Epigenetic Influences on Bovine T-helper 1 and T-helper 2 Cytokines (Interferon-gamma and Interleukin-4) in High and Low Immune Responders around the Peripartum Period

by

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ABSTRACT

EPIGENETIC INFLUENCES ON BOVINE T-HELPER 1 AND T-HELPER 2 CYTOKINES (INTERFERON-GAMMA AND INTERLEUKIN-4) IN HIGH AND LOW IMMUNE RESPONDERS AROUND THE PERIPARTUM PERIOD

Marlene Ann Paibomesai
Advisor:
University of Guelph, 2017
Professor Bonnie A. Mallard

Livestock epigenetics has applications in the areas of disease biomarkers and drug development, as well as animal breeding. The objectives of this research were: 1- investigate DNA methylation at the type 1 cytokine interferon gamma (IFN-γ) and the type 2 cytokine interleukin 4 (IL-4) promoter regions of CD4+ T-cells isolated from dairy cows, and 2- determine production of type 1 and type 2 cytokines across the peripartum period from antibody (AMIR) or cell mediated immune response (CMIR) biased cows as it relates to DNA methylation of IFN-γ and IL-4. Cows were ranked according to immune response phenotype by High Immune Response® testing, which evaluates AMIR and CMIR with type 1 and type 2 antigens, through which cows were ranked high (H), average (A), and low (L) immune responders. Cows either possessed a bias towards H-AMIR with L-CMIR (n=11) or H-CMIR with L-AMIR (n=10). Peripartum CD4+ T-cells were isolated at -28 days, +4 days and +21 days from calving and stimulated with ConcanavalinA for 24 hours. CD4+ T-cell cytokines were determined for IFN-γ, IL-4, and IL-17A from cultured cells, and DNA samples were taken for assessment of DNA methylation by bisulfite pyrosequencing. Cows that were biased towards an H-CMIR phenotype produced more cytokine (IFN-γ, IL-4, and IL-17A) at +21 days from calving than H-AMIR cows and
generally more cytokine than prepartum CD4+ T-cell samples. There were differences in cytokine methylation at individual CpG sites across the promoter regions for IFN-γ or IL-4, including: IFN-γ CpG+57 from the transcription start site (-6.6%), IFN-γ CpG+72 (-15%) and IL-4 CpG+128 (-9%) between H-CMIR and H-AMIR cows. Individual CpG site differences were evident in CD4+ T-cells sampled pre- (-28 days) and post-partum (+21 days): IFN-γ CpG+57 (-13%), IFN-γ CpG+72 (-17%), IL-4 CpG+128 (-15%), IL-4 CpG+175 (-23%) and IL-4 CpG+193 (-11%). This was associated with elevated cytokine production postpartum. This thesis supports an association of DNA methylation with bovine CD4+ T-cell cytokine production and that immune response phenotype is associated with changes in DNA methylation at IFN-γ and IL-4 promoters.
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Declaration of Work Performed

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I, Marlene Ann Paibomesai, declare that all work in this thesis was performed by me with the exception of laboratory work and analysis of Appendix A and immune phenotyping protocol and estimation of phenotypic parameters for the cows used in Chapter 3, 4, 5, and 6; sample collection and ELISA in Chapter 2.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Average</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AMIR</td>
<td>Antibody-mediated immune response</td>
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<tr>
<td>BACT</td>
<td>Beta Actin</td>
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<tr>
<td>BMC</td>
<td>Blood mononuclear cell</td>
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<tr>
<td>BoLA</td>
<td>Bovine lymphocyte antigen</td>
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<tr>
<td>CDS</td>
<td>Coding sequencing</td>
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<tr>
<td>CL</td>
<td>Corpus luteum</td>
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<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CM</td>
<td>Clinical mastitis</td>
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<tr>
<td>CMIR</td>
<td>Cell-mediated immune response</td>
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<tr>
<td>CNS</td>
<td>Conserved non-coding sequence</td>
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<tr>
<td>ConA</td>
<td>Concanavalin-A</td>
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<tr>
<td>CpG</td>
<td>Cytosine phosphate guanine</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic lymphocyte</td>
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<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
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<tr>
<td>DEX</td>
<td>Dexamethasone</td>
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<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
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<tr>
<td>EBV</td>
<td>Estimated breeding value</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>GC</td>
<td>Glucocorticoid</td>
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<tr>
<td>H</td>
<td>High</td>
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<td>HEWL</td>
<td>Hen Egg White Lysozyme</td>
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<td>HIR</td>
<td>High Immune Response</td>
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<tr>
<td>HS</td>
<td>Hypersensitivity</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Interleukin</td>
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<tr>
<td>IR</td>
<td>Immune response</td>
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<tr>
<td>iTregs</td>
<td>Induced T-regulatory cells</td>
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<td>L</td>
<td>Low</td>
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<tr>
<td>Me5</td>
<td>Cytosine methylation</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain-like receptors</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>P4</td>
<td>Progestrone</td>
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<td>Pathogen associated molecular pattern</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PGF</td>
<td>Prostaglandins</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RLR</td>
<td>RIG-I-Like receptors</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<td>Th</td>
<td>T helper</td>
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<td>TLR</td>
<td>Toll like receptor</td>
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<td>TSS</td>
<td>Transcription start site</td>
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<td>UTR</td>
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INTRODUCTION

Livestock breeding, management and health are entering a new era of study with the discovery of epigenetic mechanisms. Epigenetics has gained much attention over the last decade in the investigation of health and disease, and complex traits (Petronis, 2010; Funston and Summer, 2014; Skinner et al, 2010; Heyn, et al., 2013). In brief, epigenetic mechanisms such as DNA methylation, histone modification and microRNA, influence gene expression without a change in DNA sequence. Acting like a switch, epigenetic modifications have impact on cellular and individual phenotypes by turning on and off genes in various cell types and over time (Ho et al., 2010). Environmental stimuli experienced both in utero and ex utero can influence epigenetic status in an individual with potential transgenerational influences (Heard and Martienssen, 2014; Grossniklaus et al., 2013; Blake and Watson, 2016). Epigenetic studies in dairy cattle have been limited, but have included assessment of milk production (Singh et al., 2010), reproduction ( Su et al. 2013; Doherty et al., 2013 Walker et al., 2013; Saadi et al., 2016), ageing (Green et al., 2015) and health (Doherty et al., 2013; Doherty et al., 2016; Green and Kerr, 2014, Paibomesai et al., 2013). The dynamic field of epigenetics has the potential to better describe complex traits, lead to the discovery of new drugs and treatments, offer new breeding tools, and change current management practices on dairy farms. Therefore, epigenetic studies that focus on health traits and production traits are warranted in dairy cattle.

There are numerous epigenetic mechanisms that work in synergy with one another to influence overall chromatin accessibility; the focus of this thesis is on DNA methylation that may affect bovine CD4+ T-cell cytokine production. DNA methylation mainly occurs on cytosine-phosphate-guanine (CpG) motifs distributed across the genome in promoter regions, within genes, and untranslated regions (Jones, 2012). CpG motifs are found both as individual CpG
motifs and CpG rich regions that are referred to as CpG islands and work to assist in opening and closing chromatin (Jones, 2012). Generally, the presence of DNA methylation causes chromatin compaction leading to gene silencing, while absence of DNA methylation opens the chromatin allowing for access of the DNA binding proteins. DNA methylation has been described in limited detail in cattle and warrants further investigation.

Over the years, the dairy industry in Canada and worldwide has focused its breeding goals on higher milk production. As a result, this has led to a decline in reproductive performance and health status in the modern day Holstein breed (Berry et al., 2011; Koeck et al., 2012a; Inchaissri et al., 2010; Neuenschwander et al., 2012). Immune response (IR) phenotyping offers a tool through which dairy cattle breeders can enhance health traits in a balanced non-disease specific method (Mallard et al., 2015). Cattle can be IR phenotyped using the High Immune Response (HIR™) test (US Patent #7,258,858; Wagter-Lesperance and Mallard, 2007). It has been previously shown that a cow’s ability to mount an immune response is dependent upon their IR phenotypic status as a high (H), average (A) or low (L) immune responders (Wagter et al., 2000; Thompson Crispi et al., 2012a,b; Hine et al., 2011; Heriazon et al., 2013). Cows can be ranked according to their antibody- (AMIR) and cell- (CMIR) mediated immune response to an immune stimulation with type 1 and type 2 antigens. IR phenotypes that may exist include, but not limited to: H-AMIR/ H-CMIR; H-AMIR/L-CMIR; L-AMIR/H-CMIR; L-AMIR/L-CMIR, as well as average immune responder phenotypes (Hernández et al. 2003; Hine et al., 2011; Heriazen et al., 2013). High immune response cows have reportedly less disease occurrences than average or low immune responders (Thompson-Crispi et al., 2013; Wagter et al., 2000). Intrinsic IR imbalances can leave individuals more susceptible to intracellular and extracellular pathogens (Pinedo et al., 2009; Thompson-Crispi et al., 2013).
The peripartum period in dairy cows, which is described as three weeks before to three weeks after calving, represents a high stress and energy demanding period that is often accompanied by an increase in disease occurrences (Koeck et al., 2012a). This period has been observed as a period of immune dysfunction, often involving immune suppressive effects. Cell populations, function and trafficking are all affected throughout the peripartum period in cattle (Mallard et al., 1998; Sordillo et al., 2009; Wathes, et al. 2009; Esposito et al., 2014; LeBlanc, 2012; Burton et al., 2000). Most notable are the influences on both the innate and adaptive immune responses. More specifically, a bias in T-helper (Th) cell responses shift from a Th1 dominated response prepartum to a Th2 dominated response postpartum (Shafer-Weaver et al., 1999; Oliveria et al., 2013). This shift in immune response could be partially responsible for the increase in health incidents throughout the peripartum period, as it may affect the ability of an individual to mount an appropriate immune response to an invading pathogen. Specifically, CD4+ Th cells mediate AMIR and CMIR and influence successful clearance of specific pathogens from the host. Therefore, further investigation is warranted into the influence of Th cells throughout the peripartum period in dairy cattle.

