2). Three cows developed clinical mastitis only, seven cows developed metritis only, and

three cows developed both mastitis and metritis in the first two weeks postpartum. Blood samples were collected by puncture of the jugular vein into heparinized vacutainer tubes at days 42 and 14 prior to predicted calving date. Only two cows calved more than 2 weeks prior to predicted parturition, their prepartal sample was assigned to the nearest predicted day (one cow day -42, one cow day -14). This study was approved by the Lower Saxony State Office for Customer Protection and Food Safety (33.9–42502–04–11/0444). All procedures involving animals were carried out in accordance with the German legislation on animal welfare.

2.2. Separation of blood leukocytes and characterization of monocyte subsets

Blood was diluted with the same volume of PBS and centrifuged at 1000 x g for 10 min. Erythrocytes were lysed by adding 20 mL aqua dest. for 20 sec and subsequent addition of 20 mL double concentrated PBS. This was repeated twice until complete erythrolysis. Cells were centrifuged and washed with PBS (500 x g, 250 x g and 100 x g for 10 min each) and finally adjusted to 1 x 10^7 cells/mL in PBS. Leukocytes were suspended in PBS containing 5 g/L bovine serum albumin and 0.1 g/L NaN_3 (MIF buffer) and stained with a combination of three directly conjugated monoclonal antibodies: mouse anti-bovine CD172a-PECy5, mouse anti-human CD14-PE and mouse anti-human CD16-FITC (all from AbD Serotec, Oxford, UK) for 20 min at 4 °C. Thereafter cells were washed with MIF buffer and analyzed by flow cytometry (Accuri C6 Flow Cytometer®, Becton Dickinson GmbH, Heidelberg, Germany). Dead cells were excluded by adding propidium iodide (2 µg/mL, Calbiochem, Bad Soden, Germany). Mononuclear cells (MNC) and granulocytes (PMN) were gated according to their forward (FSC) and side scatter (SSC) properties (Gu et al., 2011). Among $CD172a^+$ MNC, three bovine monocyte subsets were defined based on their CD14 and CD16 expression: cM were $CD14^+/CD16^-$, intM were $CD14^+/CD16^+$ and ncM $CD14^-/CD16^+$. Appropriate compensation was applied for

fluorochromes used in multi-color flow analysis of monocyte subsets in order to distinguish between PI and PE. Cell doublets were gated out in dot plots SSC-A vs SSC-H. Cell counts of monocyte subsets and PMN were calculated by multiplying the absolute leukocyte count, determined in EDTA whole blood using an automatic analyzer (Celltac α MEK-6450, Nihon Kohden, Qinlab Diagnostik, Weichs, Germany), with percentages determined by flow cytometry.

2.3. Data analysis and statistical methods

All data were entered into a database and double checked for entry errors or outliers. Data were described using descriptive and graphical techniques. Descriptive analysis of raw data included the computation of median cell counts with interquartile range for individual cell populations measured at each sample time point and frequency tables of categorical study design variables (vaccination status, BCS, parity) and grouped by postpartum disease status. The small sample size precluded univariable statistical analyses of any associations between disease presence and BCS, parity, or vaccination status. Spearman's correlation coefficients were calculated to identify correlations between counts of different myeloid cell populations to assess for possible collinearity. Further analysis was performed using multivariable regression analyses. The general logistic regression model was formulated as: $\text{Logit}(Y) = \alpha + \beta_i X_i + e$, where Y is the absence or presence of postpartum disease, α is the intercept, β_i is the regression coefficient of predictor variable X_i. The term e is an independently, identically distributed binomial error term. Statistical significance was defined at $P < 0.05$. All Statistical analyses were preformed using SAS version 9.4 (SAS Institute). The dataset supporting the conclusions of this article is included within the article as an additional file (see Additional file 2).

2.4. Multivariable analysis

Multiple multivariable logistic models were used to assess the relationship between absolute counts of different myeloid cell populations circulating in the peripheral blood during the third trimester of pregnancy (42 days and 14 days prior to calving) and development of disease within the first two weeks postpartum. Multivariable logistic models were constructed using generalized estimating equations and a binary distribution for the outcome variable (postpartum disease, yes vs. no). Potential confounding variables included in the model were the original study design variables parity (parity=2, parity >2), body condition score (>3.0, <3.0), and vaccination schedule (vaccinated prepartum, yes=1 vs. no=0). These potential confounders were included in all multivariable logistic models as binary variables. Due to differences observed in counts of multiple myeloid cell populations between 42 days and 14 days prior to calving date, two logistic models were constructed based on time of data collection as a minimal model irrespective of individual contributions. The two models included data from either 42 days or 14 days prior to calving; any animals with missing data from either time point were excluded. Final models were generated using a forward stepwise selection of variables. Statistical significance was determined based on the likelihood ratio statistic of nested models, and model fit was described using Akaike's Information Criterium (AIC). Likelihood ratios and odds ratio estimates with profile-likelihood confidence intervals were used to determine significance due the small sample size. With the data analyzed here, a Wald test is not preferable over the likelihood ratio test because the estimates for the coefficient and its standard error may have unreliable normal approximation of its distribution when the sample size is small; likelihood ratio test and profile-likelihood confidence intervals do not assume normality of the estimator (Dohoo et al., 2003).

3. Results

3.1. Descriptive analysis

Characteristics of the enrolled cows are summarized in table 2. The animals were grouped by parity, BCS and vaccine status, and the model controlled for these host characteristics though addition of forced variables. Out of the 27 enrolled cows 13 developed clinical disease postpartum. There were comparable numbers of cows within each category of BCS (either > or < 3), parity (=2, >2), and vaccinated between cows who did not develop clinical disease postpartum ("healthy") and those cows who did develop clinical disease postpartum ("postpartum disease") as determined by the chi-square test (Table 2). The gating strategy used to determine absolute count of $CD14^-$ monocytes ($CD172a^+CD14^-$ mononuclear cells (MNC)), $CD14^+$ monocytes ($CD172a^+CD14^+$ MNC), and granulocytes (PMN) in blood is presented in figure 13. The counts of $CD14^+$ monocytes, $CD14^-$ monocytes, and PMN in blood at 42d and 14d prior to the expected calving date within healthy cows and postpartum disease cows are presented in table 3 and figure 14. Cows that developed postpartum disease had a median $CD14^+$ subset count at 14 days prior to expected calving date of 863 cells/µl blood whereas counts of $CD14^+$ subsets in cows that did not develop disease postpartum had median count of only 289 cells/µl blood (Fig. 14 A). The healthy and postpartum disease group had comparable median counts of $CD14^-$ subsets of the for both time points (Fig. 14 B). Descriptive analysis of monocyte subset counts based on parity revealed that cows in parity 2 that developed postpartum disease had median $CD14^+$ and $CD14^-$ counts of 677 cells/µl and 44 cells/µl, respectively whereas cows in parity 3 or greater had median CD14+ and CD14- counts of 174 cells/µl and 9 cells/µl, respectively, validating the need to utilize a model to elucidate relationships between disease and monocyte populations so that these cofounders can be controlled for (see Additional file 2). The

counts of PMN showed similar dynamics in healthy and postpartum disease cows in blood at 42 and 14 days prior to expected calving date (Table 3, Fig. 14 C)

Table 2. Descriptive analysis & characteristics of enrolled population. *Median cell counts with interquartile range (IQR) presented.*

Parameter	**Healthy (n=14)**		**Postpartum disease (n=13)**	
	Frequency		***Frequency***	
Parity=2	5		7	
Parity >2	9		6	
BCS <3	7		6	
BCS >3	7		7	
Unvaccinated	8		8	
Vaccinated	6		5	
42 days prior to calving	***Median***	***IQR***	***Median***	***IQR***
Neutrophil absolute count (cells/µl)	3075.5	2149	2851	1790
CD172a+ absolute cell count (cells/µl)	517.90	171.16	552.15	616.21
CD14+ monocyte absolute count (cells/µl)	469.95	170.13	510.12	557.28
CD14- monocyte absolute count (cells/µl)	33.45	27.06	32.73	37.63
14 days prior to calving				
Neutrophil absolute count (cells/µl)	3884.75	2303	4326.05	2202
CD172a+ absolute cell count (cells/µl)	389.86	680.35	1003.21	399.70
CD14+ monocyte absolute count (cells/µl)	341.98	602.29	915.14	358.69
CD14- monocyte absolute count (cells/µl)	36.21	60.53	53.46	49.16

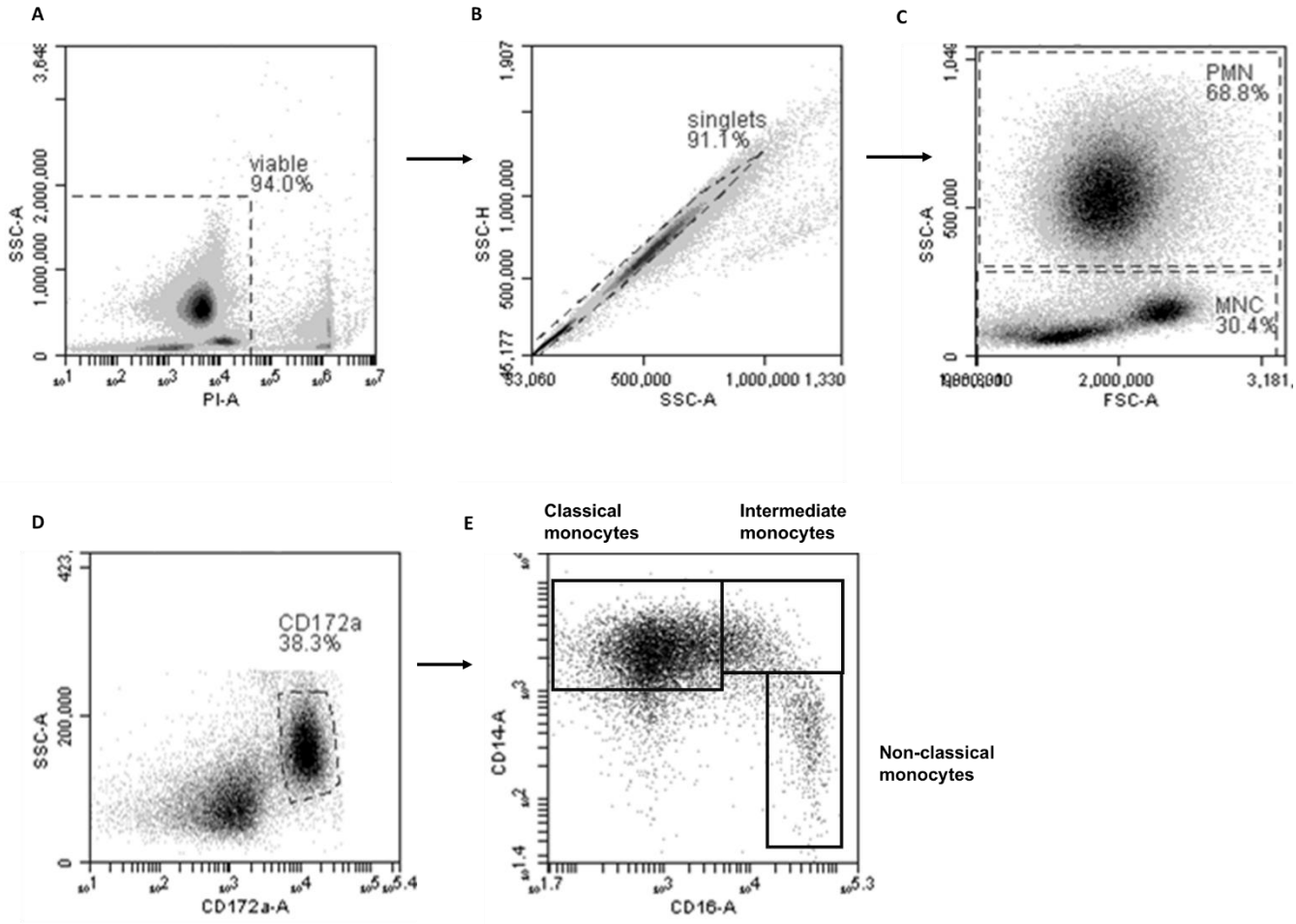

Figure 13. Flow cytometric Gating strategy for bovine peripheral blood leukocytes. Representative data are shown for cells of one animal. After gating on viable (propidium-iodide-negative) cells (A), cell doublets were excluded in SSC-A /SSC-H dot plots (B). Bovine mononuclear cells (MNC) and granulocytes (PMN) were identified based on their characteristic forward and side scatter characteristics and their percentages were calculated (C). Three-color immunofluorescence of bovine MNC labelled with mAbs specific for CD172a, CD14 and CD16 defines three monocyte subsets in peripheral blood: Among viable MNC, CD172a-positive cells were identified (D). Correlated dot plots (CD14 vs CD16) of CD172a-positive MNC display classical monocytes (CD14+CD16−, upper left), intermediate monocytes (CD14+CD16+, upper right) and nonclassical monocytes (CD14-CD16+, lower right) (E).

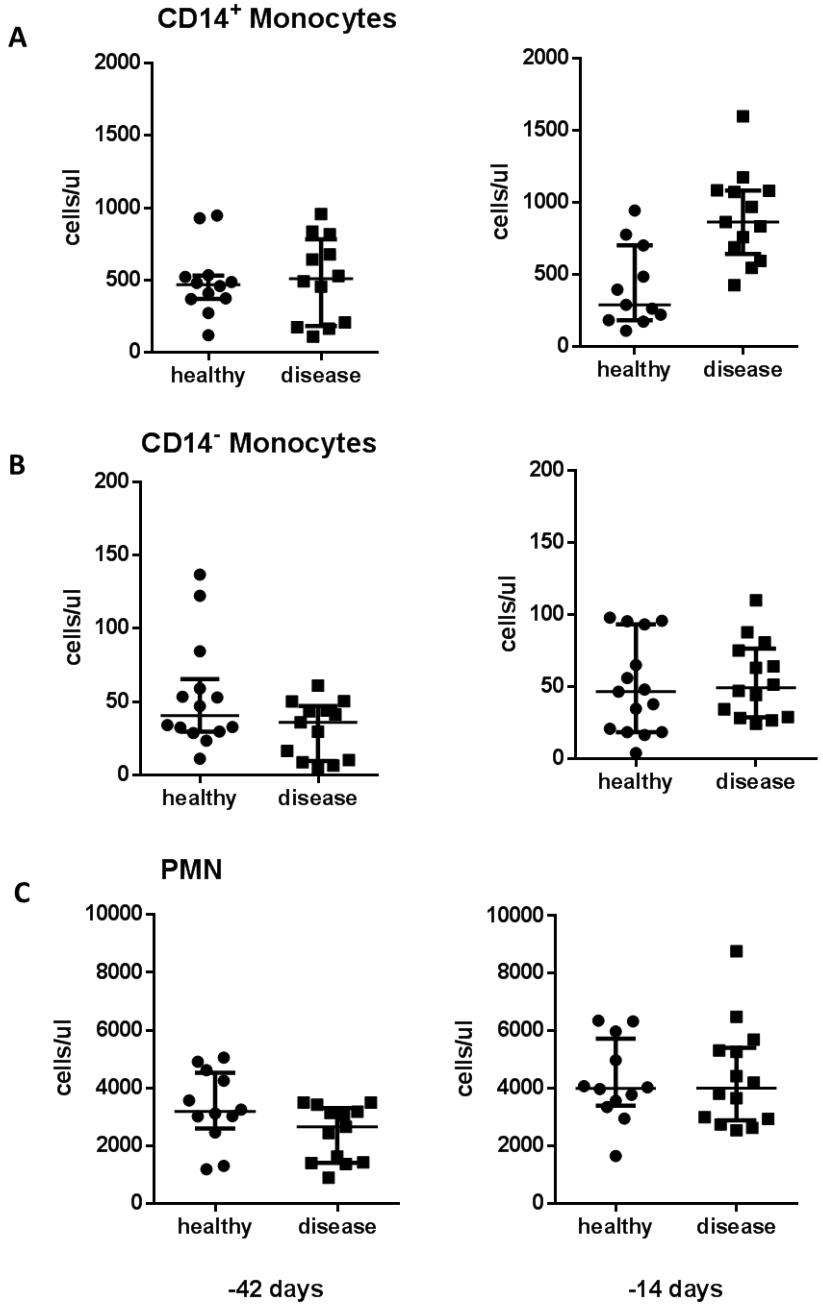

Figure 14. Monocyte and neutrophil counts in blood prepartum. Absolute counts of $CD14^+$ monocytes (A) and $CD14^-$ monocytes (B) and PMN (C) per µl blood at 42 and 14 days prior to expected calving date in 27 multiparous cows. 13 cows developed mastitis and/or metritis within 14 days postpartum (square symbols), 14 cows did not develop either disease (circular symbols). Individual values are shown with median and interquartile range.

Based on collinearity of cM and intM identified by Spearman's correlation coefficients and similar biological function (Hussen et al. 2013) between these two $CD14^+$ subsets, we chose to group $CD14^+$ subsets into a single variable of absolute count of all $CD14^+$ monocytes. Only ncM are $CD14^-$, and thus absolute count of $CD14^-$ monocytes included only ncM.

3.2. Multivariable regression models

Based on these descriptive analyses two separate models were generated for data from 42d prior to expected calving date and 14d prior to expected calving date and changes in cell counts over the days were also analyzed. At 42d prior to the expected calving date, the final model selected with a forward stepwise selection included the change in absolute counts of CD14+ monocytes from 42d to 14d prior to expected calving date ($\Delta_{14d\text{-}42d}$ CD14+), and absolute count of CD14- monocytes at 42d prior to expected calving date (Table 3-5, Fig. 15). At 14d prior to the expected calving date, the final model selected with a forward stepwise selection included the absolute count of CD14+ monocytes and absolute count of CD14- monocytes at 14d prior to expected calving date (Table 6-8, Fig. 16). A flow chart summarizing the models findings on the statistically significant relationships between 42d and 14d prepartum absolute cell counts and postpartum disease are depicted in figures 15 and 16, respectively; parameters which decreased the risk of acquiring a postpartum disease are indicated with '-' and those which increased the risk of acquiring postpartum disease are indicated with '+'.

Table 3. Multivariable analysis, Model 1 selection: only data collected at 42 days prior to calving date used to generate model. Model 1b is the best fitting model.

42d prior to calving model	-2Loglikelihood	AIC	Likelihood ratio	P-value
Base Model: Parity + BCS + Vaccination status	28.89	36.89	-	-
Model 1a: Parity + BCS + Vaccination Status + ΔCD14+	20.63	30.63	8.27	0.004
Model 1b: Parity + BCS + Vaccination Status + ΔCD14+ + CD14-	11.80	23.80	8.83	0.003
Model 1c: Parity + BCS + Vaccination Status + CD14+/CD14- ratio	22.54	32.54	6.36	0.012

Table 4. Parameter estimates for final model selected for 42d prior to expected calving date. Exact p-values calculated using a $\chi 2$ distribution; significance based on the likelihood ratio test.

Parameter	β	Standard Error	χ^2	P-value
Intercept	-6.2125	2.953	-	-
Parity (>2)	5.9317	2.8295	8.91	0.0028
Parity (=2, reference)	0	0	-	-
BCS (low, <3)	-0.4746	1.9196	0.06	0.8
BCS (high, >3, reference)	0	0	-	-
Vaccine (yes)	0.6141	1.5264	0.16	0.6879
Vaccine (no, reference)	0	0	-	-
CD14- monocyte count (cells/µl)	-0.1135	0.0615	8.83	0.003
$\Delta_{14d-42d}$ CD14+	0.0059	0.0032	7.75	0.0054

Table 5. Odds ratio estimates and profile-likelihood confidence intervals for significant explanatory variables from final model selected for 42d prior to expected calving date. Unit refers to the change in number of units the odds ratio estimate was based.

Parameter	Unit	Odds Ratio Estimate	95% Confidence Limits	
CD14- monocyte count (cells/µl)	5.0000	0.567	0.213	0.860
$\Delta_{14d-42d}$ CD14+	200.0	3.270	1.304	18.950

Table 6. Multivariable analysis, Model 2: Only data collected at 14 days prior to calving date used to generate model. Model 2b was the best fitting model.

14d prior to calving model	-2Loglikelihood	AIC	Likelihood ratio	P-value
Base Model: Parity + BCS + Vaccination status	28.89	36.89	-	-
Model 2a: Parity + BCS + Vaccination Status + CD14+	17.68	27.68	11.21	0.0008
Model 2b: Parity + BCS + Vaccination Status + CD14+ + CD14-	8.45	20.45	9.23	0.0024
Model 2c: Parity + BCS + Vaccination Status + CD14+/CD14- ratio	20.82	30.82	8.087	0.0045

Table 7. Parameter estimates for final model selected for 14d prior to expected calving date. Exact p-values calculated from likelihood ratio using a $\chi 2$ distribution; significance based on the likelihood ratio test.

Parameter	β	Standard Error	χ^2	P-value
Intercept	-7.7997	5.2934	-	-
Parity (=2)	1.5204	2.1404	0.61	0.4346
Parity (>2, reference)	0	0	-	-
BCS (low, <3)	-11.753	7.5015	8.86	0.0029
BCS (high, >3, reference)	0	0	-	-
Vaccine (yes)	-9.9861	6.845	5.67	0.0173
Vaccine (no, reference)	0	0	-	-
CD14- monocyte count (cells/µl)	-0.2536	0.1658	9.23	0.0024
CD14+ monocyte count (cells/µl)	0.044	0.0272	20.44	<.0001

Table 8. Odds ratio estimates and profile-likelihood confidence intervals for significant explanatory variables from final model selected for 14d prior to expected calving date. Unit refers to the change in number of units the odds ratio estimate was based.

