Adoptively Transferred Maternal Colostral Cells Impact Immune Status and Development in Dairy Calves

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ABSTRACT

Mortality and decreased weight gain resulting from infection and disease in dairy calves is a problem within the dairy industry. Colostrum is the sole source of maternal immunity for the calf, having a substantial impact on health and survival. To date, colostrum quality is determined by concentration of antibodies. Colostrum also contains proteins and cells, which may enhance immune development in the neonate. Our goals were to determine the impact of colostral immune cells on (1) immune status during the first month of life and (2) immune development over time. To determine the impact of adoptively transferred colostral immune cells, calves were fed either whole colostrum (WC) or cell-free colostrum (CFC) at birth. During the first month of life, calves fed CFC had decreased numbers of $CD4^+$ T cells when compared to WC-fed calves. However, CFC-fed calves had a greater percentage of monocytes during the first month of life. To determine the influence of colostral immune cells on immune development, cellular blood parameters were measured in response to two series of vaccinations (A and B). After vaccination series A, CFC-fed calves had decreased numbers of B cells when compared to WC-fed calves. After vaccination series B, CFC-fed calves had decreased levels of interleukin-2 gene expression and numbers of $CD4^+$ and $\gamma\delta$ T cells when compared to WC-fed calves. This study demonstrates that colostral immune cells impact immune status and development in dairy calves.

Key words: colostrum, adoptive transfer, leukocytes, vaccination, dairy calves

Dedication

Dedicated to my wonderful family Steve, Kathy and Stevie Neal and John Langel.

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"You win with people." –Coach Woody Hayes. I believe this sentiment goes beyond the football field. I am infinitely grateful to everyone who helped me over the past two years.

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List of Abbreviations

Full name	Abbreviation
Whole colostrum	WC
Cell-free colostrum	CFC
Peripheral blood mononuclear cell(s)	PBMC
Enzyme linked immunosorbent assay	ELISA
Immunoglobulin	Ig
Interleukin	IL
Interferon	IFN
Tumor growth factor	TGF
Tumor necrosis factor	TNF
T cytotoxic cell	T_c
T helper cell	T_h
Antigen presenting cell(s)	APC
Major histocompatibility complex	MHC
T helper cell type-1	Th1
T helper cell type-2	Th2
Bovine herpesvirus	BHV
Bovine viral diarrhea virus	BVDV
Mycobacterium avium subspecies *paratuberculosis*	MAP
Bovine respiratory syncytial virus	BRSV
Bovine leukemia virus	BLV
Foot and Mouth Disease Virus	FMDV

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Chapter 1: Literature Review

1.1 Introduction

Neonatal animals are immunologically naïve and highly susceptible to bacterial and viral pathogens. The most recent National Animal Health Monitoring System report states that 56.5% of all unweaned heifer deaths are due to scours/diarrhea and 22.25% are due to respiratory problems (USDA-APHIS:VS, 2007). Infection and disease associated with scours and respiratory ailments results in mortality and decreased weight gain. Calf morbidity has a negative impact on profitability of the dairy operation and decreases animal well-being. Raising animals with a more effective immune system lowers the incidence of disease; potentially increasing average daily gain, decreasing age of first calving, and increasing first milk production (Correa et al., 1988, Virtala et al., 1996, Heinrichs and Heinrichs, 2011).

Neonatal ruminants rely on maternal colostrum as their first and most important source of nutrients needed for survival (Quigley and Drewry, 1998). The bovine syndesmochorial placenta forms a multinucleated mass between the maternal endometrium and fetal trophectoderm. This prevents transfer of immunoglobulins (Ig), i.e. antibodies, to the fetus *in utero* (Arthur, 2001). Therefore, calves are born hypogammaglobulinemic; having inadequate levels of circulating Ig. Colostrum serves as the sole source of Ig during the neonatal period. The role of Ig in neonatal immune protection is well documented (Gay et al., 1965, Logan et al., 1974b).

Colostrum also contains leukocytes, cytokines, antimicrobial proteins, and hormones (Reiter et al., 1975, Hagiwara et al., 2000, Blum and Baumrucker, 2002). Colostral leukocytes and cytokines are able to pass through the intestinal epithelium into circulation (Schnorr and Pearson, 1984, Williams, 1993, Hagiwara et al., 2001). It is suggested that these components have immunomodulatory and protective effects in the neonate (Riedel-Caspari, 1993). Little is known of

the exact role of colostral leukocytes and cytokines in neonatal immune development. Our objectives for this project were to (1) determine the impact of colostral components on neonatal calf health and immune status and (2) analyze the impact of colostral components on long-term immune development.

1.2 Overview of the Immune System

The following will provide an overview of the immune system based on the organization and material from texts by Goldsby (2003), Abbas (2000) and Tizard (2000). It is important to understand the basic constituents and mechanisms of the immune system prior to discussing immunity in the calf. The immune system has the ability to respond to 10^9-10^{11} different antigenic determinants, or types of pathogens. This is achieved through two different but interconnected 'arms' of the immune system; the innate and the adaptive immune response. When soluble molecules, proteins and leukocytes are responding appropriately, the immune system can effectively clear a pathogen from the host.

The cells of the innate immune system provide a first line of defense against invading microbes. Phagocytes, like neutrophils, monocytes and macrophages, are innate immune cells, which rapidly respond to infection. Phagocytes engulf an invading pathogen and degrade it into smaller piece, i.e. antigens, by hydrolytic enzymes. After degradation, the antigen is expressed on the surface of the phagocyte and used to activate other cells. Natural killer cells are lymphocytes of the innate immune system. When natural killer cells are activated, cytoplasmic granules release proteins and proteases that lyse pathogen-infected cells. While these innate immune cells are quick to respond, they cannot develop memory to previously encountered pathogens.

Soluble factors, like cytokines, acute phase proteins and antimicrobial factors are also key mediators in the innate immune response. Cytokines are communication molecules secreted from

activated cells. Interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor α (TNF-α) are involved in the inflammatory response. These cytokines increase phagocyte migration and vasodilation, enhancing pathogen clearance. Acute phase proteins, i.e. complement, recruit antibodies and phagocytes to the site of infection. Complement proteins can directly lyse infected cells by forming a pore in the cellular membrane. Antimicrobial factors, i.e. enzymes and oxidants, kill or inhibit growth of the pathogen by a variety of mechanisms.

Unlike the innate immune system, the cells of the adaptive immune system recognize previously encountered pathogens. The first time an adaptive immune cell binds to an antigen, the cell will go through several divisions, referred to as clonal expansion. During clonal expansion, the cell will differentiate into short-lived ‘effector’ cells or long-lived ‘memory’ cells. Effector cells have an immediate function; migrating to the site of infection and secreting cytokines. Memory cells home to lymphoid organs and proliferate into effector memory cells in response to secondary antigenic stimulation. The adaptive immune system’s ability to respond more efficiently to subsequent infections is the basis behind disease resistance and vaccine development.

The first type of response in adaptive immunity is the cell-mediated response. The cell-mediated immune response is composed of T lymphocytes, distinguished by the surface expression of either the αβ or γδ T cell receptors. The two major subsets of αβ T lymphocytes include cytotoxic T lymphocytes ($CD8^+$) and helper T lymphocytes ($CD4^+$). $CD8^+$ T lymphocytes directly kill infected immune cells by secreting cytotoxins and proteases. $CD4^+$ T lymphocytes stimulate antibody production from B cells, induce macrophage development, and recruit granulocytes. The γδ T cell can respond directly to a pathogen, similar to innate immune cells, or be activated by phagocytes, similar to adaptive immune cells.

The humoral immune response is composed mainly of B cells. B cells mature during two sequential phases; an antigen-independent and an antigen-dependent phase. The antigen-independent phase occurs in the bone marrow, where Ig gene rearrangement maturates pro-B cells into pre-B cells. At this stage all B cells express surface IgM and continue developing in the spleen. Antigen-independent maturation occurs in the lymph nodes where antigen stimulation and gene recombination result in class-switch recombination. Class-switch recombination changes surface IgM to secondary IgG, IgA and IgE B cell isotypes. Further antigen stimulation results in clonal expansion of B cells into either antibody-secreting plasma cells or long-lived memory cells. In the blood stream, antibodies circulate and neutralize pathogens. Memory B cells can reside for years in lymphoid organs such as the spleen, lymph nodes, and bone marrow.

While the innate and adaptive immune systems represent different types of responses, they are dependent on each other for activation and regulation. Monocytes, macrophages and dendritic cells of the innate immune system and B cells of the adaptive immune system are classified as antigen presenting cells (APC). As described above, after an APC engulfs a pathogen, it will digest it into smaller peptides, i.e. antigens. The antigens are then loaded on a major histocompatibility complex (MHC) on the APC cellular surface. T lymphocytes recognize the MHC and respond by either secreting cytokines or differentiating into memory cells.

For an effective immune response, the host must be able to quickly recognize the pathogen and build immunological memory to confer protection to future infections. To do this, both 'arms' of the immune system need to be working efficiently; independently and concurrently.

1.3 Overview of the Neonatal Immune System

The transfer of immunity from mother to neonate is highly regulated and species specific (Baintner, 2007). In most species, neonates at birth have an immature and underdeveloped im-

mune system when compared to adults (Kerr and Robertson, 1954). In the bovine, the dam's placenta forms six layers of multinucleated syncytia, preventing transfer of antibodies to the fetus during gestation (Arthur, 2001). Although endogenous humoral components are produced *in utero*, antibody levels are not adequate to fight bacterial and viral infections (Husband and Lascelles, 1975). Therefore, the dam's first milk, hereto referred as colostrum, is required to provide protection until the calf's immune system can produce endogenous antibody (Banks, 1982).

Neonatal susceptibility to infections does not result from an inability to mount an immune response, but rather, is because the immune system is unprimed and immature. Pronounced differences in amount and function exist in cell types between the neonate and adult. In the calf, the percentages of $CD4^+$ and $CD8^+$ T lymphocytes are lower at approximately 16% and 8% within a peripheral mononuclear cell (PBMC) population, respectively, when compared to cow percentages at 29% and 10%, respectively (Wilson et al., 1996). Neonates have a great proportion of naïve $CD4^+$ T lymphocytes than their adult counterparts. However, the calf develops memory lymphocytes with age and antigenic stimulation (Clement et al., 1990). Calf B cell proportions at birth are 4% of lymphocytes and gradually increase to adult levels, reaching 30% by 6 months of age (Kampen et al., 2006). Antibody-producing activity of B cells is decreased in calves and doesn't reach adult levels until 14-21 days of life (Nagahata et al., 1991). However, feeding of maternal colostrum can have a negative effect on endogenous antibody production by B cells (Husband and Lascelles, 1975, Aldridge et al., 1998). Gamma delta ($\gamma\delta$) T cells are highest in calves and can reach up to 40% within a PBMC population, with a gradual decrease as the animal ages (Wilson et al., 1996). Cytokines, neutrophil-secreted myeloperoxidase, and complement proteins are found in lower levels in younger animals than in adults (Banks, 1982). The dif-

ferences in both cell number and function leave the neonate with a weaker and less efficient immune response when compared to adults.

1.4 Fetal Immune Development

While there is limited development of the bovine immune system *in utero*, cellular components of the adaptive immune system are non-the-less present in fetal calves. The numbers of $CD4^+$, $CD8^+$, and γδ T cells in fetal blood are lower when compared to adult cattle. However, the distribution of $CD4^+$ and $CD8^+$ T lymphocytes is similar in bovine fetal blood when compared to adults, where $CD4^+$ and $CD8^+$ T cells are approximately 30% and 12% within a PBMC population (Wilson et al., 1996).

Despite being immunologically naïve, when infected *in utero,* the bovine fetus can produce antigen-specific immune cells. In a study by Bartley et al. (2004), cows were challenged with *Neospora* caninum tachyzoites during mid-gestation. Cows were sacrificed and fetal lymph node PBMC were isolated and stimulated with a *N. caninum* tachyzoites lysate. Mesenteric lymph node PBMC from fetuses born to *N. caninum* challenged cows had greater a interferon-γ (IFN-γ) response compared to fetuses born to non-*N. caninum* challenged cows (Bartley et al., 2004). Additionally, the bovine fetus is able to generate antibody-specific responses. Fetuses from *Leptospira* serovar saxkoebing-infected mothers have detectable levels of *Leptospira*-specific antibodies in serum prior to colostrum feeding (Fennestad and Borg-Petersen, 1957).

Disease susceptibility in the neonate is also attributed to hormones produced by the placenta during pregnancy. Pregnancy hormones, i.e. prostaglandin E_2 and progesterone, bias the fetus and subsequently the neonate to a Th2-type response (Wegmann et al., 1993). There are two distinct $CD4^+$ T cellular responses based on cytokine expression; a T helper cell type-1 (Th1) response and a T helper cell type-2 (Th2) response. The Th1 response is pro-inflammatory

and defined by the cell-mediated immune response and IFN-γ secretion. The Th2 response is anti-inflammatory and defined by the humoral immune response (Mosmann et al., 1986). Increasing Th2 cytokines suppresses the Th1 response, inhibiting cell-mediated immunity. This bias in immune response in calves should be considered in disease prevention strategies and vaccine development.

1.5 The Colostrum-Deprived Calf

Small populations of T and B lymphocytes develop in the fetus *in utero*. However, the calf is born with minimal cellular immune defenses. For example, colostrum-deprived calves have very low amounts of endogenous antibodies produced from birth to 3 days of age (Clover and Zarkower, 1980). Antibody isotype IgM is not detected in circulation until 4 days after birth, reaching appreciable levels by 8 days of age (Husband et al., 1972). Despite the lower amount of antibodies at birth, colostrum-deprived calves mount an enhanced antigen specific antibody response compared to their colostrum-fed counterparts. For instance, Husband and Lascelles (1975) vaccinated colostrum-fed and colostrum-deprived calves at varying ages from birth to 4 months. Colostrum-fed calves did not generate an antibody response to *Brucella abortus* antigen while maternal *B. abortus*-specific antibody was present. However, an antibody response was seen in colostrum-deprived calves to *B. abortus* (Husband and Lascelles, 1975).

This phenomenon is attributed to colostral antibody inhibiting endogenous antibody production. Previous research shows that endogenous antibody production is correlated to levels of maternal antibody in serum (Logan et al., 1974a). Calves fed colostrum positive for bovine herpesvirus 1 (BHV-1) maternal antibody and then subsequently infected with BHV-1, have no active antibody response. However, calves fed colostrum negative for BHV-1 maternal antibody have an enhanced antibody response to infection (Bradshaw and Edwards, 1996). The same is

seen when calves are fed colostrum that have low or high antibody titers to bovine coronavirus. Calves fed high titer colostrum, as measured by an enzyme linked immunosorbent assay (ELISA), had lower antibody responses to subsequent coronavirus infection than calves fed low titer colostrum (Heckert et al., 1991). This creates a dilemma when vaccinating colostrum-fed calves before maternal colostral antibodies have dissipated. It is maternal colostral antibodies, not endogenous antibodies, which respond to vaccination in the calf (Husband and Lascelles, 1975, Nonnecke et al., 2005). The half-life of plasma IgG1 is approximately 20-21.5 days, therefore endogenous production of IgG doesn't begin until approximately week 4 in calves with high colostral IgG (Logan et al., 1973, Sasaki et al., 1977). It is because of this that vaccinating calves during the first week of life remains controversial in neonatal calves.

Recent research shows that not all immune responses are blocked by maternal colostral antibodies. In a 2003 (Endsley et al.) study, calves with high levels of circulating maternal antibody to bovine viral diarrhea virus (BVDV)-1 and BVDV-2 were either challenged from 2 to 5-weeks of age with BVDV-2 (strain 1373) or not infected. After challenge, BVDV-2 specific $CD4^+$, $CD8^+$, and $\gamma\delta$ T cells were detected in experimentally challenged calves, even when levels of maternal BVDV-2 specific antibodies were still circulating. A second challenge was performed with BVDV-2 (strain 1373) in both groups of calves. Only the calves previously challenged to BVDV-2 in the face of maternal antibody were protected from clinical signs of BVDV when compared to non-challenged animals (Endsley et al., 2003). This demonstrates that the cell-mediated immune response remains responsive and should be considered when designing neonatal vaccinations.

1.6 Colostrum

Colostrum is a nutrient and immune-cell dense liquid produced by the dam 3-4 weeks prior to parturition (Brandon et al., 1971, Quigley and Drewry, 1998). A variety of bioactive factors in colostrum prevent mortality and morbidity in young calves (Besser and Gay, 1994). Colostrum contains antibodies, leukocytes, cytokines, antimicrobial proteins, and hormones (Reiter et al., 1975, Hagiwara et al., 2000, Blum and Baumrucker, 2002). Many studies have demonstrated the importance of colostral antibodies in neonatal health and immune status. However, there are a limited number of studies investigating the potential role for non-antibody colostral components in neonatal immunity.

Significance of Colostral Antibodies

By far the most studied component of colostrum is colostral antibodies. Bovine colostral antibody isotypes include IgG, IgA and IgM and are represented at 85-90%, 5% and 7% of the total antibodies in serum, respectively (Klaus et al., 1969, Tewari and Mukkur, 1975, Larson et al., 1980). Studies suggest that IgG is selectively transported into the mammary gland from blood while IgM and IgA are produced locally in the mammary gland from plasma cells. In non-ruminant animals, IgA is present in the greatest amounts in colostrum. However, in ruminants, IgG is represented in the highest amount. IgG1 isotype represents 80-90% and IgG2 represents 10-20% of bovine colostrum (Larson et al., 1980). Absorption of colostral antibodies into neonatal circulation is the most important factor in calf health and survival (Tyler et al., 1999, USDA-APHIS:VS, 2007). A serum concentration of 10 mg/ml is commonly associated with adequate passive transfer of antibodies. A calf with less than 10 mg/ml is defined as having failure of passive transfer (FPT). Calves with FPT have a greater risk of morbidity and mortality (Weaver et al., 2000).

Significance of Colostral Leukocytes

The “white cells”, i.e. leukocytes, of human colostrum were first observed in 1839 by biologist Alexander Donne. However, these cells were not rediscovered until 1966 when Smith and Goldman (1968) analyzed the morphology and function of colostral leukocytes. Bovine colostrum contains between 8.7 x 10^5 to 3 x 10^6 maternal white blood cells. Of those, 22-25% are lymphocytes, 25-37% are neutrophils, and 40-50% are monocytes (Lee et al., 1980, Duhamel et al., 1987, Liebler-Tenorio et al., 2002). To demonstrate the adoptive transfer of colostral leukocytes across the intestinal epithelium, Schnorr and Pearson (1984) fed lambs fluorescein isothiocyanate (FITC)-labeled colostral cells. The number of FITC-labeled cells in circulation from lambs fed FITC-labeled colostral leukocytes was greater than animals fed colostrum without FITC-labeled leukocytes at 6 and 12 hours post-ingestion. Additionally, there were no differences in the amount of FITC-labeled colostral leukocytes from unrelated ewes or natural mothers. This study demonstrates that colostral leukocytes are adoptively transferred into circulation, possibly independent of maternal-neonatal recognition factors (Schnorr and Pearson, 1984). The transfer of leukocytes across the intestinal epithelium has also been demonstrated in calves (Liebler-Tenorio et al., 2002), pigs (Williams, 1993), rats (Seelig and Billingham, 1981), and baboons (Jain et al., 1989).

Currently, there is evidence that adoptively transferred colostral leukocytes impact bacterial clearance *in vivo*. In a study conducted in 1993 (Riedel-Caspari), colostrum was collected from dams after calving and subsequently centrifuged. The supernatant was pooled and the remaining leukocyte pellet was either added back to the pooled colostrum (COL+) or removed (COL-). Calves were experimentally infected with a strain of attaching and effacing *E. coli* (AEEC) and then immediately fed either COL+ or COL-. Calves fed COL+ excreted lower CFU

of experimentally-induced *E. coli* per gram of feces during days 3-6 of life when compared to calves fed COL-. Additionally, *E. coli* secretion in calves fed COL+ reached the lower limit of detection earlier than calves fed COL-. Lastly, the concentration of *E. coli*-specific antibodies was greater during the first 48 hours post-infection in calves fed COL+, suggesting that colostral leukocytes enhanced B lymphocyte production of *E. coli*-specific antibodies. This data suggests that colostral leukocytes may have an impact on pathogen clearance in the dairy calf. (Riedel-Caspari, 1993).

Adding further evidence that colostral leukocytes enhance bacterial clearance, Pitt et al. (1977) used newborn rats as a model for colostral leukocyte clearance of *Klebsiella pneumoniae*. Newborn rats were given 2-5 × 10^7 oral dose of necrotizing enterocolitis-causing *K. pneumonia*. Then rats were fed fresh rat milk, frozen rat milk, frozen rat milk plus rat milk leukocytes, frozen rat milk plus rat peritoneal leukocytes, formula, formula plus rat milk leukocytes, formula plus rat peritoneal leukocytes or formula with rat blood leukocytes. The rats fed frozen rat milk or formula had 90 and 100% mortality rates due to necrotizing enterocolitis, respectively. The rats fed the frozen rat milk plus rat milk leukocytes and frozen rat milk plus rat peritoneal leukocytes had lower mortality rates due to necrotizing enterocolitis when compared with frozen milk. The rats fed formula plus rat milk leukocytes, formula plus rat peritoneal leukocytes and formula with rat blood leukocytes had lower mortality rates due to necrotizing enterocolitis when compared with formula (Pitt et al., 1977). This data shows that colostral leukocytes impact clearance of *K. pneumonia* in rats.

Maternal colostral cells also alter adhesion marker expression on T lymphocytes and monocytes. In a Reber et al. (2008a) study, calves were fed either COL+ or COL- colostrum. Calves fed COL- exhibited a greater percentage and number of monocytes on day 14. Monocyte

surface adhesion and migration molecules CD11a and CD11c were also analyzed. The number and percentage of monocytes expressing CD11a on day 2 and the percentage expressing CD11c on day 14 were greater in COL- calves compared to COL+ calves (Reber et al., 2008a). When T lymphocytes were analyzed, calves fed COL- had a greater percentage and expression of CD11a on day 2 and day 14 when compared to calves fed COL+, respectively. The percentage and expression of activation markers increases in individuals experiencing physiological and environmental stressors (Guzhova et al., 1998, Holthe et al., 2004). The authors suggest that COL- fed calves experienced a stress response by a lack of ingested colostral immune cells, potentially causing an increase in migration marker expression on endogenous monocytes and T lymphocytes in calves (Reber et al., 2008b).