There is limited information on IR mechanisms that are associated with H-AMIR and H-CMIR phenotypes, and specifically around the peripartum period. The overall objectives of this thesis was to determine if there are epigenetic differences between CD4+ Th1 and Th2 cytokines (i.e. interferon-gamma (IFN-γ) and interleukin 4 (IL-4)) promoter regions during peripartum period in immune response biased cattle. The data presented in this thesis will help further clarify and investigate the physiology of immune response changes throughout the peripartum period, an important period in dairy cattle production.
The central hypothesis being tested is that bovine IFN-$\gamma$ and IL-4 cytokine expression is under the control of epigenetic modifications, during the peripartum period. It is predicted that DNA methylation will change in accordance with changes in IFN-$\gamma$ and IL-4 secretion across the peripartum period and that specific DNA methylation pattern changes will be associated with IR phenotype. The rationale for this hypothesis is that DNA methylation has been shown to have influence on gene expression in cells, more specifically in IFN-$\gamma$ and IL-4 genes of Th cells in mice and human (Shih et al., 2014; Youngblood, et al. 2013; Zhu and Paul, 2010; Vahedi, 2013).

Th cells are mediators of AMIR and CMIR and display distinct cytokine secretion that is associated with immune response to specific pathogens, which may be controlled in part by DNA methylation in dairy cattle.

The specific hypotheses are: Hypothesis 1- It is hypothesized that isolated bovine Th cells from H-AMIR and H-CMIR cows will have higher concentrations of IFN-$\gamma$ and reduced IL-4 secretion early postpartum compared to prepartum samples. This is hypothesized to occur in late postpartum as the effects of pregnancy wane and the cow resumes a normal reproductive cycle decreasing the immune suppression experienced through pregnancy and during which immune response bias becomes more prominent. More specifically, Th cells isolated in late postpartum from H-CMIR cows will return to increase IFN-$\gamma$ secretion, while H-AMIR cows will return to IL-4 secretion bias as observed in mid-lactation (Martin et al., 2016).

Hypothesis 2 - It is hypothesized that increased IFN-$\gamma$ and IL-4 secretion will be associated with a loss of DNA methylation at the IFN-$\gamma$ and IL-4 promoter regions in isolated bovine Th cells that are cultured in Th1 and Th2 biased cultures. DNA methylation is hypothesized to increase at IFN-$\gamma$ and IL-4 promoter regions with decreased cytokine secretion by stimulated Th cells in
culture. DNA methylation is hypothesized to decrease at *IFN-γ* and *IL-4* promoter regions with increased *IFN-γ* and *IL-4* secretion.

Hypothesis 3 - It is hypothesized that H-AMIR and H-CMIR cows will display differences in DNA methylation patterns at *IFN-γ* and *IL-4* promoter regions in late postpartum samples, that is associated with differences in secretion of *IFN-γ* and *IL-4*. More specifically, H-CMIR cows will have decreased DNA methylation at *IFN-γ* promoter region that will be associated with increased IFNγ secretion. H-AMIR cows are predicted to have decreased methylation at *IL-4* promoter region that will be associated with IL-4 secretion.

Hypothesis 4 - It is hypothesized that DNA methylation of *IFN-γ* and *IL-4* promoter regions will change throughout the peripartum period in correspondence with cytokine secretion differences from Th cells. More specifically, as the IFN-γ or IL-4 secretion increases there will be a decrease in DNA methylation at the *IFN-γ* and *IL-4* promoter region. These temporal differences in DNA methylation will correspond with differences in cytokine secretion throughout the peripartum period. Additionally, the differences in DNA methylation of *IFN-γ* and *IL-4* promoter region will be dependent on immune response bias outside of the peripartum period of H-AMIR and H-CMIR cows.

The objectives of this thesis were to, 1) assess CD4+ Th cell cytokine production from immune biased cows throughout the peripartum period, 2) determine DNA methylation patterns at *IFN-γ* and *IL-4* promoter regions in controlled *in vitro* settings, 3) assess DNA methylation patterns at *IFN-γ* and *IL-4* promoter regions for cattle classified as H-AMIR and H-CMIR, and 4) determine the temporal differences in *IFN-γ* and *IL-4* DNA methylation patterns throughout the peripartum period for AMIR and CMIR immune response biased cows.
Experimental Design and Objectives

Chapter 2: Assess CD4+ T-cell lineage, mainly Th1 and Th2, and DNA methylation signatures of IFN-γ and IL-4 promoter regions around the peripartum period of non-immune response phenotyped cattle. Additionally, this chapter investigates the DNA methylation signatures of Th cells in response to dexamethasone treatment in cell culture.

- Blood was collected from cows with unknown IR phenotypes (n=5) at 28 days before calving and 4 days after calving. Additionally, blood was drawn from mid-lactation cows (n=3) for assessment of dexamethasone treatment, a known glucocorticoid, on Th cytokine production in cell culture and its influence on DNA methylation.
- Isolate CD4+ T-cells by Magnetic Activated Cell Sorting (MACS) and stimulated with a known T-cell mitogen, Concanavalin A (ConA), for 24 hours.
- Cell culture supernatant was collected for evaluation of IFN-γ and IL-4 cytokine production by Enzyme-linked immunosorbent assay (ELISA).
- DNA was extracted and DNA methylation was determined by direct bisulfite sequencing at the IFN-γ and IL-4 promoter region.

Chapter 3: Assess CD4+ T-cell cytokine production from immune biased cows around the peripartum period.

- Cows (n=128) were previously IR phenotyped by the patented HIR™ test system (US Patent #7,258,858; Wagter-Lesperance and Mallard, 2007) and classified as high, average and low immune responders.
- Cows were selected and enrolled if they possessed an H-AMIR/L-CMIR (H-AMIR; n=10) and H-CMIR/L-AMIR (H-CMIR; n=11) phenotype.
- CD4+ T-cells were isolated at -28 days, +4 days and +21 days from calving from H-AMIR and H-CMIR cows and subsequently stimulated with ConA for 24 hours.
- Cell culture supernatant was collected for assessment of IFN-γ, IL-4, and IL-17A cytokine production by ELISA.

Chapter 4: Determine DNA methylation patterns at bovine IFN-γ and IL-4 promoter regions in controlled in vitro settings.

- CD4+ T-cells were isolated from mid-lactation average IR dairy cows (100 days in milk; n=5) and cultured in Th1 and Th2 biased culture conditions.
- RNA was extracted from Th1 and Th2 cultured CD4+ T-cell for assessment by quantitative polymerase chain reaction (qPCR) to confirm expression of IFN-γ and IL-4.
- DNA was also extracted from Th1 and Th2 cultured cells for assessment of CpG DNA methylation by bisulfite pyrosequencing at IFN-γ and IL-4 promoter regions.

Chapter 5: Assess DNA methylation patterns at IFN-γ and IL-4 promoter regions for cattle classified as H-AMIR and H-CMIR.

- DNA was extracted from CD4+ T-cell collected from H-AMIR (n=10) and H-CMIR (n=11) cows at 21 days after calving.
- DNA methylation of IFN-γ and IL-4 promoter regions were assessed by bisulfite pyrosequencing.

Chapter 6: Assess DNA methylation patterns at IFN-γ and IL-4 promoter regions at different time points around the peripartum period.
DNA was extracted from CD4+ T-cell collected at -28 days before calving and +21 days after calving from H-AMIR (n=10) and H-CMIR (n=11)

DNA methylation of IFN-γ and IL-4 promoter regions were assessed by bisulfite pyrosequencing
CHAPTER 1

Literature Review

1.1 The Canadian Dairy Industry

1.1.1 Overview of the Canadian Dairy Industry

The dairy industry makes up a large part of the Canadian agriculture with approximately 81.7 hectoliters of milk produced in 2015 (Statistics Canada, 2016). There are 11,683 dairy farms representing approximately 1.4 million head of cattle across Canada, with the highest concentration of cattle and farms in Ontario and Quebec as of 2015 (Statistics Canada, 2016). The average herd size in Ontario is 75 dairy cows per farm, with production of 2.6 billion liters of milk equaling $2.07 billion dollars in farm gate sales for the year of 2014/2015 in Ontario (Dairy Farmers of Ontario, 2015). The economic impact of the dairy industry on Ontario agriculture is substantial. Dairy farm size has increased over the last two decades due to economic and societal pressures. New technologies are at the forefront of the modern-day farm and are essential to efficient profitable herds. New technology has the potential to decrease labour needs, offer more flexible work hours, present more consistent feeding or milking, help detect disease earlier, present new breeding strategies, and collect big data for data mining. There are many challenges that face dairy producers in the Canadian dairy system with the top three concerns in production being: reproduction management, lameness control and decreasing disease occurrence (Dairy Herd Improvement, 2016).
1.1.2 Health occurrences in dairy cattle

Upon calving, there are many transitions taking place in a cow’s physiology, management, and environment. There is the transition from being pregnant to calving and from non-lactating to lactating associated with changes in hormones and the metabolic systems of the cow (Goff and Horst, 1997; Esposito et al., 2014; Fair, 2015; LeBlanc, 2010). Changes in feed, management, housing and behaviour can also play a role in increased stress during this critical period around calving (Doepel et al., 2002; Drackely, 1999; Sordillo, et al., 2009; Sepulveda-Varas et al., 2013). For this reason, the primiparous and periparous period in dairy cows represents a time of increased stress and increased disease occurrences both metabolic and pathogenic in origin (Aleri et al., 2016; Dubuc et al., 2010; Goff, 2006). The cost of treatment of clinical disease and the reduced production in the herd can negatively impacts profitability, animal welfare, and longevity of a dairy cow (Esposito et al., 2014; Aleri et al., 2016; Mulligan et al., 2006). For instances, the average cost of clinical mastitis (CM) in the first 30 days of lactation on a dairy farm is estimated to be $444/CM case, that includes both direct and indirect costs, with the highest contributing cost being premature culling (Rollin et al., 2015). Cows that experience a post-parturient health disorder are more likely to be culled in the following lactation than those cows which do not experience a health incident (Heise et al., 2016).