Parameter	Unit	Odds Ratio Estimate	95% Confidence Limits	
CD14- monocyte count (cells/µl)	5.0000	0.281	0.016	0.811
CD14+ monocyte count (cells/µl)	50.0000	9.033	1.591	635.660

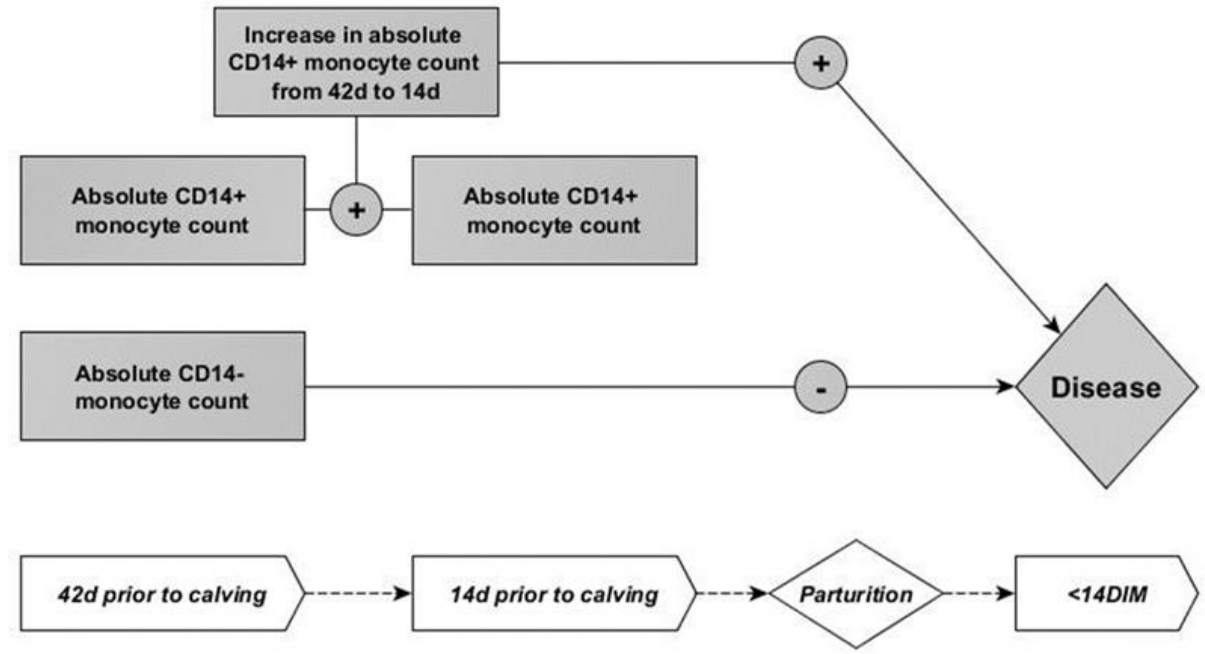

Figure 15. Results from final model selected for data from 42 days prior to calving date. Greater increase in peripheral CD14+ monocyte counts from 42 days prior to 14 days prior to expect calving date associated with increased risk of postpartum disease <14 days post-calving (increased risk indicated as '+'). Higher peripheral CD14- monocyte counts at 42 days prior to expected calving date associated with a decreased risk of postpartum disease <14 days post-calving (decreased risk indicated as '-').

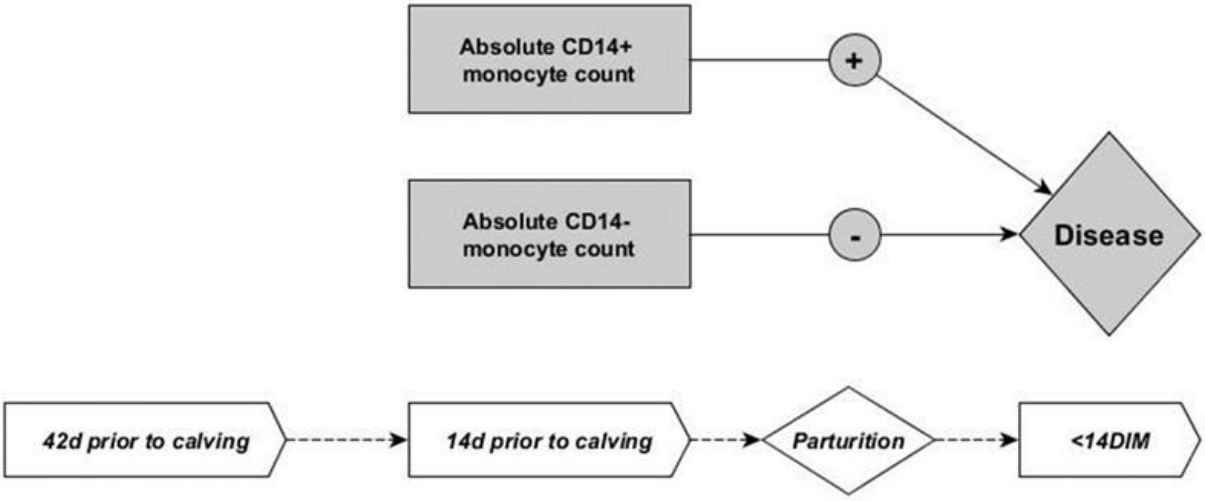

Figure 16. Results from final model selected for data from 14 days prior to calving. Higher peripheral CD14+ monocyte counts at 14 days prior to expect calving date associated with increased risk of postpartum disease <14 days post-calving (increased risk indicated as '+'). Higher peripheral CD14- monocyte counts at 14 days prior to expected calving date associated with a decreased risk of postpartum disease <14 days post-calving (decreased risk indicated as '-').

The absolute count of CD14- monocytes at 42d and 14d prior to expected calving date were significantly associated with a reduced risk of acquiring postpartum disease (Tables 3-8, Fig. 15&16). The odds ratio estimate for CD14- monocyte count was calculated based on if the count in blood were to increase by 5 cell/μl due to the relatively low numbers typically found in circulation; these odds ratio estimates were 0.567 and 0.281 at 42d and 14 d prior to expected calving dates, respectively (Tables 5&8). Models showed the increase in $\Delta_{14d\text{-}42d}$ CD14+ monocyte count and the absolute counts of CD14+ monocytes at 14d prior to expected calving date were significantly associated with increased risk of postpartum disease (Tables 3-8). The odds ratio estimate for $\Delta_{14d\text{-}42d}$ CD14+ monocyte count was calculated based on if the $\Delta_{14d\text{-}42d}$ CD14+ monocyte count were to increase by 200 cell/μl due to the relatively high numbers of CD14+ monocytes typically found in circulation and changes observed between 42 and 14d prior to the expected calving date; the odds ratio estimate was 3.270 for $\Delta_{14d\text{-}42d}$ CD14+ monocyte count at 42d prior to expected calving date (Table 5). The odds ratio estimate for CD14+ monocyte count was calculated based on if the count in blood were to increase by 50 cell/μl due to the relatively high numbers typically found in circulation; the odds ratio estimate was 9.033 for CD14+ monocyte count at 14d prior to expected calving date (Table 8). Interestingly, absolute count of PMN circulating in the blood was not found to be a significant predictor of postpartum disease (metritis and mastitis) in either model selected using data from 42d or 14d prior to expected calving date given the data on monocyte subsets (Table 3&6). The original study design variables for parity, BCS, and vaccination status included as confounding variables in the model were found to have significant relationships with disease, although these relationships were not significant at all time points; a parity of greater than 2 increased disease risk in the model for -42d samples, a BCS less than 3 decreased risk of disease in the model for -

14d samples, and vaccination in the prepartum period decreased risk of disease in the model for -14d samples (Tables 4&7).

4. Discussion

Based on the multivariable models described here, the composition of monocyte subsets in peripheral blood differs in cows that develop disease within 2 weeks postpartum compared to cows that remain healthy. Changes in the numbers of CD14- monocytes and CD14+ monocytes in the periphery predict the development of postpartum disease (mastitis, metritis). Higher counts of circulating CD14- monocytes at 42 and 14 days prior to calving reduced the probability of acquiring postpartum disease, whereas, an increase in CD14+ monocyte counts from 42 to 14 days and the CD14+ monocyte counts at 14d prior to calving increased the probability of acquiring postpartum disease (Tables 5&8, Fig 15&16). The results of the regression analyses were presented in a pictorial way in figures 15 and 16. A '+' in the figures represents a higher risk of disease whereas a '-' represents a lower risk. These positive or negative indicators of disease risk correspond to positive or negative value of the regression coefficient, β, in tables 4 and 7. The CD14+ subsets account for the majority of monocytes found in circulation with cM being the dominating cell population predicting an increased disease risk. As mentioned previously, the two bovine CD14+ subsets (cM, intM) have overlapping functions that differ from the CD14- monocyte subset (ncM). For example, both cM and intM respond to neutrophil degranulation products, migrate in presence of CCL5, and have high phagocytic capacity, whereas ncM do not (Hussen et al., 2013, Hussen et al., 2014, Hussen et al., 2016). Nonclassical monocytes are described as being the most mature of all monocytes subsets (Hussen et al., 2013). Bovine ncM have limited capacity to respond to LPS with inflammation or neutrophil degranulation products compared to the other subsets (Hussen et al., 2013, Hussen et al., 2014,

Hussen et al., 2016). In humans, ncM have a unique ability to patrol healthy tissue, and bovine ncM express adhesion molecules associated with similar behavior, suggesting that ncM are the first of the monocyte subsets to recognize and respond to infection (Geissmann et al., 2003, Hussen et al., 2016). Therefore changes in peripheral monocyte composition could lead to disturbances in primary recognition of invading pathogens within tissue as well as in the balance of specific inflammatory/anti-inflammatory responses to follow. In non-bovine species, diet composition, circadian rhythm, glucocordicoids, and vitamin D3 modulate blood monocyte subset composition, but there are currently no published studies addressing the regulation of blood monocyte composition in cattle, thus it cannot be speculated at this time what factors may cause the differences we observed (Nguyen et al., 2013, Devevre et al., 2015, Liu et al., 2015). Nonetheless, prepartum blood monocyte composition appears to be correlated with postpartum immune competency.

Peripheral monocytes migrate into tissues either in steady state or during inflammation where they may differentiate into macrophages or dendritic cells. Similar to rodents and humans, in bovine pregnancy, macrophages accumulate in the endometrium and are present in large numbers in interplacentomal and placentomal endometrium; CD14+ cells in circulation share similar gene expression profiles to macrophages in the endometrium, indicating blood monocytes likely migrate to endometrium and differentiate into macrophages (Oliveira and Hansen, 2008, Oliveira and Hansen, 2009). Tissue-resident macrophages and recruited monocytes play an important role in tissue repair in the reproductive tract postpartum. The composition of tissue-resident monocytes and monocyte derived cells in the reproductive tract and mammary gland during bovine late gestation is likely dependent on the available reservoir, migratory capacity, and functional differences of the monocyte composition in blood therefore, changes in monocyte

populations likely influence disease susceptibility (Sanchez-Torres et al., 2001, Randolph et al., 2002, Hussen et al., 2014, Hussen et al., 2016).

Though no concrete conclusions can be made on the exact role of these subsets in bovine postpartum disease at this time, this is the first piece of striking evidence which clearly shows a relationship between monocyte subset composition prepartum and postpartum disease and alludes to the different roles of CD14+ and CD14- subsets in disease susceptibility.

In contrast to others who described relationships between neutrophil function in the periparturient period, negative energy balance and metritis postpartum, this model found no relationship between neutrophil counts in the peripheral blood at 42 and 14 days prior to calving and postpartum disease (Cai et al., 1994, Hammon et al., 2006). The data used generate the models presented here do not include data on functional properties of either neutrophils or monocyte subsets from these animals. Though prepartum numbers of neutrophils readily available to migrate into tissue was not associated with postpartum disease in these animals like monocyte subsets are, the risk of disease may still be impacted by neutrophil function like described in previous studies (Guidry et al., 1976, Heyneman et al., 1990, Hammon et al., 2006). The original study design variables for parity, BCS, and vaccination status had significant relationships with disease, although these relationships were not significant at all time points the relationships were biologically plausible based on peer-reviewed literature on trained immunity from vaccination, the role of metabolic factors in disease, and relationships between parity and immune function and disease; prepartum vaccination, lower BCS (<3), and animals of a parity of 2 had decreased risk of disease (Table 4&7) (Gilbert et al., 1993, Roche et al., 2009, Saadatian-Elahi et al., 2016). For example, Gilbert et al. (Gilbert et al., 1993) found cows with a parity 4 or greater had reduced neutrophil function in the periparturient period.

Our results clearly indicate that peripheral blood monocyte composition relates to the overall immune competence of the cow postpartum. Though exact mechanisms which heighten susceptibility to postpartum disease cannot not be determined by this model, immune dysfunction postpartum seems to become apparent by blood monocyte composition as early as 42d prepartum. These findings provide new insight on maternal immune status and its relationship to postpartum disease. Prepartum monocyte composition could be a potential biomarker to identify cows at risk with compromised immune function, and therefore could aid in animal selection for immunomodulation therapies. Future research should address the specific roles of bovine monocyte subsets in disease and address findings from the model described here.

The model presented here demonstrates that changes in the numbers of CD14- monocytes and CD14+ monocytes in the periphery prepartum predict the development of postpartum disease (mastitis, metritis). We found higher counts of circulating CD14- monocytes at 42 and 14 days prior to calving reduced the probability of acquiring postpartum disease, whereas, an increase in CD14+ monocyte counts from 42 to 14 days/at 14d prior to calving increased the probability of acquiring postpartum disease. Our findings highlight the need for further investigation on the function and regulation of individual monocyte subsets in pregnancy and early lactation in order to elucidate their role involved in disease susceptibility. A greater understanding of late gestation immune status and its relationship to postpartum disease will allow for development of immunomodulators and improvements in dry cow management that will reduce the risk of postpartum disease.

5. Competing interests

The authors declare that they have no competing interests.

6. Authors' contributions

Experimental study design (HJS). Sample collection/preparation, data acquisition/processing (JH, ME, HJS). Interpretation of flow cytometric data (AS, BP, JH, HJS). Descriptive analysis of complete data set (BP, AS). Statistical analysis/logistic regression model development (BP, YHS). Writing of manuscript (BP, AS, YHS). Manuscript editing (JH, HJS).

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ADDITIONAL FILES

***Additionalfile1_dataset.dox*. Additional file 1. Complete dataset.** This file contains a table of the complete dataset from all enrolled cows used for descriptive analysis and statistical analysis (Materials and methods).

Table 2S. Complete data set used for descriptive and statistical analysis. Abbreviations: Disease (0=no disease, 1=disease recorded postpartum), individual cow identification number (Cow ID), time point (TP, -42= 42d prior to expected calving date, -14= 14d prior to expected calving date), absolute counts of classical monocytes (cM), absolute counts of intermediate monocytes (intM), absolute counts of non-classical monocytes (ncM), body condition score (BCS, 1= BCS<3, 2= BCS>3), Parity (1= parity=2, 2= parity>2), absolute counts of CD172+ CD14+ cells (CD14pos), absolute counts of CD172a+CD14- cells (CD14neg), absolute counts of PMN (Neutrophil), vaccine status (Vacc, 0= no BVD prepartum vaccination, 1= yes BVD prepartum vaccination).

Disease	CowID	TP	cM	intM	ncM	BCS	Parity	CD14+	CD14-	PMN	Vacc
1	2	-42	703	133	50	1	1	836	50	2435.3	0
1	2	-14	854	114	63	1	1	968	63	4212.1	0
0	7	-42	482	79	43	1	2	561	43	1306.5	1
0	7	-14	741	167	51	1	2	908	51	2963.6	1
1	20	-42	448	81	30	1	1	529	30	3050	0
1	20	-14	788	74	28	1	1	863	28	5282	0
1	27	-42	581	61	36	2	1	643	36	3135.6	0
1	27	-14	445	242	81	2	1	688	81	6497	0
1	30	-42	169	38	44	1	1	207	44	1400	1
1	30	-14	996	84	64	1	1	1080	64	3670.4	1
0	32	-42	759	171	53	2	1	929	53	4914	0
0	32	-14	230	32	18	2	1	262	18	6336	0
0	35	-42	490	45	47	2	1	535	47	4253.4	0
0	35	-14				2	1				0
0	37	-42	419	62	34	2	1	481	34	3019.4	1
0	37	-14	189	33	38	2	1	222	38	3785.5	1
0	38	-42	146	127	122	2	1	273	122	1189	1
0	38	-14	890	142	95	1	1	1032	95	4043.2	1
1	46	-42	823	135	41	1	1	958	41	3180.8	1
1	46	-14	912	261	24	1	1	1173	24	2752	1
0	47	-42	776	171	85	1	1	947	85	4617	0
0	47	-14	803	140	96	1	1	943	96	3984	0
1	50	-42	633	45	61	2	1	677	61	3492	0
1	50	-14	897	174	88	2	1	1071	88	4440	0
1	618	-42	98	11	9	2	2	109	9	3425	0
1	618	-14	678	79	29	2	2	757	29	5328	0
0	716	-42	344	65	33	1	2	409	33	3254.7	0
0	716	-14	97	12	4	1	2	109	4	5987.8	0

0	736	-42				2	2				1
0	736	-14	669	107	98	2	2	775	98	4089	1
1	749	-42	134	40	10	2	2	174	10	2652	0
1	749	-14	1446	149	44	2	2	1596	44	8768	0
0	802	-42	306	67	59	1	2	373	59	2460	1
0	802	-14	434	50	65	1	2	483	65	3577	1
1	810	-42	387	105	6	2	2	492	6	1625.4	0
1	810	-14	418	126	75	2	2	545	75	2953.2	0
0	825	-42	379	108	32	2	2	487	32	3031	1
0	825	-14	148	25	21	2	2	173	21	3359.4	1
0	833	-42	315	54	23	1	2	369	23	1431	1
0	833	-14	328	67	35	1	2	395	35	3013.4	1
1	861	-42	280	175	16	2	2	456	16	1374.7	1
1	861	-14	749	84	34	2	2	833	34	5700	1
0	903	-42	449	73	30	1	2	522	30	5056	1
0	903	-14	162	21	16	1	2	183	16	6363	1
0	905	-42	103	17	11	2	2	119	11	3572.8	0
0	905	-14	555	147	93	2	2	701	93	4991.3	0
1	911	-42	139	25	5	1	2	164	5	896	0
1	911	-14	465	128	27	1	2	593	27	2556	0
0	913	-42	394	65	29	1	2	459	29	3120	0
0	913	-14	256	33	18	1	2	289	18	1665	0
1	926	-42				1	2				1
1	926	-14	395	29	47	1	2	424	47	2639.4	1
1	930	-42	675	143	50	2	1	818	50	3500	1
1	930	-14	949	133	110	2	1	1082	110	3828.5	1

***Additionalfile2_parity+counts.dox*. Additional file 2. Cell counts by parity.** This file contains a table summarizing descriptive analysis of cell count by parity group (Results).

Table 3S. Peripheral blood monocyte and neutrophil cell counts (cells/µl) grouped by parity and disease status. Median counts with interquartile range (IQR) presented.

			Healthy			
Parity>2 (n=9)	Neutrophils (cells/µl)		CD14- monocytes (cells/µl)		CD14+ monocytes (cells/µl)	
Days prepartum	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
-42	3075.500	1468	31.08834	12.08982	433.7490	133.34564
-14	3468.200	2501	27.67735	40.61797	341.9845	414.47448

Parity=2 (n=5)	**Neutrophils (cells/µl)**		**CD14- monocytes (cells/µl)**		**CD14+ monocytes (cells/µl)**	

Days prepartum	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
-42	3818.200	2661	68.99881	59.69023	705.1991	561.33173
-14	4013.600	1305	66.50144	67.29404	602.5972	745.55171

Postpartum Disease

Parity>2 (n=6)	Neutrophils (cells/µl)		CD14- monocytes (cells/µl)		CD14+ monocytes (cells/µl)	
Days prepartum	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
-42	1625.400	1277	8.665826	3.84636	174.1679	291.22023
-14	5328.000	2747	34.17388	15.10259	757.2828	240.06350

Parity=2 (n=7)	**Neutrophils (cells/µl)**		**CD14- monocytes (cells/µl)**		**CD14+ monocytes (cells/µl)**	
Days prepartum	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
-42	3135.600	1057	43.90255	14.47735	677.4227	307.80345
-14	4212.100	1612	63.93440	59.29398	1070.528	219.94237

CHAPTER 6:
MATHEMATICAL MODELING OF INTRAMAMMARY *E. COLI* INFECTION DYNAMICS IN THE DRY PERIOD

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KEYWORDS: *E. coli*, dry period, modeling, bovine, intramammary infection

ABSTRACT

Dairy cows have increased risk of intramammary infections with *Escherichia coli* in the non-lactating period of late gestation (known as the ‘dry period). We expand upon an existing mathematical model to explore the role of factors unique to the dry period and late gestation immunity. We included biological knowledge of the bovine immune response in late gestation and known characteristics of the mammary gland in the steady-state portion of the dry period. The results indicate that intramammary infection dynamics of the dry period are complex and pertain to shifts in immune function in late gestation and changes to the mammary gland during involution and stead-state dry period. Only by varying growth rate of *E. coli* to that in non-lactating gland secretion, initial number of phagocytes in the gland prior to infection, rate of phagocytosis and killing of *E. coli* by phagocytes, and pro- and anti-inflammatory cytokine production reproduced the data for infections caused by persistent *E. coli* in the dry period. The changes to the mammary gland related to the process of involution and within a steady state dry period enables persistence of *E. coli* infections in mammary glands. Knowledge of the pathogenesis of persistent infections in the dry period is essential to develop preventive and treatment programs for intramammary infections throughout the dry period in dairy cows.

1. Introduction

Mastitis is one of the most prevalent and costly diseases for dairy farms (Burvenich et al., 2003, Hertl et al., 2014a, Hertl et al., 2014b). The majority of clinical mastitis cases occur within the first 60 days post-calving and cause severe losses in milk production that may span the remainder of lactation (Barkema et al., 1998, Schukken et al., 2011, Schukken et al., 2012). The non-lactating period of late gestation known as the 'dry period', primarily around dry-off and colostrogenesis, is a known time of heightened susceptibility to new intramammary infections (IMI); these IMI often persist until parturition without visible signs of inflammation, and develop into clinical mastitis following the onset of lactation (Smith et al., 1985a, Green et al., 2002, Green et al., 2005, Burvenich et al., 2007, Quesnell et al., 2012, Pomeroy et al., 2016a). Among the mastitis-causing pathogens *Escherichia coli* is the most common isolate from dry period associated with clinical mastitis in the subsequent lactation (Smith et al., 1985, Green et al., 2002, Green et al., 2005). Quesnell et al. (2012) and Gurjar et al. (2013) found when multiparous dairy cows are experimentally challenged in the dry period with *E. coli* ECC-Z, a strain known to cause mild clinical mastitis, the infection persists up until parturition without clinical signs or upregulation of pro-inflammatory cytokines (TNFα, IL-1β, IFNγ); following calving, cows develop clinical mastitis and upregulate pro-inflammatory cytokines in challenged quarters. Sipka et al. (2013) found when mid-lactation multiparous cows are given similar intramammary challenges with *E. coli* ECC-Z, cows developed clinical mastitis and upregulated pro-inflammatory cytokines within 24hr following challenge. The severity of *E. coli* mastitis and response to infection are thought to be largely dependent on cow factors (Burvenich et al., 2003, Schukken et al., 2011). The specific factors associated with late gestation and concurrently the

dry period which impact IMI dynamics and allow for persistence of infection without clinical signs in the dry period are poorly understood.