Activation markers CD25 and CD26 were analyzed on T lymphocytes in calves fed COL+ or COL-. T cells expressing CD25 increased in calves fed COL+ on day 7 when compared to calves fed COL-. Additionally, CD26 expression increased in COL+ at hour 24 and day 7 (Reber et al., 2008b). It could be hypothesized that the increase in CD25 and CD26 expression on lymphocytes is attributed to passive transfer of maternal $CD26^+$ T lymphocytes through the calf intestine and into circulation. Additionally, colostral cells could have enhanced activation status of endogenous T lymphocytes. $CD4^+CD26^+$ T cells represent a high percentage of activated mature $CD4^+$ T cells (Dong et al., 1996, Ishii et al., 2001). There are more $CD4^+CD26^+$ T cells in colostrum of fourth parity dams compared to primiparous dams (Ohtsuka et al., 2010). This data suggests that activation markers CD25 and CD26 may correlate to immunological maturity.

Colostral maternal cells influence MHC expression on lymphocytes. In the same Reber et al. (2008a) study, calves fed COL+ had a greater percentage and density of surface marker MHC-I expression on lymphocytes. APC use MHC-I to present antigen to $CD8^+$ T cells. An in-

crease in MHC-I suggests that APC from COL+ calves have a greater ability to activate $CD8^+$ T cells (Reber et al., 2008a). Two groups of Holstein calves were fed COL+ or COL- at birth. Blood was collected for 5 weeks and leukocytes were isolated and stimulated with superantigen, *Staphylococcus* enterotoxin B (SEB), which causes non-specific activation of T cells and is MHC-II dependent (Hong et al., 1996). There were less proliferating leukocytes from calves fed COL- when compared to calves fed COL+ at 3-weeks of age. The authors mention that increased proliferation of leukocytes from COL+ calves suggests an increased ability of APC to stimulate $CD4^+$ T cells by MHC-II antigen presentation (Reber et al., 2006). Increasing antigen presentation capabilities function by MHC-I or –II could aid calves fed COL+ by an enhanced ability to clear bacterial or viral pathogens.

Colostral leukocytes impact responses to vaccine antigen, *in vitro*. In a 2007 (Donovan et al.) study, pregnant dams were given a 5-way respiratory vaccine, including inactivated BVDV, prior to calving. Calves were fed whole colostrum, frozen colostrum or cell-free colostrum after birth. Calves were bled every day and leukocytes were isolated and stimulated with either a BVDV antigen or a control antigen, which the dams were not vaccinated against. None of the calves responded to control antigen, however, leukocytes from calves fed whole colostrum proliferated in response to the BVDV antigen. There was no proliferative response seen in leukocytes from calves fed frozen or cell-free colostrum to the BVDV antigen (Donovan et al., 2007). This suggests that maternal antigen-specific memory cells may migrate from blood into the mammary gland during colostrogenesis in the dam. Adoptive transfer of antigen-specific memory cells may enhance the calf's ability respond to pathogens later in life.

Significance of Colostral Cytokines

As described above, cytokines are soluble communication molecules that are involved in almost all aspects of immune function. Inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ are found in bovine colostrum (Hagiwara et al., 2000). Like cellular colostral components, cytokines can passively transfer through the intestine in neonatal calves and pigs (Yamanaka et al., 2003, Nguyen et al., 2007). When calves were given oral recombinant IL-1β (rIL-1β), an enhanced proliferative response was seen in stimulated T cells compared to pre-treatment levels. Additionally, oxidative burst response of calf neutrophils was increased post-treatment of rIL-1β when compared to pre-treatment levels. (Hagiwara et al., 2001, Nguyen et al., 2007). This suggests that inflammatory cytokines in colostrum may enhance T cell proliferative and neutrophil oxidative functions in calves.

Colostral cytokines may also function to down regulate an overactive immune response. In swine, regulatory cytokines TGF-β and IL-4 have been identified in colostrum. In this study, TGF-β (10 ng) was incubated with piglet colostral mononuclear cells and then stimulated with either lipopolysaccharide or rotavirus antigen. The addition of TGF-β decreased B cells responsiveness and antibody production (Nguyen et al., 2007). This suggests the regulatory cytokines in colostrum could aid in development of a balanced immune response, which is imperative in the neonate.

1.7 Memory T Lymphocytes; Importance in the Dairy Calf

Memory T cells develop as a part of the acquired immune system. Memory results after a lymphocytes is first exposed to an antigen, resulting in clonal expansion. A second exposure to the antigen leads to an increased proliferation of the memory cell pool and clearance of the pathogen. This phenomenon is called the anamnestic response, resulting in both immediate and long-lasting protection (Rogers et al., 2000, Veiga-Fernandes et al., 2000). T lymphocyte memory

subpopulations are distinguished by cell surface marker expression, including CD62L (L-selectin), CD45RO, and CCR7. CD62L is an adhesion surface protein on T lymphocytes, which enhances cell migration from blood to the lymph node. CD45RO is a cell surface glycoprotein, which is up regulated after activation by a pathogen or other stimulus. Lastly, CCR7 is a chemokine receptor ligand, which is responsible for lymphocyte migration through endothelial venules into lymph nodes (Sallusto et al., 1999).

Differentiating memory cell populations by surface marker expression is important in determining protection in response to infections and vaccinations. In healthy animals, central memory cells, defined as CD62L^{high}CD45RO^{+} CCR7^{+}, increase after vaccination and correlate to protection to future infections (Letvin et al., 2006). In calves 2-5 months of age, approximately 30% of CD4^{+} T cells and 18% of CD8^{+} T cells expresses activation marker CD45RO. For this age group, the 60% of CD4^{+} T cells and 69% of CD8^{+} T cells expresses CD45RA, a naïve cell marker (Bembridge et al., 1995, Hogg et al., 2011). The proportion of CD4^{+} and CD8^{+} memory cells increase while the proportion of CD4^{+} and CD8^{+} naïve cells decrease with age (Tezuka et al., 1998). This increase in memory T cell proportion suggests increased exposure to a variety of antigens (Saule et al., 2006). During diseased states, T cells phenotypes can differ between pathogens. Bovine lymphocytes have decreased expression of CD62L when infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP)(Waters et al., 2003) bovine respiratory syncytial virus (BRSV) (McInnes et al., 1999) bovine leukemia virus (BLV)(Dusinsky et al., 2000), and foot and mouth disease virus (FMDV)(Toka et al., 2011). Cattle vaccinated with *Mycoplasma mycoides* subsp. *mycoides* small colony (*MmmSC)* express two different phenotypes of memory CD4^{+} T cells based on CD62L expression (Totte et al., 2010). These memory cell phenotypes are ued to confer disease protection and overall effectiveness of the immune response.

Memory cells and antigen-specific antibodies in colostrum have not been researched in detail, however, they most likely play an important role in neonatal immune protection. Dams vaccinated during pregnancy produce antigen-specific antibodies into colostrum. Saif et al. (1983) demonstrated that cows vaccinated with a modified-live rotaviral vaccine during pregnancy had greater rotavirus-specific antibodies in colostrum compared to non-vaccinated animals. When calves were fed colostrum from either vaccinated or non-vaccinated mothers and then challenged with virulent bovine rotavirus, calves fed colostrum from vaccinated mothers had delayed onset and shortened duration of rotavirus shedding in feces (Saif et al., 1983). Additionally, as described above, calves fed colostrum from dams vaccinated with a BVDV vaccine had increased leukocyte proliferated capacity compared to leukocytes from calves fed frozen or cell-free colostrum (Donovan et al., 2007). This data combined suggests that colostrum contains memory B and T lymphocytes that may modulate neonatal immune response and enhance development.

1.8 Gamma Delta (γδ) T Cells

The γδ T cell is uniquely able to respond to infection as a cell of both the innate and the adaptive immune system. In cattle, γδ T cells can represent up to 40% of lymphocytes in the pre-weaned calf and 17% of lymphocytes in the adult cow (Wilson et al., 1996). In adult humans and mice, only 1-5% of circulating T cells are the γδ subtype (Haas et al., 1993). Furthermore, ruminant γδ T cell receptors are comprised of sixty-five gene segments while humans and mice only have eleven, suggesting an increased function of this cell-type in the bovine (Blumerman et al., 2006, Herzig et al., 2006). In addition, these may be relevant in disease resistance as memory γδ T cells have been identified in cattle (Silflow et al., 2005). While γδ T cells have been identi-

fied in colostrum, the impact of these cells on neonatal immune function has yet to be elucidated (Ohtsuka et al., 2010). The ingestion of this multifunctional cell type in colostrum may play a role in neonatal immune development.

1.9 Conclusion

While the protective role of antibodies is well documented, few studies have investigated the impact of adoptively transferred colostral immune cells. In the bovine, the placenta prevents transfer of immune components to the fetus during gestation (Arthur, 2001). This makes the calf a great model to study the impact of adoptively transferred colostral immune cells on immunity. This thesis adds compelling data to the literature that colostral immune cells impact immunity in the dairy calf. Modulating the immune response early in life may enhance long-term development of the immune system, resulting in healthier, more productive animals.

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Chapter 2. Adoptively transferred colostral immune cells impact calf immune status and health

I. Birth to Day 28

(*Formatted for* The Journal of Dairy Science)

Interpretive summary

Title: **Adoptively transferred colostral immune cells impact calf immune status and health**

Author: **Neal**

Summary: This study analyzed the impact of maternal colostral cells on neonatal immunity. Calves were fed either whole or cell-free colostrum and observational health scores and blood immune parameters were measured. We found that maternal colostral cells impact immune status in dairy calves within the first month of life. These data will provide dairy producers, the dairy industry and animal scientists with evidence that non-antibody colostral components are important in neonatal immunity. Enhancing immune function in calves could lead to improved immune development, resulting in healthier, more productive cows entering the dairy herd.

ADOPTIVELY TRANSFERRED MATERNAL IMMUNE CELLS IMPACT CALF HEALTH AND IMMUNE STATUS

Adoptively transferred maternal immune cells impact calf health and immune status

I. The neonatal period

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ABSTRACT

Mortality and decreased weight gain resulting from infection and disease in dairy calves is a problem within the dairy industry. The bovine neonate relies solely on colostrum for the acquisition of antibodies through passive transfer. To date, colostrum quality is determined by concentration of antibodies. However, colostrum also contains proteins and cells, which may enhance immune development in the neonate. To determine the impact of maternal colostral immune cells on calf health and immune status, maternal colostrum was either fed fresh or after being flash-frozen in liquid nitrogen and thawed to lysed cells. Thirty-seven female Holstein and Jersey dairy calves were bottle-fed 4 quarts total of whole colostrum (WC) or cell-free colostrum (CFC) at birth. Respiratory and fecal scores were measured from birth to d 40 of life. Calf peripheral blood samples were obtained before and after feeding colostrum as well as on d 1, 3, 7, 14, 21, and 28 of life. Peripheral blood mononuclear cells (PBMC) were collected and analyzed for cellular parameters by flow cytometry. Total respiratory scores were greater in CFC-fed calves compared to WC-fed calves on d 38 of life. There were decreased numbers of CD4$^+$ T cells and CD4$^+$CD62L$^+$CD45RO$^-$ T cells on d 1 and CD4$^+$CD62L$^+$CD45RO$^+$ T cells on d 1 and 3 in CFC-fed calves when compared to WC-fed calves. When compared to WC-fed calves, CFC-fed calves had a greater percentage of CD4$^+$CD62L$^-$CD45RO$^+$ T cells on d 7, a greater percentage of CD4$^+$CD62L$^-$CD45RO$^+$ T cells on d 1, and a greater percentage of monocytes on d 7. Our data suggests that colostral maternal cells adoptively transfer into circulation and impact neonatal immune status. By enhancing neonatal immunity during the first month of life, maternal colostral cells may impact long-term immune development and response.

Key words: colostrum, adoptive transfer, immunity, dairy calves

INTRODUCTION

The neonatal immune system is characterized as immunologically naïve, making the dairy calf highly susceptible to bacterial and viral pathogens. The National Animal Health Monitoring System reported that 56.5% of all unweaned heifer deaths were due to scours and/or diarrhea and 22.25% were due to respiratory problems (USDA-APHIS:VS, 2007). Disease incidence in calves negatively impacts profitability of the dairy operation and decreases animal well-being. To abrogate illness and disease in calves, colostrum is required for passive transfer of nutrients and antibodies (Quigley and Drewry, 1998). To date, colostrum quality is determined by antibody content alone. However, colostrum also contains leukocytes, cytokines, antimicrobial proteins and hormones (Reiter and Brock, 1975, Hagiwara et al., 2000, Blum and Baumrucker, 2002). These colostrum components may have a positive impact on neonatal status and long-term development of immune function.

Similar to antibodies, colostral leukocytes can passively transfer through the intestinal epithelium and into circulation (Schnorr and Pearson, 1984, Williams, 1993, Liebler-Tenorio et al., 2002). These transferred colostral cells may be antigen-specific, aiding in immediate pathogen clearance. Antigen-specific colostral cells have memory characteristics and may reside in calf lymphoid tissue until activated by exposure to a future pathogen (Donovan et al., 2007). Maternal colostral cells alter adhesion markers CD11a and CD11c expression on T lymphocytes and monocytes. When calves are fed cell-free colostrum (COL-), there is a greater number and percentage of monocytes expressing CD11a on d 2 and percentage expressing CD11c on d 14 compared to calves fed whole colostrum (COL+) (Reber et al., 2008a). When T lymphocytes were analyzed, calves fed COL- had a greater percentage and expression of CD11a on d 2 and d 14 when compared to calves fed COL+, respectively. An increase in adhesion markers on immune

cells from CFC-fed calves may indicate an increased pathogen insult or stress-response compared to calves fed COL+ (Reber et al., 2008a, b).

Maternal colostral cells also aid in bacterial clearance in the neonate. Reidel-Caspari (1993) experimentally-infected calves with *E. coli* and immediately fed either COL+ or COL- colostrum. Calves fed COL+ excreted less CFU of *E. coli* during the first week of infection. In addition, fecal bacteria reached the lower limit of detectability earlier and had greater concentration of *E. coli* specific antibodies during the first 48-hours post-infection (Riedel-Caspari, 1993). In an earlier study, newborn rats were given an oral dose of *Klebsiella pneumoniae* and then fed either fresh milk, frozen rat milk, frozen rat milk plus leukocytes, formula or formula plus leukocytes. Only the rat pups given fresh rat milk, frozen milk plus rat milk leukocytes and formula plus leukocytes had 90-100% survival, while the other treatments had greater mortality rates. (Pitt et al., 1977). This data suggests a role for adoptively transferred leukocytes through colostrum ingestion on neonatal immune status and function.

We investigated the impact of maternal colostral cells on health and immune status in dairy calves. We hypothesized that calves fed colostrum with maternal immune cells would enhance levels of cellular blood parameters within the first month of life compared to calves fed colostrum without maternal immune cells.

MATERIALS AND METHODS

Adoptive transfer of immune cells

Twenty-nine Holstein and eight Jersey cows from the Virginia Tech Dairy Center were equipped with a birthing monitoring system (FoAlert, Acworth, GA). All animal handling and sampling protocols were in accordance with the Virginia Tech's Institutional Animal Care and Use Committee. The FoAlert ® birth monitoring system was used for immediate notification of parturition. In brief, a veterinarian sutured a transmitter to each cow ≥ 2 weeks prior to calving to the outside of the vulva. Upon start of parturition, physical separation of the vulva triggered the transmitter to send a radio signal to a receiving unit resulting in an audible alarm and phone calls to pre-programmed numbers on an attached automated dialer. This system ensured that staff arrived prior to parturition

After parturition was complete, the cow was permitted to clean the calf. Calves were not permitted to suckle. After the calf was separated, colostrum was collected aseptically from each quarter and standard bacteriology tests were run (NMC, 2004). If clumps or blood was observed in colostrum, mastitis was assumed and the calf was not enrolled. Colostrum that was not mastitic was tested with a digital Brix refractometer to assess total serum protein. Before and after testing of colostrum, the refractometer was cleaned and calibrated with distilled H_2O. A drop of colostrum was placed on the refractometer slide to obtain the Brix reading. Quality colostrum was determined as a 23 or higher Brix score (Weaver et al., 2000).

If colostrum was determined acceptable, calves either received whole colostrum (WC) or cell-free colostrum (CFC) from their respective dams, according to the following protocols. Two, 2-quart bottles were filled with WC. One, 2-quart bottle was fed to calf within 3 hours after birth.

The second 2-quarter bottle was refrigerated (4°C) and then slowly warmed to 37° C immediately prior to the second feeding, 5-8 hours after birth.

For CFC, 4 Perfluoroalkoxy (PFA) bags (Welch Fluorcarbon, Inc., Dover, NH) were filled each with 1 liter of colostrum per bag. Each PFA bag of colostrum was placed in a Styrofoam box and liquid nitrogen was slowly poured over PFA bag. Liquid nitrogen completely covered the colostrum but did not overflow into the bag. The PFA bag was turned approximately every 3 minutes until colostrum was completely frozen. Following complete freezing of colostrum, the first 2 quarts of colostrum were slowly warmed to 37° C and fed to the calf within 3 h of life. While the first 2 quarts of colostrum were thawing, the freezing process was repeated again for the second 2 quarts of colostrum. Following complete freezing of the second 2 quarts of colostrum, the colostrum was thawed slowly at 37° C, transferred to bottles and refrigerated (4°C) until fed to the calf at 5-8 h after birth. If the calf did not suckle, an esophageal tube feeder was used. All calves were weighed at birth and administered one dose of TSV-2 nasal vaccine (Zoetis, Madison, NJ). All calves entered the Virginia Tech dairy herd and routine farm management protocols were applied.

Health scores

Observational health data was collected from birth to d 45. Coughing, nasal discharge, ear disposition, eye discharge, and fecal consistency were scored using the Calf Health Scoring Chart (University of Madison-Wisconsin School of Veterinary Medicine). Fecal consistency was scored on a ‘0’ to ‘3’ scale where ‘0’ represented normal consistency and ‘3’ represented severe scours. Coughing, nasal discharge, ear disposition and eye discharge were also scored on a ‘0’ to ‘3’ scale where ‘0’ represented healthy animals and ‘3’ represented a very ill animal. Cough, na-

sal discharge, ear disposition and eye discharge scores were combined to represent a 'Total Respiratory Score' for each day. For respiratory score analysis, data were collapsed to represent a binomial distribution where '0' represented healthy animals and any score above zero received a '1', signifying a non-healthy animal.

Isolation of colostral immune cells

After flash-freezing with liquid nitrogen and slow thawing, colostral mononuclear cells were isolated. Colostrum was pelleted by centrifugation at 1500 × g, 15° C for 30 min. Casein and free fat was carefully removed and colostral whey was discarded. If colostrum was contaminated with red blood cells, pellet was resuspended briefly in 10 μl RNAse/DNAse-free H_2O. Immediately, 2X Modified Eagle Media was added and centrifuged at 1000 RPM, 4° C for 10 min. Cells were resuspended in 10 ml PBS and a 10 μl aliquot was diluted in 1:100 in .04% Trypan Blue solution (Life Technologies, Carlsbad, CA) for counting. Colostral mononuclear cells were observed under an Axiovert 40 microscope (Zeiss, Göttingen, Germany) and no cells were detectable. Colostral leukocytes were unable to survive the rapid freezing in liquid nitrogen (data not shown).

Isolation of peripheral blood mononuclear Cells (PBMC)

Calves were bled at birth (pre-colostrum feeding), 6 h after birth and on d 1, 3, 7, 14, 21 and 28. Peripheral blood mononuclear cells were isolated on a Ficoll-Paque density gradient (1.077 g/ml; BD Falcon, Franklin Lakes, NJ, USA), from whole blood collected using 40 mM EDTA (10% vol/vol) as described previously (Shafer-Weaver et al., 1999). Mononuclear cells were resuspended at $1x10^7$ cells/ml in PBS containing 0.05% sodium azide.

Serum antibody ELISA

Bovine ELISA Quantitation Sets (Bethyl Laboratories, Inc., Montgomery, TX) were used in the assessment of IgG1 (Bethyl Laboratories, Inc., E10-116, Montgomery, TX), IgG2 (Bethyl Laboratories, Inc., E10-117, Montgomery, TX), IgM (Bethyl Laboratories, Inc., E10-101, Montgomery, TX), and IgA (Bethyl Laboratories, Inc., E10-121, Montgomery, TX) in serum samples. Each sample and standard was run in duplicate.

Nunc-Immuno MicroWell (Sigma-Aldrich, St. Louis, MO) were coated with 100 μL of bovine IgG1, IgG2, IgM, or IgA antibody and diluted in sample/conjugate diluent (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). The plate was incubated at 25°C for 1h and subsequently washed 5 times in wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Two hundred μL of blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) was added and the plates were incubated at 25°C for 30 min. Plates were washed 5 times and 100 μL of the standard or diluted sample was added into the corresponding well. Serum samples were diluted 1:100 in the sample/conjugate diluent, and all other serum samples were diluted 1:50,000 in the sample/conjugate diluent. The plate was then incubated for 1 h at 25°C and then washed 5 times. One hundred μL of diluted HRP detection antibody (1:20,000 in the sample/conjugate diluent) was then added to each well. After 1 h incubation at 25°C, the plate was washed 5 times and then 100 μL of TMB Substrate Solution (BD Biosciences, San Diego, CA) was added to each well. To stop development, 100 μL of stop solution (0.18 M H_2SO_4) was added. The absorbance of each well was read with a ELISA plate reader (BioTek μQuant Universal Microplate Spectrophotometer; BioTek, Winooski, VT) at 450 nm.

Leukocyte analysis

Flow cytometric analysis of cell surface markers was performed using bovine specific monoclonal antibodies (Table 1). Mononuclear cells were resuspended at $1x10^7$ cells/ml in PBS containing 0.05% sodium azide and 10^6 cells were aliquoted per well in 96-well plates. Cells were incubated with primary antibody for 30 min at 4°C. Cells were washed two times with PBS and subsequently incubated with isotype-specific secondary antibodies conjugated to FITC, PE, PE-Texas Red or APC for 30 min at 4°C. Cells were washed two times and in 1% formaldehyde in PBS for 20 min at RT. Cells were washed again, resuspended in 150 μL of PBS containing 0.05% sodium azide and analyzed by flow cytometry (Accuri C6, BD, San Jose, CA, USA) and FlowJo Software (TreeStar, Ashland, OR, USA).