The peripartum period, described as three weeks before to three weeks after calving in dairy cows has been documented in a numerous studies over the years and is characterized as a time of a high energy demand, increased stress, transition and immune dysfunction (Mallard et al., 1998; Sordillo et al., 2009; Wathes et al, 2009; Esposito et al., 2014; LeBlanc, 2012; Burton et al., 2000). Specific changes in physiology and metabolism of the cow during the peripartum period is related to a decrease in maintenance of both the adaptive and innate immune system,
potentially leading to a suboptimal immune response and increasing the risk of disease around this period (Shafer-Weaver and Sordillo 1997; Mallard et al., 1998; LeBlanc, 2010; Koeck et al., 2012a, Esposito et al., 2014). Koeck et al., 2012a, showed that most recorded disease occur within the first month of calving and were a combination of infectious and metabolic diseases.

1.2 Immune system overview

1.2.1 Innate host defense

The immune system is a complicated and interconnected system which roles vary from protection from pathogens to maintaining homeostasis. It is a multifaceted, dynamic system that must possess the ability to respond to external and intrinsic signals quickly and accurately. The role of the immune system is to ensure the maintenance of homeostasis in the body while reducing adverse consequences, recognize pathogens, prevent infection and minimize the impact of disease on the host. A generalized view of the immune system reveals two systems: the adaptive and innate immune system. These two systems are highly integrated and dependent upon one another to mount a successful, regulated response to a pathogen or ‘danger’ signal (Janeway and Medzhitov, 2002; Iwasaki and Medzhitov, 2015).

The innate immune response is the first line of defense against invading pathogens. It is characterized by an early response which has limited diversity and specificity, but is quick to respond to an invading pathogen or danger signal. The innate immune system does not only include early responding cells, but also represent the barriers both physical and physiological that prevent pathogens from accessing the body readily, including, temperature, pH, skin and microflora. It is using pattern recognition receptors (PRR) that the innate immune system can identify invading microbes through pathogen-associated molecular patterns (PAMPs) or danger-
associated molecular patterns (DAMPs). PRR, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain receptors (NODs), RIG-I-Like Receptors (RLRs), C-type Lectin Receptors (CLRs) and Cytosolic DNA Sensors are used for early detection of invading pathogens (Janeway and Medzhitov, 2002). These receptors recognize unique PAMPs or DAMPs, molecules which are shared by families of pathogens, for example double stranded RNA which is carried by flavivirus (Janeway and Medzhitov, 2002). The cells of the innate immune system, such as neutrophils, macrophages, and NK cells can recognize pathogens in a localized area and mount a response. PAMPs are an integral part to recognizing pathogens or danger signals in the body. DAMPs was proposed by Matzinger, is more comprehensive definition, which represents “danger” signals that exist in the body (Gallucci and Matzinger, 2001). A “danger” signal communicates to the immune system that there has been extensive damage to a cell and immune cells mediate apoptosis that helps with the maintenance of homeostasis. DAMPs brought to light that the immune systems function is much broader than protection from pathogens and is also a key system for the maintenance of homeostasis and prevention of cancer. The innate immune system is the first line of defense and communicates with adaptive host immune response when a more specific response is needed (Iwasaki and Medzhitov, 2015).

1.2.2 Adaptive host defense

The adaptive immune is composed of two branches: an antibody mediated immune response (AMIR) and a cell mediated immune response (CMIR). These two branches are both distinct and unique, and interact with one another, through the direct interaction of transcription factors, the closing of open chromatin structures, and the production of cytokine to steer the immune response to either an AMIR or CMIR response (Iwasaki and Medzhitov, 2008). The adaptive
immune response is also known as the late responder taking at least 5-7 days for initiation after recognition of a pathogen or danger signal. It has been characterized with a specific, long lasting memory, and a diverse set of receptors for specific recognition of pathogens or danger signals (Iwasaki and Medzhitov, 2008). AMIR and CMIR are both genetically and epigenetically regulated (Mallard et al., 2015; Kondilis-Mangnum and Wade, 2013; Pacis et al., 2014). AMIR, which is predominantly characterized as a type 2 immune response has typically been responsible for defense against extracellular pathogens, such as bacteria or helminthes. CMIR, which is predominantly a type 1 immune response, has been associated with defense against intracellular pathogens, such as obligated intracellular bacteria (*Mycobacterium* sp.) and viruses (Rowell and Wilson 2009; Kanno et al., 2012; Chang et al., 2014). The adaptive immune response begins with an antigen being taken up by an antigen presenting cell, such as a dendritic cell, B-cell or macrophage. The antigen is then processed for further presentation on major histocompatibility complex (MHC) to T-cells and is presented by either MHC I or MHC II which is a highly polymorphic protein that is controlled by a polygenic region in the genome (Neefjes et al., 2011). MHC I present to cytotoxic T cells (CTL) that possess CD8+ molecules and are responsible for the active killing of cells, while MHC II presents antigen to CD4+ T-cells or Th cells. The strength and avidity of the interaction of the immunological synapse between the MHC molecule and T-cell receptor complex partially determines the fate of the subsequent immune response (Neefjes et al., 2011). The pathway of the immune response is also determined by the co-stimulatory signal (CD28) and the cytokine microenvironment, as determined by not only the antigen presenting cell, but also by other immune cells and epithelial cells (Iwasaki and Medzhitov, 2015). Upon stimulation of the respective cells, several signaling pathways are activated and these signals are what steer and drive the immune response.
1.2.3 CD4+ T-helper cells lineages and differentiation

CD4+ T-helper (Th) cells are the mediators of the immune system, orchestrating AMIR and CMIR to specific pathogens by secretion of cytokine and chemokine (Zhu and Paul, 2010; Geginat et al., 2014). The Th1/Th2 paradigm was first conceptualized by Mosmann and Coffman in 1986 (Reviewed by Mosmann and Coffman, 1989) in mice, where they observed differential expression of cytokines from isolated CD4+ T-cells. Th1 and Th2 CD4+ T-cells are phenotypically characterized by cytokine expression and secretion and are typically inverse to one another (Mosmann et al., 1986). Over the past three decades this paradigm has been used to define many different phenomenon in physiology, most notable its impact on disease susceptibility, role in autoimmune mediated diseases and normal bodily function (Zhu and Paul, 2008; Crawford et al., 2014; Goverman, 2009). The discovery of new Th subtypes, such as Th17 and iTreg cells, and the presence of cells which express both Th1/Th2 cytokine profiles, have challenged this paradigm in recent years. Although not the focus of this study it is important to note that these Th subtypes are not as distinct and exclusive as previously been defined (Becattini et al., 2015; Nakayamada et al., 2012). CD4+ Th cell lineages are determined by the type of pathogen, strengthen of co-stimulatory signal and cytokine microenvironment that a naïve CD4+ T-cell experience when stimulated by an antigen presenting cell. The immune response is a very dynamic and extensive system through which many signaling pathways exist to transmit key signals which activate lineage specific transcription factors. More specifically for the CD4+ T-cell subsets there are master regulators transcription factors which define the downstream response including T-bet (Th1), Gata-3(Th2), FoxP3 (iTregs) and RORγ (Th17) (Nakayamada, 2012).
Th1 cell predominantly produce IFN-\(\gamma\) in response to stimulation. These signals are transmitted through cell surface receptors for cytokine, antigen recognition and co-stimulatory factors. Upon stimulation of the TCR in the presence of IL-12, which can be produced by dendritic cells, activates BAF (BRG1/ Brahma associated factor) that increases the expression of IL-12RB1/2 through chromatin remodeling. High level of expression of IL-12RB1/2 is achieved upon activation of STAT4 through IL-12 receptor which is expressed on cell surface, which solidifies a Th1 lineage commitment (Zhu et al., 2010). It is interesting to note that BAF is also involved in chromatin remodeling of the IFN-\(\gamma\) gene. T-bet plays the role of a master regulatory for Th1 differentiation. T-bet-/- mice lack the ability to produce a type 1 response and the ability to clear a type 1 pathogen. Upon further examination of the T-bet locus, it was observed that 13kb upstream of the transcription start site exists an enhancer region which binds STAT1/4, a transcription factor that is related to IL-12 and IFN-\(\gamma\) receptor stimulation (Aune et al., 2009). It has also been determined that nuclear factor of activated T-cells (NFAT) plays a role in increasing the expression of T-bet (Placek, et al. 2009). Th2 cell development is also determined by three key factors: TCR and MHCII peptide interaction, cytokine microenvironment and co-stimulatory signals. Stimulation of the TCR in the presence of IL-4 initiates the development of a naïve T-cell into Th2 cells. The presence of IL-4 initiates STAT-6 which is important in the transcription of the master regulator GATA-3 which binds to many sites at the Th2 locus (Zhu and Paul, 2010). Transcription of GATA-3 alone is not sufficient for Th2 locus transcription, the presence of STAT5 which is triggered by IL-2 is also necessary for Th2 differentiation (Mowen and Glimcher 2004).
1.3 Pregnancy and Parturition in Dairy Cattle

1.3.1 Pregnancy influence on bovine immune response

Throughout pregnancy there are numerous changes to the maternal immune system to establish maternal tolerance to fetal alloantigens that allow for successful delivery of offspring (Raghupathy, 2001). The cow has an epitheliochorial placental structure which limits the passage of large proteins and cells between the maternal circulatory system and the fetus. The early stages of pregnancy in the cow are defined by the establishment of corpus luteum (CL) and recognition of the conceptus and implantation. After fertilization, the peri-implantation stage, the conceptus stimulates the release of interferon tau (IFN-tau) which suppresses the expression of estrogen and oxytocin receptors in the endometrium (Brazer, 2013). The expression and secretion of IFN-tau, a type I interferon, by trophoblast is associated with fetal tolerance (Thatcher et al., 1995). The absence of IFN-tau results in fetal mortality and termination of pregnancy (Thatcher et al., 1995). The suppressed response of estrogen and oxytocin allow for the maintenance of CL and the suppression of prostaglandin F2 alpha (PGF2) which induces luteolytic phase of estrus. The CL produces high concentrations of progesterone throughout pregnancy, which maintains the pregnancy through to term, 9 months in the cow (Brazer, 2013).