The maternal immune system undergoes regulation to tolerate the growing semi-allogenic fetus, and this regulation has been shown to influence susceptibility to infection and immune response in mammalian pregnancy (Oliveira and Hansen, 2008, Rosbottom et al., 2008, Pomeroy et al., 2015). Highly inflammatory, T helper 1 (Th1) and T helper 17 (Th17)-type responses in pregnancy have been shown to be detrimental to the health of the fetus, and facets of these cell-mediated responses are greatly hindered during late gestation (Rosbottom et al., 2008, Betz, 2012, Krishnan et al., 2013). Furthermore, during the dry period there is a delay in leukocyte recruitment to the mammary gland by approximately a week following the onset of dry-off until protective levels are reached; additionally, upon reaching the gland these cells have decreased phagocytic function from ingestion of milk fat and cell debris in the early dry period (Burvenich et al., 2007). During the mid-dry period the gland remains in steady-state involution and the teat ends are sealed, and there is a high concentration of phagocytes, lactoferrin, acute phase proteins, and other soluble mediators in the gland and secretion (Todhunter et al., 1990, Stelwagen et al., 2009, Pezeshki et al., 2010). Although in the mid-dry period conditions are unfavorable for bacterial growth and this period has the lowest incidence of new IMI, existing IMI may persist (Green et al., 2002, Green et al., 2005, Gurjar et al., 2013).

Previous work has indicated that the mechanisms which enable *E. coli* IMI to persist in lactation relate to the strain's ability to invade mammary epithelial cells (Dogan et al., 2006, White et al., 2010, Dogan et al., 2012). A mathematical model developed by White et al. (2010) elucidating mechanisms which differentiate transient from persistent *E. coli* IMI in lactation adduced that the ability of *E. coli* to create intracellular reservoirs was a key determinant in

whether the IMI would be acute or chronic. It is unclear if similar mechanisms described in the model from White et al. (2010) relate to the unique differences in IMI dynamics observed in the dry period. Mathematical modeling will provide valuable insight into the potential role of maternal immune regulation in late gestation and investigate other key parameters involved in IMI dynamics specific to the dry period.

The original mathematical model was compromised of a series of differential equations based on the interaction between the bovine immune system and numbers of extracellular *E. coli* in milk and in intracellular reservoirs within a quarter (White et al., 2010). Specifically, to investigate bacterial count and somatic cell count dynamics the model from White et al. (2010) included differential equations which described 1. Exponential growth of extracellular bacteria (E), 2. Ability of bacteria to move in and out of intracellular compartments to form intracellular reservoirs (I), 3. Phagocytosis and killing of extracellular bacteria by neutrophils (PMN) and macrophages (M), 4. Pro- and anti-inflammatory cytokine dynamics (C+, C-) which influenced the numbers of PMN recruited to the gland, and 5. Milk volume in the gland based on twice daily milking which impacted population size of bacterial cells. Biological data from *in vivo* and *in vitro* bovine and murine models were used to estimate initial conditions and parameter ranges including differential cell counts in milk, killing capacity of neutrophils, growth of extracellular *E. coli*, rates of invasion and intracellular survival, and pro- and anti-inflammatory cytokine concentrations observed in *E. coli* mastitis (IL-8, TNFα, IL-10).

Here we propose to expand the model presented in White et al. (2010) to incorporate unique changes associated with the dry period in late gestating dairy cattle. We will alter the existing model with stepwise alterations to evaluate the impact of individual changes and determine which parameters may be key drivers of *E. coli* IMI dynamics in the dry period. The

sequential steps that we have taken to adjust the model to late gestation dairy cows included the following steps: 1. Remove the differential equation and factors related to milking volume, 2. Modify the estimates for extracellular *E. coli* growth to reflect slowed growth in dry cow mammary gland secretions which contain increased immune factors and decreased available nutrients for the bacteria, 3. Modify the initial number of phagocytes in the gland to reflect increased numbers of macrophages and PMN typically observed in the dry period 4. Modify the estimates for phagocytosis to reflect the inhibited phagocytosis observed at various stages of the dry period, and 5. Modify the estimates and dynamics of cytokine dynamics to reflect maternal immune regulation observed in bovine and mammalian late gestation. With this study we aim to improve the understanding of IMI dynamics in the dry period, and specifically look at the role of maternal immune regulation in enabling persistence of IMI in the dry period.

2. Materials and Methods

2.1. The model

The original mathematical model created by White et al. (2010) models the interaction between the bovine immune system and numbers of *E. coli* (E) in milk and in intracellular reservoirs within a quarter (1 of the 4 compartments of the bovine mammary gland) in lactating animals. The original model assumed that the total number of colony forming units (cfu) of *E. coli* in milk (E) grows exponentially, but these extracellular bacteria are phagocytized and killed by macrophages and PMN. Similar to what is observed in human urinary tract infections caused by *E. coli,* White et al. (2010) allowed bacteria to move between milk and an intracellular MEC reservoir (I) (Dogan et al., 2006, Olson and Hunstad, 2016). White et al. (2010) modeled production of pro-inflammatory cytokines (C+) by phagocytes following *E. coli* stimulation as contact between bacteria and immune cells. The original model accounted for the production of

pro-inflammatory cytokines being inhibited by anti-inflammatory cytokines; anti-inflammatory cytokine production was dependent on pro-inflammatory cytokine production. Macrophages were assumed to remain constant whereas rate of PMN influx was dependent on pro-inflammatory cytokine production. Milk volume and twice daily milkings were included in the original model and depletion of milk volume impacts the extracellular bacterial numbers. Time (t) was measured in days. The original model presented in White et al. (2010) was able to model differences in IMI dynamics between persistent and transient *E. coli* IMI in lactating cows based upon the rate of invasion, survival, and escape of intracellular reservoir.

To identify the primary parameters involved in *E. coli* IMI dynamics observed in the dry period we first changed the values of parameters developed for lactating infection dynamics to values typical of the dry period and/or late gestation. The original model predicted *E. coli* strains which caused transient IMI had limited ability to form intracellular reservoirs when compared to chronic IMI; this related to ability of *E. coli* to invade MEC, survive in MEC, and remain in MEC.

Eliminating parameters for milk removal by twice daily milkings had little impact on infection dynamics. Thus, parameters for milk removal were removed for subsequent interrogations of the model; the maximum volume within a quarter was set to a level typical of the dry period (V=125ml) (Smith et al., 1966). As described in White et al. (2010), this simplified description of inflammatory dynamics leads to the following series of differential equations for the concentrations of cytokines (C_+ and C_-), and the numbers of phagocytes (P) and *E. coli* (E) in milk and intracellular *E. coli* (I):

$$\frac{dC_+}{dt} = \frac{g_{cp}}{\left(1 + i_{cp}C_-\right)} E(M + P) - d_c C_+$$

$$\frac{dC_-}{dt} = g_{ca}C_+ - d_cC_-$$

$$\frac{dP}{dt} = g_pC_+ - d_pP$$

$$\frac{dE}{dt} = g_cE - kE(M+P) - aE + \frac{bI}{V}$$

$$\frac{dI}{dt} = aEV - bI - d_iI$$

The equations for the concentrations of phagocytes and cytokines remained deterministic and continuous. A stochastic version for the bacterial population section of the model was used as follows:

$$E_{\mathrm{t+dt}} = \max\{\Theta[E_{\mathrm{t}} + (g_eE_{\mathrm{t}} - kE_{\mathrm{t}}(M+P) - aE_{\mathrm{t}} + bI_{\mathrm{t}}) * dt], 0\}$$

$$I_{\mathrm{t+dt}} = \max\{\Theta[I_{\mathrm{t}} + (aE_{\mathrm{t}} - bI_{\mathrm{t}} - d_{\mathrm{i}}I_{\mathrm{t}}) * dt], 0\}$$

where Θ[x] is sampled from a Poisson distribution of mean x. The Poisson distribution was the most appropriate for this system because it is a discrete distribution and expresses the probability of a number of events (of the type that occur with a known average rate and independently of the time since the previous event) occurring in a fixed period of time. Within this model an event is defined as a change in the population size due to growth or killing.

2.2. Biological data

2.2.1. Extracellular *E. coli* growth rate (g_e) *in vitro*

Mammary gland secretions vary in composition depending on stage of lactation and of the dry period. The composition of available nutrients and host immune factors impact bacterial

growth rate (Kornalijnslijper et al., 2003). In lactation, milk secreted from the gland is nutrient rich and relatively low in immune factors in contrast to secretions from the dry period which have limited nutrients and as increase in immune factors (Smith et al., 1966, Kornalijnslijper et al., 2003, Boutinaud et al., 2016). The quantity of secretion, lactose, potassium, casein, α-lactalbumin, β-lactoglobulin, and citrate in the gland decreases in early stages of drying off (Smith et al., 1966, Boutinaud et al., 2016). In contrast, milk somatic cell count, percent total protein, percent fat content, and lactoferrin concentrations increase in mammary gland secretions as cows progress further into the dry period (Boutinaud et al., 2016).

The original model predicted a high growth rate for *E. coli* growth in milk, a nutrient rich medium. Experimental work showed both *E. coli* ECC-Z, a strain isolated from a chronic bovine IMI, and *E. coli* ECA727, a strain isolated from a transient bovine IMI, had reduced range of exponential growth when grown in mammary gland secretion from dry cows (DCS) free of cellular components; this resulted in a growth rate estimate between 25.62 and 37.17 (Fig. 17). The *E. coli* strains used for growth curves were characterized in Dogan et al. (2006) and (2012). Growth curves were setup as described in Todhunter et al. (1990). Briefly, *E. coli* strains were grown in LB broth to log phase then diluted in PBS to approximately 100 CFU/10μL, and 10 μL of bacterial suspension was added to 250 μl of pooled DCS and plated in 96-well tissue culture plates with duplicate wells for each hourly time point. Plates were incubated at 37°C and designated wells were diluted and plated each hour on LB agar plates; plates were incubated at 37°C for approximately 12-18hr before counting. Todhunter et al. (1990) found majority of *E. coli* isolates had severely reduced growth in pooled dry cow secretion; from this paper exact growth rates per isolate cannot be calculated, but it can be estimated <13.4 CFU $\log_{10}$/day, with majority of strains not able to overcome the inhibitory effects of DCS and only 9.8% of *E. coli*

isolates able to grow at rates higher than 13.4 10 CFU log_{10}/day. The effect of reducing growth rate (g_e) in the model is depicted in figure 18 for both transient strains and chronic strains. Based on Todhunter et al. (1990) and *in vitro* data, extracellular *E. coli* growth rate (g_e) was estimated to be between 13.4-37.17 CFU log_{10}/day in the dry period.

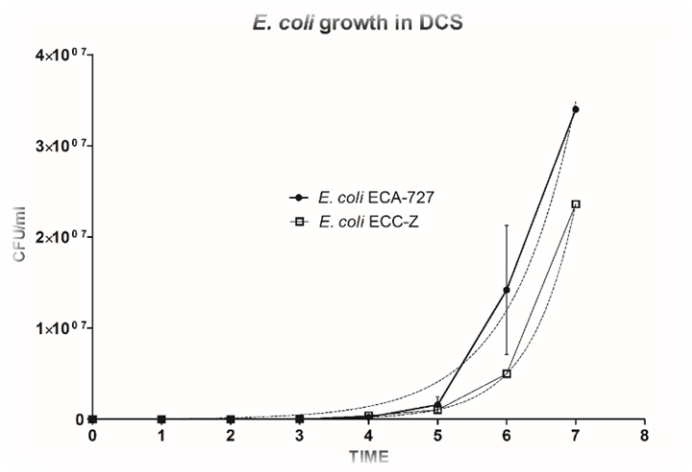

Figure 17. Growth curves of transient and persistent *E. coli* isolates in dry cow secretion. Dashed lines indicate exponential, non-linear fit to data. Mean growth with standard error represented at each hourly time point for transient strain, *E. coli* ECA-727 (black circle), and persistent strain, *E. coli* ECC-Z (white square). Experiments were run in duplicate. Time in days is shown on x-axis.

2.2.2. Initial SCC in the gland (P+M)

In lactation, healthy dairy cows have relatively low SCC (<100,000 cells/ml indicates healthy quarter); the cut-off for healthy SCC may vary depending upon stage of lactation, age/parity, season, stress, etc. (Pilla et al., 2012). The SCC rises drastically from a low SCC when a quarter is infected with *E. coli* during lactation and results in an episode of clinical mastitis; transient mastitis-causing strains often result in the greatest influx of PMN (Dopfer et al., 1999, Kornalijnslijper et al., 2003, White et al., 2010). The original model is able to recapitulate this rise in SCC following infection in transient strains, and in persistent mastitis-causing strains we see chronically high SCC with no complete clearance of extracellular bacteria.

Inchaisri et al. (2000) reported SCC of 5.43 cells $\log_{10}$/ml in milk prior to drying-off; this value was comparable to initial PMN values in the original mathematical model for lactating cows. During involution phagocytes, predominantly macrophages, are recruited to the gland as a normal process of involution (Nickerson, 1989, Inchaisri et al., 2000, Wedlock et al., 2004). Todhunter et al. (1990) found SCC rise in uninfected quarters to 7.2 cells $\log_{10}$/ml within a week following dry-off, and Inchaisri et al. (2000) found a similar increase in SCC during early stages of involution >6.5 cells $\log_{10}$/ml in healthy quarters and maintaining approximately 6.41 cells $\log_{10}$/ml in steady-state. The initial value for phagocytes in an uninfected, healthy gland in the dry period deviates drastically from that in a lactating animal and was assessed in the in model by allowing the initial value of PMN and macrophage to reach 5.40-6.19 cells $\log_{10}$/ml and 5.93-6.71 cells $\log_{10}$/ml, respectively. To maintain simplicity of the model macrophages (M) and PMN (P) were combined into one compartment representing phagocytes for as the original model stands the macrophage population is constant and relative small at 5,000 cells/ml).

2.2.3. Phagocytic killing (k) of extracellular bacteria (E)

The capacity of macrophages and PMN to phagocytose and kill bacteria can be modulated by factors including cytokines, complement, antibodies, hormones, lipids, etc. and thus, are likely to be influenced by changes to the mammary gland secretion composition reported in the dry period (Mullan et al., 1985, Subandrio et al., 2000, Denis et al., 2006, Levin et al., 2016). Due to lack of bovine data on macrophage and PMN killing rate of *E. coli,* the killing rate was originally estimated from *in vitro* data from mouse models; this value is likely to differ in the dry period. We estimated the ability for mammary gland phagocytes to phagocytose and kill *E. coli* was reduced in the dry period based on multiple *in vitro* studies which show the reduced capacity for PMN and macrophages isolated from mammary gland secretions to kill

various mastitis-causing bacteria (Mullan et al., 1985, Bassalik-Chabielska et al., 1988, Fox et al., 1988). Fox et al. (1988) reported as cows progressed into the dry period, phagocytes isolated from mammary gland secretions had reduced phagocytosis and killing of *E. coli*. Tjoelker et al. (1990) reported approximately an 8-10% decrease in phagocytosis of *S. aureus* at 2 weeks since dry-off. Paape et al. (1992) also report diminished phagocyte function with advancing stage of the dry period, with approximately 20% reduction in phagocytosis of *S. aureus* from 1-2 weeks prior to drying off to 15 to 22 days dry. Based on the literature described here, we estimated an 8-20% reduction in killing rate (k) by phagocytes in the dry period.

2.2.4. Cytokine growth rates (g_{cp}, g_{ca})

The original mathematical model made the following assumptions about cytokines and the immune response to IMI: production of pro-inflammatory cytokines is inhibited by the presence of anti-inflammatory cytokines, anti-inflammatory cytokines are rate dependent on the concentration of pro-inflammatory cytokines, and all pro-inflammatory cytokines or all anti-inflammatory cytokines share identical profiles and function (White et al., 2010). The original model also assumed macrophages present in the milk remain at a constant concentration, whereas the concentration of PMN increases at a rate dependent on the concentration of pro-inflammatory cytokines present in the milk (White et al., 2010). The model relied on *in vivo* experimental infection data from Bannerman et al. (2004) and (2008) as references.

Experimental *E. coli* IMI in the dry period with a persistent-mastitis-causing strain from Quesnell et al. (2012) and Pomeroy et al. (2016a) was used as a reference for the modified model for IMI in the dry period. Both Quesnell et al. (2012) and Pomeroy et al. (2016a) show that there are no detectable increases in pro-inflammatory cytokines in the dry period following *E. coli* challenge, but anti-inflammatory cytokine IL-10 is increased post-challenge. Pomeroy et

al. (2015) and (2016b) observed altered cytokine production by bovine monocyte-derived dendritic cells (moDC) in response to *in vitro* stimulation with *E. coli* ECC-Z in the dry period. Specifically, moDC generated from blood monocytes from dry cows had enhanced IL-10 production compared to early stages of lactation; depending on the stage of lactation or dry period compared, there is a 1-2.6 fold-increase in IL-10 production over a 24hr period. This range of fold increase was used in conjunction with the original models predicted value for anti-inflammatory cytokine production rate to investigate the impact of this parameter and estimate its value in dry period IMI dynamics. Changes to pro-inflammatory cytokines involved in cases of clinical mastitis were more difficult to estimate as not all pro-inflammatory cytokines were regulated in similar ways in the bovine moDC model in late pregnancy. For example, IL-12, a key Th1 cytokine, appeared to be downregulated in late gestation, with moDC from the first half of a 60 day dry period at approximately only 30% of production observed in moDC from early lactation (Pomeroy et al., 2015). Unlike IL-12, TNFα production was not found to be significantly different in moDC from the dry period compared to early lactation, and IL-1β had enhanced production in moDC from the dry period relative to early lactation (Pomeroy et al., 2016b). The ~30% reduction in the production of Th1-cytokine, IL-12 observed in moDC from dry cows was used as the maximum level of reduction in pro-inflammatory cytokine production from lactating values in model interrogation (Pomeroy et al., 2015). The upper estimate was the original value estimated from the original model of lactating cows.

2.2.5. Parameter estimation

The model predictions for the extracellular *E. coli* counts generated using the differential equations from White et al. (2010) were fitted to the data. The parameters were estimated within Berkeley-Madonna, by maximizing likelihood assuming a normal distribution of error. The

initial values for the parameter estimation are given in Table 9. Parameters values which were estimated by the model included rate of phagocytic killing (k), growth rate of *E. coli* in mammary gland secretion (g_e), rate of pro- and anti-inflammatory cytokine production (g_{cp}, g_{ca}), initial number of phagocytes present in the gland (int(P)), and influx rate of PMN to the gland (g_p). Although the influx rate of PMN to the gland (g_p) was not considered in sensitivity analysis, and rate of influx in non-lactating glands in the late dry period and lactating glands is unknown, this parameter value was shown to be cow-specific in the original model and thus left to be estimated with curve fitting (White et al., 2010).

3. Results

3.1. Influence of individual parameter values on model output

To understand how the uncertainty in the output of the original stochastic mathematical model can be apportioned to different sources of uncertainty in inputs known to deviate in the dry period we first changed one-factor-at-a-time. We found no major differences in dynamics e.g. time to clearance or persistence for transient or chronic-mastitis-causing *E. coli* when twice daily milk removal by milking was excluded from the model, respectively therefore, milking was excluded for subsequent deviations of the model. The discretized stochastic model was run 1000 times, and mean ±SD were calculated and are depicted in Figures 18-21 for one-factor-at-a-time sensitivity analysis.

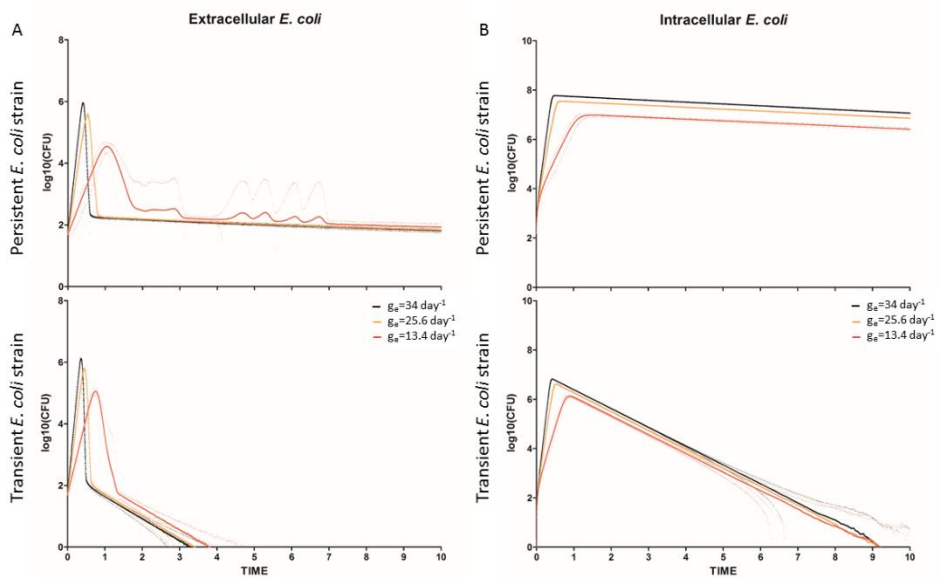

Figure 18. One-factor-at-a-time sensitivity analysis: varying growth rate of extracellular *E. coli* (g_e). Model outputs for extracellular *E. coli* (A), and intracellular *E. coli* reservoirs (B) for both persistent and transient *E. coli* strains when g_e parameter value is varied while other parameter values are fixed. Solid lines represent model output mean of 1000 actualizations of the model; dotted lines represent +/- standard deviation. Time in days is shown on x-axis.