Statistical analysis

All statistical tests were performed using the PROC GLIMMIX procedure of SAS 9.3 (SAS Institute Inc. Cary, NC). Non-normally distributed data, as determined by the studentized T residuals, were natural log transformed. A natural log transformation was applied to the number and percentage of $CD4^+$ T cells and number of $CD4^+CD62L^+CD45RO^-$ T cells, $CD4^+CD62L^-CD45RO^+$ T cells and $CD4^+CD62L^+CD45RO^+$ T cells. For transformed datasets, significance was declared using transformed data while non-transformed LSM ± SEM were reported in text. Each statistical model included the fixed effects of treatment, time, breed and the temperature at birth and the associated interactions. Each calf was designated a temperature code based on the minimum and maximum temperature the day of birth: cold (C) -born, minimum day temperature ≤ -30°C; mild (M) -born, minimum day temperature > -30° C and maximum day temperature ≤ 80° C; hot (H) -born, maximum day temperature > 80° C. The repeated statement was applied to

the day of blood collection using calf nested within treatment as the subject. If pre-treatment levels were significantly different from each other, a covariate was assigned for h 0. Error structures were tested and the one giving the lowest Akaike information criterion was chosen for analysis. Slices were evaluated for the associated interactions and a Tukey's adjustment was applied. Significance was declared at $P \leq 0.05$.

RESULTS

Antibody titers

Concentrations of serum IgG1, IgG2, IgM and IgA were analyzed prior to colostrum ingestion and on d 1 of life. Treatment did not have an impact on concentration of IgG1, IgG2, IgM, and IgA antibodies in animals (Figure 2.1). The concentration of IgA was decreascFCed in WC-fed, C-born calves ($3.17\times10^3 \pm 2.14\times10^2$) when compared to H-born calves fed the same treatment ($4.58\times10^3 \pm 2.98\times10^2$, $P = 0.0017$). The concentration of IgG2 was decreased in WC-fed, C-born calves ($1.50\times10^3 \pm 1.57\times10^2$) when compared to H-born calves fed the same treatment ($2.03\times10^3 \pm 1.51\times10^2$, $P = 0.033$). Lastly, Jersey calves fed CFC had a decreased concentration of IgG2 ($1.27\times10^3 \pm 1.58\times10^2$, $P = 0.012$) compared to Holstein calves fed the same treatment ($1.80\times10^3 \pm 1.03\times10^2$).

Health scores

Fecal consistency scores were lowest on d 1 in CFC and WC-fed calves (0.06 ± 0.14 and 0.06 ± 0.09, respectively). Fetal consistency scores peaked on d 14 (1.79 ± 0.20 and 1.78 ± 0.25, respectively) and decreased to near starting levels by d 45 in CFC- and WC-fed calves (0.21 ± 0.26 and 0.09 ± 0.13, respectively). No differences were seen between treatments in fecal consistency scores (data not shown; appendix Figure 1). Respiratory scores increased for CFC and

WC-fed calves on d 12 (0.41 ± 0.26 and 0.12 ± 0.10, respectively). On d 38, CFC-fed calves had a greater respiratory score when compared to WC-fed calves (0.33 ± 0.37 and 0.14 ± 0.11, respectively, $P = 0.030$, Figure 2.2).

Peripheral blood mononuclear cells (PBMC)

The number of PBMC (LSM ± SEM, Figure 2.3) per ml of blood peaked on d 28 for CFC-fed calves ($4.39\times10^6 \pm 4.97\times10^5$) and d 21 for WC-fed calves ($4.74\times10^6 \pm 4.68\times10^5$). Day relative to colostrum ingestion impacted the number of PBMC per ml of blood in all calves ($P < 0.0001$). There was no impact of treatment, breed of calf or temperature at birth on the number of PBMC during the first month of life.

CD4$^+$ T cells

The number of CD4$^+$ T cells (LSM ± SEM, Figure 2.4) peaked in CFC-fed calves by d 21 ($3.96\times10^5 \pm 8.38\times10^4$) and in WC-fed calves by d 14 ($2.95\times10^5 \pm 6.11\times10^4$). There was a decreased number of CD4$^+$ T cells observed on d 1 in CFC-fed calves compared to WC-fed calves (natural log transformed; 10.17 ± 0.22 and 11.40 ± 0.25, respectively, $P = 0.0003$). There were a decreased percentage of CD4$^+$ T cells in CFC-fed calves when compared to WC-fed calves at h 6 (natural log transformed; 1.14 ± 0.21 and 1.87 ± 0.24, respectively, $P = 0.021$) and on d 1 (natural log transformed; 0.92 ± 0.16 and 2.15 ± 0.19, respectively, $P < 0.0001$). Jersey calves had a higher percentage of CD4$^+$ T cells compared to Holstein calves in both CFC-fed animals (natural log transformed; 1.84 ± 0.22 and 1.25 ± 0.11, $P = 0.012$, respectively) and WC-fed animals (natural log transformed; 2.25 ± 0.23 and 1.55 ± 0.12, $P = 0.031$, respectively).

$CD4^+CD62L^+CD45RO^-$ T cells

The number of $CD4^+CD62L^+CD45RO^-$ T cells (LSM ± SEM, Figure 2.5) peaked by d 21 in CFC-fed calves ($1.75 \times 10^6 \pm 4.59 \times 10^4$) and by d 7 in WC-fed calves ($1.48 \times 10^5 \pm 3.89 \times 10^4$). A decreased number of $CD4^+CD62L^+CD45RO^-$ T cells were seen in CFC-fed calves compared to WC-fed calves on d 1 (natural log transformed; 8.90 ± 0.32 and 10.53 ± 0.36, respectively, P = 0.0008). Also, a decreased percentage of $CD4^+CD62L^+CD45RO^-$ T cells in CFC-fed calves were seen when compared to WC-fed calves on d 1 (35.32 ± 4.87 and 51.96 ± 5.45, respectively, P = 0.025) and on d 7 (33.63 ± 4.39 and 47.17 ± 4.78, respectively, $P = 0.040$). Breed and temperature and birth did not impact the number or percentage of $CD4^+CD62L^+CD45RO^-$ T cells in WC- or CFC-fed calves.

$CD4^+CD62L^-CD45RO^+$ T cells

The number (LSM ± SEM, Figure 2.6) of $CD4^+CD62L^-CD45RO^+$ T cells peaked on d 28 in both WC and CFC-fed animals ($7.31 \times 10^4 \pm 2.43 \times 10^4$ and $8.41 \times 10^4 \pm 2.35 \times 10^4$, respectively). Day relative to colostrum ingestion had an impact on the number of $CD4^+CD62L^-CD45RO^+$ T cells in all calves ($P < 0.0001$). There were a greater percentage of $CD4^+CD62L^-CD45RO^+$ T cells in CFC-fed calves when compared to WC-fed calves on d 7 (21.74 ± 2.82 and 13.50 ± 2.99, respectively, $P = 0.005$). Breed and temperature and birth did not impact the number or percentage of $CD4^+CD62L^-CD45RO^+$ T cells in WC- or CFC-fed calves.

$CD4^+CD62L^+CD45RO^+$ T cells

The number (LSM ± SEM, Figure 2.7) of $CD4^+CD62L^+CD45RO^+$ T cells peaked in CFC-fed calves at d 21 ($1.20 \times 10^5 \pm 2.70 \times 10^4$) and in WC-fed calves on d 28 (6.72×10^4 ±

1.71×10^4). There were a decreased number of $CD4^+CD62L^+CD45RO^+$ T cells in CFC-fed calf peripheral blood when compared to WC-fed calves on d 1 (natural log transformed; 9.14 ± 0.22 and 10.08 ± 0.24, respectively, $P = 0.0044$) and on d 3 (natural log transformed; 10.29 ± 0.22 and 10.94 ± 0.24, respectively, $P = 0.047$). Calves that were fed CFC had a higher percentage of $CD4^+CD62L^+CD45RO^+$ T cells on d 1 (42.44 ± 3.0) when compared to WC-fed calves (33.23 ± 3.43, $P = 0.050$). The percentage of $CD4^+CD62L^+CD45RO^+$ T cells in C-born calves was higher (39.27 ± 2.26) than in H-born calves (31.47 ± 1.97, $P = 0.039$). The range of $CD4^+CD62L^+CD45RO^+$ T cells was 30-42% over the first month of life in WC and CFC-fed animals.

Monocytes ($CD14^+$ cells)

The number and percentage of monocytes (LSM ± SEM, Figure 2.8) in calves during the first month of life was analyzed. Calves fed CFC had a higher percentage of monocytes on d 3 (41.02 ± 2.62) when compared to WC-fed calves (33.01 ± 2.89, $P = 0.041$). Day relative to colostrum ingestion had an impact on both the number and percentage of monocytes in all calves during the first month of life ($P < 0.0001$). Temperature at birth had an impact on the percentage of monocytes in CFC-fed calves where H-born calves had a decreased percentage of monocytes (20.65 ± 2.10) when compared to C-born calves (31.54 ± 2.42, $P = 0.0052$).

B ($CD21^+$) cells

The number (LSM ± SEM, Figure 2.9) of B cells in all animals peaked on d 28 for CFC and WC-fed calves ($1.45\times10^6 \pm 1.75\times10^5$ and $1.46\times10^6 \pm 1.76\times10^5$, respectively). Day relative to colostrum ingestion had an impact on the number and percentage of B cells in all calves during

the first month of life ($P < 0.0001$ and $P = 0.0002$, respectively). Treatment, breed of calf, or temperature at birth did not impact B cell numbers in calves during the first month of life. The percentage of B cells was impacted by breed of calf, where Jersey calves had fewer B cells (22.95 ± 1.99) than Holstein calves (27.56 ± 1.22, $P = 0.046$).

Gamma delta (γδ) T cells

The number (LSM ± SEM, Figure 2.10) of γδ T cells peaked on d 21 for CFC and WC-fed calves ($4.11\times10^5 \pm 6.05\times10^4$ and $3.63\times10^5 \pm 6.44\times10^4$, respectively). Day of colostrum ingestion had an impact of number and percentage of γδ T cells in calves during the first month of life ($P < 0.0001$). There are no differences between treatments, breed and temperature at birth for numbers of γδ T cells at any of the time points. Temperature at birth had an impact on the percentage of γδ T cells in CFC-fed calves where H-born calves had a decreased percentage of monocytes (20.65 ± 2.10) when compared to C-born calves (31.54 ± 2.42, $P = 0.0052$).

DISCUSSION

While the role of colostral antibodies has been defined, the impact of colostral immune cells on neonatal immune development has yet to be elucidated. Previous reports have demonstrated the ability of maternal colostral cells to affect immune cell function within the first weeks of life. The data presented in this study suggests that maternal colostral cells adoptively transfer into circulation and impact immune status in the calf. This may have an influence on immune development overtime in dairy calves.

In the current study, we found that CFC-fed calves had a decreased number and percentage of $CD4^+$ T cells within the first 24 hours of life when compared to WC-fed calves. To our knowledge, this is the first study measuring $CD4^+$ T cell levels in calves fed either CFC or WC

colostrum. Colostrum from first parity cows contains approximately 25% $CD4^+$ T cells (Ohtsuka et al., 2010). Bovine colostral leukocytes adoptively transfer through the intestine and into circulation (Liebler-Tenorio et al., 2002). Therefore, the adoptive transfer of $CD4^+$ T lymphocytes could account for the increase in $CD4^+$ T cells in WC-fed calves on d 1 of life. $CD4^+$ T cells are responsible for increasing phagocytic capacity of macrophages, recruiting neutrophils to the site of infection and enhancing antibody production by B cells (Zhu and Paul, 2008). In neonatal $CD4^{-/-}$ knockout mice, animals were more susceptible to an enteric pathogen when compared to B cell deficient or wild-type control mice. These studies suggests that enhancing $CD4^+$ T cells in the neonatal may result in increased pathogen clearance and survival (Echeverry et al., 2010).

The number of $CD4^+CD62L^+CD45RO^-$ T cells were fewer in CFC-fed calves on d 1 when compared to WC-fed calves. Additionally, the percentage of $CD4^+CD62L^+CD45RO^-$ T cells was fewer in CFC-fed calves when compared to WC-fed calves on d 1 and 7. Trafficking of leukocytes from the periphery to lymph nodes is regulated by adhesion molecules, i.e. CD62L, expressed on the cell surface (Sallusto et al., 1999). In healthy calves, CD62L expression on $CD4^+$ and $CD8^+$ T cells is highest during the first week of life and gradually decreases as the animal ages (Howard et al., 1992, Hogg et al., 2011). There is evidence that colostral cellular components are more likely to come from peripheral circulation than from the mammary gland itself (Harp et al., 1988, Sheldrake et al., 1988, Harp et al., 2004). The difference in amount and percentage of $CD4^+CD62L^+CD45RO^-$ T cells on d 1 in WC-fed calves could be explained by adoptively transferred colostral $CD62L^+$ cells which migrated through the intestine and were detected in blood. However, it is also possible that CFC-fed calf $CD4^+$ T cells are shedding CD62L from the cellular surface in response to an antigen or cytokine stimulus. Cattle have decreased expression of CD62L when infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) (Waters

et al., 2003), bovine respiratory syncytial virus (BRSV) (McInnes et al., 1999), bovine leukemia virus (BLV) (Dusinsky et al., 2000) and foot and mouth disease virus (FMDV) (Toka et al., 2011). Therefore, CFC-fed calves could have a decreased ability to clear pathogens in their body, resulting in shedding of CD62L from lymphocytes. Further research is needed to determine if the decrease of CD62L in CFC-fed calves is results in a loss of migratory function.

Glycoprotein marker CD45 exists in two isoforms; CD45RA, associated with resting, naïve cells, and CD45RO, associated with an activated phenotype (Cossarizza et al., 1996). In calves, the percentage of MAP-specific $CD4^+$ T cells expressing $CD45RO^+$ increases *in vitro* upon stimulation with MAP antigens (Waters et al., 2003). Cattle infected with BRSV had greater proportion of $CD4^+CD62L^-CD45RO^+$ T cells by 10 days post-infection in bronchial lymph nodes (McInnes et al., 1999). These *in vitro* and *in vivo* studies suggest that an increase in the number and percentage of $CD4^+CD62L^-CD45RO^+$ T cells 6 hours after birth and on days 1, 3, and 7 in CFC-fed calves may be explained by increased antigenic stimulation in the calf.

In this study, there were fewer numbers of $CD4^+CD62L^+CD45RO^+$ T cells on d 1 and 3 in CFC-fed calves when compared to WC-fed calves. Typically, $CD4^+CD62L^+CD45RO^+$ T cells have been defined as central memory cells. Antigen-specific colostral memory cells may have an impact in the calf. Donovan et al. (2007) vaccinated dams with inactivated BVDV antigen in late gestation. After parturition, calves were either fed fresh, frozen or leukocyte-free colostrum. Calves fed fresh colostrum had increased proliferative response to BVDV antigens *in vitro* when compared to calves fed frozen or cell-free colostrum. Comparatively, none of the calf PBMC proliferated to an antigen to which the dams were not vaccinated with, mycobacterium purified protein derivative (Donovan et al., 2007). The increase in number of $CD4^+CD62L^+CD45RO^+$ T cells in WC-fed calves could be attributed to the adoptive transfer of memory cells from colos-

trum. However, in our study the percentage of $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells was higher in CFC-fed calves on day 1 compared to WC-fed calves. It is likely that the greater percentage of $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells in CFC-fed calves is attributed other cell types decreasing during that time. The percentage of each cell type is determined from a whole mononuclear cell population. The increase in the percentage $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells in CFC-fed calves could be attributed to other cell types decreasing concurrently. Alternatively, the increase in $CD4^{+}CD62L^{+}CD45RO^{+}$ T cell numbers on day 1 of life could be explained by an increase in total number of cells per ml of blood. Future studies are needed to determine the function of $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells in the bovine.

CONCLUSION

In the current study, we show that CFC-fed calves have fewer $CD4^{+}$, $CD4^{+}CD62L^{+}CD45RO^{-}$ and $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells than WC-fed calves within the first month of life. However, the percentage of monocytes was higher in CFC-fed calves when compared to WC-fed calves. We concluded that adoptively transferred immune cells have a role in the immune status of neonatal dairy calves. Enhancing immune function in calves could lead to increased disease resistance resulting in healthier animals entering the dairy herd.

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Table 2.1. Primary and secondary antibodies used for flow cytometric analysis of PBMC populations.

Primary antibody target[1]	Clone	Isotype	Primary antibody dilution	Secondary antibody
$CD4^+$ T cell	IL-A11	IgG2a	1:400	IgG2a-FITC[2]
CD62L	BAQ-92A	IgG1	1:400	IgG1-PE-Texas Red[2]
CD45RO	IL-A1116	IgG3	1:400	IgG3-PE[3]
B cell ($CD21^+$)	BAQ-15A	IgM	1:400	IgM-APC[2]
Monocytes ($CD14^+$)	MM61	IgG1	1:400	IgG1-PE-Texas Red[2]
γδ TCR^+ T cell	CACTB32A	IgG1	1:800	IgG1-PE-Texas Red[2]

[1]Primary mouse-generated monoclonal antibodies purchased from VMRD, Pullman, WA.
[2]Secondary goat anti-mouse monoclonal antibodies purchased from Life Technologies, Carlsbad, CA
[3]Secondary goat anti-mouse monoclonal antibodies purchased from Southern Biotechnologies, Birmingham, AL

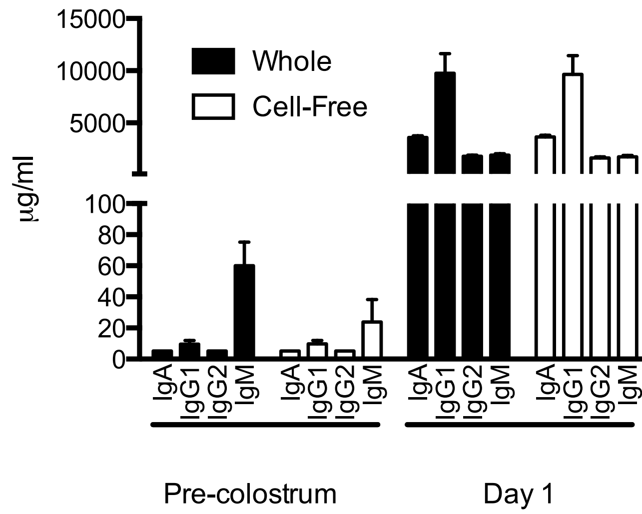

Figure 2.1. Antibody concentration in calves pre and post-colostral feeding.

Antibody concentration in calves fed whole (n = 14) or cell-free (n = 16) colostrum prior to and 1 day post-feeding.

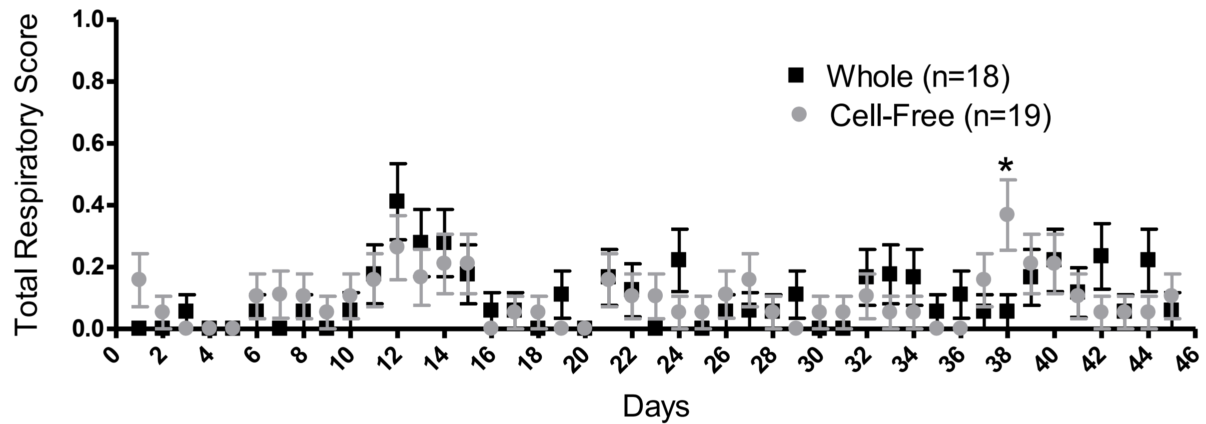

Figure 2.2. Total respiratory scores in calves during the first month of life.

Total respiratory scores of calves fed whole (n = 17) or cell-free colostrum (n = 19). Data as expressed at LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. *$P \leq 0.05$

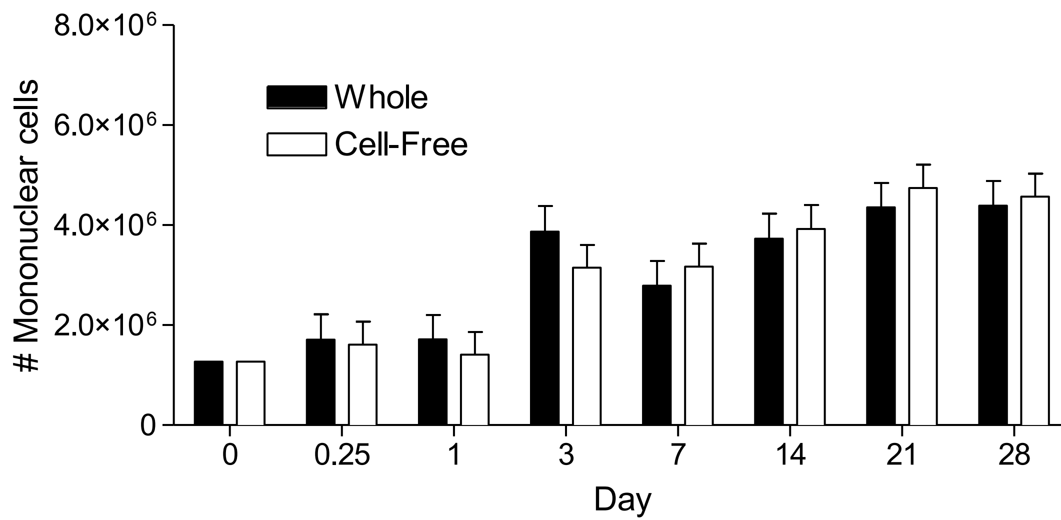

Figure 2.3. Mononuclear cells in calves during the first month of life.