Implantation occurs at day 19 in the cow with the adhesion of the caruncular, intercaruncular and trophectoderm (Fair, 2015). Prostaglandin is produced both by the conceptus and the uterus of a pregnant cow and promotes elongation and growth of the conceptus (Brazer, 2013).

Immune response regulation throughout pregnancy is an important part in the maintenance of pregnancy as shown in women (Oreshkova et al., 2012). Typically, Th2 bias and increase in FoxP3 Treg cells throughout pregnancy ensures a successful pregnancy as characterized in
women (Loewendorf et al., 2014; Xin et al., 2014). In the cow, the interaction of Th1/Th2 with pregnancy has not been well established (Fair et al., 2015). Oliveria and Hansen, 2008, showed that cows at day 33-34 days pregnant had limited changes in immune cell populations (CD8+, CD4+, γδ T cells and CD68+ monocytes) of peripheral blood mononuclear cells (PBMC) samples. Analysis of cytokine expression of CD4+ T-cells showed a preference towards Th2 in endometrial tissue in early pregnancy (Oliveria et al., 2013). Later in pregnancy, day 240 of gestation, PBMC samples showed an increase CD25+CD4+ T-cells or Treg cells and γδ T cells, as well as increased trafficking of CD68+ macrophage to the endometrium (Oliveira et al., 2010; Oliveria et al., 2008). Further characterization of bovine both adaptive and innate immune response to pregnancy in peripheral and localized to the uterus is needed.

1.3.2 Peripartum period influence on the bovine immune response

There are many physiological changes that occur around this period most notable is the interplay between hormones and glucocorticoids. It has been previously characterized that in the few weeks leading up to calving there is an observed decrease in progesterone, a hormone that predominant in pregnancy, and a peak in estrogen and glucocorticoids a few days before calving (Shenavai et al., 2012). Along with these hormonal changes there is the onset of lactation and change in net energy demand of the cow, as they transition from a non-lactational, late pregnancy state to an early lactation, non-pregnant state. The peripartum period is time of stress, with many hormonal, metabolic, and physiological changes occurring (Mallard et al., 1998; Sordillo et al., 2009; Wathes, et al. 2009; Esposito et al., 2014; LeBlanc, 2012; Burton et al., 2000). These changes may cause that suboptimal immune response that has been characterized throughout the peripartum period (Mallard et al., 1998; Aleri et al., 2016). There is evidence that
suggests that specific immune cell populations, trafficking, and function are altered in response to peripartum effects (Sordillo et al., 2009; Shafer-Weaver and Sordillo 1997; Shafer-Weaver et al., 1999; Burton et al., 2000; Karcher et al., 2008; Van Kampen and Mallard, 1997; Kimura et al, 2006). These include changes in cell proportions, such as CD4:CD8 ratio, population of neutrophils and monocytes, as well as alteration in the proliferative, trafficking and activity of these cells (Shafer-Weaver et al, 1999; Van Kampen and Mallard, 1997; Meglia et al., 2005).

Megila et al. (2005) looked at specific populations of lymphocytes during the transitional period in dairy cows. They observed a general decrease in lymphocyte and eosinophil populations just prior to and after calving, returning to prepartum numbers two weeks after calving. Megila et al., (2005), also observed an increase in monocytes prior to calving and an increase in B-cells in circulation after calving. It was also observed that CD8+ T-cells act in an immunosuppressive manner, and had predicted that this may be due to the suppressive response of other cell types such as CD4+ T-cells (Shafer-Weaver et al., 1997). This overall immunosuppression of lymphocyte populations was also observed by other researchers during the peripartum period (Mehrzad and Zhao 2008; Van Kampen and Mallard 1997). Additionally, cows that developed endometritis after calving were shown to have a decrease in peripheral Treg and monocytes (Brodzki, et al., 2014).

Although there has been substantial evidence of overall decrease in several key immune cell populations and decrease in function in both the adaptive and innate response, the direct cause of the immune dysfunction through the peripartum period has not been determined. In cattle, there is a lack of research on specific changes in T-cell lineages (Th1/Th2 or other Th cell types) around calving and how this may influence a cow’s overall health in the peripartum period and beyond (Fair, 2015). However, there is evidence of changes in the bovine immune response in
response to vaccination through the peripartum period at the protein and transcriptional level (Wagter et al., 2000; Weikard et al., 2015). Overall, CD4+ T-cell populations have been shown to decrease during the peripartum period (Kimura, et al., 1999; Brodzki et al. 2014). Shafer-Weaver et al., 1999 did observe a bias towards Th2 post-parturient compared to those in mid-lactation. It has been determined in women and mice that pregnancy represents a Th2 bias or phenomenon (Chaouat et al. 2003; Oreshkova et al., 2012). T-cell lineage biases through the periparturient period and how this relates to cow health has not been determined.

1.4 Genetic regulation of the disease resistance and immune response

1.4.1 Breeding for disease resistance in the dairy industry

Increase milk production and intensive management strategies have decreased reproductive performance, increased disease occurrence and decreased longevity of dairy cows worldwide (Berry et al., 2011; Koeck et al., 2012a; Inchaisri et al., 2010; Neuenchwander, et al., 2012). Through the foundation of previously established quantitative genetics and estimated breeding values, disease resistance can be selected for in a dairy herd. Although disease resistance is a desired trait, it often difficult to select for as it is dependent upon accurate and consistent health records as recorded by producers (Koeck et al., 2012). In addition to this, low disease prevalence (less than 5%), incomplete exposure, and imperfect diagnostics (low sensitivity/specificity) lowers disease resistance heritability (Bishop and Woolliams, 2010). For these reasons, it is challenging to select for a board based disease resistance that accounts for numerous diseases that are pathogenic and metabolic in origin. This can leave an individual susceptible to other types of pathogens or disease if selecting for only one disease resistance trait. When dealing with a disease that has a variety of etiologies, such as mastitis or bovine respiratory disease, it is best
to possess a broad base disease resistance compared to resistance to one specific pathogen (Glass et al., 2012). This broad-based disease resistance had left researchers with the obstacle of determining a system that selects livestock for disease resistance and resilience.

Disease records that were collected by producers in the field were used to determine genetic estimated of heritability to common disease in Canadian Holsteins (Koeck et al., 2012a). Disease frequencies were as follows: mastitis (12.6%), displaced abomasums (3.7%), ketosis (4.5%) retained placenta (4.6%), metritis (10.8%), cystic ovaries (8.2%), and lameness (9.2%) (Koeck et al., 2012a). The corresponding heritability ranged from $h^2=0.02-0.6$ with phenotypic correlations between displaced abomasums (0.27) and ketosis and retained placenta and metritis (0.14) (Koeck et al., 2012a). The heritability ranges are typical of most disease resistance genetic studies with some improvement in heritability with accurate health records (Rupp and Boichard, 2003; Gernand et al., 2012; Heringstad et al., 2005; Koeck et al., 2012a). There have been continued efforts to use genomics to identify key single nucleotide polymorphism (SNP) and genes that infer disease resistance with some success (Kadri et al., 2015; Sahana et al., 2014). Others have suggested that selection through genomic approaches to identify candidate variants which contribute to disease resistance and vaccine response. Most notable, Glass et al., 2012, identified candidate variants (bovine lymphocyte antigen (BoLA) DRB3, TLR4, TLR 8) in response to vaccination to bovine respiratory syncytial virus (BRSV). Interestingly, BoLA DQ variation (Class II genes) has been shown to have a crucial role in T-cell activation and response (Gerner et al. 2009). In addition to these BoLA-DR variations have also been suggested to have influence on clinical mastitis occurrences (Rupp et al., 2007; Sharif et al., 2000). Bovine tuberculosis resistance was investigated using SNP Chip data and found a predicted heritability of 0.23 with a prediction accuracy of 0.33 (Tsairidou et al., 2014). Despite these advances in
disease resistance genetics, there is still considerable challenge in collecting accurate field data for analysis and some issues with interpreting the results (Bishop and Woolliams, 2014).

1.4.2 Immune response phenotype, heritability, and genomics

The innate and adaptive immune responses are an important part in disease resistance and pathogen clearance. There are individual variations in immune response that has been demonstrated through difference in response to vaccination in cattle (Wagter et al., 2000). Initially, Mallard et al. 1989 had shown in miniature pigs that were classified for different MHC genotypes had different AMIR and CMIR (Mallard et al., 1989; Mallard et al., 1992). This set the stage for further investigation into classification of immune response to determine high and low with methods assessing adaptive immune response. Furthermore, pigs that were bred for high and low immune responses were observed to have different responses to infection (Mallard et al., 1992). Those pigs classified as high (H) AMIR and H-CMIR produced an earlier and more robust antibody concentration in serum than those pigs that were low for both adaptive IR traits. It is interesting to note that H-AMIR/H-CMIR pigs also showed severe clinical signs to infection challenge, but achieved less disease score overall compared to low (L) L-AMIR/L-CMIR phenotypes (Magnusson, et al. 1998). There is evidence as well that AMIR and CMIR response in pigs is influenced by the swine lymphocyte antigen genetic variations (Lumsden, et al. 1993).