The value of maximum *E. coli* bacteria numbers and the time at which this peak occurs is sensitive to the growth rate of extracellular bacteria (g_e) for both transient and chronic *E. coli* strains (Fig. 18). As the growth rate of extracellular *E. coli* (g_e) is reduced to estimated rates of *E. coli* growth in dry cow secretion (g_e= 13.4, g_e=25.6) the peak in extracellular bacteria and intracellular bacteria declines and the time at which maximum counts occurs is delayed. Decline in extracellular *E. coli* coincides with a decline in SCC, pro- and anti-inflammatory cytokine production (not shown). Changes to growth rate yields similar changes for both transient and chronic-mastitis causing *E. coli* strains. However, even at the lowest growth rate (g_e=13.4) we do not see bacterial numbers as low as observed *in vivo*. This parameter influences the model output but reduced g_e alone cannot explain differences in dynamics between lactating and dry period according to our mathematical model.

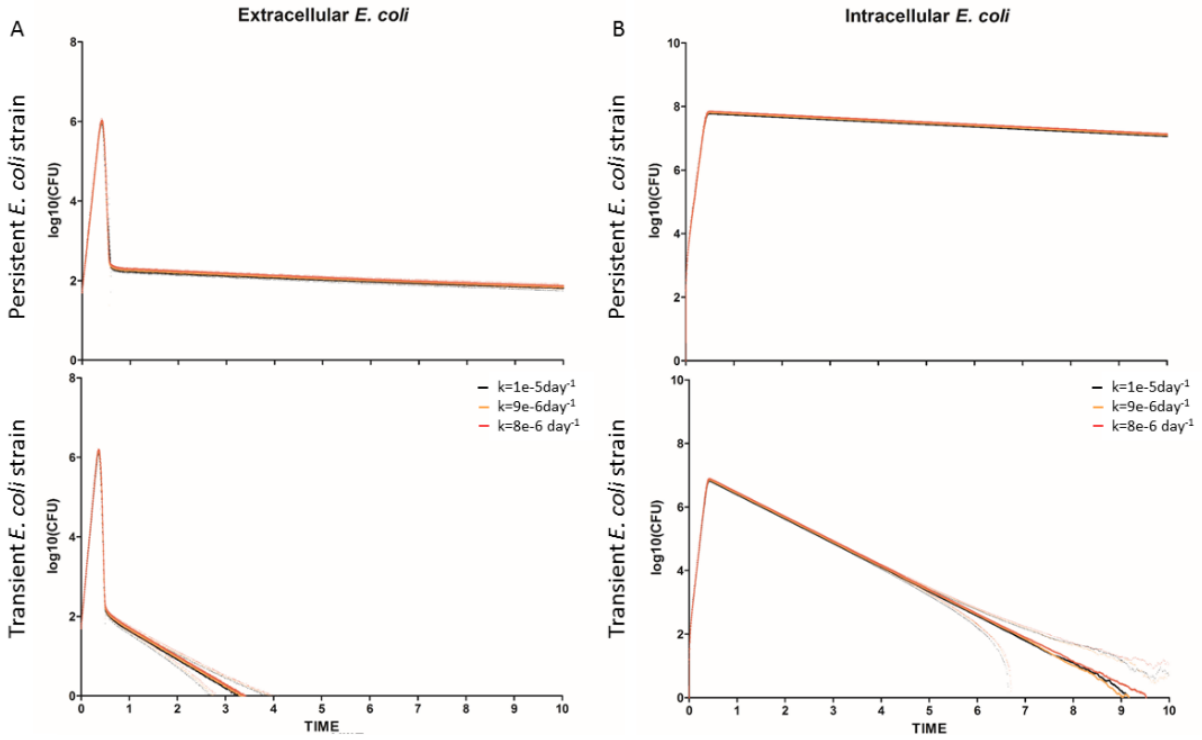

Figure 19. One-factor-at-a-time sensitivity analysis: varying rate of killing of extracellular *E. coli* by phagocytes (k).Model outputs for extracellular *E. coli* (A), and intracellular *E. coli* reservoirs (B) for both persistent and transient *E. coli* strains when k parameter value is varied while other parameter values are fixed. Solid lines represent model output mean of 1000

actualizations of the model; dotted lines represent +/- standard deviation. Time in days is shown on x-axis.

The value of maximum *E. coli* bacteria numbers and the time at which this peak occurs is not highly sensitive to the killing rate of extracellular bacteria by phagocytes (k) for both transient and chronic *E. coli* strains (Fig. 19). There was only a minute increase in *E. coli* bacteria numbers with reduced killing rates, but time to peak bacterial counts was not effected nor was overall shape of model output. Sole changes to this parameter minimally influences the model output but may be involved in IMI dynamics in the dry period as an interaction with other dry period-altered model inputs.

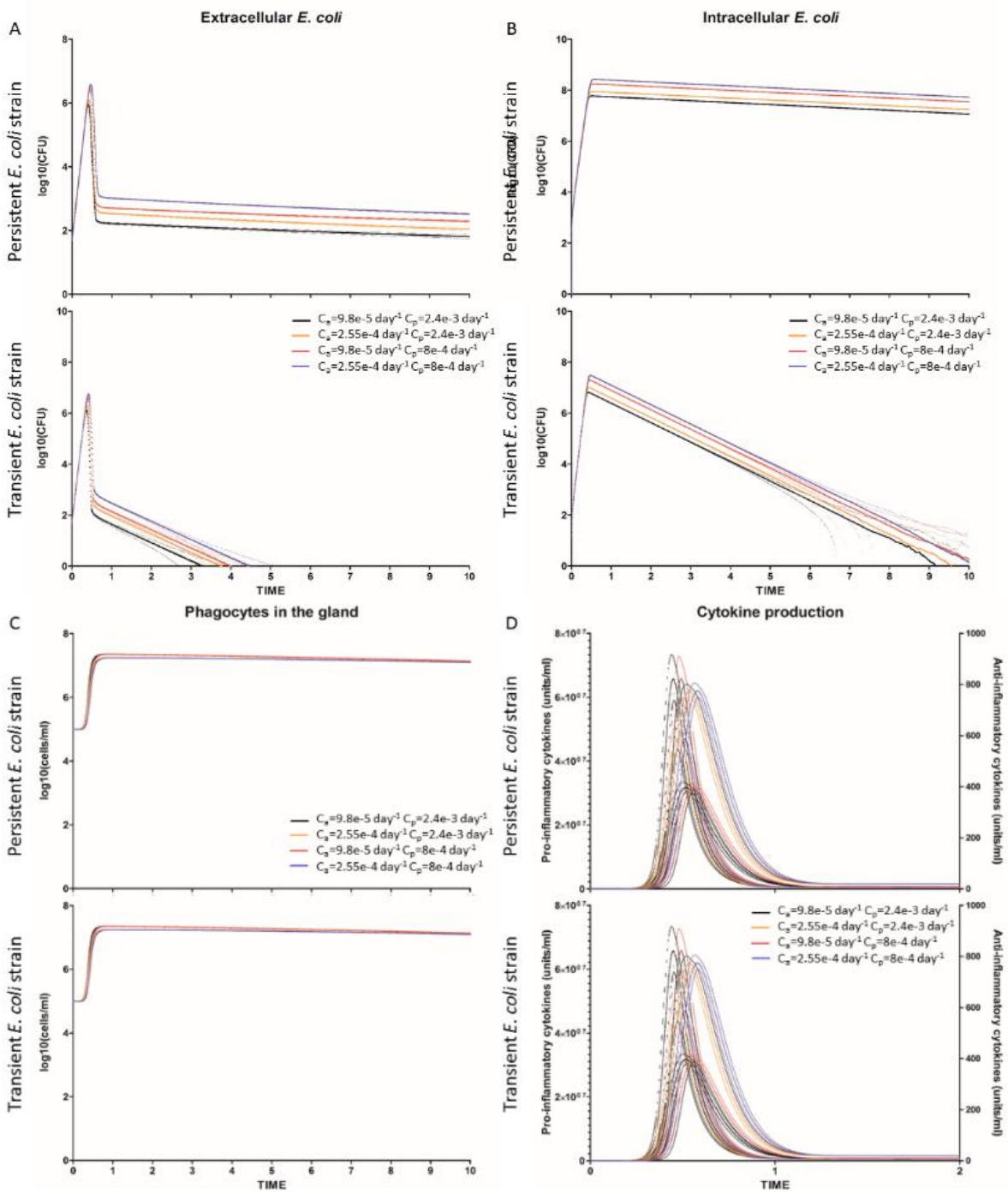

Figure 20. One-factor-at-a-time sensitivity analysis: varying rate of pro- and anti-inflammatory cytokine production by phagocytes (g_{cp}, g_{ca}). Model outputs for extracellular *E. coli* (A), intracellular *E. coli* reservoirs (B), PMN numbers (C), and pro- and anti- inflammatory cytokine production (D) for both persistent and transient *E. coli* strains when g_{cp} and g_{ca} parameter values are varied while other parameter values are fixed. Solid lines represent model output mean of 1000 actualizations of the model; dotted lines represent +/- standard deviation. Time in days is shown on x-axis.

The value of maximum *E. coli* bacteria numbers is sensitive to the growth rate of pro- and anti-inflammatory cytokine production (g_{cp}, g_{ca}) in model outputs for both transient and chronic *E. coli* strains (Fig. 20). As the rate of anti-inflammatory cytokine production is increased to estimated rates in late gestation cows the peak extracellular bacteria and intracellular bacteria numbers increases, however, the time at which maximum counts occurs was not affected. Increases to rate of pro-inflammatory cytokine production also increased *E. coli* bacteria numbers, and when both input values for pro- and anti-inflammatory cytokines were altered to late gestation-estimated levels the effect on *E. coli* bacteria numbers was additive. The impact of cytokine production rate values on other outputs is minimal, and only related to changes in anti-inflammatory cytokine production (Fig. 20). These model inputs influence the model output but cannot recapitulate the cytokine profiles in the dry period or bacterial counts

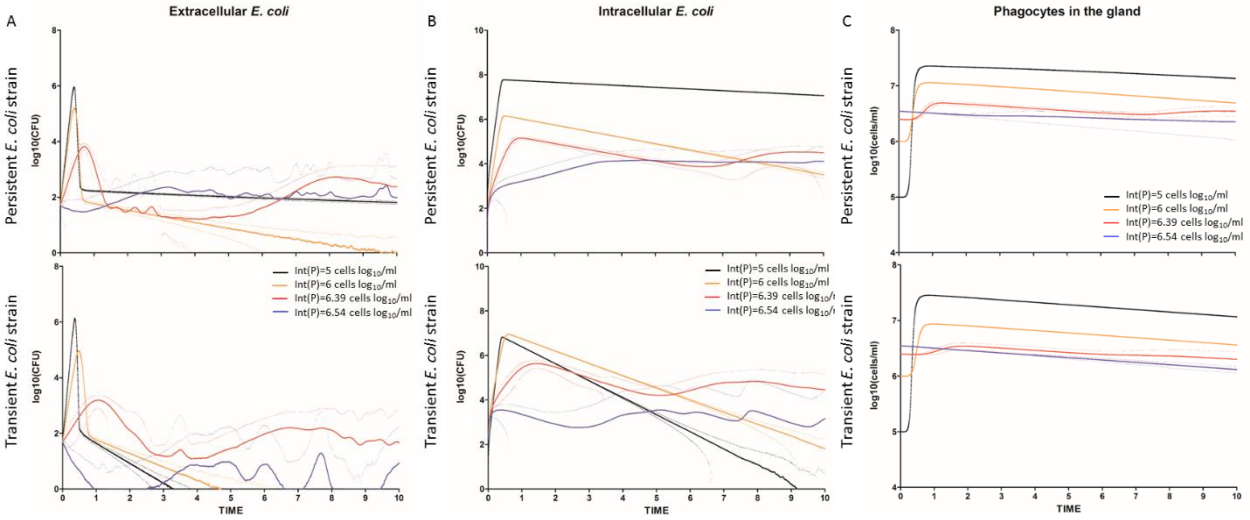

Figure 21. One-factor-at-a-time sensitivity analysis: varying initial number of phagocytes in the gland (int(P)). Model outputs for extracellular *E. coli* (A), intracellular *E. coli* reservoirs (B), and PMN numbers (C) for both persistent and transient *E. coli* strains when int(P) value is varied while other parameter values are fixed. Solid lines represent model output mean of 1000 actualizations of the model; dotted lines represent +/- standard deviation. Time in days is shown on x-axis.

The peak *E. coli* bacteria numbers, persistency of bacteria in the gland, and overall shape of extracellular and intracellular *E. coli* bacteria count outputs were highly sensitive to the initial number of phagocytes in the mammary gland. Of the model inputs tested in the one-factor-at-a-time sensitivity analysis using estimated dry period values, initial number of phagocytes in the gland showed the greatest source of uncertainty. As the initial number of phagocytes in the gland was increased in the model input, there was a dramatic decrease in peak extracellular *E. coli* bacteria numbers, and a delay in time for extracellular *E. coli* bacteria numbers to reach peak values. There exists a threshold between 6.00 CFU $\log_{10/ml}$ and 6.39 CFU $\log_{10}$/ml initial number of phagocytes at which above this threshold the intracellular *E. coli* bacteria numbers do not decline nor reach 0; this is observed even in transient strains which have minimal capacity to invade and survive in MEC according to the original model. There also exists a threshold between 6.39 CFU $\log_{10/ml}$ and 6.54 CFU $\log_{10}$/ml initial number of phagocytes at which extracellular bacteria no longer experience an initial rise and peak within the first few days of the infection, but rather decline.

3.2. Curve fitting to *in vivo* data of experimental *E. coli* IMI in the dry period

In vivo data on bacteria counts at quarter level following experimental *E. coli* IMI from Pomeroy et al. (2016a) (n=3) were used to estimate input parameter values hypothesized to deviate in the dry period. The model reproduced the data with the estimated parameter in Table 9. Values for parameters for phagocytic killing rate (k), extracellular *E. coli* growth rate (g_e), pro- and anti-inflammatory cytokine production (g_{cp}, g_{ca}) following curve fitting to *in vivo* data of extracellular *E. coli* counts in mammary gland secretion were estimated (Table 9).

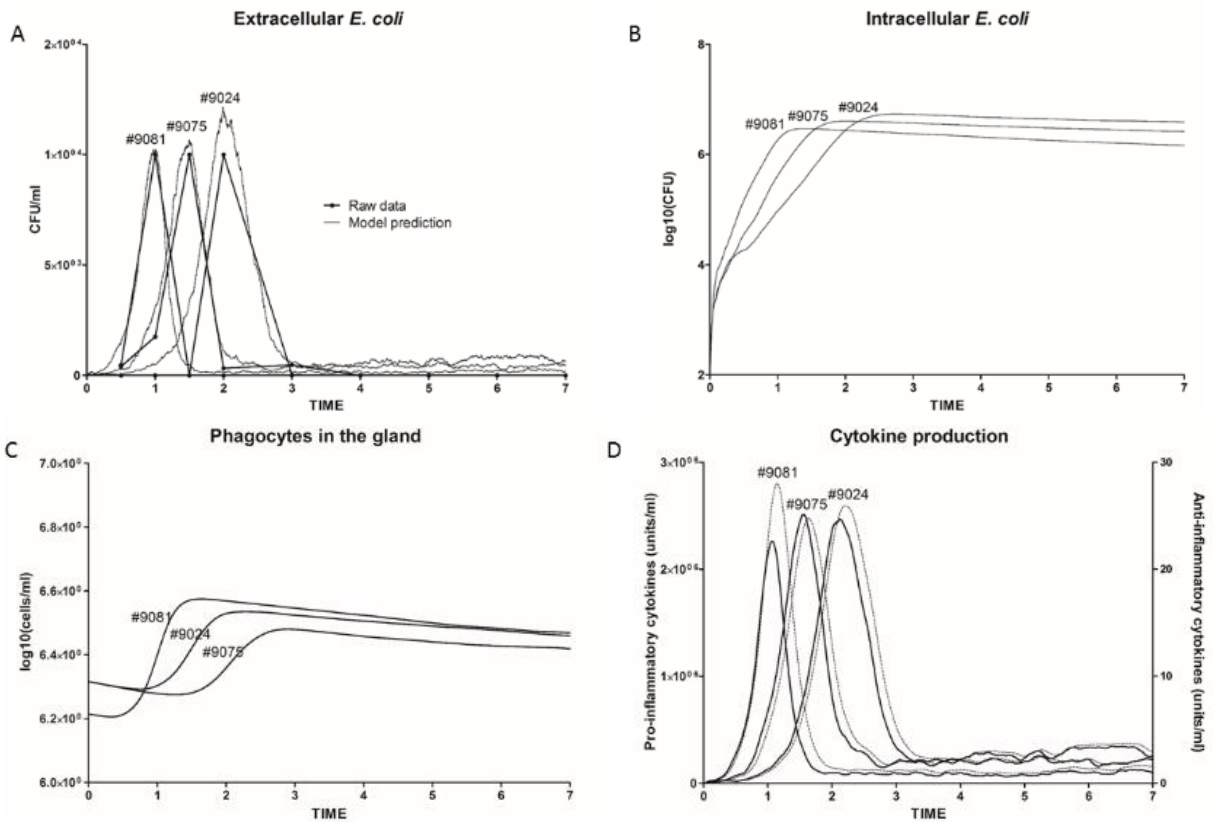

Figure 22. Curve fitting to in vivo data. Model predictions for extracellular *E. coli* (A), intracellular *E. coli* reservoirs (B), PMN numbers (C), and cytokine production (D) for persistent *E. coli* strain intramammary infection using data from in vivo experimental infection from Pomeroy et al. 2016 on extracellular *E. coli* (A). Raw data is indicated in (A) as solid dots; excluding raw data shown in (A), the remaining curves in (A-D) represent a single realization of the stochastic model. Pro-inflammatory cytokine production is represented by solid line, and anti-inflammatory cytokine production is represented by dashed line. Time in days is shown on x-axis.

The original model predicted lactating cows had peaks in pro-inflammatory cytokine levels between 13 to 22 h after infection. When model is fit to data from experimental infection in the dry period the peak in pro-inflammatory cytokine levels occurs later ranging from 26 to 50h after infection, varying by cow (Fig. 22). The peak in anti-inflammatory cytokine levels was predicted to occur approximately 1 to 2h following the peak in pro-inflammatory cytokines which is a more rapid onset than with model predictions in lactating cows. Though peaks in cytokine production were predicted by the model, the peak levels for both pro- and anti-

inflammatory cytokine production (units/mL) were reduced by approximately 2 logs compared to model predictions for lactating cows in (White et al., 2010). The rate of production of pro-inflammatory cytokine production was estimated to be reduced by approximately 30% comparable to reduced production of IL-12 from *in vitro* cultured bovine moDC following UV-killed *E. coli* ECC-Z stimulation from dry period compared to those from lactation (Table 9) (Pomeroy et al., 2015). The anti-inflammatory production rate was estimated to be increased but only by approximately 6 to 27% compared to the value predicted for lactating animals which is within range from values found from *in vitro* cultured bovine moDC following UV-killed *E. coli* ECC-Z stimulation from dry period compared to those from lactation (Table 9) (White et al., 2010, Pomeroy et al., 2016b)

The model is able to predict a small peak in extracellular *E. coli* with a peak in intracellular bacteria following peak in extracellular *E. coli* population, and the intracellular reservoir persists at levels between 1.5 to 3.5E+06 CFU while extracellular bacteria remain at below approximately 200 CFU following peak counts (Fig. 22). The curve fitting estimated growth rate to be reduced and this was consistent between model predictions based on different cow data. The model is unable to show complete elimination of extracellular bacteria as seen in *in vivo* IMI data. Changes to the *E. coli* behavior under non-lactating mammary gland conditions may occur and impact the rate of invasion of MEC, rate of exiting intracellular reservoir to extracellular compartment, and/or death rate of intracellular *E. coli*; we were unable to model here due to lack of existing data (Kalita et al., 2014).

The model predicted higher numbers of phagocytes initially present in the non-lactating gland prior to infection (Table 9). The rate of killing of extracellular *E. coli* by phagocytes estimated from *in vivo* dry period *E. coli* IMI was reduced by 13.1-18.5% from the rate estimated

for lactating cows (White et al., 2010) (Table 9). Recruitment of PMN to the gland (g_p) varied more by individual cow and were within range of those from lactating cows, but were comparatively high to majority of values estimated for lactating cows; despite higher estimates for rate of PMN infux to the gland, the peak number of phagocytes in the gland was only between 0.1 to 0.4 log10 cells/mL increase from the initial number present in the gland.

Table 9. Model parameter definitions, initial values and model estimates.