The number of mononuclear cells per ml of peripheral blood from calves fed whole (n = 14) or cell-free (n = 17) colostrum. Data are expressed as LSM ± SEM.

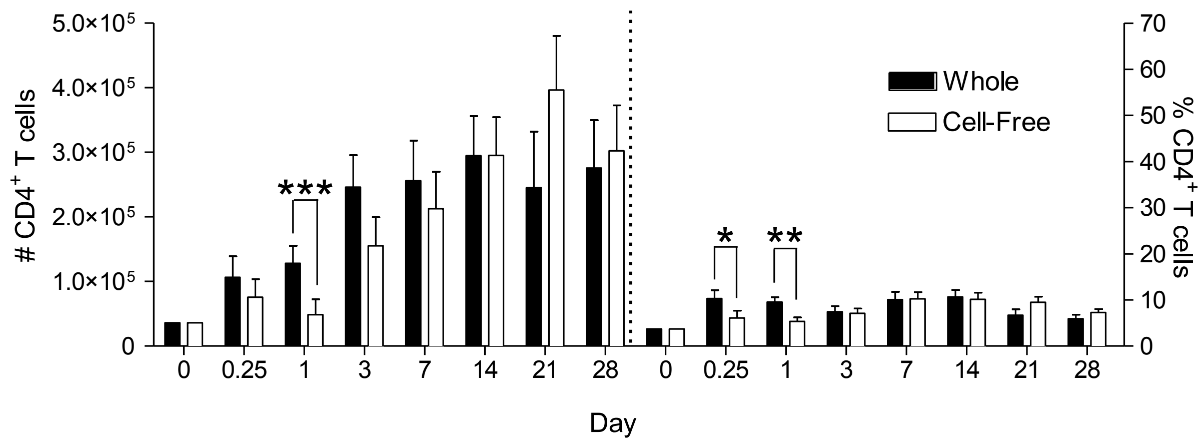

Figure 2.4. CD4+ T cell in calves during the first month of life.

The number (left) per ml of blood and percentage (right) of CD4+ T cells are expressed as LSM ± SEM in calves fed whole (n = 14) or cell-free (n = 17) colostrum. Asterisks indicate a difference between treatment groups within a time-point. Test of significance determined using natural log transformed data for both number (D 1: cell-free: 10.12 ± 0.23; whole: 11.40 ± 0.26) and percentage (Hour 6: cell-free: 1.14 ± 0.21; whole: 1.87 ± 0.24; D 1: cell-free: 0.92 ± 0.16; whole: 2.15 ± 0.19) of $CD4^{+}$ T cells. *$P\leq0.05$ **$P\leq0.01$ ***$P\leq0.001$

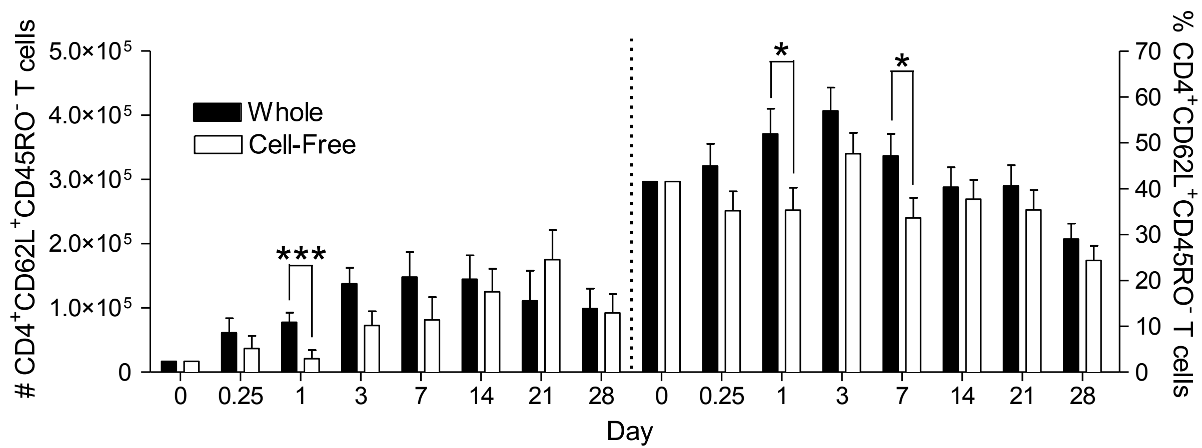

Figure 2.5. $CD4^+CD62L^+CD45RO^-$ T cells in calves during the first month of life.

The number (left) per ml of blood or percentage (right) within the peripheral blood mononuclear cell population of $CD4^+CD62L^+CD45RO^-$ T cells from calves fed whole (n = 14) colostrum or cell-free (n = 17). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. Significances were determined using natural log trans-formed data for number (D 1: cell-free: 8.90 ± 0.32; whole: 10.53 ± 0.36) of $CD4^+CD62L^+CD45RO^-$ T cells. *$P \leq 0.05$, ***$P \leq 0.001$

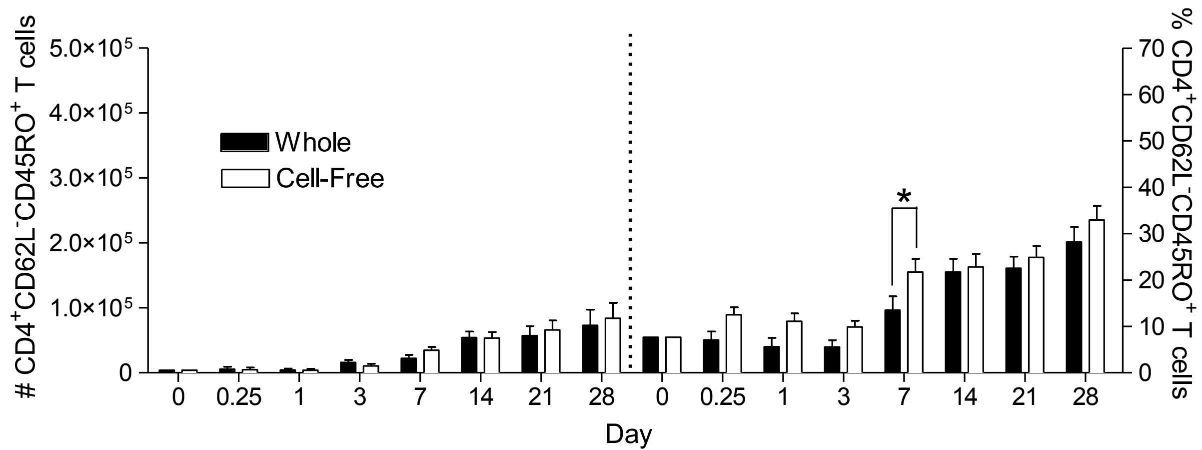

Figure 2.6. $CD4^+CD62L^-CD45RO^+$ T cells in calves during the first month of life.

The number per ml of blood (left) or percentage (right) within the peripheral blood mononuclear cell population of $CD4^+CD62L^-CD45RO^+$ T cells from calves fed whole (n = 14) colostrum or cell-free (n = 17) colostrum. Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. *$P \leq 0.05$

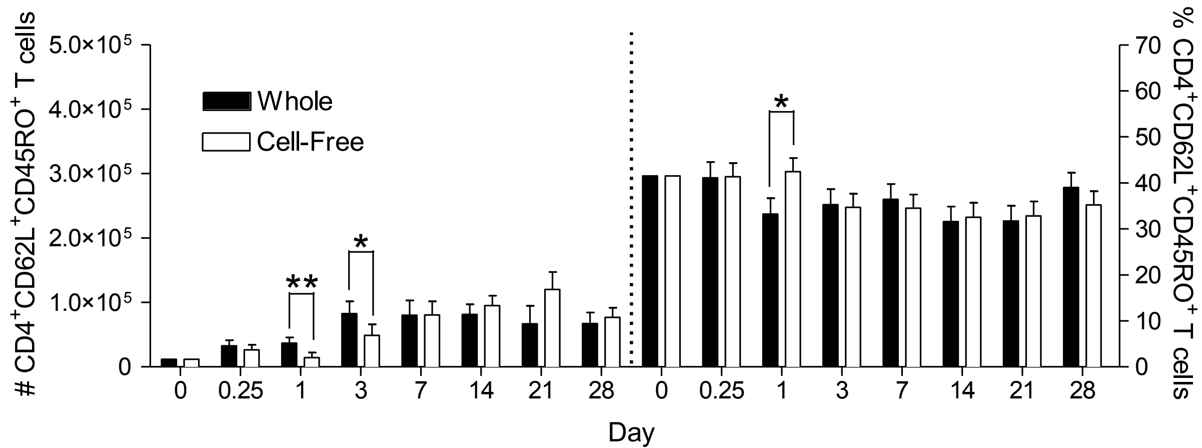

Figure 2.7. $CD4^+CD62L^+CD45RO^+$ T cells in calves during the first month of life.

The number (left) per ml of blood or percentage (right) within the peripheral blood mononuclear cell population of $CD4^+CD62L^+CD45RO^+$ T cells from calves fed whole (n = 14) or cell-free (n = 17) colostrum. Data are expressed as LSM ± SEM. Asterisks indicated a difference between treatment groups within a time-point. Significances were determined using natural log transformed data for number (D 1: cell-free: 9.14 ± 0.22; whole: 10.08 ± 0.24, D 3: cell free: 10.29 ± 0.22, whole: 10.94 ± 0.24) of $CD4^+CD62L^+CD45RO^+$ T cells. *$P \leq 0.05$ **$P \leq 0.01$

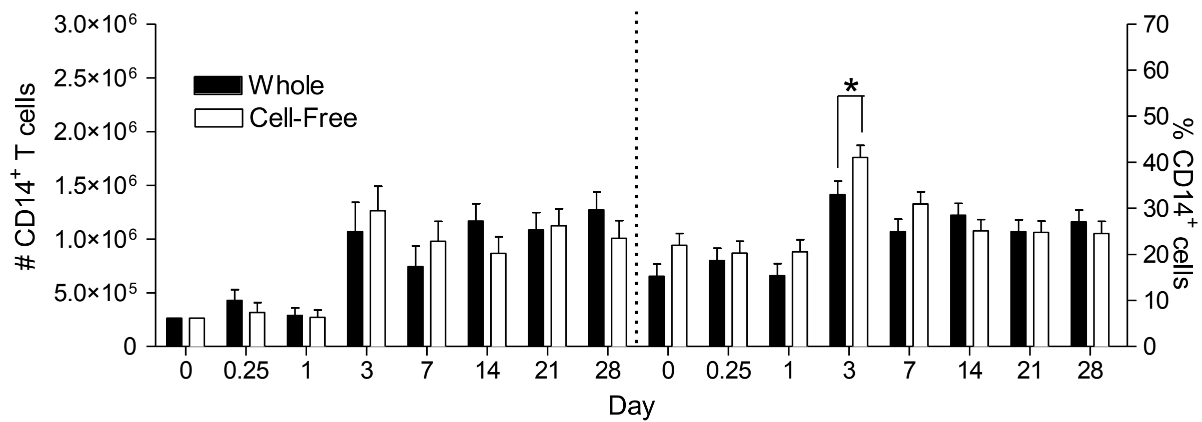

Figure 2.8. Monocytes ($CD14^{+}$) in calves during the first month of life.

The number per ml of blood (left) or percentage (right) within the peripheral blood mononuclear cell population of monocytes from calves fed whole (n = 14) and cell-free (n = 15) colostrum. Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. *$P \leq 0.05$

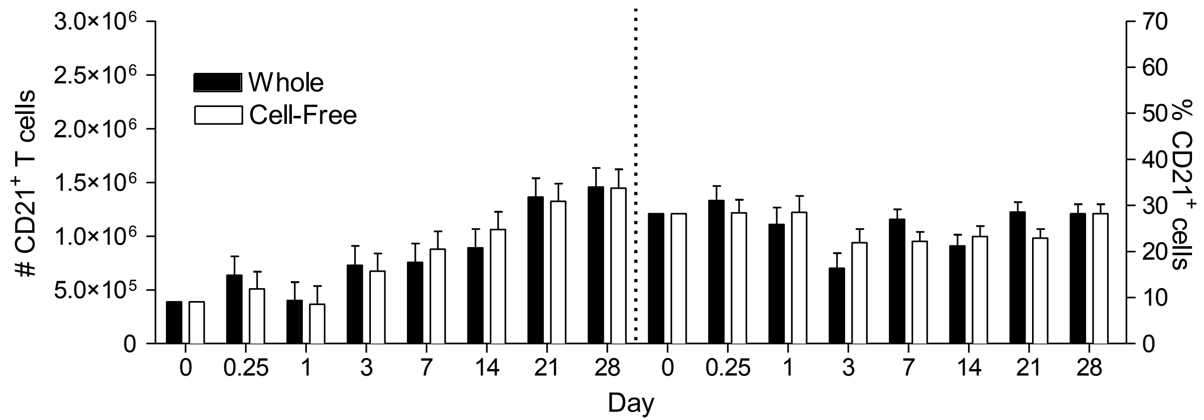

Figure 2.9. B cells ($CD21^+$) in calves during the first month of life.

The number (left) per ml of blood or percentage (right) within the peripheral blood mononuclear cell of B cells ($CD21^+$) from calves fed whole (n = 14) and cell-free (n = 15) colostrum. Data are presented as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point.

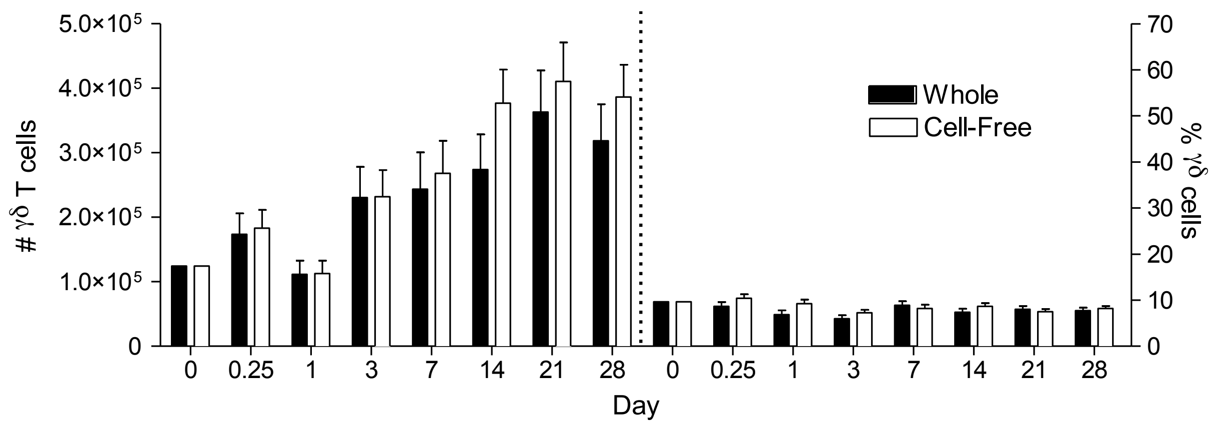

Figure 2.10. γδ T cells in calves during the first month of life.

The number (left) per ml of blood or percentage (right) within a peripheral blood mononuclear cell population of γδ T cells from calves fed whole (n = 17) or cell-free (n = 18) colostrum. Data are presented as LSM ± SEM.

Chapter 3. Adoptively transferred colostral immune cells enhance vaccine response in dairy calves. II. Vaccination Response

***(Formatted for the* Journal of Dairy Science)**

Interpretive summary

Title: **Adoptively transferred maternal colostral immune cells enhance vaccine response in dairy calves**

Author: **Neal**

Summary: The impact of adoptively transferred maternal colostral cells on calf immune development was determined. Calves were fed either whole colostrum (WC) or cell-free colostrum (CFC) and blood immune cell parameters and cytokines were measured in response to two vaccination series. WC-fed calves have an enhanced cellular and cytokine response to vaccines for up to nine months of age compared to CFC-fed calves. These data provide information on how adoptive transfer of immune cells at birth could lead to improved immune development.

ADOPTIVELY TRANSFERRED COLOSTRAL IMMUNE CELLS ENHANCE VACCINE RESPONSE IN DAIRY CALVES

Colostral maternal immune cells impact calf health and immune status

II. Vaccination response

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ABSTRACT

Vaccination in the bovine contributes to improved herd health and production. Enhancing immune development at a young age may increase vaccine efficacy later in life. In the bovine, colostrum is the sole source of maternal immunity, having a substantial impact on health status in the neonate. To date, colostral antibody concentration is used to evaluate colostrum quality. Colostrum also contains proteins and cells, which may enhance immune development and future responses to vaccines. To determine the impact of maternal colostral cells on immune development, thirty-seven female Holstein and Jersey dairy calves were bottle-fed 4 quarts total of whole colostrum (WC) or cell-free colostrum (CFC) at birth. Calves were vaccinated with two series of multivalent vaccines. Series A consisted of vaccines given between month 1 and 4 months of life. Series B consisted of vaccines given between 5 and 10 months of life. Calf peripheral blood samples were obtained before each vaccination series and subsequently monthly for 3 months after each vaccination series. Cellular blood parameters were determined by flow cytometry. Quantitative real-time PCR was used to determine cytokine gene expression before vaccination series B and once a month for 2 months after vaccination series B. Calves fed CFC had fewer numbers of B cells within 1 month and 2 months after vaccination series A when compared to WC-fed calves. Calves fed CFC had decreased gene expression levels of interleukin-2 and numbers of $CD4^+$, $CD4^+CD62L^+CD45RO^-$, $CD4^+CD62L^-CD45RO^+$, $CD4^+CD62L^+CD45RO^+$ and $\gamma\delta$ T cells within 2 months after vaccination series B when compared to WC-fed calves. Our findings show a greater response to vaccines up to nine months post-feeding of WC. This indicates a role for maternal colostral cells in development of the neonatal immune system.

Key words: colostrum, adoptive transfer, leukocytes, vaccination dairy calves

INTRODUCTION

The bovine neonate is born with deficiencies in amount and functioning capacity of non-specific and specific immune cells. This results in a decreased ability to combat infections associated with scours and respiratory diseases. The dairy calf does not experience cross-placental transfer of immune cells during gestation. Therefore, calves rely solely on colostrum for transfer of maternal immunity. Colostrum contains cells, proteins, and soluble molecules that may impact neonatal immune development. Colostral cells traffic through the intestine into neonatal circulation (Schnorr and Pearson, 1984, Williams, 1993, Liebler-Tenorio et al., 2002). Previous work shows that adoptively transferred colostral immune cells increase levels of $CD4^{+}$ T cells within the first month of life in dairy calves (Neal et al., 2013).

Conferring successful vaccine protection in cattle is important in preventing new or recurring infections. Adoptive transfer of ingested colostral immune cells can enhance antigen-specific responses in calves. Calves born from vaccinated dams have enhanced leukocyte proliferative responses to the vaccinate antigen *in vitro*. However, this increase was not seen in calves fed either frozen colostrum or cell-free colostrum (CFC) (Donovan et al., 2007). This could be attributed to maternal colostral memory cells, which may enter the mammary gland during colostrogenesis (Saif et al., 1983, Bandrick et al., 2008). Previous studies show that leukocytes from whole colostrum (WC) fed calves have enhanced activation markers and major histocompatibility complex II (Reber et al., 2008a, b). Together, these studies suggest that calves fed WC have a greater ability to activate immune cells, potentially enhancing responses to antigen exposure.

The objective of this research was to determine the impact of adoptively transferred maternal colostral cells on the immune response to vaccinations in dairy calves. We hypothesize

that WC-fed calves would have a great increase in cellular parameters after vaccination when compared to CFC-fed calves.

MATERIALS AND METHODS

Adoptive transfer of immune cells

Twenty-nine Holstein and eight Jersey cows from the Virginia Tech Dairy Center were equipped with a birthing monitoring system (Foalert, Acworth, GA). All animal handling and sampling protocols were in accordance with the Virginia Tech's Institutional Animal Care and Use Committee. The FoAlert ® birth monitoring system was used for immediate notification of parturition. In brief, a veterinarian sutured a transmitter to each cow ≥ 2 weeks prior to calving to the outside of the vulva. Upon start of parturition, physical separation of the vulva triggered the transmitter to send a radio signal to a receiving unit resulting in an audible alarm and phone calls to pre-programmed numbers on an attached automated dialer. This system ensured that staff arrived prior to parturition

After parturition was complete, the cow was permitted to clean the calf. Calves were not permitted to suckle. After the calf was separated, colostrum was collected aseptically colostrum from each quarter according to standard bacteriology tests were run (NMC, 2004). If clumps or blood was observed in colostrum, mastitis was assumed and the calf was not enrolled. Colostrum that was not mastitic was tested with a digital Brix refractometer to assess total serum protein. Before and after testing of colostrum, the refractometer was cleaned with distilled H_2O. A drop of colostrum was placed on the refractometer slide to obtain a Brix reading. Quality colostrum was determined as 23 Brix score (Weaver et al., 2000).

If colostrum was determined acceptable, calves either received whole colostrum or cell-

free colostrum from their respective dams, according to the following protocols. Two, 2-quart bottles were filled with WC. One, 2-quart bottle was fed to calf within 3 hours after birth. The second 2-quarter bottle was refrigerated (4°C) and then slowly warmed to 37° C immediately before the second feeding at 5-8 hours after birth.

For CFC, 4 Perfluoroalkoxy (PFA) bags (Welch Fluorcarbon, Inc., Dover, NH) were filled each with 1 liter of colostrum per bag. Each PFA bag of colostrum was placed in a Styrofoam box and liquid nitrogen was slowly poured over the PFA bag. Liquid nitrogen completely covered the colostrum but did not overflow into the bag. The PFA bag was turned approximately every 3 minutes until colostrum was completely frozen. Following complete freezing of colostrum, the first 2 quarts of colostrum were slowly warmed to 37° C and fed to the calf within 3 h of life. While the first 2 quarts of colostrum were thawing, the freezing process was repeated again for the second 2 quarts of colostrum. Following complete freezing of the second 2 quarts of colostrum, the colostrum was thawed slowly at 37° C, transferred to bottles and refrigerated (4°C) until fed to the calf at 5-8 h after birth. If the calf did not suckle, an esophageal tube feeder was used. All calves were weighed at birth and administered one dose of TSV-2 nasal vaccine (Zoetis, Madison, NJ). All calves entered the Virginia Tech dairy herd and routine farm management protocols were applied.