In dairy cattle, IR phenotypes can be determined by stimulation with type 1 and type 2 antigens through the patented HIR® method (US Patent #7,258,858 Wagter and Mallard 2007). Initially, this testing method was performed on cattle in selected dairy herds in Ontario with ovalbumin (OVA) that was dissolved in the Escherichia coli J5 vaccination. From this test group, cows were classified into three groups; high (H), average (A) and low (L) immune responders, based
on IgG1 and IgG2 specific for OVA and *E. coli* *J*5 around and during the peripartum period (week -8, -3, 0, 3, 6 relative to calving). There was a notable influence of week from calving on the heritability calculated for antibody (IgG1 and IgG2) response to OVA test antigen ($h^2$=0.32 to 0.64) and *E. coli* *J*5 vaccination ($h^2$=0.13 to 0.88) (Wagter et al., 2000). The development of a test that would evaluate CMIR was more complicated as previous testing antigens used to induce a delayed type hypersensitivity response (DTH) (*Bacillus Guerin* (BCG)-induced purified protein derivative) cross reacted with the tuberculin tests that are utilized in cattle (Hernandez et al. 2005). Through the testing of various type 1 test antigens, *Candida albicans* was identified as a suitable antigen for the HIR® testing system and was used to determine CMIR variation and heritability in cattle (Hernandez et al., 2005; Heriazon et al., 2009; DeLapaz, 2008). CMIR heritability in dairy cattle was estimated to be 0.17. Cows that possess a high AMIR and CMIR had relatively less disease occurrences compared to average and low responders for mastitis (2x less), retained placenta (6x less) and ketosis (2x less) (Thompson-Crispi et al., 2012c). It was also observed that cows which possessed H-CMIR phenotype were less seropositive for *Mycobacterium paratuberculosis* (Pinedo et al., 2009). It was demonstrated that AMIR/CMIR traits are phenotypically and genetically inverse to one another and therefore consideration of both AMIR and CMIR traits are important in the selection of a balanced board based disease resistance (Wilkie and Mallard, 1999; Heriazon et al., 2013; Thompson-Crispi et al., 2012a; Mallard et al., 2015).

Following the characterization of AMIR and CMIR traits, Thompson-Crispi et al., 2012a,b, evaluated IR traits, heritability, and association with disease occurrence with adaptive immune response traits as determined by HIR test in dairy cattle (n=445) across Canada with the help of the Canadian Bovine Mastitis Research Network. Briefly, cows IR variation, heritability, and
disease occurrence were evaluated by testing with a type 1 (*C. albicans*) and type 2 (Hen egg white lysozyme (HEWL) antigens in a 21-day protocol. Blood samples were collected at Day 0, Day 14 and Day 21 from the date of injection to determine AMIR by the evaluation of IgG1 and IgG2 and DTH stimulation with *C. albicans* was performed on Day 21 with skin fold thickness taken at 0 and 48 hours from injection. Subsequently, cows were ranked according to the resulting AMIR and CMIR response. There was a notable difference in IR traits (AMIR and CMIR) across Canada, with increased DTH responses and lower day 14 antibody responses in the province of Alberta (Thompson-Crispi and Mallard, 2012b). In this study, CMIR and AMIR heritability and genetic association were calculated and the resulting heritability estimates were 0.19±0.10 (CMIR) and 0.16-0.41±0.09-0.11 (AMIR) with a genetic correlation between the two IR traits ranging from -0.13 to -0.45±0.32-0.46 (Thompson-Crispi et al., 2012a). Overall, cows with high AMIR and CMIR had less disease occurrences than those cows that were classified with an average and low AMIR and CMIR with reduced incidence of mastitis, metritis, displaced abomasums, and retained placenta (Thompson-Crispi et al., 2012b; Thompson-Crispi et al., 2013a). Natural antibody to key hole limpet hemocyanin was also evaluated in these herds with estimated heritability of 0.32 (IgG) and 0.18 (IgM), which is similar to the AMIR (IgG1 and IgG2) traits described above. Cows that were ranked with a high production of natural antibody to key hole limpet hemocyanin had lower rate of clinical mastitis (Thompson-Crispi et al., 2013b). In addition to improved disease resistance high immune response cattle have improved colostrum quality as determined by the presence of heightened IgG concentration and lactoferrin concentrations (Wagter et al., 2000; Fleming et al.; 2016). Overall, the studies confirm and support that selectively breeding for IR traits (AMIR and CMIR) confer disease resistance to a multitude of diseases. In addition, to the phenotypic and genetic association studies of IR traits in
dairy cattle, Thompson-Crispi et al., 2014 conducted a genome-wide association study (GWAS) on animals that possessed high AMIR (n=81) and low AMIR (n=82) phenotype and high CMIR (n=75) and low CMIR (n=65) phenotype with Illumina Bovine SNP50 Bead Chip. In summary, 186 SNPs were associated with AMIR trait and 21 SNPs were associated with the CMIR trait. A majority of the SNPs identified were located on chromosome 23, which contains the BoLA. In addition to candidate SNPs in BoLA complex, there were also significant association with complement and the cytokines IL17A, IL17F, IL17RA, tumor necrosis factor (TNF), galectins 1, 2, and 3, BCL2 and β-defensin (Thompson-Crispi et al., 2014). Further work needs to be completed on verifying SNP differences between high AMIR and high CMIR compared to those cows with a low AMIR and low CMIR and the biological significance of those SNPs.

There remained more questions than answers as to what the biological cause of these diverse IR traits in cattle, thus requiring further assessment of specific immune responses. Overall, there is a clear influence of age and pregnancy status on the adaptive immune response in cattle. There is a clear relationship of IR phenotype with specific immune cell populations in H-AMIR and H-CMIR cows when tested outside the peripartum period as heifers (Hine et al., 2011). In this study, Hine et al., 2012 showed that H-AMIR cows possessed a higher population of B-cells while H-CMIR cows possessed a higher population of γδ T-cells. Further investigation, into specific immune cell reaction to stimuli in vitro and in vivo is required to determine inherent differences between cows that possess high or low immune response traits. Studies focused on those inherent immune differences could unlock the biological significance of IR traits, the development of possible intervention to increase immune response of low and average immune responders, improve vaccine response in cattle, and decrease disease occurrence.
1.5 Epigenetic regulation of immune response

1.5.1 Definition of epigenetics and the epigenome

Conrad Hal Waddington first coined the term epigenetics in the 1960’s to describe changes in gene expression or phenotype that cannot be described by genetic differences between individuals (Handel et al., 2010). Epigenetics, meaning “above genetics” is defined as modifications that are important regulators of gene expression and is a distinguishing factor in cell differentiation and cell lineage commitment (Wilson et al., 2009). Playing a large role in developmental biology, epigenetic modifications have been shown to play a role in gene by environment interactions and may have a crucial role in disease phenotypes (Smith and Meissner, 2014; Funston and Summers, 2016; Petronis, 2010). Epigenetic modifications encompass several different mechanisms and are generally described as any modification to DNA which changes gene expression without changes to the germline sequence (Kim et al., 2009). These modifications can include DNA methylation, histone acetylation, phosphorylation, ubiquitination and methylation, and microRNA (Handel et al., 2010). These modifications are known as the epigenome. The epigenome determines cell phenotype through the influence of gene expression and is heritable from cell to cell, giving these modifications relative stability throughout the lifetime of an animal. Acting like a switch, the epigenome is dynamic in its response to environmental stimuli allowing flexibility and adaptability of a species to change to its surrounding environment (Funston and Summers, 2016).

Epigenetic modifications possess the ability to be reversed in some cases which allows for a certain degree of plasticity in cell lineage and differentiation. These epigenetic modifications at regulatory loci can act in both local and distant proximity to one another and some elements are
capable of working in repressive and activator states (Skora and Spradling, 2010). In general, active transcription is accompanied by histone acetylation and DNA demethylation, producing an open chromatin state, making the DNA more permissive to the binding of transcription factors and transcription regulators. On the other hand, a closed or compacted chromatin structure can generally be defined as an area that contains more DNA methylation and less histone acetylation. These areas are compacted into a heterochromatin structure to which there is limited access to DNA by transcription regulators, as the DNA is wrapped tightly around the histone protein. In early epigenetic studies, researchers determined areas of the chromatin that were accessible using DNase I digestion, the sites identified in this method were termed DNase I hypersensitivity sites. In a DNase I hypersensitivity test, areas of heterochromatin structure that would not be digested and thus not be cleaved were considered to be in a repressive state (Wilson et al., 2009).

Together the opening and closing of the DNA tertiary structures help create a dynamic process through which environmental stimuli can be transmitted to the transcription of RNA and translation of protein. Today, epigenetic methods are focused mainly on identification of epigenetics marks at both a global level and down to a specific site of DNA methylation in the genome.

Currently, it has not conclusively shown that epigenetic marks can be transmitted transgenerationally from parent to offspring (Ho and Burggren, 2010; Heard and Martienssen, 2014; Grossniklaus et al., 2013; Blake and Watson, 2016). Due to the vast impact of epigenetic mechanisms on biological processes in vertebrates, one can appreciate the recent surge in literature on this timely topic. Therefore, this portion of the literature review will focus on a brief overview of DNA methylation mechanisms, epigenetics in the context of immune cell signatures, and finally recent advancements in livestock epigenetics.
1.5.2 DNA methylation

DNA methylation is defined by the presence of a methyl group covalently bound on the fifth carbon of the cytosine nucleotide in the mammalian genome (Jones, 2012). DNA methylation preferentially occurs on CpG motifs in the genome (Kim et al., 2009), but has been discovered on other dinucleotide base pairs (CpT, ApT) to a lesser amount (Kim et al., 2009). The frequency of $\text{Me}^{5}\text{Cytosine}$ in the mammalian genome is estimated to be approximately 1% with methylation occurring on 70-80% of the CpG motifs (Kim et al., 2009). Distribution of CpG motifs in the genome occur relatively evenly throughout the mammalian genome, although to a lesser extent than what was expected (Bestor et al., 2014). CpG islands are portions of DNA where there is a high density of CpG motifs and are typically unmethylated in the adult genome (as reviewed by Bestor et al., 2014). In comparison to other locations in the genome CpG are found a lesser frequency in introns, 3’ untranslated regions, and intragenic sequences (Bestor et al., 2014). Most promoter regions (~75% of genes) possess a high frequency of CpG motifs or CpG islands, which are relatively undermethylated compared to other region in the genome and do not correlate with expression of their respective genes (Edwards et al., 2010). In contrast to this, heterogeneous methylation tends to occur in areas with low to moderate CpG densities and tend to correlate with gene expression (Bestor et al., 2014; Law et al., 2010). Global methylation is well conserved across the genome and does not change relative to the adult cell types; however, there are localized differences in methylation patterns around cell specific genes (Edwards et al, 2010). The cause and effect of the presence of DNA methylation and gene transcription has not been determined, however there is growing evidence that loss of DNA methylation is correlated with the binding of transcription factors and subsequent transcription (Matsuo et al., 1998; Bestor et al., 2014). Interestingly, the presence of aberrant global demethylation causes cell