Symbol	Definition	Initial Value (Dry)	Estimated Value (Dry)	White et al. (2010)	Units
Parameters with same value for all cows					
i_{cp}	Inhibitory effect of anti-inflammatory cytokines on growth of pro-inflammatory cytokines	0.035	-	0.035	none
d_p	Rate of loss of PMN from milk and/or death of PMN	0.1	-	0.1	day^{-1}
d_c	Rate of loss of cytokines	10	-	10	day^{-1}
M	Number of macrophages in milk	5000 (fixed)	-	5000 (fixed)	cells/mL
V_{max}	Maximum volume of mammary gland secretion or milk in a quarter	0.125 (fixed)	-	2.5 (fixed)	Liters
a	Rate of movement of *E. coli* from milk to intracellular reservoirs	5.5 (persistent), 0.51 (transient)	-	5.5 (persistent), 0.51 (transient)	day^{-1}
b	Rate of movement of *E. coli* from intracellular reservoirs to milk	7.5E-03 (persistent), 0.55 (transient)	-	7.5E-03 (persistent), 0.55 (transient)	day^{-1}
d_i	Death rate of *E. coli* in intracellular reservoirs	0.1 (persistent), 1.2 (transient)	-	0.1 (persistent), 2.1, 0.9, 1.4, 1.2, 1.4, 1.8, 1.9 (transient)	day^{-1}
Parameters with different values for individual cows (persistent *E. coli* IMI a, b, and d_i values used)					
g_e	Growth rate of *E. coli* in non-lactating mammary gland secretum or milk	13.4-37.17	24.92, 25.49, 25.65	34	day^{-1}
k	Killing rate by macrophages (P and M)	8.00E-06–9.20E-06	8.34E-06, 8.69E-06, 8.15E-06	1.00E-05	cell/day
g_{cp}	Baseline growth rate of pro-inflammatory cytokines in absence of anti-inflammatory cytokines stimulated by contact between *E. coli* and macrophages	0.00168–0.0024	0.00173, 0.00169, 0.00169	0.0024	day^{-1}
g_{ca}	Rate of growth of anti-inflammatory cytokines (dependenton concentration of pro-inflammatory cytokines)	9.80E-05–2.55E-04	1.35E-04, 1.09E-04, 1.04E-04	9.80E-05	day^{-1}
Int(P)	Initial number of PMN in milk or phagocytes in non-lactating mammary gland secretum (P + M)	1.10E+06–6.68E+06	1.64E+06, 2.07E+06, 2.07E+06	1.00E+05	cells/mL
g_p	Rate of influx of PMN into the milk in presence of pro-inflammatory cytokines	0.02-8	1.9, 0.7, 1.0	0.02, 0.04, 0.02, 0.03, 0.03, 0.50, 2.0, 0.8, 6.0, 2.0, 0.3	day^{-1}

4. Discussion

The dry period is a time of increased susceptibility to new IMI, of which, coliforms bacteria have been shown to be a common cause of these IMI (Green et al., 2002). *E. coli* IMI do not elicit severe clinical mastitis in the dry period, but rather IMI acquired in the dry period persist until calving, and often cause clinical mastitis in early lactation (Quesnell et al., 2012, Gurjar et al., 2013, Pomeroy et al., 2016a). The bovine mammary gland experiences drastic changes during involution and steady-state of the dry period. The dry period also overlaps with immunological changes occurring in late gestation apart of healthy pregnancy. These major physiological and immunological changes to the gland are likely to impact *E. coli* IMI dynamics, however the specific factors responsible for allowing infection to establish and persistent into the subsequent lactation are unknown. The objective of this work was to interrogate the mathematical model for lactating cows developed by White et al. (2010) to elucidate the role of factors known to change in the dry period in IMI dynamics.

The model suggests lower growth rate of extracellular bacteria does reduce total bacterial counts in the mammary as expected and consequently reduces the cytokine response and recruitment to the gland, but change to this variable alone was not sufficient to recapitulate the IMI dynamics observed *in vivo.* Reduced growth rates, even those less than half the rate from the original model for lactating cows, still have a dramatic, rapid rise in SCC, pro- and anti-inflammatory cytokines, and extracellular *E. coli* CFU/ml ranges between 5-6 CFU $\log_{10}$/ml which is 1-3 CFU $\log_{10}$/ml higher than previously document in *in vivo* experimental challenge models (Quesnell et al., 2012, Pomeroy et al., 2016a).

During involution, phagocytes are recruited to the mammary gland, and the number of phagocytes remains high in the steady-state of the dry period (Nickerson, 1989, Inchaisri et al.,

2000, Wedlock et al., 2004). Increasing the initial number of phagocytes in the gland prior to *E. coli* challenge had a drastic effect on the model output and IMI dynamics. Increasing the number of phagocytes resulted in reduced numbers of *E. coli* bacteria, with extracellular bacteria numbers reaching 0 during the course of infection, but in conjunction this also often resulted in enhanced bacterial persistency in the gland through the formation of intracellular reservoirs, and reduced inflammatory response. Pathogen load is known to influence the severity of clinical signs of mastitis (Burvenich et al., 2003). Burvenich et al. (2003) found that the concentration of environmental *E. coli* in the mammary gland was the primary determinant in the severity of the clinical signs in early lactation cows. This immediate reduction in extracellular *E. coli* numbers by increased phagocytes likely played a role in reducing immune response though reduction in antigen load. Previous experimental work in lactating cows demonstrated increased clinical mastitis severity with deficiencies in both neutrophil function and concentrations, further suggesting our model is biologically relevant. Furthermore, in this model, the influx rate of PMN to the gland (g_p) was allowed to vary by cow in data fitting, according to the model influx rate was relatively high compared to many of the lactating animal values from White et al. (2010) (Table 9). The role of phagocyte composition, number, and rate of influx to the mammary gland in dry, late gestation cows needs further investigation *in vivo* to support model findings.

When fit to *in vivo* data from an experimental IMI with chronic-mastitis-causing *E. coli* ECC-Z, the model estimated increased initial numbers of phagocytes which reflected biologically relevant values documented in the literature, however, if other immune parameters were fixed at lactating values (k, C_a, C_p) the peak *E. coli* numbers were underestimated. The model fit was improved with these other immune parameters were estimated concurrently, and the model predictions showed reduced in inflammatory responses and killing capacity in

conjunction with increased initial phagocyte numbers and reduced bacterial growth. This suggests there is an interaction between changes specific to dry period and changes associated with maternal immune regulation in late gestation (Todhunter et al., 1990, Collier et al., 2012, Pomeroy et al., 2015, Pomeroy et al., 2016b). Though peaks in both pro- and anti-inflammatory cytokines were predicted by the model for dry cows, it is possible that with approximately 2 log reduction in units, these peaks may have been missed due to low sensitivity of protein measurements by ELISA in *in vivo E. coli* experimental IMI in the dry period observed by Quesnell et al. (2012). Furthermore, this drastic reduction in peak production of pro-inflammatory cytokines is hypothesized to directly relate to lack of clinical signs following IMI in the dry period.

Another intriguing finding was the ability of transient strains to persist when initial numbers of phagocytes in the gland were high despite minimal capacity to invade and survive in MEC. This poses a question on the involvement in cell-mediated responses in persistent *E. coli* IMI. White et al. (2010) found the model output for *E. coli* bacteria numbers and persistency was highly sensitive to survival of bacteria in the intracellular compartment, but there was no input in the model for Th1-type, cell-mediated responses such as killing of infected MEC by the NK cells and antigen-specific cytotoxic T-cells. These Th1-type responses and formation of antigen-specific responses are hypothesized to be hindered in bovine late gestation, and it is likely the reduced ability of host immune cells to generate Th1-type responses and kill infected MEC contributes to IMI persistency in late gestation. In mammalian pregnancy, the maternal immune system is regulated to protect the semi-allogenic fetus. Furthermore, the current mathematical model has an oversimplified equation is used to describe all pro-inflammatory cytokines despite the known, unique roles of individual cytokines in immune response. We lack *in vitro* and *in vivo*

data to properly model cell-mediated immune response, and future work should investigate cell-mediated responses in the mammary gland during the dry period and lactation.

Intramammary infection dynamics are complex and involve multiple factors which our model is not able to describe such as cell-mediated responses/killing of infected MEC, antigen specific responses, role of individual cytokines/chemokines in response, functional differences amongst phagocytes i.e. neutrophils vs. macrophages. Further the available data for parameter estimation only relates to chronic-mastitis-causing strains. It may also be possible that *E. coli* strains may respond to the limited nutrients and other soluble factors in the non-lactating gland environment which cause changes to invasion of MEC, rate of exiting these intracellular compartments, and/or survival within MEC (Blomfield, 2001, Kalita et al., 2014). The modifications to the original model developed by White et al. (2010) presented here suggest dry period IMI dynamics and persistency relate to interactions between maternal immune regulation in late gestation and changes to the mammary gland during involution and steady-state of the dry period. Future experimental work should enhance our understanding of the relationship between maternal immune regulation mechanisms in late gestation and immune response in the mammary gland. Future work should also investigate changes to mammary tissue-resident immune cell populations and cell-mediated responses in IMI in the dry period with *E. coli* strains varying in ability to form intracellular reservoirs.

5. Conclusions

The key findings from this study indicate that *E. coli* IMI infection dynamics relate to both factors associated with involution and steady-state period in the non-lactating mammary gland and changes to immune function in late gestation. Specifically, reduced *E. coli* growth rate in non-lactating mammary gland secretion, phagocytic function, and pro-inflammatory cytokine

production, increased anti-inflammatory cytokine production and number of phagocytes in the gland all influence the IMI dynamics in the dry period according to the model predictions. This study has limited *in vivo* data to confirm these model predictions and can only be used to guide future *in vivo* and *in vitro* work pertaining to IMI dynamics in the dry period. Future *in vivo* work should investigate the role of late gestation factors vs. factors related to changes to the mammary gland during involution and steady state dry period and other hypotheses generated from this model interrogation. More data should also be collected for to improve precision of parameter value estimation to improve the accuracy of the model and biological relevance.

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CHAPTER 7:
INTRAMAMMARY IMMUNIZATION WITH UV-KILLED *ESCHERICHIA COLI* SHOWS PARTIAL PROTECTION AGAINST LATE GESTATION CHALLENGE WITH A HOMOLOGOUS STRAIN

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KEYWORDS: *E. coli*, Vaccination, Mastitis, Late gestation

ABSTRACT

The objective of this study was to evaluate the efficacy of intramammary immunization with UV-killed *E. coli* ECC-Z on prevention of intramammary colonization after a challenge with a dose of the homologous *E. coli* ECC-Z live bacteria.

A total of 10 cows were included in a study to evaluate the efficacy of intramammary immunization. All 10 cows received then an intramammary immunization of 100 CFU of UV-killed *E. coli* ECC-Z bacteria into one hind quarter at the time of dry off. Approximately 2 weeks before the anticipated calving date both hind quarters of all cows were challenged with 100 CFU of live *E. coli* ECC-Z bacteria. Five of the cows were vaccinated parenterally with a commercial J5 bacterin, and 5 cows served as no parenteral vaccination controls. The cows were then followed over time and infection risk, clinical scores, somatic cell count and milk production were observed over time.

The results of these 10 cows showed partial protection of intramammary immunization on the outcome of a subsequent homologous intramammary challenge. Immunization resulted in a lower probability of infection, a lower bacteria count, lower somatic cell counts and milk conductivity, a lower clinical mastitis score and increased milk production compared to unimmunized control quarters. Once the analysis was corrected for immunization, parenteral J5 vaccination had no significant impact on any of the measured parameters. These results provide the first evidence that intramammary immunization may improve the outcome of an intramammary *E. coli* infection in late gestation and onset of mastitis immediately following parturition. Unlike systemic vaccination, which generally does not reduce the intramammary infection risk, the intramammary immunization did show a five-times reduced odds of an established intramammary infection after challenge. Cytokine profiles indicated a local return of

pro-inflammatory response after challenge as the data showed a more pronounced increase in in IFN-γ with a subsequent negative feedback due to a spike in the level of IL-10 in immunized quarters relative to non-immunized quarters.

Although these results are preliminary and obtained on only 10 cows, the results provide insight into the biological benefits of triggering mucosal immunity in the mammary gland.

1. Introduction

Clinical mastitis is one of the most common, and costly challenges to the dairy industry (Grohn et al, 2004). Approximately 40% of clinical mastitis cases are attributed to gram-negative, mostly coliform, bacterial infection (Barkema et al. 1998, Grohn et al. 2004, Oliveira et al. 2013). Bovine coliform mastitis is generally considered to be caused by opportunistic infections (Bradley and Green, 2001), although evidence for a host-adapted sub-population is growing (Bradley and Green, 2001, Dogan et al. 2006, Shpigel et al. 2008, Lippolis et al. 2014). The incidence of clinical mastitis peaks on most farms immediately following parturition (Barkema et al., 1998), although several observational and experimental challenge studies indicate the presence of the mastitis-causing bacteria isolated from the dry gland before parturition without clear inflammatory indicators of mastitis (Green et al., 2007, Quesnell et al. 2012). The late gestation challenge study using *E. coli* ECC-Z by Quesnell and co-workers (2012) showed that cows in late gestation respond to challenge with an IL-10 dominated response and a minimal response of pro-inflammatory cytokines such as IFN-γ and IL-1β. This suggested shift towards a predominantly anti-inflammatory, highly regulated response during the dry period likely reflects an adaptation in maternal immune signaling during late gestation to protect the semi-allogenic fetus (Quesnell et al. 2012, Pomeroy et al. 2015).

Dairy cows may be vaccinated parentally against coliform mastitis using a core J5 bacterin that has been repeatedly reported to/ reduce clinical severity of response to these infections (Hogan et al. 1992; Hogan et al. 1995; Wilson 2008). However, the data of these vaccination studies have not shown a reduction in incidence of clinical coliform cases. Local immune responses are important for protection against pathogens which predominately infect and cause disease at mucosal sites. Mucosal vaccines have shown to be effective both in the

case of viral (i.e. oral polio vaccine, intranasal infectious bovine rhinotracheitis (IBR) vaccine, oral rabies vaccine) and bacterial (i.e. oral cholera vaccine, oral *E. coli* vaccine, *S. aureus* imm vaccine) pathogens (Pavot et al. 2012, Vilte et al. 2012, Gogoi-Tiwari et al. 2015). Hogan et al. (1997) and Smith et al. (1999) studied the effect of a systemic vaccination in combination with an intramammary *E. coli* J5 bacterin immunization schedule on responses of antibody titers. The results showed that intramammary immunization enhanced IgG titers in serum in the dry period and in lactation and in whey in early lactation compared to subcutaneous immunizations (Hogan et al. 1997, Smith et al. 1999). However, the immunization schedule had minimal effect on systemic and local signs of clinical mastitis following challenge. The authors concluded that the reason for this lack of protection was due to the 4 hour boiling of the bacteria in the creation of the bacterin-vaccine (Smith et al. 1999).

More recent studies have used so called 'ghost' bacteria as vaccine organisms. These 'ghost' organisms are killed bacteria where the internal structures of the bacteria are destroyed but the bacterin have the same cell surface composition as their living counterparts (Mayr et al. 2005). Such ghost organisms display all surface components in a natural non-denatured form, even highly sensitive and fragile structures like pili are well protected. It has been shown that ghost organisms are able to induce a strong mucosal immune response (Jalava et al. 2003). The potency, safety and relatively low production cost of bacterial ghosts also offer a significant technical advantage (Mayr et al. 2005, Yuli and Kiyono 2003, Lubitz et al. 2009).

Therefore, the objective of this study was to evaluate the efficacy of intramammary immunization with UV-killed *E. coli* ECC-Z on prevention of intramammary colonization after a challenge with a dose of the homologous *E. coli* ECC-Z bacterium prepartum, a period of heightened susceptibility to intramammary infection. The *E. coli* ECC-Z strain used for this

study has been used in previous challenge trials in both the dry period and lactation, is well-characterized, and known to cause mild, clinical mastitis (Dogan et al. 2006, Quesnell et al. 2012, Sipka et al. 2013, Lippolis et al. 2014). The impact of immunization on clinical signs, milk production, somatic cell counts and cytokine profiles was also evaluated.

2. Materials and methods

2.1. Cows

Ten adult Holstein cows were selected from the Cornell University Teaching and Research Dairy herd (T&R). Selection was based on the following criteria: cows had completed at least 1 previous lactation, and they were expected to have an approximate 45-48 days dry period. Also, cows with any signs of detectable illness including but not limited to clinical mastitis at time of dry-off or with any of the last six monthly individual cow's SCC before dry-off greater than 250,000 cells/mL were excluded. Cows were culture negative at time of enrollment, and culture negative prior to challenge with *E. coli* ECC-Z as determined by bacteriology described within the section, 'Mammary gland secretion and blood sample collection'. Finally, cows with any major traumatic injury to the teat ends were excluded.

2.2. Housing

Cows in both groups were housed, fed, and managed identically throughout the dry period and postpartum. Three weeks prior to the anticipated calving date, cows were transferred from free stalls to the Cornell University Large Animal Research and Training Unit (LARTU) facility. Here the cows were housed in individual maternity pens and had unlimited access to water and feed. Cows remained in the study throughout the non-lactating period, calving and first 7 days of the ensuing lactation. No adverse reactions to the vaccine prior to the intramammary *E. coli* challenge were observed in any of the cows. The study

protocol was approved by the Cornell University committee on Animal Use and Care (IACUC protocol 2006-0158).

2.3. Parenteral vaccination and intramammary immunization

The selected cows were paired by expected calving date so they were approximately the same and then were randomized to receive parenteral J5 vaccine or serve as control. The J5 vaccine (*Escherichia coli* Bacterin, J5 strain; Zoetis, Kalamazoo, MI) was administered by the investigators (5mL) subcutaneously on the upper part of the rib cage just posterior to the scapula. The cows in the control group were unvaccinated controls. Vaccinated cows were immunized 15 days prior to dry-off followed by a second dose at dry-off and a third dose 15 days post dry-off. Cows in both the groups were dried off by abrupt cessation of milking and all four quarters were left untreated with also no internal teat sealant.

In addition to the systemic vaccination, all cows received an intramammary immunization of the equivalent approximately 100 CFU of UV irradiated *E. coli* ECC-Z at the time of dry-off in one of the two hind quarters. The production of the UV-killed *E. coli* is described here in brief. The *E. coli* ECC-Z bacterial strain was grown in Luria Bertani (LB) broth until a concentration of approximately 10E6 bacteria per mL of broth was obtained. Approximately 1 liter of broth was then irradiated. The UV irradiation device is composed of a stainless steel outer unit containing three inner chambers of quartz tubes connected in sequence. The bacterial broth was pumped through a thin layer between the outer steel unit and inner quartz tubes. Eight germicidal low pressure mercury lamps were used as the source of UV light exposure. Every 50 milliseconds, two UVX-25 UV light sensors monitored the desirable amount of UV energy required for consistent radiation. The resulting UV radiated bacterial solution was checked for bacterial growth. No live bacteria were recovered. The sterility of the UV irradiated

broth was checked before each intramammary application. Throughout this manuscript, the intramammary application of these ghost bacteria will be referred to as immunization rather than vaccination.

2.4. Intramammary bacterial challenge

Escherichia coli strain ECC-Z (Dogan et al. 2006), a strain originally isolated from a cow with mild persistent clinical mastitis (Lippolis et al. 2014), was used as the intramammary challenge strain. The challenge inoculum was prepared by inoculating a frozen stock culture of the ECC-Z strain into LB broth. The LB broth was incubated for 18h at 37°C. A total of 100µL of this culture was inoculated into fresh LB broth and incubated for approximately 2.5h at 37°C. The log phase LB broth culture was used for inoculation. A 1:10 dilution of the log phase culture was made in phosphate buffer saline (PBS) and adjusted to 100 colony forming units (CFU) of inoculation dose. The CFU per mL of the challenge bacteria was determined by plating 100µL in duplicate on LB agar plates. Two hind quarters of each control or parenterally vaccinated cow was challenged by infusion of approximately 100 CFU of *E.coli* ECC-Z strain suspended in PBS. The cows were challenged approximately 10 days before the expected calving date, a third quarter received a similar infusion of vehicle (PBS) only, while the fourth quarter remained unchallenged.

2.5. Mammary gland secretion and blood sample collection

Milk or dry cow secretion samples were collected at the time of vaccinations, at the time of intramammary immunization and daily thereafter for seven days, from one week before to the day of challenge on a daily basis, at the time of challenge and every 12 hours thereafter until 72h after challenge, and from the day of calving until 7d thereafter at every milking (12h apart).

Previous work demonstrates that frequency of sampling in the dry period, when using aseptic technique as done here, is not associated with clinical mastitis (Green et al. 2002).

Milk, dry cow secretions (hereafter referred as dry cow mammary gland secretions, DCS) were aseptically collected in sterile vials for microbiological culture and cytokine analysis. All four quarters were sampled individually. For cytokine analyses, milk samples were centrifuged at 20,000×g and 4°C for 30 min. The fat layer was removed and milk whey was collected and stored in aliquots at −20°C until ELISA analyses. For microbiological culture, samples were transported immediately on ice to the microbiology laboratory for culture according to the protocols recommended by the National Mastitis Council and described in detail by Hogan et al. 1992). Briefly, 100µL of milk or DCS were plated on Columbia Sheep Blood agar (Oxoid) and MacConkey's agar plate. Plates were incubated for 24 – 48h at 37°C and were examined daily for bacterial growth. As previously described in Gurjar et al. 2013, representative individual colonies were isolated and stored at −70°C for molecular strain typing by random amplification of polymorphic DNA (RAPD) analysis to identify challenge strain *E. coli* ECC-Z. In brief, individual isolates from bacterial culture plates were grown in LB broth at 37°C for 12 h. The DNA was isolated from samples using a QIAquick DNeasy isolation kit (Qiagen Inc., Valencia, CA). The RAPD primers designed specifically for RAPD typing of gram-negative bacteria were as follows: forward 5′-AGTAAGTGACTGGGGTGAGCG-3′ and reverse 5′-TACATTCGAGGACCCCTAAGTG-3′. These primers have previously been shown to provide discernment between mastitis *E. coli* bacterial strains (Dogan et al., 2006). The PCR products were evaluated using gel electrophoresis in a 1.5% agarose gel at 60 V for 1.5 h. Bacteria counts were obtained through three serial 10-fold dilutions. Where necessary additional dilutions were performed for peak CFU counts which occurred roughly around C+18h. The results of the

bacteriological analysis were expressed as log10 CFU per mL of milk. An aliquot of each milk sample was sent to Dairy One® (Ithaca, NY) for SCC determination using a fossomatic cell count analyzer. Data were counted as SCC per mL of milk and expressed as a linear score. In the post calving period, the animals were milked with a quartermilker and four Lactocorders®, each connected to a single quarter. This enabled determining the milk production and conductivity from individual quarters with the aid of the Lactocorder® device.

2.6. Clinical observation for local and system signs

Systemic and local symptoms of inflammation were assessed throughout the trial period as described earlier by Petzl et al. (2012). All time periods indicated above for collection of blood and milk or DCS samples were also used for recording clinical signs. Rectal temperature, appetite and general attitude were evaluated. The systemic signs were scored on a three point scale, 1 = no signs to 3 = severe systemic signs. The udder was palpated for soreness, swelling, and hardness. The udder was scored on a 1 to 4 scale with 1 being normal and 4 being the gland swollen, warm, sore, firm, and with a red discoloration. The milk appearance, consistency and color were scored daily in the post calving period at every 12 hour interval. The milk appearance was scored on a 4 point scale with 1 being normal white homogenous milk and 4 being dark yellowish milk with clots as observed on the Lactocorder® filter. The three scores, cow, udder and milk score, where then added to an overall clinical score. Cows with overall clinical score of 3 or less were recorded as having no clinical mastitis, those with scores between 4 and 7 as having mild to moderate mastitis and those with scores of $\geq$ 8 as having severe mastitis.