Vaccinations

Animals were given two series of vaccinations according to protocols put forth by the Virginia Tech Dairy Center and the herd veterinarian (Table 1). Vaccination series A was administered between 1 and 4 months of age and included a label dose of a multivalent killed vaccine Ultrachoice 7 (Zoetis, Madison, NJ) and a multivalent modified live vaccine Vira Shield (Novar-

tis, Basel, Switzerland). Vaccination series B was administered between 5 and 10 months of age and included a second dose of Ultrachoice 7 and Vira Shield. A dose of modified live RB-51 vaccine (Professional Biological Company, Denver, CO) also was given between months 5 and 10.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Each calf was bled before each vaccination series and subsequently once a month for 3 months after each vaccination series. Peripheral blood mononuclear cells were isolated on a Ficoll-Paque density gradient (1.077 g/ml; BD Falcon, Franklin Lakes, NJ, USA), from whole blood collected using 40 mM EDTA (10% vol/vol) as described previously (Shafer-Weaver et al., 1999). Mononuclear cells were resuspended at $1x10^7$ cells/ml in PBS containing 0.05% sodium azide.

Flow Cytometry

Flow cytometric analysis of cell surface markers was performed using bovine specific monoclonal antibodies (Table 1). Mononuclear cells were resuspended at $1x10^7$ cells/ml in PBS containing 0.05% sodium azide and 10^6 cells were aliquoted per well in 96-well plates. Cells were incubated with primary antibody for 30 min at 4°C. Cells were washed two times with PBS and subsequently incubated with isotype-specific secondary antibodies conjugated to FITC, PE, PE-Texas Red or APC for 30 minutes at 4°C. Cells were washed twice and fixed in 1% formaldehyde in PBS for 20 minutes at RT. Cells were washed again, resuspended in 150 μl of PBS containing 0.05% sodium azide and analyzed by flow cytometry (Accuri C6, BD, San Jose, CA, USA) and FlowJo Software (TreeStar, Ashland, OR, USA).

RNA Isolation and cDNA synthesis

Isolated PBMC were resuspended in TRIzol ® (Invitrogen; Carlsbad, CA) at 5×10^6 for RNA isolation and stored in -80°C. Total RNA was extracted from leukocytes using TRIzol ® reagent according to manufacturer's instructions with the exception that a second EtOH wash was completed. RNA was resuspended into 30μl of deionized H_2O and the concentration of nucleic acid was determined on a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were frozen at -80°C.

Samples that were stored in -80°C were thawed on ice. Complimentary DNA (cDNA) was synthesized from mRNA (2.2µg). This was done by heating at 70°C for 10min with 250ng of random hexamer primers (Invitrogen, Carlsbad, CA). Samples were cooled to 4°C and centrifuged at 350 x g for 30 sec, 4°C. A 20.5%/vol Buffer, 10%/vol Dithiothreitol (DTT), 5%/vol deoxyribonucleotides (DNTPs) (VWR, Suwanee, GA) , and 2.5%/vol Superscipt II (Reverse transcriptase) solution was added to each sample. All reagents were purchased from Invitrogen, Carlsbad, CA. To replicate, samples were incubated at 42°C for 50 minutes and then incubated at 70°C for 15 minutes. Samples were diluted to 10ng/μl of cDNA with DNase/RNase free water (Qiagen; Valencia, CA).

qRT-PCR

The total reaction volume of qRT-PCR analysis was 25 μl containing 300nM of both forward and reverse primers (Integrated DNA Technologies; Coralville, IA), 50nM of 6-carboxyfluorescein (FAM) probe (Biosearch Technologies Inc.; Novato, CA), 2X TaqMan® Universal PCR Master Mix (Applied Biosystems; Foster City, CA), and 50ng cDNA. Samples

were heated at 50°C for 2 min once, 95°C for 10 min once, 95°C for 15 sec for 40 cycles, and cooled at 60°C for 1 min. All samples were run on the ABI 7300 Real-Time PCR System (Applied Biosystems; Foster City, CA) and normalized using housekeeping gene GAPDH. Primer Express 3 software (Applied Biosystems; Foster City, CA) was used to design qRT-PCR primer sets spanning exon junctions for interferon γ (IFNγ), interleukin 2 (IL-2) and tumor growth factor β (TGF- β) genes. A custom primer probe mix was used for interleukin 10 (IL-10) (Applied Biosystems; Foster City, CA). GAPDH forward GACCCCTTCATTGACCTTCA, GAPDH reverse GATCTCGCTCCTGGAAGATG, GAPDH probe TTCCAGTATGATTCCACCCACGG-CA. IFN-γ forward TTGAATGGCAGCTCTGAGAAA, IFN-γ reverse GATCATCCACCG-GAATTTGAA, IFN-γ probe AGGACTTCAAAAAGCT, IL-2 forward CCAAGGTTAACGC-TACAGAATTGA, IL-2 reverse ATTGTCCATTGAATCCTTGATCTC, IL-2 probe AGCTCCAAGCAAAAACCTGAACCCCA, IL-10 Primer/Probe mix (Applied Biosystems; Foster City, California).

Statistical Analysis

All statistical tests were performed using the PROC GLIMMIX procedure of SAS 9.3 (SAS Institute Inc. Cary, NC). Non-normally distributed data, as determined by the studentized T residuals, were natural log transformed. For vaccination series A data, a natural log transformation was used for the number and percentage of $CD4^{+}$ T cells, number of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells and number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells. For vaccination series B data, a natural log transformation was used for the number and percentage of $CD4^{+}$ T cells, number of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells, number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells. For transformed datasets, significance was declared using transformed data while non-transformed

LSM ± SEM were reported in text. Each statistical model included the fixed effects of treatment, time, breed and the temperature at birth and the associated interactions. Each calf was designated a temperature code based on the minimum and maximum temperature the day of birth: cold (C)-born, minimum day temperature ≤ -30°C; mild (M)-born, minimum day temperature > -30° C and maximum day temperature ≤ 80° C; hot (H) born, maximum day temperature > 80° C. The repeated statement was applied to the day of blood collection using calf nested within treatment as the subject. If pre-treatment levels were significantly different from each other, a covariate was assigned for period -1. Error structures were tested and the one giving the lowest Akaike information criterion was chosen for analysis. Slices were evaluated for the associated interactions and a Tukey's adjustment was applied. Significance was declared at $P \leq 0.05$.

RESULTS

Peripheral Blood Mononuclear Cell (PBMC) Response to Vaccination

Two months after vaccination series A, the number of mononuclear cells (LSM ± SEM; Figure 3.1) peaked in WC-fed (month 2: $8.87 \times 10^6 \pm 6.87 \times 10^5$) and CFC-fed calves (month 2: $7.27 \times 10^6 \pm 6.76 \times 10^5$). Temperature at birth had an impact on the number of PBMC in WC-fed calves where C-born calves had an increased number of PBMC ($8.91 \times 10^6 \pm 5.01 \times 10^5$) when compared to M-born calves ($6.01 \times 10^6 \pm 5.90 \times 10^5$, $P = 0.0021$) and H-born calves ($5.34 \times 10^6 \pm 7.77 \times 10^5$, $P = 0.0015$).

One month after vaccination series B, the number of mononuclear cells (LSM ± SEM; Figure 3.1) peaked in WC-fed calves (month 1: $1.14 \times 10^7 \pm 9.81 \times 10^5$) where CFC-fed calves did not peak until two months post-vaccination (month 2: $9.69 \times 10^6 \pm 1.09 \times 10^6$). Within 1 month ($P = 0.037$) and at 1 month ($P = 0.018$) after vaccination series A, there were fewer numbers of

mononuclear cells in WC-fed calves (month 0: $1.13\times10^{7} \pm 1.01\times10^{6}$ and month 1: $1.14\times10^{7} \pm 9.81\times10^{5}$) when compared to CFC-fed calves (month 0: $8.15\times10^{6} \pm 1.06\times10^{6}$ and month 1: $7.90\times10^{6} \pm 1.02\times10^{6}$).

$CD4^{+}$ T Cell Response to Vaccination

The number of $CD4^{+}$ T cells per ml of blood (LSM ± SEM; Figure 3.2) peaked 2 months after vaccination series A in WC-fed calves (month 2: $5.01\times10^{5} \pm 1.23\times10^{5}$) and CFC-fed calves (month 2: $4.89\times10^{5} \pm 1.27\times10^{5}$). There was no treatment effect in response to vaccination series A. However, there was an overall breed effect where Jersey calves had a higher number (natural log transformed; 12.71 ± 0.24) and percentage (natural log transformed; 1.78 ± 0.18) of $CD4^{+}$ T cells when compared to Holstein calves (natural log transformed number; 12.01 ± 0.13, P = 0.013 and percentage; 1.03 ± 0.10, P = 0.0008).

The number of $CD4^{+}$ T cells per ml of blood (LSM ± SEM; Figure 3.2) in WC-fed calves peaked within 1 month after vaccination series A (month 0: $9.74\times10^{6} \pm 1.26\times10^{5}$) where CFC-fed calves did not peak until 2 months post-vaccination (month 2: $8.97\times10^{5} \pm 1.41\times10^{5}$). Within 1 month (month 0; P = 0.03) after vaccination series B, CFC-fed calves had a decreased number of $CD4^{+}$ T cells ($5.70\times10^{5} \pm 1.32\times10^{5}$) compared to WC-fed calves ($9.74\times10^{6} \pm 1.26\times10^{5}$). The number of $CD4^{+}$ T cells in C-born calves fed WC ($1.01\times10^{6} \pm 1.22\times10^{5}$) were greater compared to M-born calves fed the same treatment ($5.83\times10^{5} \pm 1.19\times10^{5}$, P = 0.05). The percentage of $CD4^{+}$ T cells in C-born calves fed WC (8.61 ± 0.82) were higher compared to M-born calves fed the same treatment (5.45 ± 0.84, P = 0.033).

$CD4^{+}CD62L^{+}CD45RO^{-}$ T Cell Response to Vaccination

The number of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells per ml of peripheral blood (LSM ± SEM; Figure 3.3) peaked 2 months after vaccination series A in WC-fed calves (month 2: 8.23×10^{4} ± 2.60×10^{4}) and CFC-fed calves (month 2: 5.74×10^{4} ± 2.68×10^{4}). At 1 month post-vaccination, calves fed CFC had a lower percentage of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (8.52 ± 1.59) when compared to WC-fed calves (13.27 ± 1.79, P = 0.050). The same was seen at 2 months post-vaccination, where CFC-fed calves had a lower percentage of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (7.97 ± 2.24) when compared to WC-fed calves (15.77 ± 2.22, P = 0.015). The percentage of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells in H-born calves fed WC (28.4 ± 2.51) was higher than M-born calves (16.48 ± 2.00, P = 0.0012) and C-born calves fed the same treatment (15.32 ± 1.70, P = 0.0002).

The number of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (LSM ± SEM; Figure 3.3) in response to vaccination series B peaked within 1 month post-vaccination in WC-fed calves (month 0: 2.05×10^{5} ± 5.56×10^{4}) where CFC-fed calves did not peak until 2 months post-vaccination (month 2: 1.63×10^{5} ± 5.52×10^{4}). Within 1 month (month 0; P = 0.044) and at 1 month (month 1; P = 0.002) after vaccination series B, CFC-fed calves had a fewer number of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (month 0 natural log transformed: 9.54 ± 0.38; month 1 natural log transformed: 9.88 ± 0.36) when compared to WC-fed calves (month 0 natural log transformed: 11.22 ± 0.36; month 1 natural log transformed: 10.95 ± 0.38). Additionally, within 1 month after vaccination series B (P = 0.008) CFC-fed calves had a fewer percentage of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (5.92 ± 2.58) when compared to WC-fed calves (15.42 ± 2.41). Jersey calves had a greater percentage of $CD4^{+}CD62L^{+}CD45RO^{-}$ T cells (12.70 ± 1.50) when compared to Holstein calves (9.04 ± 0.92) in response to the second vaccination (P = 0.040).

$CD4^{+}CD62L^{-}CD45RO^{+}$ T cells Response to Vaccination

The number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells per ml of blood (LSM ± SEM; Figure 3.4) peaked 2 months after vaccination series A in WC-fed calves (month 2: $2.13\times10^{5} \pm 5.05 \times10^{4}$) and CFC-fed calves (month 2: $2.21\times10^{5} \pm 5.17\times10^{4}$). Month of vaccination impacted the number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells in all calves in response to vaccination series 1 ($P = 0.0004$). Jersey calves fed WC had a greater number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells (natural log transformed; 11.83 ± 0.37) compared to Holstein calves fed CFC (natural log transformed 10.94 ± 0.19; $P = 0.041$). Calves fed CFC had a greater percentage of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells when compared to WC-fed calves within 1 month (40.22 ± 3.06 and 28.41 ± 3.39, respectively, $P = 0.013$), at 1 month (54.60 ± 2.93 and 44.54 ± 3.17, respectively, $P = 0.025$), and at 2 months (43.31 ± 3.01 and 55.01 ± 3.25, respectively, $P = 0.012$). Jersey calves fed CFC had a greater percentage of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells (55.39 ± 3.54) compared to Holstein calves fed the same treatment (44.50 ± 1.65, $P = 0.0088$).

The number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells per ml of blood (LSM ± SEM; Figure 3.4) peaked within 1 month after vaccination series B in WC-fed calves (month 1: $5.49\times10^{5} \pm 1.07\times10^{5}$) where CFC-fed calves did not peak until 2 months post-vaccination (month 2: $4.62\times10^{5} \pm 7.04\times10^{4}$). Within 1 month after vaccination series B, CFC-fed calves had a decreased number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells (month 0 natural log transformed: 12.24 ± 0.21) compared to WC-fed calves (month 0 natural log transformed: 12.82 ± 0.20, $P = 0.049$). Day of vaccination impacted the percentage of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells in response to vaccination series A in all calves ($P = 0.017$). Treatment, breed and temperature at birth did not have an effect on the percentage of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells.

$CD4^+CD62L^+CD45RO^+$ T cell Response to Vaccination

The number of $CD4^+CD62L^+CD45RO^+$ T cells (LSM ± SEM; Figure 3.5) in response to vaccination series A peaked within 1 month post-vaccination in WC-fed calves (month 0: $1.95\times10^5 \pm 3.65\times10^4$) where CFC-fed calves did not peak until two months post-vaccination (month 2: $1.68\times10^5 \pm 4.53\times10^4$). Jersey calves fed WC had a greater number of $CD4^+CD62L^+CD45RO^+$ T cells (natural log transformed; 11.89 ± 0.29) compared to Holstein calves fed the same treatment (natural log transformed; 11.18 ± 0.15, $P = 0.036$).

The number of $CD4^+C62L^+CD45RO^+$ T cells (LSM ± SEM; Figure 3.5) in response to vaccination series B peaked within 1 month post-vaccination in WC -calves (month 0: $3.97\times10^5 \pm 6.13\times10^4$) where CFC-fed calves did not peak until two months post-vaccination (month 2: $2.80\times10^5 \pm 5.04\times10^4$). Within 1 month (month 0; $P = 0.035$) and at 1 month (month 1; $P = 0.029$) after vaccination series B, CFC-fed calves (month 0 natural log transformed: 11.90 ± 0.23; month 1 natural log transformed: 11.58 ± 0.22) had a fewer number of $CD4^+CD62L^+CD45RO^+$ T cells when compared to WC-fed calves (month 0 natural log transformed: 12.57 ± 0..21; month 1 natural log transformed: 12.28 ± 0.22). Month of vaccination impacted the percentage of $CD4^+CD62L^+CD45RO^+$ T cells in all calves in response to vaccination series 1 ($P = 0.0003$). Jersey calves fed CFC had a decreased percentage of $CD4^+CD62L^+CD45RO^+$ T cells (26.31 ± 0.36) compared to Holstein calves fed CFC (34.96 ± 1.75; $P = 0.019$).

Monocyte Response to Vaccination

The number per ml of peripheral blood and percentage within a PBMC population of monocytes (LSM ± SEM; Figure 3.6) were analyzed. Month of vaccination impacted the number of monocytes in all calves in response to vaccination series 1 ($P = 0.006$). Jersey calves fed CFC

had a decreased number of monocytes ($7.07\times10^5 \pm 2.72\times10^5$) compared to Holstein calves fed CFC ($1.57\times10^6 \pm 1.34\times10^5$, $P = 0.0075$). Additionally, Jersey calves fed WC had a decreased number of monocytes ($1.23\times10^6 \pm 2.63\times10^5$) compared to Holstein calves fed WC ($1.95\times10^6 \pm 1.43\times10^5$, $P = 0.021$). Lastly, Jersey calves fed CFC had a decreased percentage of monocytes (15.77 ± 3.45) compared to Holstein calves fed CFC (27.57 ± 1.75, $P = 0.0044$) in response to vaccination series A.

The number per ml of peripheral blood and percentage within a PBMC population of monocytes (LSM ± SEM; Figure 3.6) were analyzed. Jersey calves fed CFC had a decreased number of monocytes ($1.17\times10^6 \pm 1.77\times10^5$) compared to Holstein calves fed CFC ($1.82\times10^6 \pm 9.52\times10^4$, $P = 0.0038$). Jersey calves had a decreased percent of monocytes (10.35 ± 1.64) compared to Holstein (18.38 ± 0.77, $P = 0.0002$). Calves fed CFC had a higher percentage of monocytes within 1 month after vaccination series B (20.48 ± 1.72) compared to WC-fed calves (12.38 ± 1.77, $P = 0.0014$).

B Cell Response to Vaccination

The number of B cells (LSM ± SEM; Figure 3.7) in response to vaccination series A peaked 2 months post-vaccination in WC (month 2: $2.99\times10^6 \pm 2.50\times10^5$) and CFC-fed calves (month 2: $1.73\times10^6 \pm 2.55\times10^5$). At 2 months ($P = .0004$) after vaccination series A, CFC-fed calves had fewer B cells ($1.73\times10^6 \pm 2.55\times10^5$) when compared to WC-fed calves ($2.99\times10^6 \pm 2.50\times10^5$). The number of B cells was greater in C-born calves fed WC ($2.95\times10^6 \pm 2.26\times10^5$) compared to M-born calves ($1.61\times10^6 \pm 2.47\times10^5$, $P = 0.0004$) and H-born calves ($1.84\times10^6 \pm 2.93\times10^5$, $P = 0.014$) fed the same treatment. Additionally, 1 month after vaccination series A, CFC-fed calves (month 1: 26.92 ± 1.91) had a decreased percentage of B cells when compared to

WC-fed calves (month 1: 34.35 ± 1.93, $P = 0.0075$).

The number per ml of peripheral blood and percentage within a PBMC population of B cells (LSM ± SEM; Figure 3.7) were analyzed. There were not any overall treatment differences at any of the sampling time-points for the number or percentage of B cells in response to vaccination series B. Breed and temperature at birth impacted the percentage of B cells in calves. Jersey calves fed WC had a higher percentage of B cells (31.78 ± 1.98) when compared to Holstein calves fed the same treatment (24.66 ± 1.26, $P = 0.0063$). The percentage of B cells was fewer in C-born calves fed WC (21.27 ± 1.76) compared to M-born (32.17 ± 1.91, $P = 0.0003$) and H-born calves (31.23 ± 1.93, $P = 0.0025$) fed the same treatment.

Gamma Delta (γδ) T cell Response to Vaccination

The number of γδ T cells (LSM ± SEM; Figure 3.8) in response to vaccination series A peaked 2 months post-vaccination in WC (month 2: $7.04 \times 10^5 \pm 9.25 \times 10^4$) and CFC-fed calves (month 2: $7.07 \times 10^5 \pm 9.13 \times 10^4$). Month relative to vaccination affected the number ($P \leq .0001$) of γδ T cells in response to vaccination series A. There was an increase in the number of γδ T cells in WC-fed, C-born calves ($6.48 \times 10^5 \pm 6.58 \times 10^4$) when compared to H-born calves fed the same treatment ($3.82 \times 10^6 \pm 9.62 \times 10^4$, $P = 0.046$). Two months post-vaccination series A, calves fed CFC had a greater percentage of γδ T cells (10.46 ± 0.85) compared to WC-fed calves (7.25 ± 0.81, $P = 0.0093$). Jersey calves fed WC had a decreased percentage of γδ T cells (4.87 ± 1.21) compared to Holstein calves fed WC (8.61 ± 0.70, $P = 0.014$). Additionally, the percentage of γδ T cells was greater in C-born calves fed CFC (11.55 ± 1.24) compared to M-born calves (7.20 ± 1.08, $P = 0.014$) fed the same treatment.

The number of γδ T cells (LSM ± SEM; Figure 3.8) in response to vaccination series B

peaked 2 months post-vaccination in WC (month 2: $1.42\times10^6 \pm 1.56\times10^5$) and CFC-fed calves (month 2: $1.22\times10^6 \pm 1.62\times10^5$). Treatment had an effect ($P = 0.038$) on the overall numbers of γδ T cells in response to vaccination series B in WC ($1.34\times10^6 \pm 1.06\times10^5$) and CFC-fed calves ($1.01\times10^6 \pm 1.06\times10^5$). Within 1 month after vaccination series B, γδ T cell percentage was decreased ($P = 0.031$) in CFC-fed calves (month 0: 8.53 ± 1.17) when compared to WC-fed calves (month 0: 12.17 ± 1.13).

Cytokine Response to Vaccination

Cytokines IFN-γ, IL-2, IL-10 and TGFβ were analyzed in response to vaccination series B (Table 3). One month after vaccination series B, IL-2 mRNA expression was decreased in CFC-fed calves when compared to WC-fed calves ($P = 0.048$). IL-10 mRNA expression was greater in C-born calves fed WC (4.13 ± 0.36) compared to M-born calves (6.51 ± 0.59) fed the same treatment ($P = 0.0004$).

DISCUSSION

To our knowledge, this is the first study demonstrating a role for adoptively transferred colostral cells in vaccination responses of dairy cattle. While much is known about the passive transfer of antibodies in the calf, little research has been done to elucidate the role of adoptively transferred lymphocytes in neonatal immune development. Previous work shows that colostral immune cells enhance pathogen clearance and increase lymphocyte activation markers and major histocompatibility complex II expression (Reber et al., 2005, Reber et al., 2006). Colostral cells can also be antigen specific. Piglets fed colostrum from sows vaccinated against *Mycoplasma hyopneumoniae* had a greater delayed-type hypersensitivity response compared to piglets fed colostrum from unvaccinated sows (Bandrick et al., 2008). Additionally, calves fed colostrum from

dams vaccinated against bovine viral diarrhea virus (BVDV) had enhanced responses to BVDV antigen when compared to calves fed frozen or cell-free colostrum (Donovan et al., 2007). Adoptively transferred cellular immunity may help to overcome the inhibitive effects of maternal antibodies in the newborn allowing for enhanced vaccination response later in life.