Typically, DNA methylation is irreversible in the adult genome except in the case of imprinted genes, silencing transposons, and X chromosome inactivation in females (Kim et al., 2009). DNA methylation is maintained by DNA methyl transferases (DNMT), a highly conserved protein in eukaryotes (Arand et al., 2012; Zhong, 2016). Three different DNMT exist in mammalian species including DNMT1, DNMT3a and DNMT3b. DNMT1 is involved in the maintenance of DNA methylation across the genome, with a preference towards newly synthesized DNA. DNMT1 is often associated with other proteins that are involved in condensation of chromatin conformation and the silencing of transcription (Kim et al., 2009; Matske and Mosher, 2014; Zhong 2016; Gamper et al., 2009). In contrast to this, DNMT3 establishes de novo DNA methylation in unmethylated DNA in the genome (Matske and Mosher, 2014; Zhong, 2016). DNA methylation is recognized by proteins that contain methyl-CpG binding domains (MBD), although there is rising evidence that this is not always the case as reviewed by Zhu et al., 2016. There is evidence to suggest that underlying DNA sequence has influence on regions of methylated DNA and is an area that warrants further investigation (Schubeler, 2015; Gibbs et al., 2010). There are many questions that still surround DNA methylation role in activation of gene expression and how this pertains to development, disease and complex traits. This is also complicated by the gaps in the fundamental DNA methylation mechanisms and how this may differ across species. In addition, it has not been demonstrated that DNA methylation can transmitted from generation to generation after an environmental stimulus has ceased (Gertz et al. 2011).
1.5.3 Epigenetics and CD4+ Th cells

The adaptive immune response is a dynamic system that can respond to several different stimuli in a specific manner. Epigenetic mechanisms have been identified in numerous cells that are a part of the immune system. Due to the specific nature of the studies presented in this thesis, we will focus on the current literature on epigenetic modifications association with CD4+ T-cell plasticity in type 1 and type 2 cytokine production in the context of DNA methylation. A further review of epigenetic influences on the transcriptome and the epigenome in T-cells differentiation is presented by Shih et al., 2014. Committed Th1 cells express high concentrations of IFN-γ and limited IL-4 production. In contrast Th2 cells produce high concentrations of IL-4 and little to no IFN-γ (Youngblood, et al. 2013). The IFN-γ and IL-4 loci undergo epigenetic changes in response to stimuli and processes are highly dependent on specific transcription factors for the Th1 and Th2 lineages (Youngblood, et al. 2013; Zhu and Paul, 2010; Vahedi, 2013; Janson et al., 2009). Th cell lineage specification requires the presence of both DNMT1 and DNMT3 to maintain specificity after Th commitment and plasticity in the initial stages of commitment (Makar et al, 2003; Gramper et al., 2009; Youngblood, et al. 2013) There lacks information on how Th are able to maintain lineage specification through to memory cells, what the degrees of plasticity is in memory cells for specific effector function, and what epigenetic marks confer effector function vs. memory Th cell and do these differ based upon the presented stimulus (Komori et al., 2015; Li, 2012).

The promoter region of the IFN-γ gene has been described by numerous studies in correlation to disease states. Briefly, DNase I hypersensitivity (HS) analysis revealed differential DNA accessibility sites between Th1 and Th2 cells. Notably, conserved non-sequencing (CNS) sites had increased DNA accessibility and possessed DNA methylation marks that were associated
with IFN-γ. The CNS of the *IFN-γ* include: CNS-34, CNS-22, CNS-6, CNS+18-20, CNS+29 from the transcription start site (TSS). These CNS DNase I HS are present in Th1 cells and not observed in Th2 or naïve T-cells. CNS-6 which is 6kb upstream of *IFN-γ* TSS has been proposed to play a role in early differentiation of a Th1 cell, as it contains a DNase I HS site that is present in naïve T-cells (Aune et al., 2009). It was determined that a number of transcription factors bind to this location including Th1 specific transcription factors: T-bet, STAT 5, and NFAT-1 (Janson et al., 2009). Further upstream exists CNS-2 which is located 18-20kb upstream of *IFN-γ* locus, this region also contains a DNase I HS site only present in Th1 differentiated cells and is important for enhancer activity of CNS-6 (Aune, et al. 2009; Wilson et al., 2009; Lee, et al., 2006). There is substantial evidence that DNA methylation of key areas in the *IFN-γ* loci are influenced by DNA methylation changes that associate with IFN-γ transcription and secretion (Winders, et al. 2004; Schoenborn et al., 2007; Balasubramani et al., 2010; Dong et al., 2013; Sun et al., 2013; reviewed by Aune, et al. 2013) The *IFN-γ* promoter region has been used in numerous clinical studies to determine DNA methylation status of the *IFN-γ* gene in mice and humans (Deaton, et al., 2014; Brand et al., 2012). To date there has been limited information on specific DNA methylation of Th1 and Th2 cytokine promoter regions in cattle.

The Th2 locus as described above is a large region that is composed of 140kb that contains a number of regions that influence transcription, including enhancer, silencers, and locus control regions (Lee et al., 2006; Ansel, et al., 2006). DNase I hypersensitivity analysis revealed three DNase I HS sites located between *IL-4* and *IL-13* gene, two of which are associated with Th2 polarized cells (Santangelo et al., 2002). Both of these CNS sites were determined to be associated with high level of expression of *IL-4*, *IL-13*, and *IL-5* transcription, but were not necessary for the initiation of transcription (Lee et al., 2006; Wilson et al., 2009). Although these
sites appear to not be important for initiation of transcription, these sites may play a critical role in Th2 commitment as opposed to initial development of these cells (Lee et al., 2006). In addition to these DNase I HS sites, the second intron of the *IL-4* gene in combination with enhancer CNS1 increased the transcriptional activity of the Th2 locus. In contrast to this at the 3’ end of the *IL-4* gene is another DNase I HS site that has been shown to bind HM1, a transcription factor, and acts as a silencer of *IL-4*. This site is unique in that is it present in both naïve and effector Th1 and Th2 cells (Lee et al., 2002). *IL-4* is transcribed in conjunction with *IL-5* and *IL-13* and has a unique control region which is located far upstream to the Th2 locus. In mice and humans, this region is known as RAD50, it contains four DNase I hypersensitivity regions, three of which are only present in Th2 cells (Lee, et al., 2006; Wilson et al., 2009; Williams et al., 2013). These DNase I HS sites have been shown to bind important transcription factors, such as GATA-3 which is the master regulator of the Th2 lineage (Lee et al., 2006). DNA methylation in *IL-4* gene promoter and enhancer sites is associated with gene expression and has been proposed as regulator of cytokine gene expression and is associated with clinical signs in allergy and infection (Deaton et al, 2014; Canani et al., 2015; Kwon et al. 2008; Mi et al., 2008; Guan et al., 2011). There continues to be studies that investigate epigenetic modifications of selected Th1 and Th2 locus regions including *IL-4*, *IL-13*, *IL-5* and *IFN-γ* locus in mice and human in context of disease and complex traits (Russ et al., 2013).

1.6 Livestock Epigenetics

Epigenetic research in domestic livestock species is growing each year with interest of its application in breeding, management, and health of various livestock species: chicken, sheep, and cattle (Goddard et al., 2014; Triantaphyllopoulos et al., 2016; Pitel, 2014). Despite this recent increase in published work in livestock epigenetics, there is still a great need for further
characterization and comparison between difference species. It remains unclear how epigenetics studies will impact livestock breeding practices and management (Feeny et al., 2014; Triantaphyllopoulos et al., 2016; Gonzalez-Recio et al., 2015; Goddard et al., 2014; Ibeagha-Awemu et al., 2015)

In the field of bovine epigenetics, there lies very few published studies, but the number grows each year. Epigenetic studies in dairy cattle have been limited in nature, but include assessment of milk production (Singh et al., 2010, 2012; Gonzalez-Recio et al., 2012), nutrition (Dong et al., 2014; O’Doherty et al, 2014) reproduction (Liu et al., 2013; Grundberg et al., 2013; Walker et al, 2013; Saadi et al., 2016), aging (Green et al., 2015), health and immunity (Doherty et al., 2013; Doherty et al., 2016; Green and Kerr, 2014; Paibomesai et al., 2013). The field with the most completed work is in embryonic development in cloned cattle where aberrant epigenetic marks have been identified and are associated with a successful live birth (Urrego et al., 2014; Smith et al., 2015) For this reason, there is novelty in the research proposed to study epigenetic profiles in the bovine immune response. Epigenetic research is a large area of study; therefore, we will highlight a few studies in bovine epigenetics as it applies to this thesis. There is evidence for temporal differences in DNA methylation in bovine fibroblasts in the context of ageing where samples were compared at 5 months of age and 16 months of age from the same animal (Green et al., 2015). There was a remarkable difference in LPS response of the fibroblast cells in regards to cytokine mRNA transcription, which correlated with an overall hypomethylation of promoter regions across the genome, however DNA methylation did not vary between the ages collected suggesting stability of DNA methylation across the genome (Green et al., 2015). In mastitis research, CpG methylation of CD4 promoter region was 16% more methylated in cows with CM compared to controls, which corresponded to decrease in CD4 expression in PBMC (Wang et al.,
Walker et al., 2013 looked the relationship of global DNA methylation with RNAseq data and showed that 39% of DNA methylation probes mapped to genes with differential transcription with the onset of pregnancy. Most recently, Doherty et al., 2016 observed differential CpG methylation exist between infected and tuberculosis infected animals and that a negative relationship between 5’ UTR methylation of IFN-γ gene and IFN-γ expression of CD4+ T-cells in cattle infected with M. bovis. In cattle, CpG methylation is contained mainly in intragenic regions with substantial representations in promoters, exons, and intragenic regions which agree with other mammalian DNA methylation distribution (Doherty, et al., 2016). The field of bovine epigenetics grows each passing day and the information collected will be critical in understanding the mechanism of epigenetics in cattle and how this might influence future breeding, management, and treatment.
1.7 References