2.7. Cytokine analysis

Ninety-six well sample plates (Thermo-Fisher, Pittsburg, PA) were coated with a primary capture antibody for IFN-γ (1µg/mL, CLONE, Serotec, Inc., Raleigh, NC), IL-10 (5µg/mL,

CLONE, Serotec, Inc., Raleigh, NC) in a 0.05M carbonate coating buffer, pH 9.6. Plates were allowed to incubate overnight at 4°C. All plates, washes, and blocking reagents were allowed to equilibrate to room temperature. Plates were washed 3 times with wash buffer (50mM tris-buffered saline (TBS) w/ 0.05% tween-20, pH 8.0) and blocked with 1:10 Seablock:TBS (Pierce, Rockford, IL) for one hour. Subsequently, plates were washed again 3 times with wash buffer. Recombinant proteins for cytokine IFN-γ were diluted to appropriate concentrations and included as a standard curve for each assay. Due to unavailability of recombinant bovine IL-10 at the time this study was conducted, we could not run standards for IL-10 with this assay; IL-10 was therefore expressed as measured optical density (OD) values. For each assay 100µl of standards and samples were incubated at room temperature for at 2 hours. Plates were washed 3 times with wash buffer. Biotin-labeled secondary antibodies: IFN-γ (5µg/mL, CLONE Serotec, Inc., Raleigh, NC), IL-10 (5µg/mL, CLONE, Serotec, Inc., Raleigh, NC) diluted in blocking buffer were added to the wells (100µL/well). Plates were incubated for 1 hour at room temperature in the dark. Plates were washed 3 times in wash buffer, and following washing steps streptavidin:HRP complex diluted 1:1000 in blocking buffer was added to wells (100µL/well), and incubated in the dark for 1 hour at room temperature. Plates were washed 3 times in wash buffer and 100µl/well TMB was added to each well. Plates were incubated for 10 – 25 minutes at room temperature in the dark and 100µL/well of 2M H_2SO_4 was added to wells. The concentrations of IFN-γ in the whey samples were calculated by extrapolating from the respective standard curves, and the values expressed as biological units of activity per milliliter. A background correction reading at 565nm was subtracted from the 450nm absorbance readings.

2.8. Data analysis

Data were entered into spreadsheets and double checked for entry errors. All data was analyzed using the SAS v 9.4 statistical analysis program (SAS Institute Inc., Cary, NC). Initially data was graphed and evaluated for outliers and unlikely observations. Outliers were studied in detail and re-analyzed to confirm where necessary. Differences in linear score of SCC, milk production, conductivity, clinical score, log10(bacteria count) were analyzed using linear mixed models (SAS, PROC MIXED), adjusting for repeated measures within animals and using Bonferroni for post hoc tests. The general format of the model was:

$Y = \alpha + \beta_1 *$ time point $+ \beta_2 *$ immunization $+ \beta_3 *$ vaccination $+ \beta_4 *$ immunization*vaccination + Cow (random) + Re.

Where Y is the outcome variable, α is the intercept, β_i are regression coefficients, time point is a categorical variable for each time-point of measurement, immunization is a dummy variable (1=yes, 0= no), vaccination is a dummy variable (1=yes, 0= no), immunization*vaccination is the interaction term, Cow is a random cow effect and Re is a complex error term, where R is the within cow correlation and e is an identically, independently normally distributed error term. A very similar model was used for the IMI outcome variable, but this was analyzed in a logistic regression term where logit(probability of IMI) was the outcome variable. A somewhat more complex model was used for cytokine profile analysis. The model used for analysis was:

$Y = \alpha + \beta_1 *$ time point $+ \beta_2 *$ immunization $+ \beta_3 *$ vaccination $+ \beta_4 *$ immunization*vaccination $+ \beta_5 *$ prechallenge average $+ \beta_6 *$ postchallenge $+ \beta_7 *$ immunization*postchallenge + Cow (random) + Re.

Where all terms are the same as previous and prechallenge average is the average cytokine level of the three samples taken immediately before challenge (C-4d, C-7d, C),

postchallenge is an indicator variable taking on the value of 1 for the time points C+12h to C+7d and all time points in between, the final interaction reflects the impact of immunization on the cytokine profile in the time periods immediately after challenge. Differences were considered statistically significant when the probability of a type I error is < 0.05.

3. Results

3.1. Clinical data

No major clinical disease issues were encountered with the 10 cows that were enrolled into the trial. All cows went through the trial, eventually recovered from challenge infection and were all returned to the teaching and research dairy facility. Clinical scoring of cows and milk of the cows was done at every milking. After challenge with *E. coli* ECC-Z, clinical abnormalities were observed in almost all cows, but none of the cows became systemically sick due to clinical mastitis. The result of the regression analysis [results not shown] of the clinical score indicated that the quarters that were immunized had a lower score by an average of .09 score points and this reduction was borderline significant with a p-value of .07. All challenged quarters, immunized and non-immunized, showed a significantly increased score that had increased with an average of .15 compared to non-challenged control quarters (P<.01). The least square means obtained from the regression model are shown in Figure 23. The clinical score in the challenged but non-immunized control quarters started out higher immediately after calving and then became approximately identical to the immunized quarters after three days. Body temperature of the cows throughout the trial was on average 38.6ºC (SD .52), with no significant differences observed between the vaccinated and control cows, at 38.6ºC (SD .57) and 38.5ºC (SD .45) respectively.

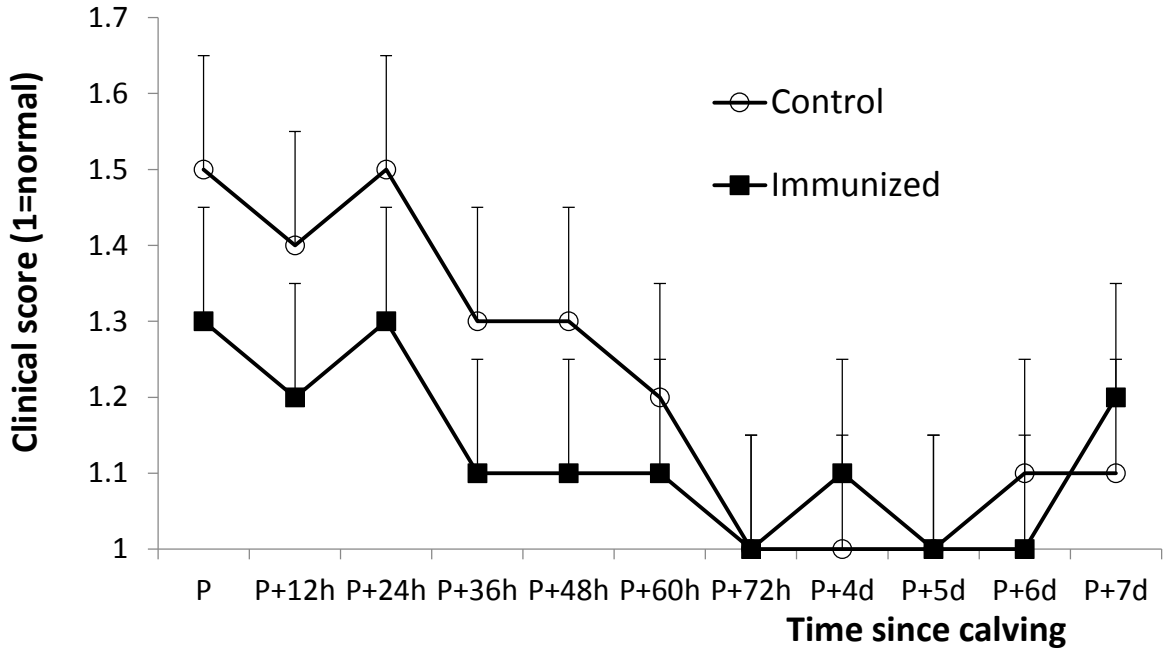

Figure 23. Least square means of the clinical score in unimmunized control quarters and quarters that were immunized. Time points refer to time (hours 'h', days 'd') relative to parturition 'P'. Error bars represent the SE of the LSM estimates.

3.2. Intramammary infection

The effect of immunization on bacteria counts is shown in table 10. Intramammary infection was defined as a sample being culture positive for *E. coli* ECC-Z as determined by RAPD of isolates. In table 10 the 10 logarithm of bacteria counts were used as the outcome variable for linear mixed model regression. Of the challenged, unimmunized control quarters, 10 out of 10 quarters were at least once positive for *E. coli* ECC-Z, one out of these 10 quarters was only positive at the very first post-challenge sampling. Of the challenged, immunized quarters, 7 out of 10 quarters were at least once positive, 5 out of these 7 quarters were only positive at the very first post-challenge sampling. The results indicate that the log10 bacteria counts are significantly lower in the immunized quarters relative to the control quarters. The average drop in bacteria counts was .33 log units and this was highly significant (P<.0001). Parenteral vaccination did not contribute to a further reduction in bacteria counts once the effect of

intramammary immunization was accounted for (P=.13). The least square means of the linear mixed model regression are shown in figure 24a. A very clear distinction between immunized and control quarters is evident. There is also a clear bi-modality in the graphs, where bacteria counts are dropping at 5 days after challenge before calving, but then increase again as soon as the cow has calved. The logistic mixed model regression results on the probability of a new IMI is also shown in table 10. Again, immunized quarters had a significantly lower probability of being infected. The odds ratio of being infected can be estimated as .2 (= $e^{-1.63}$), or control quarters are 5 times more likely of being infected compared to immunized quarters. In figure 24b, the probability of a quarter being culture positive is calculated and plotted over time. Immunized quarters had a higher probability of becoming culture negative immediately after challenge (P<.05). The immunized quarters also had a lower probability of being infected post-calving.

Table 10. Impact of immunization on bacterial counts and probability of IMI in the challenged quarters. Bacterial counts were 10Log transformed and analyzed in a linear model, probability of IMI was binary and analyzed used a logistic regression model.

		Bacterial counts			Probability of IMI		
Effect		**Estimate**	**Standard Error**	**Pr > |t|**	**Estimate**	**Standard Error**	**Pr > |Z|**
Intercept		-0.30	0.26	0.28	-2.36	0.47	<.0001
Time point		n/a	n/a	0.00	3.06	0.99	0.00
Vaccine	Yes	0.41	0.27	0.13	0.99	0.73	0.17
Immunization	Yes	-0.33	0.08	<.0001	-1.63	0.60	0.01

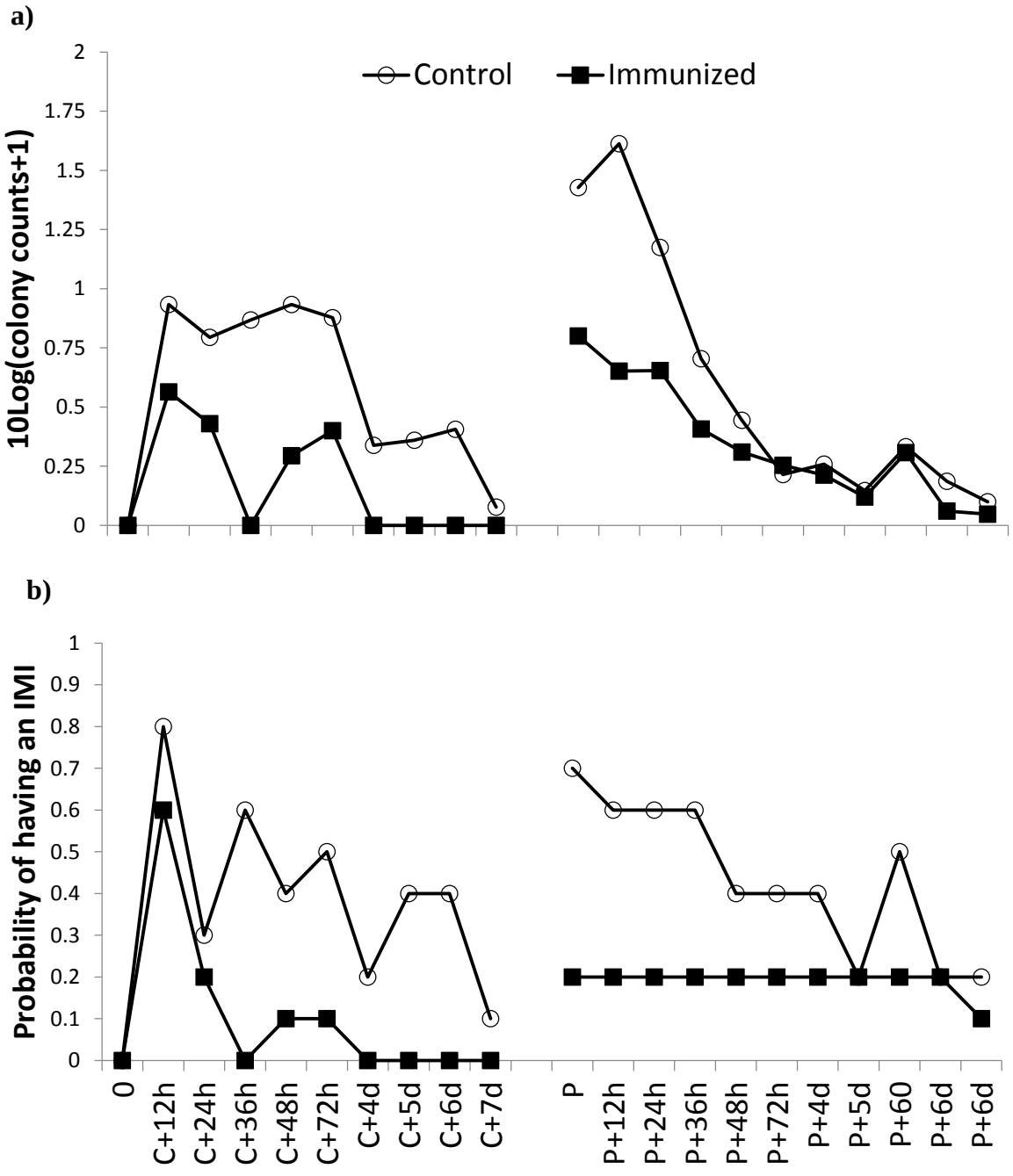

Figure 24. (a) Least squares means of mixed model regression on log10 bacteria counts and (b) probability of having an IMI in the immunized and unimmunized control quarters. Time points refer to time relative to challenge (C) or parturition (P).

3.3. Milk production, SCC and conductivity

Quarter milk production was measured using the lactocorder device. Milk production regression results indicated that the immunized quarters had an increased quarter milk production

of .58 kg/milk per day (P<.01). Parenteral vaccination did not have a significant impact on milk production once immunization was accounted for (P=.40). The least square means resulting from the final regression model are shown in figure 25. Milk production started out approximately equal between the unimmunized, challenged control quarters and immunized, challenged quarters but in the days after calving milk production in immunized quarters increased relative to unimmunized quarters for the duration of the trial. In table 11, the linear mixed model regression of the linear score of the somatic cells counts at quarter level are shown. Immunized quarters had a significantly lower linear score by .40 linear score units. Challenge increased the linear score. The least square means of this model are shown in figure 26a. In table 11, the linear mixed model regression results of milk conductivity are shown. The average conductivity in immunized, challenged quarters was significantly lower compared to unimmunized, challenged quarters. Least square means from the regression model on milk conductivity are shown in figure 26b.

Table 11. Linear mixed model results of linear score and electrical conductivity in immunized and control quarters. Both models used were linear regression models.

	Linear score			Conductivity		
Effect	**Estimate**	**Standard Error**	**Pr > |t|**	**Estimate**	**Standard Error**	**Pr > |t|**
Intercept	2.45	0.23	<.0001	4.34	0.27	<.0001
Time point	n/a	n/a	<.0001	n/a	n/a	<.0001
Challenge	0.18	0.07	0.01	0.92	0.10	<.0001
Vaccine	0.40	0.29	0.17	0.23	0.31	0.46
Immunization	-0.40	0.08	<.0001	-1.43	0.12	<.0001

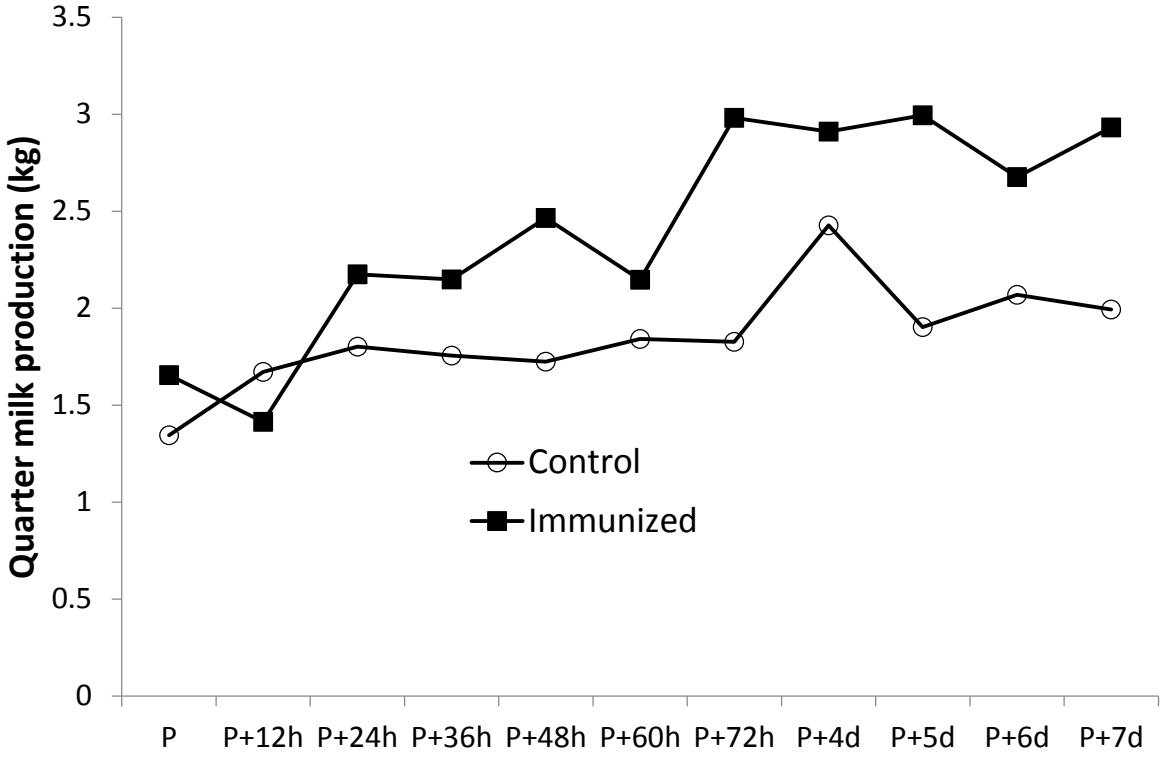

Figure 25. Least squares means of quarter milk production in immunized and unimmunized control quarters. Time points refer to time relative to parturition (P).

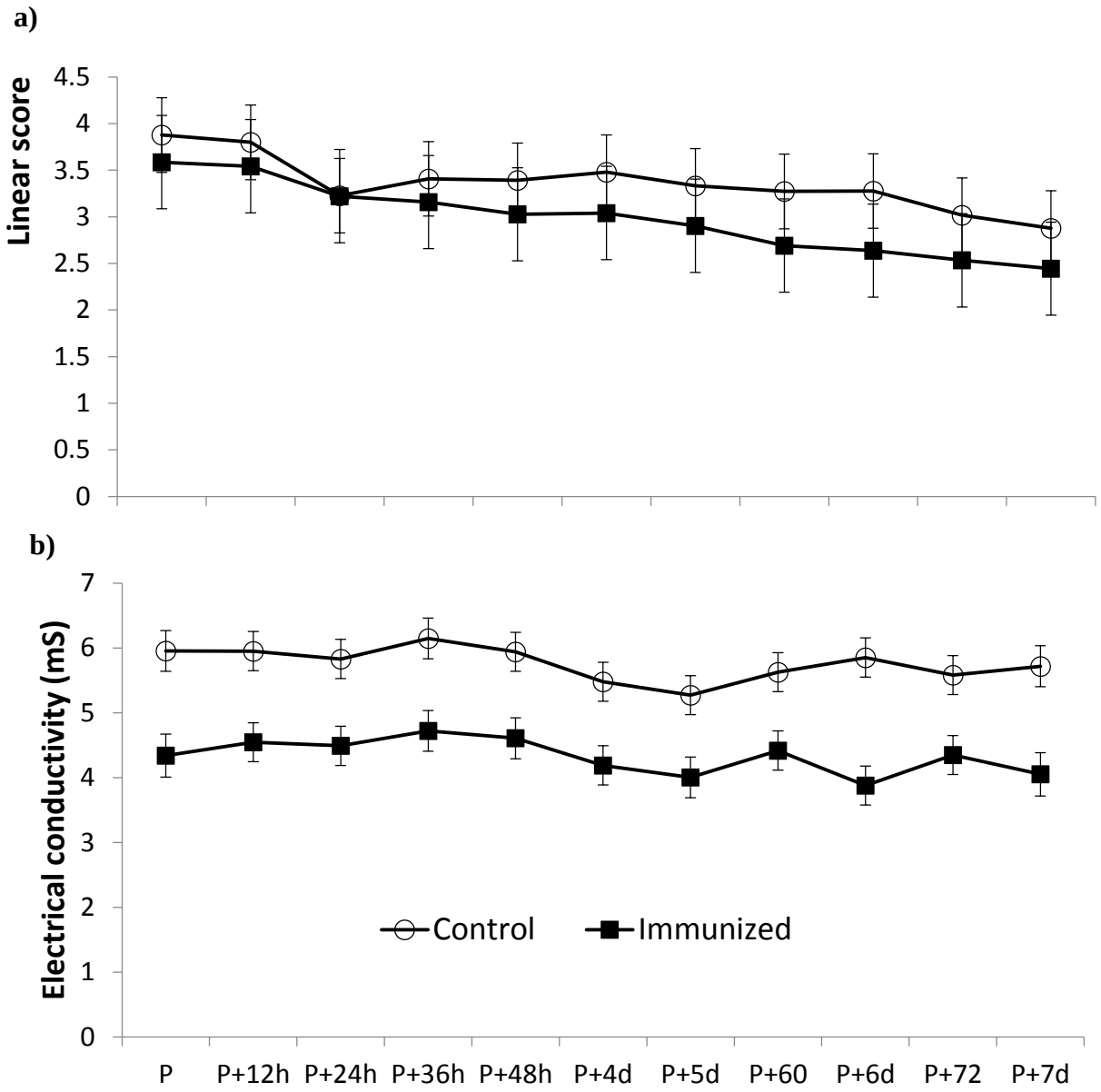

Figure 26. Least squares means of (a) linear score and (b) electrical conductivity in immunized and unimmunized control quarters. Time points refer to time relative to parturition (P). Error bars represent the SE of the LSM estimates.