In our study, there were no differences in $CD4^+$ T cell levels in response to the first vaccine exposure. However, within 1 month after the second vaccine exposure CFC-fed calves had a decreased number of $CD4^+$ T cells when compared to WC-fed calves. Previous studies show a role for IFN-γ secreting $CD4^+$ T cells in vaccine responses in cattle. Oh et al. (2012) injected naïve cattle with an inactivated foot and mouth disease (FMDV) vaccine and animals were subsequently challenged with FMDV 21 days post-vaccination. IFN-γ levels were determined after restimulation of PBMC in culture with the vaccine antigen. Cattle that were categorized as clinically protected had greater IFN-γ levels *in vitro* when compared to cattle categorized as clinically unprotected. When $CD4^+$ T cells subsets were depleted *in vitro*, IFN-γ production decreased (Oh et al., 2012). Other studies show that when lymphocytes from vaccinated cattle are stimulated with the respective viral antigen *in vitro*, $CD4^+$ T cells are the major proliferating cell type (Brown et al., 1998, Naiman et al., 2002, Zuerner et al., 2011). This together with our data suggests an important role for colostral cells in memory $CD4^+$ T cells responses. Previous research shows that memory cells from the dam may traverse to the mammary gland during colostrogenesis (Saif et al., 1983, Donovan et al., 2007, Bandrick et al., 2008). Our previous study showed that WC-fed animals had an increased number of $CD4^+$ T cells within the first month of life compared to CFC-fed calves (Neal et al., 2013). It is possible that adoptively transferred maternal colostral cells either assists in maturation of endogenous $CD4^+$ T cells, contribute a memory $CD4^+$ T cell population in the calf, or a combination of both.

There was a decreased percentage of $CD4^+CD62L^+CD45RO^-$ T cells in CFC-fed calves in response to vaccination series A when compared to WC-fed calves. Additionally, in response to vaccination series B, CFC-fed calves had a decreased number and percentage of $CD4^+CD62L^+CD45RO^-$ T cells when compared to WC-fed calves. These results were unexpected as CD62L expression decreases on activated $CD4^+$ T cells (Jung et al., 1988, Waters et al., 2003a). Additionally, $CD62L^+$ $CD45RO^-$ T cells are traditionally described as naïve, non-activated T cells (Geginat et al., 2003, Lefrancois, 2006). However, recent investigations into heterogeneous populations of memory T cells identified small populations of T cells with stem cell-like memory (T_{scm}). This population shares similar life span and proliferative capacities to hematopoietic stem cells (HSCs) and are $CD62L^+$, $CD45RO^-$ (Muranski et al., 2011). It is possible that the greater number of $CD4^+CD62L^+CD45RO^-$ T cells in WC-fed calves can be attributed to an increase in $CD4^+$ T_{scm} populations. Additionally, our previous work showed an increase in $CD4^+CD62L^+CD45RO^-$ T cells 1-d post-colostrum ingestion (Neal et al., 2013). It is not known whether the increase in CD62L expression on $CD4^+$ T cells within the first week of life could enhance future responses to antigens in the host. Further research is needed to determine if bovine $CD4^+CD62L^+CD45RO^-$ T cells have a role in vaccine responses.

When analyzing activation marker CD45RO, a treatment difference was seen after vaccination series A. CFC-fed calves had a greater percentage of $CD4^+CD62L^-CD45RO^+$ T cells over the two months after vaccination series A when compared to WC-fed calves. CD45RO is associated with cellular activation and memory response (Mackay, 1999). However, our lab showed a greater percentage of $CD4^+CD62L^-CD45RO^+$ T cells on d 7 of life and a numerically greater number until d 28 of life in CFC-fed calves when compared to WC-fed calves (Neal et al., 2013). It is possible that this effect carried over into vaccination series A as many calves

were vaccinated around or right after 28 d of life. WC-fed calves had a greater number of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells within 1 month after vaccination series B. Previous studies show an increase in CD45RO on $CD4^{+}$ T cells after vaccinations in both young and adult cattle (Stabel and Robbe-Austerman, 2011, Totte et al., 2013) .The increase in $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells after vaccination series B could be attributed to enhanced antigen presentation and lymphocyte activation capabilities in WC-fed calves when compared to CFC-fed calves. Additionally, $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells may have memory cell characteristics that increase cellular proliferation to a vaccine antigen.

$CD4^{+}CD62L^{+}CD45RO^{+}$ T cells were decreased within 1 month and at 1 month after vaccination series B in CFC-fed calves when compared to WC-fed calves. Studies show that $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells have central memory cell capabilities and are important in conferring vaccine protection (Sallusto et al., 1999, Vaccari et al., 2005). Central memory cells are very sensitive to antigenic stimulation and have an increased ability to sustain a memory T cell population when compared to effector cells (Wu et al., 2002, Wherry et al., 2003). This increase in $CD4^{+}CD62L^{+}CD45RO^{+}$ T cells may suggest that WC-fed calves have either an increase in memory cell population or an increased ability of memory cells to proliferate when compared to CFC-fed calves. Animals that exhibit an increased central memory cell population in response to vaccination may have enhanced abilities to clear invading pathogens.

When monocyte levels were analyzed, the only difference seen was within 1 month after vaccination series B. The percentage of monocytes was greater 1 month post-vaccination in CFC-fed calves when compared to WC-fed calves. It is likely that the greater percentage of monocytes in CFC-fed calves is attributed other cell types decreasing during that time. The percentage of each cell type is determined from a whole mononuclear cell population. The increase

in monocyte percentage in CFC-fed calves could be because other cell types decreased concurrently. This is further supported by our data showing no differences in number of monocytes per ml of blood within the same time point after vaccination series B.

We found that CFC-fed calves had a decreased number and percentage of B cells at 2 months and 1 month after the first vaccine exposure, respectively. However, no differences were seen after vaccination series B. The role of B cells in vaccine response is to generate long-lived immune responses to subsequent stimulation. This protection can be attributed to the ability of B cells to differentiate into either memory B cells or antibody-producing plasma cells (Tangye and Tarlinton, 2009). B cells are found in bovine colostrum, thus, similar to $CD4^+$ T cells, memory B cells could have passively transferred into neonatal circulation. It is interesting to note that of the cell types investigated; only B cells were different between treatments at a sampling time-point after vaccination series A. This could be because B cells directly interact with and uptake antigen, potentially responding more quickly than cells that cannot, like $CD4^+$ T cells (Batista and Neuberger, 2000). Further research is needed to see if the proliferation in B cells is correlated with an increase in antigen-specific antibody production.

We show that the percentage of γδ T cells was decreased in WC-fed calves 2 months post-vaccination when compared to CFC-fed calves. As described above, this could be contributed to another cell type increasing in WC-fed calves while the percentage of γδ T cells was decreasing. This is further supported by not having any differences between treatments for the number of γδ T cells. In response to vaccination series B, CFC-fed calves had a decreased number of γδ T cells within 1 month and at 1 month compared to WC-fed calves. Bovine γδ T cells proliferate in response to a variety of vaccines (Waters et al., 2003b, Silflow et al., 2005, Blumerman et al., 2006). Memory γδ T cells have been identified in humans, mice and non-

human primates (Hiromatsu et al., 1992, Eberl et al., 2002). It is possible that memory γδ T cells found in colostrum passively transferred into neonatal circulation and trafficked lymphoid tissue in the calf. These memory γδ T cells may have proliferated in response to vaccination series B. Additionally, endogenous populations of γδ T cells may be responding to the viral antigen directly. Unlike $CD4^+$ T cells, γδ T cells have the capacity to respond directly to viral antigens and present it to other T cells (Brandes et al., 2005, Chien and Konigshofer, 2007). This would allow for immediate response after vaccination, possibly accounting for the greater number of γδ T cells in WC-fed calves. Lastly, the increase in γδ T cells may enhance antigen presentation to $CD4^+$ T cells, increasing proliferation post-vaccination. Understanding the response of bovine γδ T cell to specific antigens would be beneficial for future vaccine development in cattle.

Lastly, vaccination did not induce a difference in mRNA expression for inflammatory cytokine IFN-γ or anti-inflammatory cytokines IL-10 or TGFβ between treatments at any of the sampling time-points. However, CFC-fed calves produced fewer IL-2 mRNA when compared to WC-fed calves ($P \leq 0.05$). IL-2 is a cytokine crucial for activation and differentiation of $CD4^+$ and $CD8^+$ memory and effector T cells (Boyman and Sprent, 2012). While the majority of cytokine-secreting $CD4^+$ T cells produce IFN-γ post-immunization, IFN-γ levels do not always correlate to protection. IFN-γ producing $CD4^+$ T cells are short-lived and have limited ability to sustain as central memory cells (Wu et al., 2002). However, studies show IL-2 secreting $CD4^+$ T cells serve as a reservoir for central memory $CD4^+$ T cells with effector capacities. The greater level of IL-2 at 1 month after vaccination series B suggests a functional difference in $CD4^+$ T cells in WC-fed calves compared to CFC-fed calves.

CONCLUSIONS

In the current study, we determined that calves fed CFC colostrum had fewer B cell numbers after vaccination series A and fewer gene expression of IL-2 and numbers of $CD4^+$ T cells and γδ T cells after vaccination series B. It can be concluded that adoptively transferred immune cells do impact the cellular response to vaccinations in the dairy calf. Novel methods to enhance immune function in calves through adoptive transfer of immune cells at birth could lead to healthier and more productive animals entering the dairy herd.

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Table 3.1 Vaccination series A and B protocols.

	Ultrachoice 7 (Zoetis)	*Vira Shield (Novartis)*	*RB51 (Professional Biologicals)*
Series A	1-4 months	1-4 months	**-**
Series B	5-10 months	5-10 months	5-10 months

Table 3.2. Primary and secondary antibodies used for flow cytometric analysis of PBMC populations.

Primary antibody target[1]	Clone	Isotype	Primary antibody dilution	Secondary antibody
$CD4^+$ T cell	IL-A11	IgG2a	1:400	IgG2a-FITC[2]
CD62L	BAQ-92A	IgG1	1:400	IgG1-PE-Texas Red[2]
CD45RO	IL-A1116	IgG3	1:400	IgG3-PE[3]
B cell ($CD21^+$)	BAQ-15A	IgM	1:400	IgM-APC[2]
Monocytes ($CD14^+$)	MM61	IgG1	1:400	IgG1-PE-Texas Red[2]
γδ TCR^+ T cell	CACTB32A	IgG1	1:800	IgG1-PE-Texas Red[2]

[1]Primary mouse-generated monoclonal antibodies purchased from VMRD, Pullman, WA.
[2]Secondary goat anti-mouse monoclonal antibodies purchased from Life Technologies, Carlsbad, CA
[3]Secondary goat anti-mouse monoclonal antibodies purchased from Southern Biotechnologies, Birmingham, AL

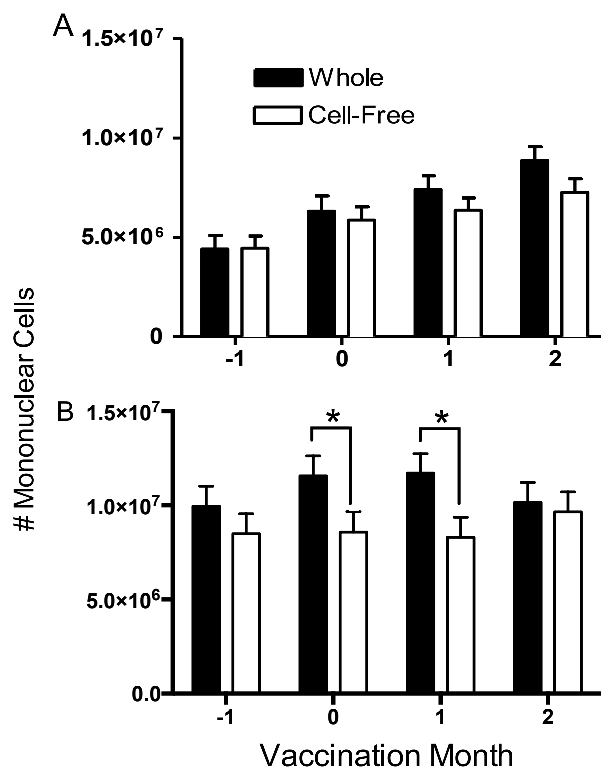

Figure 3.1. Mononuclear cells in calves in response to vaccination series A and B.

The number per ml of blood of mononuclear cells in response to vaccination series A and B from calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16, vaccination series B: n = 12). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. $*P \leq 0.05$

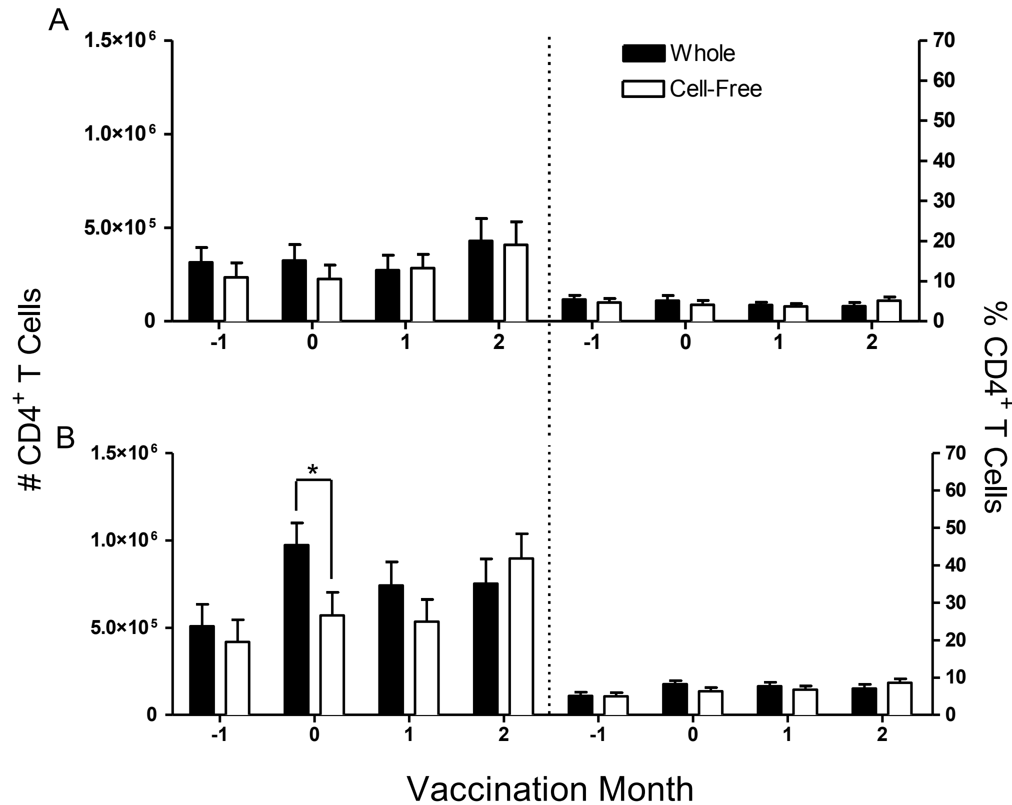

Figure 3.2. CD4+ T cells in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of CD4+ T cells in response to vaccination series A and B in calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16; vaccination series B: n = 12). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. Significances were determined in response to vaccination series A and B using natural log transformed data for number (vaccination series B, month 0: whole: 13.61 ± 0.21; cell-free: 12.82 ± 0.23, vaccination series B, month 1: whole: 13.54 ± 0.21; cell-free: 12.93 ± 0.21) and percentage of CD4+ T cells. *$P \leq 0.05$

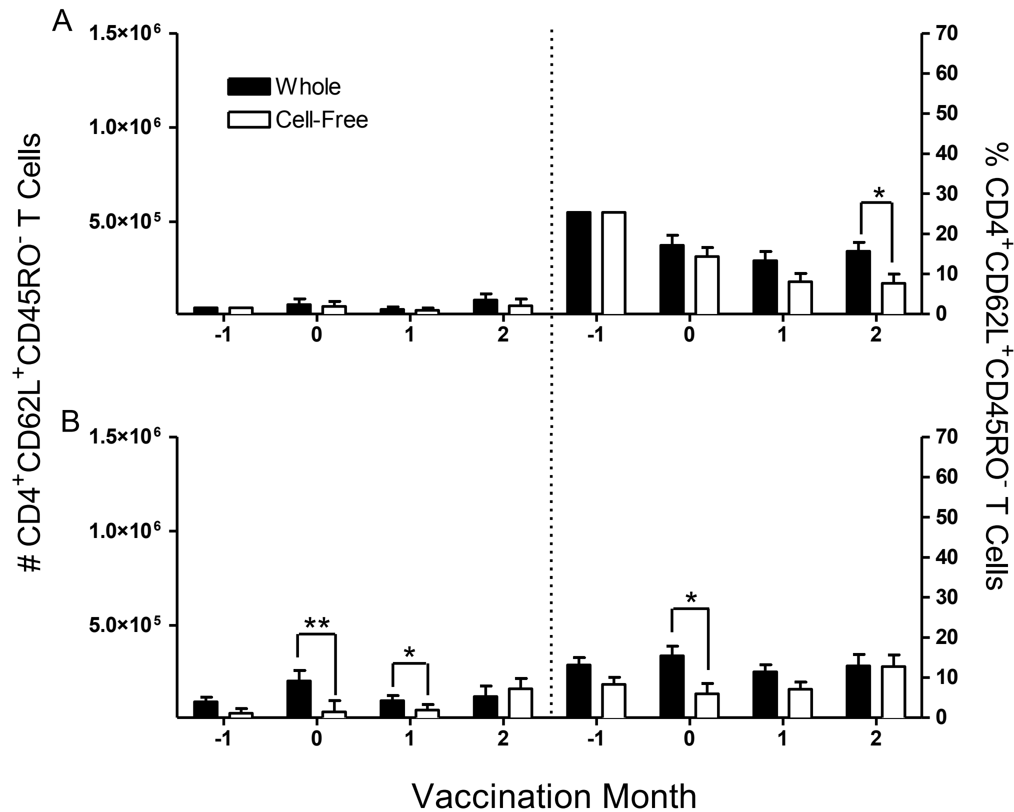

Figure 3.3. CD4$^+$CD62L$^+$CD45RO$^-$ T cells in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of CD4$^+$CD62L$^-$CD45RO$^+$ T cells in calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16; vaccination series B: n = 12). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. Significances were determined in response to vaccination series A and B using natural log transformed data for number (vaccination series B, month 0: whole: 11.22 ± 0.36; cell-free: 9.54 ± 0.38, vaccination series A, month 1: whole: 10.95 ± 0.38; cell-free: 9.88 ± 0.36) and percentage (vaccination series B, month 0: whole: 2.21 ± 0.21; cell-free: 1.30 ± 0.23) of CD4$^+$CD62L$^+$CD45RO$^-$ T cells. *$P \le 0.05$ **$P \le 0.01$

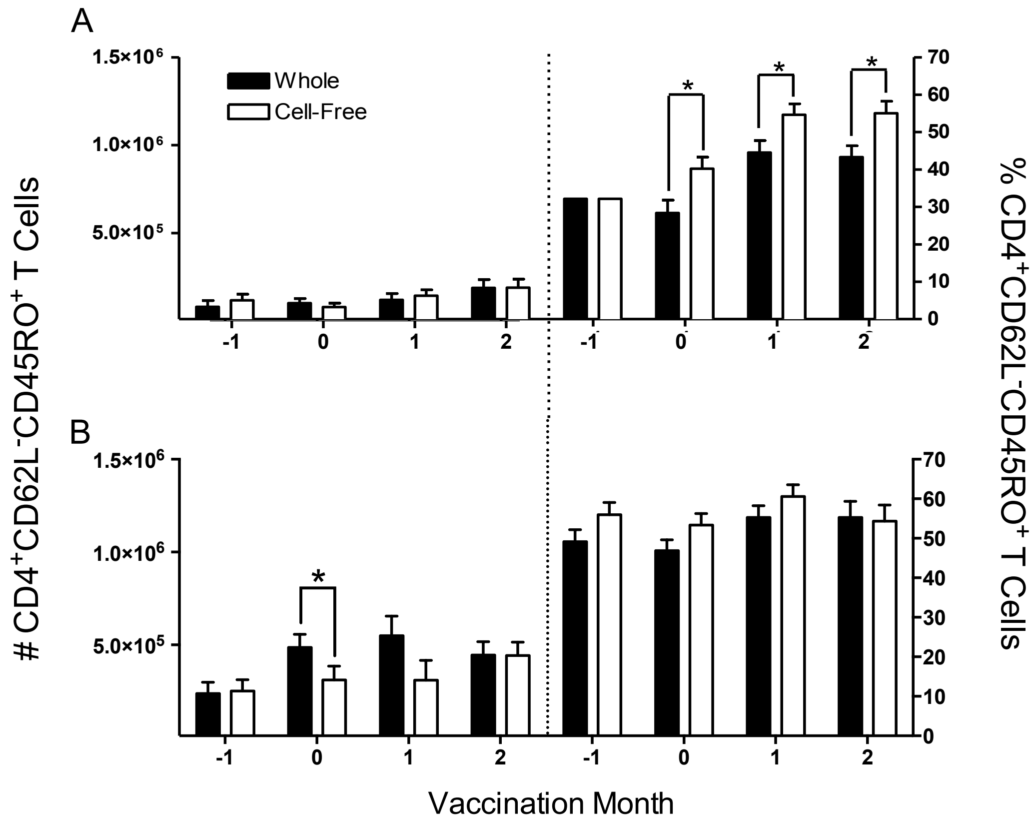

Figure 3.4. $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of $CD4^{+}CD62L^{-}CD45RO^{+}$ T cells in calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16; vaccination series B: n = 12). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. $^{*}P \leq 0.05$