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CHAPTER 2

Effects of Parturition and Dexamethasone on DNA methylation Patterns of IFN-γ and IL-4 promoters in CD4+ T lymphocytes of Holstein Dairy Cows

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CHAPTER 2

Effects of parturition and dexamethasone on DNA methylation patterns of IFN-γ and IL-4 promoters in CD4+ T lymphocytes of Holstein dairy cows

2.1 Abstract

This study investigated epigenetic mechanisms by which DNA methylation affects the function of bovine adaptive immune system cells, particularly during the peripartum period, when shifts in type 1 and type 2 immune response (IR) biases are thought to occur. Stimulation of CD4+ T-lymphocytes isolated from 5 Holstein dairy cows before and after parturition with concanavalin A (ConA) and stimulation of CD4+ T-lymphocytes isolated from 3 Holstein dairy cows in mid lactation with ConA alone or ConA plus dexamethasone (Dex) had significant effects on production of the cytokines interferon gamma (IFN-γ, type 1) and interleukin 4 (IL-4, type 2) that were consistent with DNA methylation profiles of the IFN-γ gene promoter region but not consistent for the IL-4 promoter region. ConA stimulation increased the production of both cytokines before and after parturition. It decreased DNA methylation in the IFN-γ promoter region but increased for IL-4 promoter region. Parturition was associated with an increase in IFN-γ production in ConA-stimulated cells that approached significance. Overall, DNA methylation in both promoter regions increased between the prepartum and postpartum periods, although this did not correlate with secreted cytokine concentrations. Dexamethasone treated cells acted in a manner consistent with the glucocorticoid’s immunosuppressive activity, which mimicked the change at the IFN-γ promoter region observed during parturition. These results support pregnancy as type 2 IR biased, with increases of IFN-γ occurring after parturition and an
increase in IL-4 production before calving. It is likely that these changes may be epigenetically controlled.
2.2 Introduction

The peripartum period, generally defined as three weeks before to three weeks after calving, is a time of stress, transition, high energy demand, and suboptimal immune response (IR) (Sordillo et al., 2009). At this stage dairy cows experience hormonal, management, behavior, and feed changes, as well as the stress of the transition into high-volume lactation (Huzzey et al., 2005; Doepel et al., 2002). Some specific hormonal changes include a decrease in progesterone production and an increase in estrogen and glucocorticoid (GC) production just before calving (Weber et al., 2001; Kimura, et al., 1999). These changes and stressors have marked this period as one of a high incidence of disease, both infectious and metabolic (Drackley, 1999; Kim et al., 2005), which can be costly in terms of disease treatment, decreased milk production, and animal welfare.

Changes in IR during pregnancy are correlated with alterations of leukocyte populations and their function (Shafer-Weaver et al., 1999; Karcher et al., 2008; Kimura, et al., 1999; Ishikawa et al., 2004). A study by Van Kampen et al., 1997, showed that cells of the immune system change before and after calving. Meglia et al., 2005, found large changes in cell populations and function, including decreased lymphocyte and eosinophil counts just before calving, the counts returning to normal by two weeks after calving. Meglia also observed an increase in the monocyte count just before calving and in the B-cell count early in the postpartum period. Others have noted a decline in the proliferation of T lymphocytes during the peripartum period, along with a shift between a type 1 and a type 2 IR (Shafer-Weaver et al., 1999; Kimura et al., 1999). This effect on T-lymphocyte proliferation and T-helper (Th) cell phenotypes can be simulated in vitro in a dose-dependent manner by the synthetic GC dexamethasone (Dex), which tends to promote a type 2 IR (Ramirez et al., 1996; De et al., 2002). As such, Dex treatment of bovine
CD4+ cells in vitro can offer insight into the role of GCs in the differentiation of Th cells and hence the IR during the peripartum period (Preisler et al., 2000).

Epigenetic modifications in the form of histone modifications and DNA methylation are highly involved in the induction, maintenance, and heritability of gene expression and subsequent protein production in various cell types (Karrow et al., 2011). Epigenetic modifications play a role in cell differentiation throughout development and play a crucial role in immune cell phenotypes. In general, DNA methylation typically represses gene expression, whereas DNA demethylation typically increases gene expression by opening the chromatin structure. The extent to which DNA methylation has an effect on gene expression depends not only on the gene of interest but also on which regulatory factors may control subsequent transcription of that gene. For this study, the Th lineage decisions of key interest were those in which DNA methylation appears to have modulating effects on the promoters of the genes for interferon gamma (IFN-γ) and interleukin 4 (IL-4) in humans and several other species, including mice and cattle (Sanderse et al., 2006; Reiner, 2005; Wilson et al., 2009). Expression of these cytokines is critical for the induction and maintenance of Th cell subtypes. Epigenetics is now commonly thought to represent a critical connection between gene expression and the environment contributing to sustained changes in cellular phenotypes that are acquired during development (Petronis, 2010). As such, DNA methylation serves as an important subject for investigation of molecular, cellular, and physiological responses to internally and externally induced biochemical states.

There is still some debate concerning the exact changes that occur in bovine Th-cell populations during the peripartum period, particularly in relation to the shifts in type 1 and type 2 IR before, during, and after calving. For instance, in a study by Shafer-Weaver et al., 1999, mid-gestation was characterized by a type 1 IR bias, whereas cows sampled 3 d after parturition showed a type
2 IR bias. These results were in contrast to others in the literature indicating that pregnancy tends to be dominated by type 2 IRs, as shown in several species, including humans and mice (Shimaoka et al. 2000; Marzi et al. 1996). Additionally, although there are numerous studies that focus on changes in peripartum IR, the causal mechanism of the observed immunodepression, particularly any epigenetic contribution, remains largely unknown.

The objectives of this study, therefore, were: 1) to assess the effects of the T-cell mitogen concanavalin A (ConA) and the synthetic GC Dex on 2 key type 1 and 2 cytokines, IFN-γ and IL-4, respectively, as secreted from bovine CD4+ T cells before and after parturition; and 2) to evaluate any changes in DNA methylation patterns within the IFN-γ and IL-4 promoters of bovine CD4+ T cells before and after ConA stimulation, with and without Dex treatment. Our hypotheses were as follows: 1) Dex treatment of CD4+ T cells in vitro will abrogate any stimulatory effects of ConA and promote DNA methylation, consequently reducing cytokine expression in a manner consistent with a type 2 IR bias; and 2) changes in IFN-γ and IL-4 production by CD4+ T cells during the peripartum period will be associated with changes in DNA methylation of the gene promoters of these cytokines.

2.3 Materials and methods

Animals and blood collection

Holstein dairy cows were housed at the University of Guelph dairy research farm. All animal handling was approved by the Animal Care Committee of the University of Guelph (AUP #04R063). Blood was collected from 5 of the cows 4 wk before calving and 4 d after calving for peripartum analysis. The dates for prepartum collection were chosen according to the predicted calving dates. The Dex-treatment blood samples were collected from 3 cows in mid-lactation (at
~100 d in milk); these cows were different from those used for the peripartum analysis. The blood (80 to 100 mL) was collected by caudal vein venepuncture into 10-mL Vacutainer tubes (BD, Franklin Lakes, New Jersey, USA) containing ethylene diamine tetraacetic acid.

Isolation of blood mononuclear cells (BMCs)

Cells were isolated by carefully overlaying 15 mL of Histopaque 1107 (Sigma, Oakville, Ontario), according to the manufacturer’s instructions, with an equal volume of blood. After centrifugation in a 50-mL conical Falcon tube [400 × g, 30 min, room temperature (RT)], cells were collected at the gradient interface. Phosphate-buffered saline (PBS; 0.01 M, pH 7.4) was added to the cells, for a total volume of 45 mL. After washing by centrifugation (250 × g, 10 min, RT) the remaining erythrocytes were lysed with 2 mL of sterile water. The BMCs were pelleted and washed again with 40 mL of PBS (0.01 M, pH 7.4) and centrifuged (250 × g, 15 min, RT). Viable BMCs were counted by means of a hemocytometer with the use of trypan blue exclusion dye (Sigma).

Selection of CD4+ T lymphocytes

CD4+ T cells were isolated with the MiniMACS system (Miltenyi Biotech, Auburn, California, USA) according to the manufacturer’s instructions. The cells (1 × 10⁷) were incubated with 100 μL of mouse antibody against bovine CD4+ T lymphocytes (ILA-11, VMRD, and diluted 500-fold) for 30 min at 4°C. The cells were washed with PBS (300 × g, 10 min, rt), suspended, and incubated in 80 μL of MiniMACS buffer (PBS, 2 mM EDTA, 0.5% bovine serum albumin) and 20 μL of magnetic microbeads coated with goat against mouse IgG (Miltenyi Biotech) per 1 × 10⁷ cells (15 min, 4°C). Cells were counted as previously described, washed in 40 mL of PBS (250 × g, 10 min, RT), and then suspended in 1 mL of MiniMACS buffer or, if necessary, an increased
volume proportional to $2 \times 10^8$ cells/μL of MiniMACS buffer. The cell suspension (500 μL) was added to the magnet-bound column. The column was washed 3 times with 500 μL of MiniMACS buffer, and the bovine CD4+ cells were eluted in 1 mL of the buffer. The column separation was repeated to improve purity. Purity was confirmed by flow cytometry as greater than 99% (data not shown).