3.4. Cytokine profiles

Cytokine profiles for IFN-γ and IL-10 are shown in figure 27a and 27b respectively. For both cytokines the concentrations in milk were already higher immediately before the challenge relative to the levels at the time of the vaccinations and the intramammary immunization. For that reason a covariate was added into the linear model that corrected for the mean level of the

cytokine immediately before challenge (pre-challenge average: the average of d-7, d-4 and d0). Results of the linear models are shown in Table 12. The key variable to evaluate in this table is the interaction between immunization and post-challenge. In the case of IFN-γ this interaction has a parameter value of 1.68, indicating that immediately after challenge the IFN-γ level in immunized quarters increased by 1.68 units compared to the control quarters. In the case of IL-10, the opposite was shown. In immunized quarters, IL-10 levels dropped by 0.74 units in immunized quarters immediately after challenge compared to control quarters.

These two effects are shown in detail in figure 27. Interferon-γ levels increased significantly post-challenge but only in the immunized quarters, non-immunized quarters showed no increase due to challenge (Fig. 27a). This increase in IFN-γ levels after challenge was of relative short duration and the levels were back to the level of non-immunized quarters at approximately 4 days post challenge. After calving, IFN-γ levels decreased again and went back to the levels of the period before dry-off and during vaccination. Figure 27b shows the levels of IL-10 in immunized and control quarters. There was again a slight, but in this case non-significant, increase in IL-10 immediately before challenge. The level of IL-10 was significantly lower after challenge in quarters that were immunized compared to the control quarters. Immediately after calving IL-10 levels in immunized quarters spiked and then returned to pre-challenge levels. After calving no difference in IL-10 levels were observed between immunized and control quarters.

Table 12. Linear mixed model for IFN-γ and IL-10; both models used were linear mixed models.

	IFN-γ			IL-10		
Effect	**Estimate**	**Standard Error**	**Pr > |t|**	**Estimate**	**Standard Error**	**Pr > |t|**
Intercept	-0.57	0.53	0.31	0.03	0.36	0.95
Time point	n/a	n/a	0.90	n/a	n/a	0.006
Immunization	0.06	0.17	0.70	-0.09	0.17	0.59
Vaccine	-0.66	0.67	0.33	0.29	0.20	0.15
Prechallenge average	0.57	0.10	<.0001	0.15	0.09	0.14
Postchallenge	2.51	0.29	<0.001	0.01	0.28	0.98
Immunization*postchallenge	1.68	0.39	<0.001	-0.74	0.32	0.02

The t- and Z-critical values to determine if coefficients are significantly different from 0 are based on a 2-tailed test with an alpha of 0.05

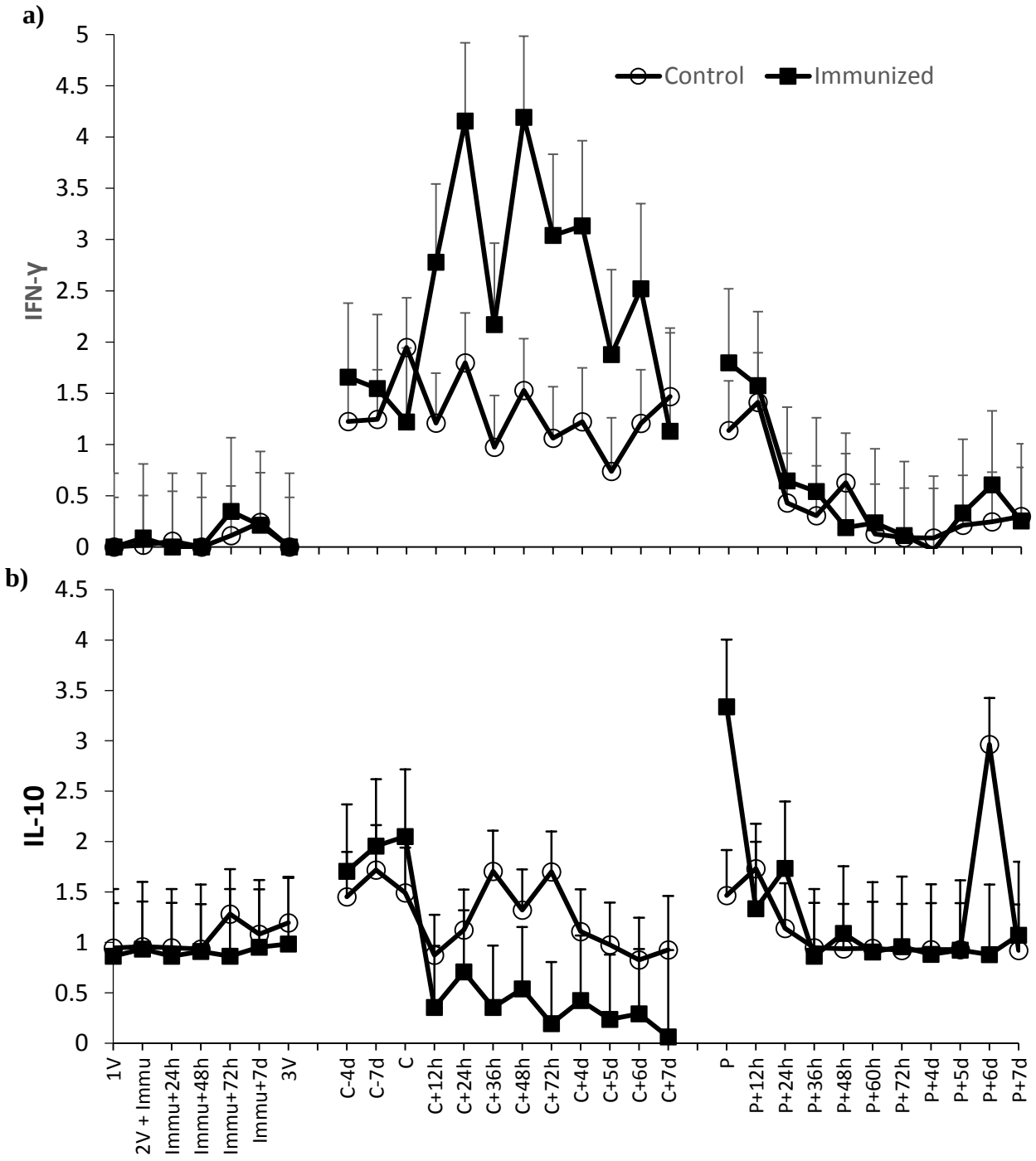

Figure 27. Least squares means of (a) IFN-γ cytokine values and (b) IL-10 in unimmunized control and immunized quarters. Time points refer to time relative to challenge (C) or parturition (P). Time of peripheral vaccination (V) is indicated. Error bars represent the SE of the LSM estimates.

4. Discussion

The results of the intramammary immunization study in 10 cows showed a partially protective effect of intramammary immunization at dry-off on the outcome of a subsequent intramammary challenge in the late dry period. Immunization resulted in a fivefold lower probability of infection (odds ratio of .2), and a lower bacterial load. These results provide the first strong evidence that intramammary immunization with UV-killed *E. coli* bacterin in dairy cows may improve the outcome of an intramammary infections acquired in the dry period. The results of intramammary immunization appear to be more protective compared to systemic vaccination, which most often does not reduce the intramammary infection risk (Hogan et al. 1992, Wilson et al. 2008). The precise mechanism that would lead to this protection against infection is not known, but it may be hypothesized that immunization with UV-killed *E. coli* is protective. This is in line with a series of studies on protective effects of mucosal immunization with oral administration of *E. coli* ghost bacteria in defense against experimental infection in mice (Mayr et al. 2005, 2012). In a recent study on intramammary immunization of mice against a subsequent *S. aureus* challenge, it was also shown that intramammary immunization significantly reduced bacterial load after challenge when compared to subcutaneous vaccination or a non-treated control group (Gogoi-Tiwari et al. 2015, 2016). The authors suggested that the enhanced generation of antigen-specific humoral and cell-mediated immunity observed following intramammary immunization lead the superior protection against *S. aureus* challenge (Gogoi-Tiwari et al. 2015).

The second important finding was that intramammary immunization resulted in lower SCC and milk conductivity, a somewhat lower clinical mastitis score, and increased milk production compared to challenged, unimmunized quarters. Part of this effect may be attributed to the much lower risk of infection and lower bacteria counts in quarters that did get infected.

However, even in infected quarters, the severity of signs was numerically, but not statistically, lower in immunized quarters (data not shown). Reduction in clinical severity caused by IMI present in early lactation by UV-killed bacterin intramammary immunization in at dry-off needs further investigation in order to differentiate between cases in which there was full protection vs. partial protection.

Another valuable finding in this study was the difference in cytokine profile observed between immunized and challenged versus unimmunized and challenged quarters. The unimmunized control quarters responded as shown before by Quesnell and co-workers (2012); these quarters showed no significant increase in pro-inflammatory cytokine production following challenge, in this case IFN-γ, and increased concentration of IL-10 after challenge relative to immunized, challenged quarters. In contrast, the immunized quarters showed a modified pro-inflammatory response with a moderate increase in IFN-γ and a decrease in IL-10 concentrations following challenge. This study indicates the suggested maternal immune regulation of pro-inflammatory, Th1-type responses described in late gestation may be modified in the mammary gland with intramammary immunization (Rosbottom et al. 2008, Quesnell et al. 2012, Maeda et al., 2013; Paibomesai et al., 2013; Pomeroy et al. 2015). This may suggest that mucosal immunity in the mammary gland is more flexible during this period of maternal immune regulation. Previous studies on mucosal immunization against *E. coli* using ghost bacteria have also shown in addition to improved humoral immunity enhanced antigen-specific IFN-γ response following challenge with target antigen (Mayr et al. 2005, 2012). Additionally, non-specific priming of innate immune cell populations at mucosal sites such as monocytes and macrophages which induce epigenetic changes to generate what is known as "trained immunity'' has been well-documented in other species and these effects have been shown to improve the efficacy of

immunization and the protection against secondary, non-target infections (Benn et al. 2013, Arts et al. 2015). Since antigen-specific immunity was not determined by either antibody titers or *in vitro* recall responses, it cannot be determined from this study which cell types were affected by the immunization and whether the enhanced protection from *E. coli* IMI was due to the generation of primed tissue-resident innate immune cells and/or antigen-specific lymphocytes by intramammary immunization. The mechanisms involved can only be speculated at this point based on current understanding in immunomodulation and immune programming.

The mechanism of action of such a local immunization of the mucosa in the mammary gland is not fully understood. Although few studies have been published on intramammary immunization, already in 1985, Colditz and Watson (1985) reported on intramammary immunization of sheep to prevent IMI with *S. aureus* bacteria. Glands locally immunized with killed *Brucella abortus* provided a greater neutrophil response to staphylococcal infection. Secretions of these immunized glands contained elevated concentrations of mononuclear cells. The authors concluded that humoral and cellular characteristics of the locally immunized mammary gland influences the kinetics of the neutrophil influx during staphylococcal infection (Colditz and Watson, 1985). The studies on intramammary immunization in dairy cows by Smith et al. (1999) did not show any protective effect of the immunization i.e. there was no difference in clinical signs, bacterial load, or cell influx observed between immunized and control quarters, despite the enhanced antibody response in the mammary secretum. The authors hypothesized that the extensive boiling of the organisms prior to use as a vaccine may have resulted in low antigenic properties of this solution. It has been suggested that ghost bacteria alone are able to stimulate both the innate and adaptive immune system (Lubitz et al. 2009). The use of ghost

bacteria may have been a key difference between the earlier studies of Smith et al. (1999) and the current studies using intramammary application of ghost bacteria such as UV-killed bacterin.

Although these results are preliminary and obtained on only 10 cows, the results certainly appear to provide insight into the biological benefits of triggering mucosal immunity in the mammary gland. The current study uses 10 adult cows, and showed a significant impact on bacterial infection risk after challenge. Still, this first study with 10 cows will need to be repeated to be able to provide more solid evidence that intramammary immunization with UV-killed bacterin is indeed efficacious in reducing intramammary infections. The use of a homologous challenge provides the best possible scenario to show efficacious protection of intramammary immunization. Obviously, such homologous challenge is not a very realistic scenario for commercial dairy farms. Further experimental work with heterologous organisms will be necessary to show a broad spectrum of efficacy (Schukken et al. 2011). Ultimately, such intramammary immunization with UV-killed bacterin will need to be effective under field conditions. Not only is it essential to show further efficacy in repeated studies with heterologous challenge, it is also necessary to obtain a better mechanistic understanding of the immune response that is stimulated using this novel vaccination approach (Bharathan et al. 2011). Finally, it will be essential to develop an application system that under all management circumstances will be both effective and safe. Application of UV-killed bacterin into the mammary gland certainly also provides a risk for contamination at the time of heightened susceptibility to infection. Therefore, further investigations into the mechanisms and clinical efficacy of intramammary immunization are warranted based on the promising observations in the study reported here.

5. Conclusions

Intramammary immunization at the time of dry-off with UV killed *E. coli* bacterin without addition of adjuvant proved to be partially effective in preventing subsequent intramammary infection after a homologous live *E. coli* challenge. Immunization also resulted after calving in a lower linear score, lower electrical conductivity and a higher milk production. The small size of the study warrants careful conclusions and further studies on intramammary immunization, to show it efficacy with heterologous challenge and eventually its efficacy and safety under field conditions.

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CHAPTER 8: DISCUSSION

1. Introduction

Cows have an increased susceptibility to *E. coli* IMI during the early and late stages of the dry period (Smith et al., 1985a, Green et al., 2002). Intramammary infections acquired in the dry period often persist until calving and cause clinical mastitis in the subsequent lactation (Green et al., 2002, Pyorala, 2008). Clinical signs of mastitis typically do no accompany infection within the dry period, not even IMI caused by *E. coli* which typically elicit highly inflammatory responses during lactation (Quesnell et al., 2012, Sipka et al., 2013, Pomeroy et al., 2016a). Available perinatal vaccines targeted at *E. coli* mastitis, the predominant isolate found in IMI acquired in the dry period, are currently only labeled to reduce clinical severity of the disease. The work presented here sought to identify and characterize key parameters involved in the heightened risk to *E. coli* IMI in the dry period and subsequent postpartum mastitis. The major findings from the studies show: 1) the dam's immune system has a dynamic regulation of peripheral blood monocyte composition and moDC function by stage of lactation and pregnancy (chapters 2&3); 2) cows have reduced populations of inflammatory monocytes, antigen presentation, and Th1-type cytokine production and enhanced anti-inflammatory cytokine production in late gestation (chapters 2-4); 3) moDC function can be regulated by pregnancy-associated hormones 17β-estradiol and progesterone (chapter 4); 4) deviations in prepartum peripheral blood monocyte composition can increase the risk of postpartum infectious disease (chapter 5); and 5) the persistence and unique dynamics of IMI in the dry period appears to be an interaction between hindered maternal immune function in pregnancy and the drastic changes to the gland related to involution/steady-state of the dry period (chapter 6). However despite the changes to immune function in late gestation, the mammary gland is still able to generate protective immunity in the dry period to eliminate *E. coli* IMI effectively and reduce clinical

severity following UV-killed *E. coli* intramammary immunization (chapter 7). The collective work from this dissertation suggests the relationship between maternal immune regulation and immune response in the non-lactating mammary gland is complex. Our understanding of both maternal immune regulation in dairy cattle and the mucosal immunity in the non-lactating mammary gland are still limited, but this work may guide future work in this area and provide insight for the design of vaccines, biotherapeutics, and immunomodulators and identify when such interventions would be beneficial.

2. Maternal immune regulation of myeloid cells in bovine late gestation

A primary objective of this dissertation was to characterize changes in immune function in late gestation in order to elucidate the underlying mechanisms in *E. coli* IMI dynamics in the dry period. The maternal immune system is regulated during pregnancy in order to tolerate the developing semi-allogenic fetus, but these changes also impact the ability to respond to invading pathogens (Betz, 2012, Krishnan et al., 2013, Zenclussen, 2013, Schumacher et al., 2014, Fair, 2015). Maternal-fetal tolerance involves multiple mechanisms to prevent highly inflammatory, cell-mediated responses against fetal antigens however, these mechanisms are not well-characterized in dairy cattle. Oliveira and Hansen (2008), Oliveira et al. (2010), and Mansouri-Attia et al. (2012) described the pivotal role of monocytes (Mo), macrophages (Mφ), and dendritic cells (DC) in early bovine pregnancy, and they observed an increase in these mononuclear phagocytic cells in the endometrium in early stages of pregnancy that were maintained at high numbers into late gestation; these cells played a role in regulating the cytokine environment. The cross-sectional study presented in chapter 2 and longitudinal study presented in chapter 3 both showed that late gestation, non-lactating cows have unique profile of myeloid cell composition and function likely to promote tolerance though reduced antigen

presentation, enhanced anti-inflammatory responses, and reduction in certain inflammatory responses. Maternal immunity varies by stage of pregnancy based on unique roles the immune system plays in pregnancy recognition, placentation, maintenance, and labor. The results from the longitudinal study presented in chapter 3 provide evidence that cattle, similar to humans and rodents, experience dynamic shifts in the maternal immune system as pregnancy progresses until calving. Specifically, the work presented here indicates late gestation, concurrent to the dry period, contains the most dramatic changes to monocyte composition and moDC function.

2.1. Monocyte composition in late gestation and postpartum infectious disease

Cows maintained higher proportions of cM and lower proportions of intM in the peripheral blood from mid-gestation up until calving, after which the cM decreased and intM increased in the postpartum period as cows progressed further into lactation (chapter 3). A nadir in the proportion of ncM was observed approximately 2 weeks prior to calving (chapter 3). Analogous to human monocytes, three bovine monocyte subsets are classified by expression of CD14 and CD16, however, only certain functional characteristics overlap between human and bovine monocyte subsets primarily between cM and intM (Hussen et al., 2013, Hussen et al., 2014, Ziegler-Heitbrock, 2014, Corripio-Miyar et al., 2015, Thomas et al., 2015, Hussen et al., 2016). Classical monocytes arise from the bone marrow and are thought to mature into ncM via intM (Sunderkotter et al., 2004, Ziegler-Heitbrock et al., 2010). Bovine intM generate comparatively greater pro-inflammatory responses to LPS and neutrophil degranulation products than cM and ncM (Hussen et al. 2013; Corripio-Miyar et al. 2015; Hussen et al. 2016). Limiting the population size of the more mature, more inflammatory intM subset that expresses high levels of MHC II relative to cM may be one mechanism of maintaining fetal tolerance in bovine pregnancy and reduce highly inflammatory responses (Hussen et al., 2013, Hussen et al., 2014,

Hussen et al., 2016). The absolute count of monocyte population in the blood peaked immediately following calving, but declined as cows progressed in lactation (chapter 3). This dramatic increase in monocyte numbers observed postpartum may potentially relate to the damaging, prolonged, highly inflammatory responses that most often accompany *E. coli* IMI in the postpartum period (Ziegler-Heitbrock, 2007).

Unlike the findings presented here on monocyte composition in bovine pregnancy, healthy human pregnancy has been characterized by increases in intM and decreases in cM in the blood, and an overall rise in monocyte numbers with a steady increase in monocyte activation as pregnancy progresses (Luppi et al., 2002, Faas and de Vos, 2016). Cattle and humans differ in placentation, humans require a substantial increase in uterine NK cells and inflammatory monocytes to the reproductive tract in the early vascular remodeling and maintenance of such an invasive placenta whereas this need may not be as prominent in non-invasive cotyledon placentation in cattle (Smith et al., 2009, Lash et al., 2010, Peter, 2013). Again, these differences reiterate the need to characterize bovine pregnancy immunology as these production animals have distinct differences from humans and other mammalian species.

Findings from the longitudinal study also show that approximately 2 weeks prior to calving cows experience a nadir in ncM proportions (chapter 3). As the function of ncM is not well-defined in bovine species it is difficult to say their exact role in pregnancy, but these changes may relate to preparation for labor. Bovine ncM have also been shown to have different function from their human counterparts, making extrapolations from human pregnancy difficult. These cells are thought to have patrolling function, and more anti-inflammatory properties. Hussen et al. (2013), (2014), and (2016) found ncM to show little to no response to neutrophil degranulation products, LPS, and CCL5. Thus, it is also not easy to decipher their role in bovine

E. coli IMI and mastitis with the available peer-reviewed literature. Further research needs to be conducted on ncM function to interpret the importance of this nadir 2 weeks prepartum with regards to maternal immune regulation, labor, and implications for immune response to IMI and other infectious postpartum diseases.

Monocytes play multiple roles in shaping the immune response from polarization and expansion of primary and memory lymphocytes to activation, recruitment of other myeloid cells, and can exert microbicidal effects (Geissmann et al., 2008, Xiong and Pamer, 2015). Monocytes may also differentiate into Mφ or DC of varying phenotypes with in the tissue depending upon the initial monocyte subset and signals received from other tissue-resident cells and soluble factors present in the tissue (Tacke and Randolph, 2006, Geissmann et al., 2008, Dominguez and Ardavin, 2010). The fate of circulating monocyte subsets upon reaching the mammary gland is not entirely known in dairy cattle. Hussen et al. (2014) showed that CCL5 induced selective migration of bovine cM and differentiation of these cells towards LPS-hypo responsive Mφ *in vitro* reiterating functional differences in these subsets, and also suggesting *in vivo* migration to and differentiation in tissue may vary between subsets; this may relate the increased cM in blood to the lack of inflammatory following *E. coli* intramammary challenge observed in the dry period. Oliveira et al. (2010) found a subpopulation of endometrial Mφ that differentiated via the M2 activation pathway during pregnancy and based on similarity in gene expression between endometrial and blood $CD14^+$ cells, suggested that the immense infiltration of $CD14^+$ cells in the pregnant endometrium is a result of the recruitment from blood monocytes. As dry cows also experience a substantial increase in Mφ and other phagocytes in the mammary gland during involution, this is likely comparable to the findings on endometrial Mφ. This monocyte composition in the blood likely shapes the monocyte and macrophage differentiation in the tissue

to give rise to more M2-like macrophages with reduced antigen presentation. Given the importance of late gestation and *E. coli* IMI in the dry period, these changes noted in blood cells presented here in conjunction with previous work on bovine monocyte subset differentiation and endometrial Mφ, future work needs to address these hypotheses in an *in vivo* model analyzing cells in the mammary gland.