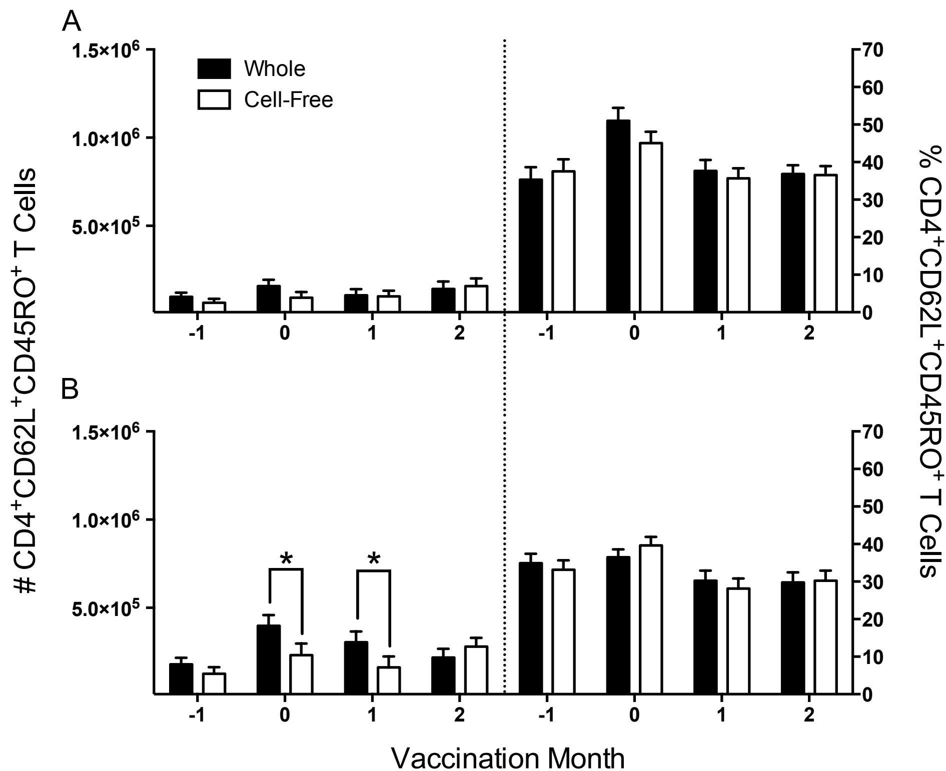

Figure 3.5. $CD4^+CD62L^+CD45RO^+$ T cells in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of $CD4^+CD62L^+CD45RO^+$ T cells in response to vaccination series A and B in calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16; vaccination series B: n = 12). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. Significances were determined in response to vaccination series A and B using natural log transformed data for number (vaccination series B, month 0: whole: 12.58 ± 0.22; cell-free: 11.9 ± 0.23, vaccination series B, month 1: whole: 12.28 ± 0.22; cell-free: 11.58 ± 0.22) of $CD4^+CD62L^+CD45RO^+$ T cells. *$P \leq 0.05$

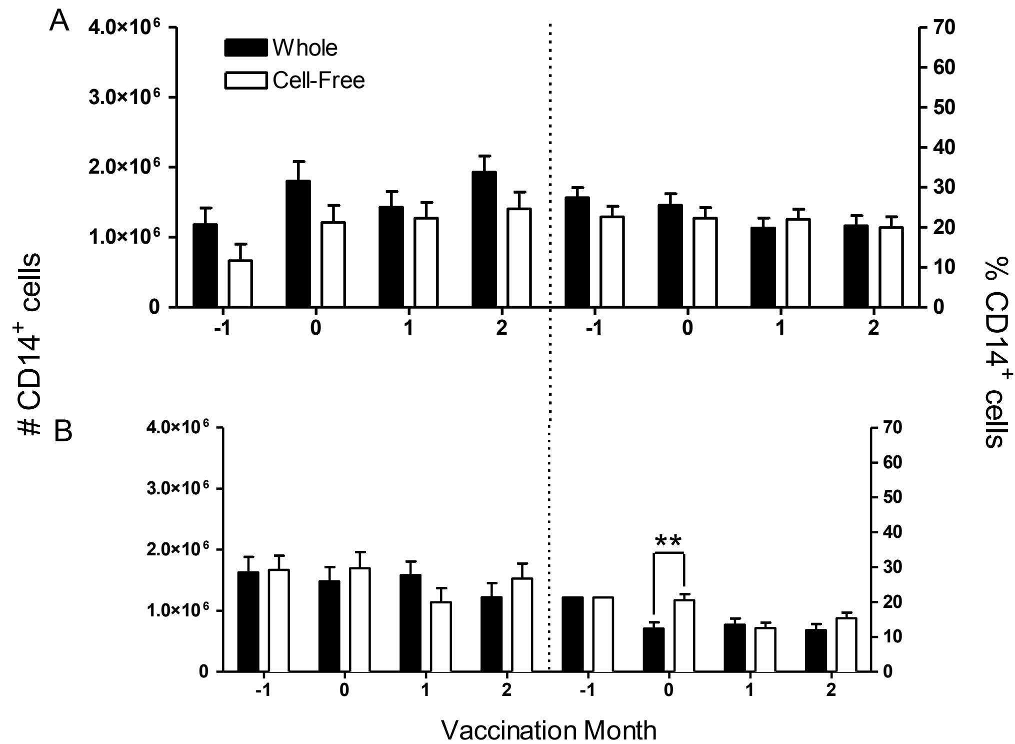

Figure 3.6. Monocytes (CD14⁺ cells) in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within the mononuclear cell population of monocytes (CD14⁺ cells) in response to vaccination series A and B in calves fed whole (vaccination series A: n = 11; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 15; vaccination series B: n = 12).Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. $^{*}P \leq 0.05$

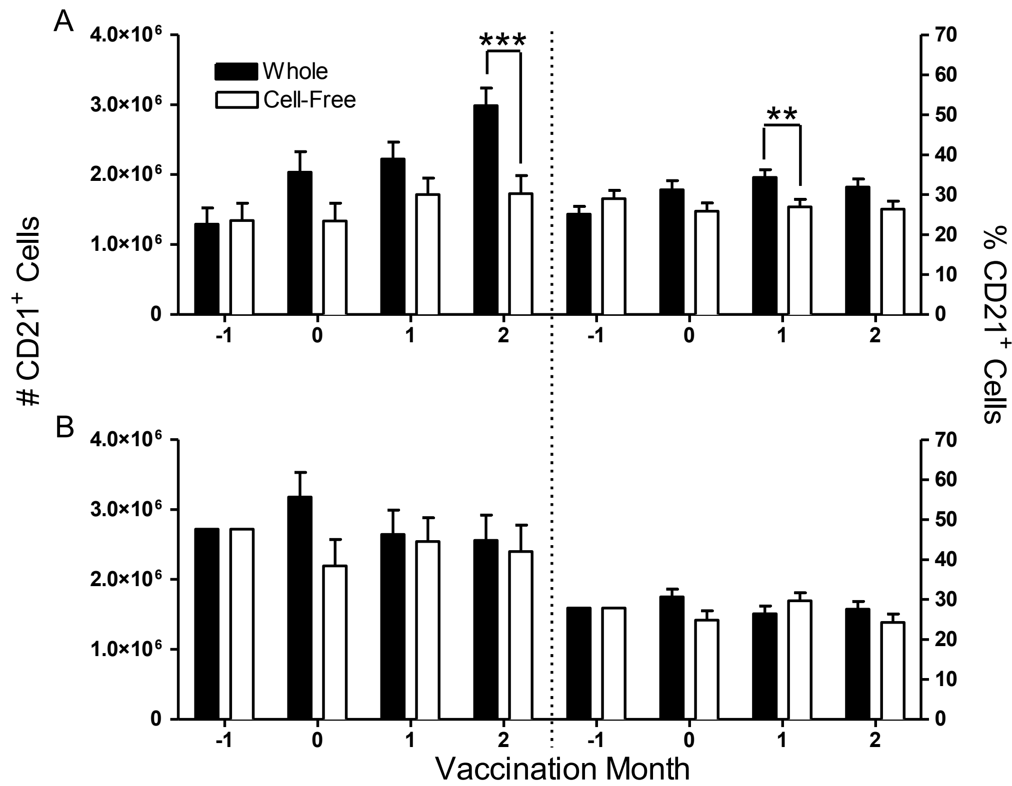

Figure 3.7. B cells ($CD21^+$) in calves in response to vaccination series A and B.

The number per ml of blood and percentage within a mononuclear cell population of B cells ($CD21^+$) cells in response to vaccination series A and B in calves fed whole (vaccination series A: n = 11; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 15; vaccination series B: n = 12). Data are expressed as LSM ± SEM. $^{**}P \leq 0.01$ $^{***}P \leq 0.001$

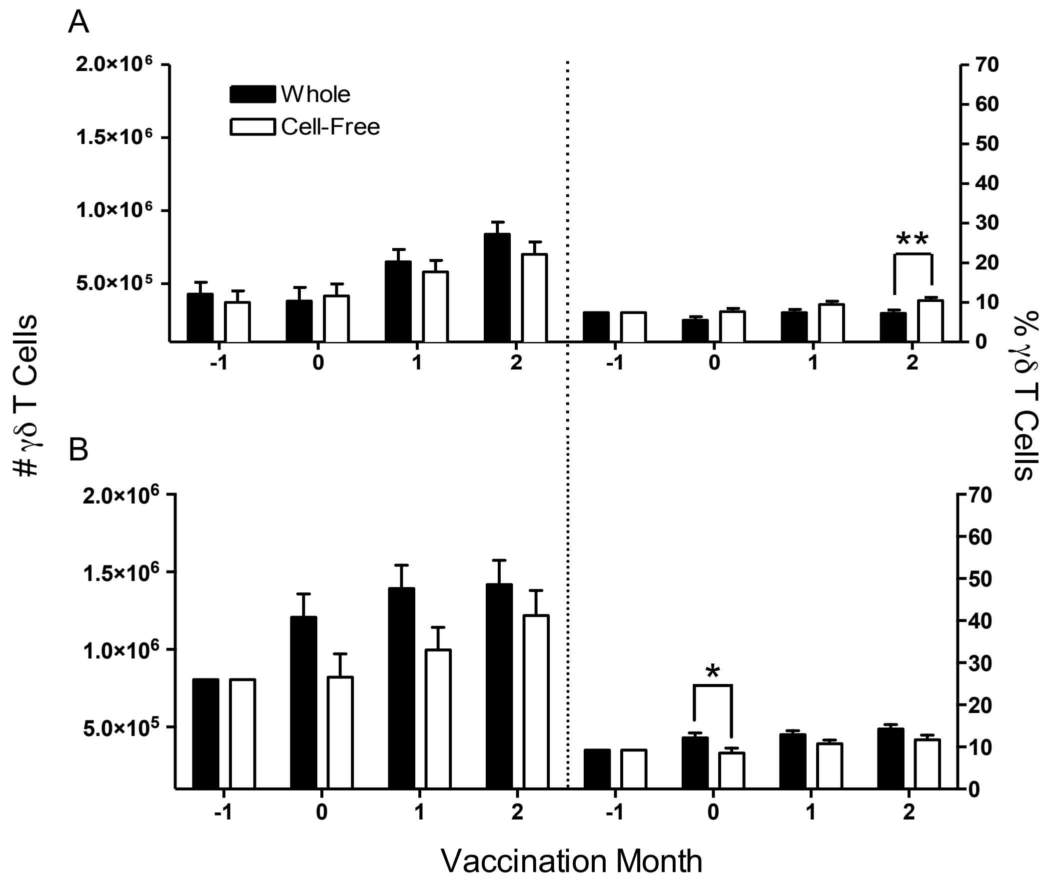

Figure 3.8. γβ T cells in calves in response to vaccination series A and B.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of γβ T cells in response to vaccination series A and B in calves fed whole (vaccination series A: n = 13; vaccination series B: n = 12) or cell-free colostrum (vaccination series A: n = 16; vaccination series B: n = 12). $*P \leq 0.05$ $**P \leq 0.01$

Table 3.3. Relative mRNA expression of interferon-γ, interleukin-2, interleukin-10, tumor growth factor-β in response to vaccination series B.

Cytokine (dCT)	Treatment	>1	0	1	*P*-value
IFNγ	Whole	9.01	9.25 ±0.32	9.08 ±0.35	NS
	Cell-free	9.01	8.96 ±0.33	8.91 ±0.34	
IL-2	Whole	12.29	11.94 ±0.48	12.18 ±0.50	* *P* = 0.048 (month 1)
	Cell-free	12.29	12.35 ±0.48	13.60 ±0.46 *	
TFGβ	Whole	13.91 ±0.42	13.67 ±0.45	14.15 ±0.48	NS
	Cell-free	13.85 ±0.45	13.73 ±0.50	13.98 ±0.49	
IL-10	Whole	6.21	5.70 ±0.39	5.43 ±0.42	NS
	Cell-free	6.21	6.46 ±0.36	5.54 ±0.36	

Chapter 4. Conclusion and Future Research

Morbidity and mortality of dairy calves is a large problem on U.S. dairy operations (USDA-APHIS:VS, 2007). One way to address this problem is by feeding quality maternal colostrum. Many investigations have proven the benefits of colostral antibodies. The focus of this thesis was to characterize the impact of adoptively transferred colostral immune cells on immune status and development in calves. The bovine placenta is 6-layers thick and inhibits transfer of immune components to the fetus (Arthur, 2001). By using the bovine neonate, we were able to characterize cellular responses without maternal-fetal transfer *in utero* affecting the results. A novel finding of this study is that adoptively transferred colostral immune cells play a role in vaccine responses up to nine months post-treatment.

Our research did not definitively prove that colostral immune cells adoptively transfer through enterocytes in the intestine and into circulation. To do this, two methods could be approached (1) cells could be labeled with either a fluorescent dye or by a radioactive tracer. Previous groups have labeled colostral immune cells with a fluorescent dye, fed the animal the labeled cells, and then successfully detected the fluorescent dye in circulation (Schnorr and Pearson, 1984, Jain et al., 1989, Williams, 1993, Liebler-Tenorio et al., 2002). Another way to prove that colostral immune cells reach circulation is (2) to vaccinate the dam during gestation with an antigen to which that dam is naïve to, or has never encountered. The dam's colostrum would be collected and tested to determine if memory cells specific for the vaccinate antigen are present. If the vaccinate antigen stimulates cellular proliferation more from colostral immune cells from vaccinated dams when compared to colostral immune cells from unvaccinated dams, it could be suggested that there are memory cells in colostrum. This colostrum would be fed to the calf and

the same detection methods could be used on blood immune cells to identify cells specific for the vaccinate antigen.

The decreased number and percentage of $CD4^+$ T cells and $CD4^+$ T cell subsets in CFC-fed calves compared to WC-fed calves was either attributed to (1) an increase in adoptively transferred colostral $CD4^+$ T cells detectable in circulation (2) colostral cytokines and growth factors which stimulated proliferation of endogenous $CD4^+$ T cells in the calf or (3) a combination of (1) and (2). To answer this question, soluble molecules, like cytokines and growth factors, need to be analyzed in addition to immune cells. Cytokines and growth factors have been identified in colostrum and can transfer into circulation (Hagiwara et al., 2000, Yamanaka et al., 2003). However, the role of cytokines in neonatal immune development has yet to be elucidated. Therefore, analyzing other colostral components will enhance our understanding of the impact of maternal colostrum on the neonate.

Another concern with our study is that cytokines and antibodies could not be measured for particular time-points. In November of 2011, the -80°C freezer malfunctioned and serum samples from calves at birth to approximately 10 months of age thawed and could not be used for analysis. Analysis had been previously completed for IgA, IgG1, IgG2 and IgM antibody levels for the first month of life. However, cytokine analysis was not completed for either of the sampling time points presented in this study. Analyzing cytokine levels in calves during the first month of life from these samples would have been very beneficial. Cytokines may have played a role in the increase in $CD4^+$ T cells on d 1 of life. Also, analyzing antibody and cytokine levels in animals after vaccinations would have provided information to characterize the impact of adoptively transferred colostral immune cells on immune development.

Initially, we had hoped to analyze MHC-II expression on B cells and monocytes. This would have allowed us to characterize the antigen presentation capacity during the first month of life as well as in response to vaccination. However, the primary MHC-II antibody sent to us from the manufacturer was contaminated with a *Streptococcus* species of bacterium. This was not discovered until months after the initial shipment of the antibody. The contamination may have lowered the binding affinity of the antibody to the MHC-II surface receptor, leaving us with inconsistent results. The MHC-II data is presented in the appendix of this thesis, however this data is not publishable. There is no way of knowing if the MHC-II for a particular sample is accurate.

Secondly, $CD8^+$ T cells could not be analyzed for the two sampling time periods presented here. Our flow cytometry machine is able to distinguish up to 4 fluorescent dyes in one sample. At the beginning of the study, CD4, CD8, CD62L, and CD45RO surface markers were tagged with a primary antibody specific for the particular cell type. These surface markers were then distinguished by four different secondary fluorochromes, FITC, APC, PE-TR and PE, respectively. When I began analyzing samples from this study in fall of 2011, I noticed that the APC fluorochrome was not appropriately differentiating from the other colors. After consultation with Dr. William Davis at Washington State University, I decided that the fluorochrome APC cannot be run on the flow cytometer at the same time as fluorochrome PE-TR. By looking at the emission spectra of these two fluorochromes, one would not expect there to be any problems with overlapping emission wavelengths, which would cause spectral differentiation problems. However, this particular APC fluorochrome used did not differentiate appropriately from the other emission spectra. Therefore, because APC was used as a secondary antibody for $CD8^+$ T cells, this cell type was not used in analysis.

After treatment, calves entered the Virginia Tech dairy herd and followed standard farm protocols. Calves received more than 1 vaccination at the same time. Additionally, administration of vaccinations varied by 1-3 months between animals. There were some animals which were vaccinated at 5 months of age and some which were vaccinated at 9 months of age. This caused variation in the time between vaccinations and when our animals were sampled. Some animals were sampled 1 d after vaccination and some were sampled 3 weeks after vaccination. Lastly, because vaccines were given together, we could not determine if the response induced by vaccination was attributed to one individual vaccine or the combination of all vaccines given.

An important next step for this research is to see where adoptively transferred colostral immune cells migrate after ingestion. Our results and previous literature would suggest that if colostral memory immune cells traffic into circulation, some could reside in lymphoid tissue. By feeding fluorochrome-labeled colostral immune cells to calves and then performing a necropsy on the animal, one could look for labeled cells in lymphoid organs like Peyer's patches, spleen and other peripheral lymph nodes. Another important question to address is if the impact of adoptively transferred maternal colostral cells on vaccination response correlates to future protection. Animals could be challenged with live a bacteria or virus in WC and CFC-fed animals and disease progression could be analyzed. This type of experiment would be beneficial in our understanding of the role of colostral immune cells in memory development.

Our research has provided exciting results and left us with many questions to be answered. The chapters of this thesis are focused on two important time points in relation to neonatal immune status and development; the first month of life and in response to vaccinations. The data generated from this study gives our lab a starting point for future research in understanding the function of colostral immune cells.

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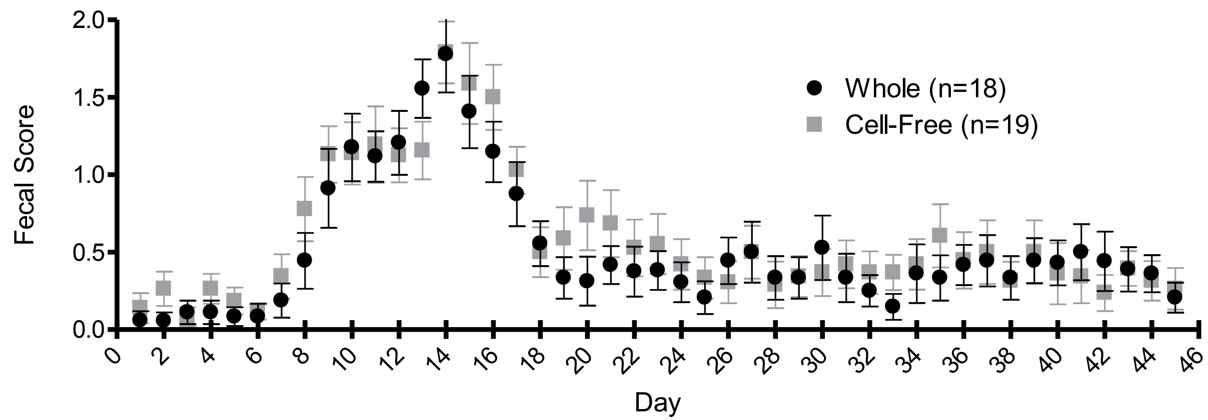
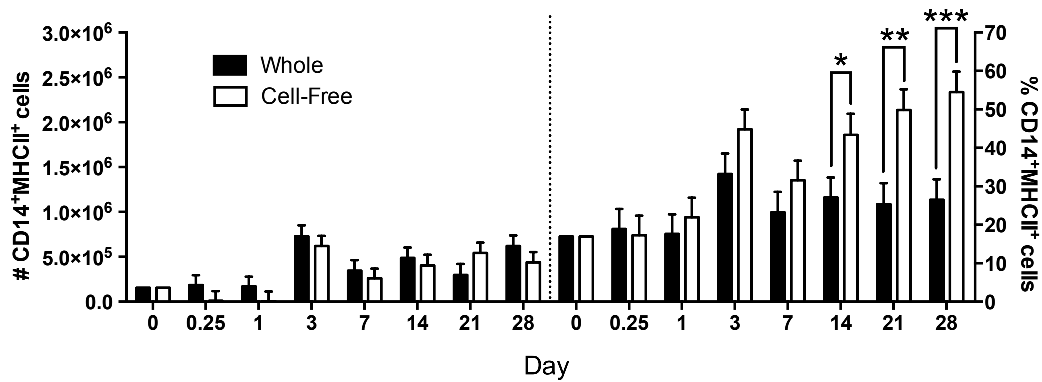
Appendix A: Supporting Data



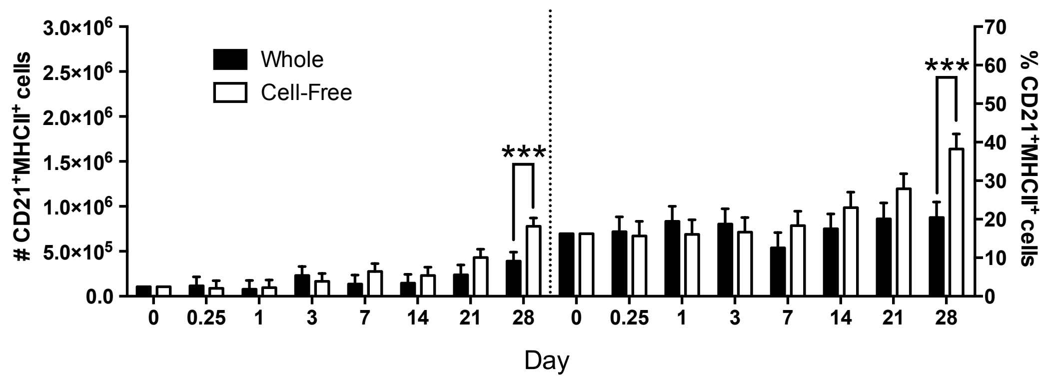
Appendix Figure A.1. Chapter 2: Fecal scores in calves during the first month of life.

Fecal scores from calves fed whole (n = 18) or cell-free (n = 19) colostrum.



Appendix Figure A.2. $CD14^+MHCII^+$ cells in calves during the first month of life.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of $CD14^+MHCII^+$ cells in calves fed whole (n = 14) or cell-free colostrum (n = 17). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. *$P \leq 0.05$ **$P \leq 0.01$ ***$P \leq 0.001$



Appendix Figure A.3. $CD21^+MHCII^+$ cells in calves during the first month of life.

The number (left) per ml of blood and percentage (right) within a mononuclear cell population of $CD21^+MHCII^+$ cells in calves fed whole (n = 14) or cell-free colostrum (n = 17). Data are expressed as LSM ± SEM. Asterisks indicate a difference between treatment groups within a time-point. ***$P \leq 0.001$

Appendix B: Statistical Analysis

SAS Code for immune cells, cytokines and antibodies

```
dm 'clear log'; dm'clear output';
option ls=70;
ods rtf;
ods graphics on;
data CD4; set birthd28cd4;
data p0; set CD4;
if day=0;
covcalc=CD4num;
keep calf covcalc;
proc print data=p0;
proc sort data=CD4; by calf;
proc sort data=p0; by calf;
data CD4; merge CD4 p0; by calf;
proc print data=CD4;
data reduced; set CD4;
if day=0 then delete;
```

This section of the code is for the covariate. If pre-treatment levels were significantly different from each other, a covariate was assigned and included in the model. If pre-treatment levels were not significantly different between WC and CFC treatments, then the covariate was not assigned and was not included in the model statement.

```
proc glimmix data= reduced plots=studentpanel;
class calf treatment day breed temp_code;
model CD4num= treatment covcalc day breed temp_code treatment*day treatment*breed treatment*temp_code / solution ddfm=bw;
random day/ subject=calf(treatment) type=csh residual;
lsmeans treatment;
lsmeans day;
lsmeans breed;
lsmeans temp_code;
lsmeans treatment*day / slicediff=day slice=day adjust=tukey lines;
lsmeans treatment*breed / slicediff=treatment slice=treatment adjust=tukey lines;
lsmeans treatment*temp_code/ slicediff=treatment slice=treatment adjust=tukey lines;
lsmeans treatment / at covcalc=8;
output out=mydiagnostics resid=residual pred=predicted student=student;
data potentialOutliers;set mydiagnostics;
if abs(student) > 2;
proc sort; by student;
proc print data=potentialOutliers;
var calf treatment day breed temp_code CD4num predicted residual student;
run;
ods graphics off;
ods rtf close;
```

SAS Code for Total Respiratory Scores

```
dm 'clear log'; dm'clear output';
option ls=70;
ods rtf;
ods graphics on;
data RespScores; set RespScores;
data p0; set RespScores;
if day=0;
covcalc= RespScores;
keep calf covcalc;
proc print data=p0;
proc sort data= RespScores; by calf;
proc sort data=p0; by calf;
data RespScores; merge RespScores p0; by
calf;
proc print data= RespScores;
data reduced; set RespScores;
if day=0 then delete;
```

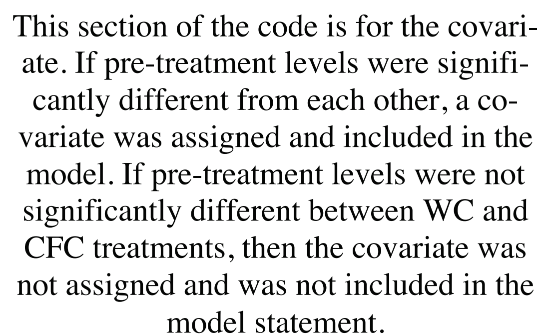
This section of the code is for the covariate. If pre-treatment levels were significantly different from each other, a covariate was assigned and included in the model. If pre-treatment levels were not significantly different between WC and CFC treatments, then the covariate was not assigned and was not included in the model statement.

```
proc glimmix data=RespiratoryScore plots=studentpanel;
class animal treatment day breed temp_code;
model total_score (descending) = treatment|day treatment|breed treatment|temp_code/
dist=binary;
random day / subject=animal(treatment) type=vc residual;

lsmeans treatment / oddsratio ilink cl;
lsmeans day / oddsratio ilink cl;
lsmeans breed / oddsratio ilink cl;
lsmeans temp_code / oddsratio ilink cl;
lsmeans treatment*day / slice=day slicediff=day oddsratio ilink cl;
lsmeans treatment*breed / slice=treatment slicediff=treatment oddsratio ilink cl;
lsmeans treatment*temp_code/ slice= treatment slicediff= treatment oddsratio ilink cl;
run;
ods graphics off;
ods rtf close;
```

SAS Code for Fecal Scores

```
dm 'clear log'; dm'clear output';
option ls=70;
ods rtf;
ods graphics on;
data FecalScores; set FecalScores;
data p0; set FecalScores;
if day=0;
covcalc= FecalScores;
keep calf covcalc;
proc print data=p0;
proc sort data= FecalScores; by calf;
proc sort data=p0; by calf;
data FecalScores; merge FecalScores p0; by
calf;
proc print data= FecalScores;
data reduced; set FecalScores;
if day=0 then delete;
```




```
ods graphics on;
ods rtf on;

proc glimmix data=TotalHealthScores;
   class animal treatment day breed temp_code;
   model total_score=treatment|day treatment|breed treatment|temp_code/dist=mult link=glogit s;
   random day/ subject=animal(treatment) group=total_score;

run;
ods graphics close;
ods rtf close;
```

Appendix Table B.1. Birth to day 28 error structures for immune cell datasets

Cell Type	Number or Percent	Error structure (type)	
		Untransformed	Transformed
Total PBMCs	Number	CS	N/A[1]
$CD4^+$ T cells	Number	CSH	CS
$CD4^+$ T cells	Percent	CSH	CS
$CD4^+CD62L^+CD45RO^-$ T cells	Number	CSH	CS
$CD4^+CD62L^+CD45RO^-$ T cells	Percent	CS	N/A[1]
$CD4^+CD62L^-CD45RO^+$ T cells	Number	CSH	CS
$CD4^+CD62L^-CD45RO^+$ T cells	Percent	CS	N/A[1]
$CD4^+CD62L^+CD45RO^+$ T cells	Number	CSH	CS
$CD4^+CD62L^+CD45RO^+$ T cells	Percent	CSH	N/A[1]
$CD14^+$ cells (monocytes)	Number	SP(POW)	N/A[1]
$CD14^+$ cells (monocytes)	Percent	CSH	N/A
$CD21^+$ cells (B cells)	Number	CSH	N/A[1]
$CD21^+$ cells (B cells)	Percent	CSH	N/A[1]
γδ T cells	Number	CSH	N/A[1]
γδ T cells	Percent	CSH	N/A[1]

[1]Cell type did not have to be transformed, therefore error structure is not applicable

Appendix Table B.2. Vaccination series A error structures for immune cell datasets

Cell Type	Number or Percent	Error structure (type)	
		Untransformed	Transformed
Total PBMCs	Number	Ar(1)	N/A[1]
$CD4^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+$ T cells	Percent	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^-$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^-$ T cells	Percent	Ar(1)	N/A[1]
$CD4^+CD62L^-CD45RO^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^-CD45RO^+$ T cells	Percent	Ar(1)	N/A[1]
$CD4^+CD62L^+CD45RO^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^+$ T cells	Percent	Ar(1)	N/A[1]
$CD14^+$ cells (monocytes)	Number	Ar(1)	N/A[1]
$CD14^+$ cells (monocytes)	Percent	Ar(1)	N/A[1]
$CD21^+$ cells (B cells)	Number	Ar(1)	N/A[1]
$CD21^+$ cells (B cells)	Percent	Ar(1)	N/A[1]
γδ T cells	Number	Arh(1)	N/A[1]
γδ T cells	Percent	Ar(1)	N/A[1]

[1]Cell type did not have to be transformed, therefore error structure is not applicable

Appendix Table B.3. Vaccination series B error structures for immune cell and cytokine datasets

Cell Type/Cytokine	Number or Percent	Error structure (type)	
		Untransformed	Transformed
Mononuclear Cells	Number	Ar(1)	N/A[1]
$CD4^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+$ T cells	Percent	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^-$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^-$ T cells	Percent	Ar(1)	N/A[1]
$CD4^+CD62L^-CD45RO^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^-CD45RO^+$ T cells	Percent	Ar(1)	N/A[1]
$CD4^+CD62L^+CD45RO^+$ T cells	Number	Arh(1)	Ar(1)
$CD4^+CD62L^+CD45RO^+$ T cells	Percent	Ar(1)	N/A[1]
$CD14^+$ cells (monocytes)	Number	Ar(1)	N/A[1]
$CD14^+$ cells (monocytes)	Percent	Ar(1)	N/A[1]
$CD21^+$ cells (B cells)	Number	Ar(1)	N/A[1]
$CD21^+$ cells (B cells)	Percent	Ar(1)	N/A[1]
γδ T cells	Number	Arh(1)	N/A[1]
γδ T cells	Percent	Ar(1)	N/A[1]
IFN-γ	Number	Ar(1)	N/A[1]
IL-2	Number	Ar(1)	N/A[1]
IL-10	Number	Ar(1)	N/A[1]
TGF	Number	Ar(1)	N/A[1]

[1]Cell type did not have to be transformed, therefore error structure is not applicable

Isolation of Mononuclear/Lymphocytes Cells From Whole Blood

Purpose: To isolate mononuclear/lymphocytes cells from whole blood samples

Reagents and Materials:

40 mM EDTA (VWR, Suwanee, GA)
PBS (pH 7.42)
Ficoll-paque (1.077 g/ml; BD Falcon, Franklin Lakes, NJ, USA)
Sterile ddH_2O
2 X MEM (2 X MEM, from 10 X MEM, Sigma M0275) (pH 7.4)
50 ml CellStar conical tubes

Procedure:

Procedure:

1. Collect 50 ml blood using EDTA (10% of 2x citrate) or 10% of 40 mM EDTA.

2. Transfer blood into 50 ml centrifuge tubes.

3. Spin at 2000 rpm for 30 min at 15° C with brake turned off.

4. Remove 5 ml buffy coat layer using 10 ml pipette in the smallest possible volume with the least amount of RBC and transfer into a 50 ml centrifuge tube containing 20 ml PBS. Then layer over 12.5 ml ficoll-paque in a 50 ml centrifuge tube.

    - Pick up 25 ml of cells in 25 ml pipette.
    - Tip tube so that ficoll-paque almost reaches front edge of the tube, and carefully place one drop of cells just in front of the ficoll. Tip the tube so the cells run onto the ficoll, and then slowly add the remaining cells into the tube. As you add more cells, bring the tube back to a near vertical position.

OR for smaller volumes of blood:

- Take off 5 ml of buffy coat and mix with 5 ml PBSE then layer over 5 ml ficoll- paque in a 15 ml centrifuge tube.

NOTE: Do one way or the other, depending on volume of blood.

5. Centrifuge for 30 min at 1300 rpm, 25° C with brake off.

6. Take off mononuclear cell band and add to 20 ml PBS in a 50 ml centrifuge tube with a 10 ml pipette. Try not to pick up much ficoll.

7. Resuspend in PBS to 45 ml, and spin 10 min at 1000 RPM, 15° C.

    a. If RBC are present in pellet: Resuspend cells in 5 ml PBS. Add 10 ml ddH_2O, pipette for 18 sec, then add 10 ml 2x MEM and mix

b. Top off tube with PBS, then spin 10 min at 1000 RPM, 4° C. May repeat if necessary.

8. Resuspend cells in 10 ml PBS, take an aliquot for counting.

9. Centrifuge cells at 10 min at 1000 RPM, 4° C.

Isolation of Mononuclear/Lymphocytes Cells From Colostrum

Purpose: To isolate mononuclear/lymphocytes cells from colostrum

Reagents and Materials:

PBS (pH 7.42)
Sterile ddH_2O
2 X MEM (2 X MEM, from 10 X MEM, Sigma M0275) (pH 7.4)
40 µm Nylon Cell Strainer (BD Falcon, Franklin Lakes, NJ, USA)
Small metal spatula
50 ml CellStar conical tubes

Procedure:

1. Collect 200 µl of colostrum.

2. Transfer colostrum into 4-50 µl conical tubes.

3. Centrifuge colostrum at 1500 g, 40 minutes at 26° C.

4. Remove fat layer with small metal spatula.
    a. Do this by scrapping around edges of tube to loosen fat layer.
    b. Dump supernatant by continuously using scrapper to get fat off the sides without letting any of the fat slide down into cell pellet.

5. Add 5 ml of PBS directly into each of the 4-50 µl conical tubes. Pipet up and down to re-homogenize pellet.

6. Combine 4 of the 50 µl conical tubes into 2-50 µl conical tubes with 10 µl per tube
7. Spin at 1000 RPM, 10 minutes, 4° C.

8. Remove supernatant and add 10 ml of PBS to each pellet. Pipet up and down to reho-mogenize pellet.

9. Combine 2 of the 50 conical tubes into 1-50 µl conical tubes with 20 µl in the tube.
10. Spin at 1000 RPM, 10 minutes, 4° C.

11. Remove supernatant and add 20 ml of PBS to each pellet. Pipet up and down to reho-mogenize pellet.

12. Filter 20 ml of PBS + cells thru a 40 µm Nylon Cell Strainer.
    a. Repeat if colostrum is extra dirty.

13. If RBCs are present in pellet: Resuspend cells in 5 ml PBS.

14. Add 10 ml ddH_2O, pipette for 18 sec, then add 10 ml 2x MEM and mix.

15. Top off tube with PBS, then spin 10 min at 1000 RPM, 4° C. May repeat if necessary.

16. Centrifuge cells at 10 min at 1000 RPM, 4° C.

FACS Staining

Purpose: To identify cell types by FACS (Fluorescence Activated Cell Sorter)

Reagents and Materials:

Primary antibody
Secondary antibody
FACS wash
FACS Wash (PBS + 2% FCS + .05% NaAz)
1% Formaldehyde in PBS
    20 ml PBS
    0.55ml of 36.5% Formaldehyde

Procedure:

1. Place 100 μL of 1×10^6 cells per well in a V bottom 96 well plate
2. Seal with plate sealer.
3. Centrifuge at 1200 rpm, 3 min, 4° C. Flick out liquid.
4. Resuspend in 50 μL of FACS wash (unstained control) or 50 μL of primary antibody.
5. Incubate for 1 hour at 4°C in the dark.
6. Centrifuge. Flick out liquid.
7. Wash 3x with 200 μL of FACS wash.
8. Add secondary antibody in 50 μL.
9. Incubate for 30 minutes at 4° C in the dark.
10. Wash 3x in FACS wash.
11. Resuspend in 1% PBS formaldehyde (100 μl).
12. Incubate for 20 minutes at 25° C in the dark.
13. Wash 2x in FACS wash.
14. Resuspend in 100-200 μl FACS wash.
15. Read on FACS machine.

Isolation of RNA from TRIzol

Purpose: The isolation of RNA from peripheral blood mononuclear cells

Reagents and Materials:

TRIzol (Gibco BRL 5596 VA)
Chloroform (Sigma C2432)
Ethanol
Isopropanol (Sigma I9516)
1.7 ml microcentrifuge tubes (RNASE/DNASE free)

Procedures:

1. Homoegenization

a. After protocol A.1 (Isolation of Mononuclear/Lymphocytes Cells From Whole Blood), 500 μl of cells at 1×10^7 were pelleted by centrifugation at 1,000 RPM, 15° C for 10 minutes.
b. Lyse cells in 1ml TRIzol Reagent (warmed to room temperature) by repetitive pipetting.
c. Transfer the homogenate to a 1.7 ml microcentrifuge tube. Store in -70° C (no longer than 1 month) or go directly to the next step.

2. Phase Separation

A. Incubated the homogenized samples for 5 min. at room temperature to permit the complete dissociation of nucleoprotein complexes.

a. Add 0.2 ml choloform per 1 ml TRIzol Reagent. Cap tube securely. Vortux tube for 30 sec. and incubate at room temperature for 2-3 min.
b. Centrifuge the sample at 7,500 RPM at 4° C for 15 min. Following centrifugation, the mixture separates into a lower red, phenol-choloform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Protein is in the lower phase.

3. RNA Precipitation

a. Transfer the acqueous phase to a fresh microcentrifuge tube.
b. Precipitate the RNA from the aqeous phase by mixing with 500 μl of isopropanol per 1 ml of TRIzol Reagent used in Step 1.
c. Invert tube 3-5 times to mix.
d. Incubate samples at room temperature for 10 min and centrifuge 7,500 RPM at 4° C for 15 min. The RNA precipitate, often invisible before centrifugation, forms a gel-like

pellet on the side and bottom of the tube.

4. RNA Wash

a. Remove the supernatant and discard.
b. Wash the RNA pellet once with 75% EtOH, adding at least 1 ml of 75% EtOH per 1 ml of TRIzol Reagent used in Step 1.
c. Mix the sample by vortexing and centrifuge at 5,000 RPM for 5 min. at 4° C.
d. Repeat EtOH wash by repeating step 4 a-c.
   i. NOTE: The RNA precipitate can be stored in 75% EtOH for 1 week at 4° C, or 1 year at -20° C. After storage, repeat centrifugation to pellet RNA.

5. Redissolving the RNA

a. Remove the supernatant and briefly air-dry the RNA pellet (5-10 min). It is important to not let the RNA pellet dry completely as this will greatly decrease its solubility.

b. Dissolve the RNA in 30 μl nuclease free ddH_2O by heating for 10 min at 60° C and 1150 RPM on thermomixer. Remove 3 μl aliquot for OD reading.

6. Determine the RNA quantity and purity as follows: (For RNA, an optical density of 1 is 40 μg/ml and DNA is 50 μg/ml.

a. Take 2 μl of sample and read on Nanodrop at 260 nm.

b. The concentration of the RNA sample will be:
   i. (OD) (40μg/ml)(dilution factor) = μg/ml, divide by 1000 to get μg/ml

7. Assess the purity by reading the OD at 280 nm and calculate the OD_{260nm}:OD_{280nm}. If the ratio is 2, then the sample is extremely pure; if the ratio is below 1.6, it is likely contaminated with protein.

8. Store RNA sample at -70° C until needed

Making cDNA

Purpose: To make cDNA from single-stranded RNA

Reagents and Materials:

Random Hexamer Primers (Invitrogen, Carlsbad, CA)
20.5%/vol (5x) Buffer (Invitrogen, Carlsbad, CA)
10%/vol (0.1mM) Dithiothreitol (DTT) (Invitrogen, Carlsbad, CA)
5%/vol (10 mM) deoxribonucleotides (DNTPs) (VWR, Suwanee, GA)
2.5%/vol Superscript II (Reverse transcriptase) (Invitrogen, Carlsbad, CA)
DNase/RNase free water (Qiagen; Valencia, CA)
1.7 ml microcentrifuge tubes (RNASE/DNASE free)

Procedure:

1. In 1.7 ml tube mix
   a. 1 µl random hexamer primers (125 ng/µl) + 11 µl RNA (2.2 µg total) for 'normal' samples.
   b. 1 µl random hexamer primers (125 ng/µl) + 28.8 µl RNA (2.2 µg total) for 'low' samples.
   c. 2 µl random hexamer primers (125 ng/µl) + 22 µl RNA (2.2 µg total) for 'FALSE' samples

2. Heat 70°C for 10 min.

3. Make up buffer mix (per sample).

Reagent	'Normal sam-	'Low samples'	'FALSE' samples
5x buffer	4.0 μl	8.76 μl	9.10 μl
0.1 mM DTT	2.0 μl	2.0 μl	4.0 μl
10 mM DNTP	1.0 μl	1.0 μl	2.0 μl
Superscript	0.5 μl	0.5 μl	1.0 μl

4. Place samples on wet ice for 2 min.

5. Centrifuge 4°C for 30 sec.

6. Add buffer mix per sample on wet ice.
   a. 7.5 µl for 'normal' samples
   b. 14 µl for 'low' samples
   c. 21.5 µl for 'FALSE' samples

7. Incubate 25°C for 10 min.

8. Incubate 42°C for 50 min.

9. Incubate 70°C for 15 min.

10. Add RNase/DNase free H_2O per sample to get 10ng/µl cDNA.

11. Store at -20°C.

Reverse Transcriptase Real-Time PCR for mRNA Quantification (qRT-PCR)

Purpose: Quantify gene expression.

Reagents:

Primers
Probes
Taqman ® Universal PCR Master Mix (Applied Biosystems, Foster City, CA)
DNase/RNase free water (Qiagen; Valencia, CA)
1.7 ml microcentrifuge tubes (RNASE/DNASE free)
ABI 7300 Real-Time PCR System

Procedure:

1. For quantitative Real-Time PCR analysis, use TaqMan® Universal PCR Master Mix and the ABI 7300 Real-Time PCR System.

2. Add 300nM of both forward and reverse primers, 50nM of 6-carboxyfluorescein (FAM) probe, 2X TaqMan® Universal PCR Master Mix, and 50ng cDNA per well for a total of reaction volume of 25µl.

3. Run the ABI 7300 Real-Time PCR System using the follow program.

4. Compare cycle threshold (Ct) values of a stable housekeeping gene to Ct values of desired gene by subtracting values (ΔCt).

5. If there is an experimental control, compare the difference in Ct values (ΔCt) to the unstimulated cell ΔCt values (ΔΔCt) by subtracting. The reported value is then calculated for fold induction ($2^{-\Delta\Delta Ct}$).

6. If there is no experimental control, reported value is the ΔCt.