In human and rat pregnancy, deviations from maternal immune regulation of monocyte and Mφ composition and function lead to complications and increased risk of preeclampsia. Although healthy human and rat pregnancy is accompanied with increased monocyte activation, there is a limit, beyond which an inflammatory disorder may develop or other complications may arise (Tang et al., 2015a, Faas and de Vos, 2016). Findings from the prospective study presented in chapter 5 show deviations from maternal immune regulation of monocyte composition prepartum poses as a risk for postpartum infectious diseases including metritis and mastitis. In the case of mastitis, these deviations may potentially exacerbate inflammation in the postpartum period without effective clearance of the pathogen to IMI acquire in the dry or postpartum. It is also possible these prepartum immune dysfunctions may increase risk of acquire IMI in the dry period. Galvao et al. (2012) showed blood monocytes from cows which developed metritis had lower IL-10 and TNFα gene expression following *in vitro E. coli* stimulation; the monocyte subset composition from these animals were unknown. Without functional analysis of these monocytes in pre- and postpartum periods it is not possible to determine if these animals' monocytes were functionally comparable, nor can the role of peripheral blood monocyte composition in postpartum disease be determined. Nonetheless, these findings do indicate individual monocyte subsets are regulated in healthy pregnancy, and deviations in their regulation in late gestation, specifically the balance between CD14+ and CD14- subsets, increase

risk for incidence of infectious disease following parturition. This places precedence on future research to investigate the various roles of bovine monocyte subsets play in both pregnancy and disease. In addition, future work should elucidate factors which may cause monocyte composition and maternal immune regulation to deviate and increase risk of disease.

2.2. Regulation of moDC response to *E. coli* in late gestation and the dry period

Dendritic cells are potent antigen presenting cells which play a critical role in maternal immune tolerance (Blois et al., 2007, Gomez-Lopez et al., 2014). Dendritic cells have a unique ability to induce both antigen-specific immunity and tolerance through their prominent role in naïve T cell activation (Hivroz et al., 2012). In addition to initiation of antigen-specific responses, DC are also involved in initiating and directing innate immunity, this includes crosstalk with NK cells to induce NK cell activation, IFNγ production, and cytotoxic function (Zanoni et al., 2007, Sipka et al., 2016). In mammalian pregnancy, DC are regulated to hinder development of cell-mediated, inflammatory responses against fetal antigens and cells by promoting the generation of Treg cells, inhibiting NK cell cytotoxicity, and hindering generation of Th1 and Th17-type responses (Saito et al., 2005, Lin et al., 2007, Leno-Duran et al., 2014). Tolerance generated by DC play a crucial role in preventing pregnancy complications such as preeclampsia and preterm birth in humans and rodents (Negishi et al., 2012, Hsu and Nanan, 2014, Wang et al., 2014, Prins et al., 2015). Studies presented here indicate there are comparable dynamic changes occurring in gestating dairy cows which had not been described previously (chapters 2&3).

In both the cross-sectional study and longitudinal study presented in chapters 2 and 3, respectively moDC were found to have impaired maturation in response to *E. coli* stimulation in late gestation within the early dry period. Phenotypic changes following *E. coli* stimulation

suggest moDC in late gestation are least mature and likely have a reduced ability to activate naïve T helper cells due to hindered CD80 and MHC II upregulation when compared to moDC from early lactation (chapters 2&3). Monocyte-derived DC from the dry period have reduced IL-12 production, increased IL-10 production, increased IL-1β production with no major changes to TNFα production; these profiles vary throughout pregnancy and lactation, but greatest changes from the studies presented here were observed in the first week of dry-off. These results support a the initial hypotheses that DC have reduced ability to generate antigen-specific, Th1-type responses with greatest regulation of these responses in late gestation compared to other stages of pregnancy and lactation. The peak production in pro-inflammatory cytokine, IL-1β observed in the first week of the dry period concurrent to late gestation, and the unhindered TNFα production throughout pregnancy in bovine moDC are comparable to the selective, tightly regulated increase in specific inflammatory cytokines observed in human pregnancy; bovine pregnancy is not a general state of suppressed inflammatory responses (Rusterholz et al., 2007). The dynamic changes to phenotypic maturation and cytokine production following *E. coli* stimulation throughout pregnancy likely reflect the complex and differing needs and unique factors present at each stage of pregnancy and lactation. For example, IL-1β, among other cytokines may relate to the recruitment of phagocytes to the mammary gland during involution and/or cervical ripening and preparation for labor in late stages of pregnancy and may be balanced with heightened IL-10 production to mitigate risk of preterm birth (Peltier, 2003, Wedlock et al., 2004, van Engelen et al., 2009, Dubicke et al., 2010, Nagamatsu and Schust, 2010).

The observations made in the study presented in chapter 4 show moDC have impaired maturation in response to *E. coli* stimulation following *in vitro* treatments of late gestation levels of progesterone and estradiol. Comparable to other mammalian species, findings presented here

indicate high levels of estradiol and progesterone in circulation observed in late gestation dairy cows regulate moDC and likely other myeloid immune cells which express progesterone and estradiol receptors (chapter 4) (Hunter et al., 1970, Echternkamp and Hansel, 1973, Pioli et al., 2006, Segerer et al., 2009, Lasarte et al., 2013, Schumacher et al., 2014). Specifically, estradiol and progesterone hindered CD80 and MHC II expression, and enhanced IL-10 production in moDC from lactating, non-pregnant cows following *in vitro E. coli* stimulation (chapter 4). These changes induced by progesterone and estradiol in DC populations are comparable to what has been described other mammalian species (Bachy et al., 2008, Butts et al., 2008, Hughes et al., 2008, Della Bella et al., 2011, Papenfuss et al., 2011, Xu et al., 2011, Cordeau et al., 2012, Lasarte et al., 2013). However, these estradiol and progesterone treatments did not recapitulate the enhanced CD14 expression observed in both the cross sectional and longitudinal study, and the effects on the production of other cytokines is unknown. As described in murine and human pregnancy, there are numerous pregnancy-associated factors that extend beyond progesterone and estradiol which regulate myeloid cells including fetal-derived factors, pregnancy-associated glycoproteins, human chorionic gonadotropin, etc. (Segerer et al., 2009, Della Bella et al., 2011, Warning et al., 2011, Schumacher et al., 2013, Schumacher et al., 2014). Regulation by pregnancy-associated factors likely differs between the periphery and tissue due to interactions between various cell populations, and unique composition and level of pregnancy-associated at various sites in the body. Future work needs to further investigate other pregnancy-associated factors and factors specific to the dry period, and their interactions to understand how moDC phenotype and function are regulated dynamically throughout pregnancy. The effects on the production of other cytokines such as IL-12 and IL-1β, and on other functional traits are unknown, and the mechanism by which these hormones regulate bovine moDC have yet to be

determined. Furthermore, the pregnancy-associated factors which regulate monocyte composition described earlier also remain unknown, but these items should addressed in future research.

Metabolism and metabolic factors such as adipocytokines, glucose levels, ketone bodies, and vitamin D3 vary by stage of lactation and pregnancy; these factors have all be shown to influence immune function across mammalian species (Valsamakis et al., 2010, Contreras and Sordillo, 2011, Rulle et al., 2012, Kabara et al., 2014). These metabolic factors are known to influence the effects of other immunomodulatory factors for example, inhibition of mTOR, a pathway which integrates growth factors, energy status, oxygen and amino acid signals to regulate multiple cell growth processes, can inhibit the anti-inflammatory, immunosuppressive effects of glucocorticoids on immune cell function (Weichhart et al., 2011). The onset lactation following parturition comes with high energy demands and metabolic changes that are known to influence immune function in dairy cows (Contreras and Sordillo, 2011, Zarrin et al., 2014, Sundrum, 2015). In addition to steroid hormones such as progesterone and estradiol, differentiation of monocytes into DC and DC maturation is regulated by lipid metabolism, ketone bodies, glucose metabolism and the mTOR pathway, and vitamin D3 and specifically play a large role in generation of tolerogenic DC in non-bovine, mammalian species (Ferreira et al., 2015, Kelly and O'Neill, 2015, Pearce and Everts, 2015, Youm et al., 2015, Sim et al., 2016, Sukhbaatar et al., 2016). Recently, Eger et al. (2016) found bovine monocytes had limited ability to take up glucose following parturition compared to monocytes from the prepartum period due to downregulation of glucose transporter expression as a function of lactose production. Eger et al. (2016) suggested changes to monocyte glucose metabolism might relate to their impaired macrophage differentiation; this work supports the likely a role of metabolism in the

differentiation and maturation of bovine monocytes into moDC. Perkins et al. (2001) found that diet-induced negative energy balance caused an increase in MHC II expression on blood leukocytes in steers further supporting potential metabolic induced changes in moDC over stages of pregnancy and lactation. Myeloid cells are dependent on glycolysis to generate more pro-inflammatory responses like IL-1β production suggesting changes decreases in glucose following parturition may be one factor related to cytokine profiles from moDC observed at various stages of pregnancy and lactation (Orlinska and Newton, 1993, Freemerman et al., 2014). Furthermore, β-hydroxybutyrate (BHB) negatively regulates human monocyte IL-1β production, and given levels of BHB in serum are high postpartum in early lactation but low in the dry period this emphasizes the connection between metabolic state of a non-lactating period and immune regulation in conjunction with effects by pregnancy-associated steroid hormones (Bernier-Dodier et al., 2011, Youm et al., 2015). Cessation of lactation also causes a reduction in hormones related to milk production such as prolactin, which has been shown to enhance inflammatory responses of various PBMC (Brand et al., 2004, Boutet et al., 2007). Given the unique energy demands of a dairy cow, the effects of metabolic and lactation-related factors on bovine moDC and myeloid cell populations as they relate to changes throughout lactation and the dry period need to be addressed in future work. These metabolic changes associated with the energy demands of the growing fetus and the transition from lactation to the dry period likely relate to the unique in moDC differentiation and maturation in the early dry period in addition to effects of estradiol and progesterone. The interaction of these metabolic changes from non-lactating to lactating stages, such as mTOR inhibition or activation, with other immunomodulatory pregnancy-associated factors and/or metabolic factors are not well-characterized in cattle.

The mechanisms by which these pregnancy-associated factors regulate immune cells is not well-described in dairy cattle. In non-bovine species, pregnancy is known to involve epigenetic regulation and improper epigenetic regulation may lead to pregnancy complications like preeclampsia (Chen and Wang, 2013, Brooks et al., 2016). Paibomesai et al. (2013) found that both parturition and dexamethasone treatment were able to regulate the DNA methylation patterns of IL-4 and IFNγ promoters of CD4+ T cells in dairy cattle related to an increase of IFN-γ production following parturition and an increase in IL-4 production prior to calving. It is known that environmental factors which induce differential epigenetic regulation of immune cell populations creating lasting, heritable impacts on immune response to disease (Jirtle and Skinner, 2007). Epigenetic mechanisms including DNA methylation, histone modification, and microRNA can be induced by endocrine factors, endocrine-disruptors, and metabolites and nutritional status including estrogen, phytoestrogens, and Acetyl-CoA respectively through the regulation of epigenetic-modifying gene expression (Zhang and Ho, 2011, Etchegaray and Mostoslavsky, 2016). Epigenetic regulation of maternal immune cells by pregnancy-associated factors is another area of research worth investigating to assist in the development of novel immunomodulators by identifying targets and improve understanding of mechanisms generating maternal immune tolerance and altered immunity in the dry period.

3. Drawing relationships between late gestation immune regulation and *E. coli* IMI dynamics in the dry period

The work presented in chapters 2-5 investigated changes in blood myeloid cell populations in pregnancy. These changes in blood cells may relate to the unique IMI dynamics observed in the dry period, but these *in vitro* studies cannot account for the specific changes which occur during involution and steady-state of the dry period within the mammary gland.

Findings from the mathematical modeling of IMI dynamics in the dry period presented in chapter 6 recapitulate both the persistency of IMI in the dry period and the reduction in cytokine response which likely relates to the lack clinical signs observed with IMI occurring in the dry period. The *E. coli* IMI dynamics are more complex than initially hypothesized, and the involvement of unique physiological and immune changes to the mammary gland with the cessation of lactation is likely to play a larger role than initially thought. The model findings suggest the persistency and limited pro-inflammatory response is related to effects from physiological and immunological changes in the dry period and late gestation maternal immune regulation which overlaps with the dry period. The non-lactating mammary gland has an increased numbers of phagocytes with reduced phagocytic abilities, and secretions from the gland are unfavorable for bacterial growth i.e. reduced nutrients, increased soluble immune factors, etc. relative to milk (Todhunter et al., 1990, Inchaisri et al., 2000, Boutinaud et al., 2016). The role of maternal immune regulation i.e. suppression of Th1-type immunity and highly inflammatory responses appears to contribute to dampened cytokine responses, and may relate to inability to kill infected host cells however this cannot be confirmed with the mathematical model or experimental work (chapters 2-6). The *in vivo* work presented in chapter 7 shows the mammary gland is able to clear extracellular bacteria yet the IMI persists post-calving which suggests the formation of intracellular reservoirs is crucial (Dogan et al., 2006, White et al., 2010). Future work should investigate changes to mammary tissue-resident immune cell populations and cell-mediated responses in IMI in the dry period with different *E. coli* strains that vary in their ability to form intracellular reservoirs. Furthermore, future work should differentiate between dry period and late gestation factors involved in the unique IMI dynamics observed in late gestation, dry cows.

4. Targeting mucosal immunity in the dry period for *E. coli* IMI

The long term goal of the work presented here is to provide necessary insight to progress preventive medicine for postpartum *E. coli* mastitis through the development and/or optimization of immunomodulators and vaccination in the dry period. The work presented in chapter 7 indicate that it is possible to elicit protective Th1-type immunity against *E. coli* IMI occurring in the dry period with an intramammary immunization of a homologous strain at the start of dry off. Local immunization resulted in enhanced IFNγ production and reduced IL-10 production following live *E. coli* challenge approximately 10-12d prior to calving; this coincided with reduced bacterial numbers and probability of being infected, and effectively reduced cases of clinical mastitis and clinical severity postpartum relative to unimmunized, challenged quarters (chapter 7) (Pomeroy et al., 2016a). This further suggests the late gestation maternal immune regulation observed in previous work in chapters 1- and in peer-reviewed literature functions differently from immune cells in the blood and/or can be manipulated locally in the mammary gland to produce Th1-type responses (Rosbottom et al., 2008, Maeda et al., 2013, Pomeroy et al., 2015, Pomeroy et al., 2016b). The lack of crossover of protective immunity between unimmunized and immunized quarters within a cow further emphasizes the quarters act as individual compartments which is relevant when designing a locally administered immunomodulator or vaccine.

The presence of DC and other myeloid cell populations in the bovine mammary gland has been confirmed in previous work, and work presented here emphasizes the need to continue research on the function of myeloid cell populations resident in mammary tissue to elucidate the relevancy of the moDC *in vitro* model in local immune response to IMI in the dry period (chapters 2-4). It is not possible to decipher the mechanisms that were involved in generating

protective immunity from the work presented in chapter 7, and it is unknown if the protective immunity related to non-specific trained immunity of innate cells, and/or the generation of a local antigen-specific lymphocyte cell population from effective activation of DC (van der Meer et al., 2015, Jensen et al., 2016). Future work should also include measurements of antigen-specific antibody production and T cell via recall responses by cells within an immunized quarter. In addition to measurements on antigen-specific immunity, future work should also investigate the formation of trained immunity for example, by analyzing epigenetic changes to local immune cell populations and/or by investigating the effect of an intramammary immunization on a non-homologous *E. coli* challenge.

Findings from the mathematical modeling study presented in chapter 6 indicated local changes to the mammary gland during involution and cessation of milk production were important parameters in shaping IMI dynamics. Like the mathematical model, the results from dry period intramammary immunization reiterate the need to investigate pregnancy-associated changes to tissue resident DC to understand the role of maternal immune regulation in intramammary immunization and IMI infection dynamics in the dry period. Although mechanisms cannot be deduced here, targeting mucosal immunity in late gestation with intramammary immunization appears to be more effective than systemic vaccination in generating protective responses. Furthermore the lack of crossover of protective immunity from an immunized quarter to a neighboring unimmunized quarter within a cow emphasizes localization and lack of migration of these trained and/or antigen specific cells. Thus, route of administration and should be considered when developing immunomodulators and vaccinations to prevent IMI.

5. Conclusions and future directions

Pregnancy occurs regularly and frequently in dairy cattle, and the impact of pregnancy immune regulation should not be ignored when considering disease management and the development of vaccines, immunomodulators, and therapeutics. Findings in this dissertation, items from current literature, and hypotheses and ideas developed from these two sources are summarized in figure 28 of the general discussion. Mechanisms pertinent to fetal tolerance may compromise immune response to certain invading pathogens and effective response to vaccination. In order to develop successful preventative medicine and treatment for *E. coli* IMI that aim to manipulate and boost the host immune response, target pathways must be identified, and timing and route of administration of immunomodulators and vaccines must be selected to optimize efficacy without compromising safety or milk and meat withholding time. Species differences including placentation and unique energy demands of a production animal render human and rodent models of pregnancy suboptimal for understanding maternal immune regulation in dairy cattle. Work presented here indicates that dairy cattle undergo dynamic maternal immune regulation of mononuclear phagocytic cells which are likely to contribute to the unique *E. coli* IMI dynamics in the dry period in conjunction with physiological and immunological changes specific to cessation of lactation. Future work should expand upon the role of maternal regulation in later stages of pregnancy with regards to tolerance of the fetus and relationship with response to vaccination and invading pathogens. The unique changes to immune function within the mammary gland in the dry period and interplay with maternal immune regulation must also be addressed in future work. Expediting the development of safe and effective immunomodulators and vaccines for late gestation dairy cows impinges on mechanisms of maternal-fetal immune tolerance involved in a healthy, uncomplicated pregnancy

and negative effects on pregnancy and calf health when there are deviations in the maternal **immune system.**

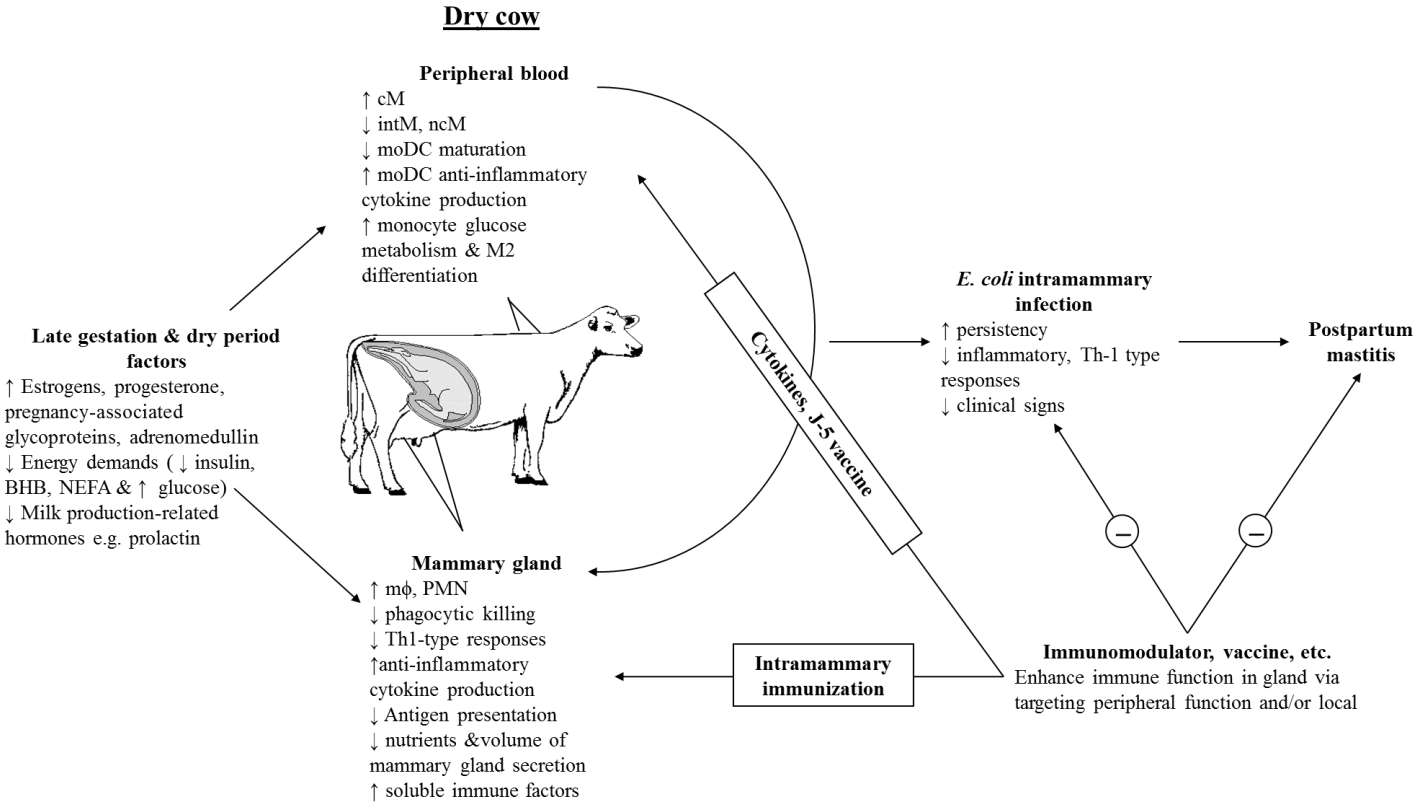

Figure 28. Summary of dissertation findings with relevant literature. Aspects of immune function of dry cows, late gestation and dry period factors which may regulate these changes, and the proposed relationship with *E. coli* IMI, postpartum mastitis risk, and ability to enhance immunity via immunomodulators and vaccination.

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