**CD4+ T-cell cultures**

For peripartum analysis — The isolated CD4+ T cells were cultured in 200 μL of RPMI medium with 300 mg/L of glutamine, 10% fetal calf serum (FCS), and a 1/250 dilution of penicillin and streptomycin (Invitrogen Canada, Burlington, Ontario), at a concentration of $2.5 \times 10^6$ cells/mL, in a Costar 96-well round-bottom plate (Sigma, Oakville, ON) at 37°C for 24 h in an atmosphere of 5% CO2. Half of the plated cells were stimulated with ConA (2.5 μg/mL); the other half of the cells served as unstimulated controls.

For Dex analysis — CD4+ T lymphocytes were cultured in phenol-red-free + glutamine RPMI medium and 10% charcoal-stripped FCS (Invitrogen Canada) on a similar plate, at the same concentration, under the same conditions, for 72 h. All the cells were stimulated with ConA (2.5 μg/mL); half were also stimulated with 10 μM of Dex, a dose shown in preliminary experiments to cause the maximum decrease in CD4+ T-cell proliferation in vitro (data not shown). The T-cell medium was made with phenol-red-free RPMI because phenol red can act as a weak estrogenic agonist, which was pertinent for projected downstream applications (Berthois, 1986). For the same reason, the FCS was charcoal-stripped.
Enzyme-linked immunosorbent assay (ELISA)

An aliquot (150 μL) of supernatant was collected from each culture well after the designated incubation period to evaluate production of the cytokines IFN-γ and IL-4 when the cells were unstimulated, stimulated with ConA, or stimulated with ConA and Dex. The aliquots were pooled and then stored at −20°C for ELISA.

The IFN-γ concentration was determined with a bovine IFN-γ ELISA kit (Mabtech, Cincinnati, Ohio, USA) according to the manufacturer’s instructions. For the IL-4 ELISA, Immulon 2HB 96-well flat-bottom plates (Fisher Canada, Nepean, Ontario) were coated with a 1 μg/μL dilution of mouse antibody against bovine IL-4 (AbD Serotec, MorphoSys US, Raleigh, North Carolina, USA) in carbonate–bicarbonate buffer (pH 9.6), 100 μL/well, and incubated for 48 h at 4°C. The coating solution was aspirated and 200 μL of blocking buffer [PBS (pH 7.4) + 3% Tween 20] added to each plate. The plates were incubated for 90 min at RT. Samples and standards were added after removal of the blocking buffer. A recombinant bovine IL-4 (AbD Serotec) was used as the positive control starting with a dilution of 40 000 pg/mL to prepare a working dilution of 2000 pg/mL that was serially diluted from 1/2 to 1/256. Blocking buffer was used as the negative control. All controls and sample dilutions were added to the plates in duplicate and incubated for 150 min at RT on a shaker. The plates were washed 4 times with washing buffer [PBS (pH 7.4) + 0.05% Tween 20], 300 μL/well, in an ELx405 Autoplate Washer (BioTek Instruments, Winooski, Vermont, USA). For antibody detection a 1/8000 dilution of biotinylated mouse monoclonal antibody against bovine IL-4 (AbD Serotec) in washing buffer was added, 100 μL/well, and the plates were incubated for 60 min at RT on a shaker. Next, the plates were washed 4 times as previously described, 100 μL of a 1/10 000 dilution of a conjugate of streptavidin and horseradish peroxidase (Invitrogen Canada) in washing buffer was added to
each well, and the plates were incubated for 45 min at RT on a shaker. After incubation the
plates were washed 5 times as previously described, 100 μL of 3,3',5,5'-tetramethylbenzidine
substrate (IDEXX Laboratories, Westbrook, Maine, USA) was added to each well, and the plates
were incubated in the dark for 45 min on a shaker. After incubation 100 μL of 1 M H2SO4 was
added to each well to stop the reaction. The optical density of the individual wells was obtained
at 450 nm with an EL808 plate reader (BioTek Instruments) and the KCjunior software package
(Bio-Tek Instruments).

Genomic DNA (gDNA) extraction

After collection of the culture supernatant, 200 μL of PBS was added to the CD4+ T cells
remaining in the culture plate. The cell suspension was mixed and washed (300 × g, 5 min, RT)
and either stored at −80°C for future DNA extraction or used immediately for DNA extraction
with the DNeasy Tissue Kit (Qiagen, Mississauga, Ontario) according to the manufacturer’s
instructions.

Bisulfite treatment and DNA amplification

To evaluate DNA methylation, gDNA (500ng) was bisulfite-treated with the EZ DNA
methylation kit (Zymo Research, Orange, California, USA) according to the manufacturer’s
instructions. Specific primers (Table 2.1) for both converted and unconverted promoter regions
of the bovine IFN-γ (Gi:23821137) and IL-4 (GI:555892) genes were designed with the use of
BiSearch Software 24 from the Bos_taurus_UMD_3.1.1 assembly (NCBI). The promoter region
selected for IFN-γ contained 6 CpG sites, and the promoter region selected for IL-4 contained 5
CpG sites. The selected regions were amplified by polymerase chain reaction (PCR) with the use
of Platinum Taq polymerase (Invitrogen Canada) in 20-μL reactions with 2 μL of template
converted or unconverted DNA, 2 μL of 10× PCR buffer, 0.6 μL of 50 mM MgCl2, 0.5 μL of 10 mM deoxynucleotide triphosphates (Invitrogen Canada), and 1 μL of the respective forward and reverse primers at a concentration of 15 mM. For both analyses a touchdown PCR program was used with annealing temperatures going from 60°C to 54°C in the first part of the program after a denaturation step at 95°C for 2 min, 6 cycles of 95°C for 30 s, 60°C for 30 s, and then 72°C for 45 s, 23 cycles of 95°C for 30 s, 54°C for 30 s, and then 72°C for 45 s, and a final extension step of 72°C for 20 min. The PCR products were run on a 2% agarose gel to verify band size. For IFN-γ and IL-4, gel extraction was done with the PureLink Quick Gel Extraction Kit (Invitrogen Canada) on the band corresponding to the IFN-γ or the IL-4 promoter region, whose lengths were 609 and 684 base pairs (bp), respectively.

**Cloning and sequence analysis**

Cloning was conducted with the TOPO TA Cloning Kit (Invitrogen Canada) according to the manufacturer’s instructions. Two Luria–Bertani (LB) plates per sample were prepared at different concentrations (suspensions of 20 and 40 μL of cells) and incubated at 37°C overnight. Ten individual colonies were selected from the 2 plates for each treatment and cultured in 5 mL of LB liquid broth overnight. Plasmids were extracted with the GenElute Plasmid Miniprep Kit (Sigma), and insertion of IFN-γ and IL-4 was verified by PCR and 1.5% agar gel electrophoresis. The PCR conditions were as follows: denaturation at 95°C for 10 min, 34 cycles of 95°C for 45 s, 59°C for 45 s, and then 72°C for 45 s, and extension at 72°C for 20 min. The verified insert-containing plasmid preparations were sequenced at the Robarts Research Institute, London, Ontari with an Applied Biosystems 3730 Analyzer. The sequences were annotated and aligned in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and analyzed with BiQ Analyzer.
software (Bock et al. 2005). Seven clones per condition were collected for the peripartum period and 10 clones per condition for the Dex treatment analysis.

**Statistical analysis**

Statistical differences were reported as significant at $P \leq 0.05$, highly significant at $P \leq 0.01$, and a trend at $P \leq 0.1$. The treatment effect of Dex on the cytokine production of ConA-stimulated CD4+ T cells, as measured by ELISA, was calculated with a 2-tailed, paired t-test with the program R 2.11.1 (http://www.r-project.org). The significance of the ELISA data was determined with analysis of variance between the 4 treatment groups (prepartum unstimulated, prepartum ConA-stimulated, postpartum unstimulated, postpartum ConA-stimulated) with R 2.11.1 for both IFN-γ and IL-4. The significance of differences in DNA methylation between unstimulated and stimulated cells from prepartum and postpartum samples was also calculated with a 2-tailed, paired t-test with R 2.11.1.

The percentage methylation in IFN-γ was analyzed by comparing the 6 CpG sites within the promoter region for each of the treatments. The overall change in methylation from prepartum to postpartum samples was calculated for both unstimulated and stimulated cells. The same procedure was completed for the 5 CpG sites of the IL-4 promoter region. Bioinformatic analysis for DNA element identification and conservation estimates was conducted in MultiTF (Ovcharenko et al. 2005). Subsequent CpG island prediction was done with the use of CpG Island Searcher (Takai and Jones, 2003).

**2.4 Results**

**Effect of parturition on cytokine production**
The isolated bovine CD4+ T-cells showed a significant increase in IFN-γ production upon ConA stimulation in both prepartum and postpartum samples (Figure 2.1a). The comparisons for peripartum effects were determined for ConA-stimulated cells only, as there was no detectable level of IFN-γ in the supernatant of the unstimulated CD4+ T-cells at 24 h. There was an overall increase in the concentration of IFN-γ in the supernatant of the ConA-stimulated CD4+ T cells between the prepartum (mean 961 pg/mL) and postpartum (1498.7 pg/mL) blood samples, which were obtained from the same cows, and this difference approached significance (P = 0.08).

For IL-4 there was also an overall increase in production upon ConA stimulation in both prepartum and postpartum samples (Figure 2.2a). There was a higher concentration of IL-4 in the prepartum stimulated samples (mean 124 pg/mL) than in the postpartum stimulated samples (90.2 pg/mL), but this difference was not significant (P = 0.36).

**Effect of parturition on DNA methylation of cytokine gene promoters**

Upon stimulation with ConA, DNA methylation in the IFN-γ promoter region of isolated CD4+ T cells decreased by 3.0% for prepartum samples and 9.5% for postpartum samples, as determined from the overall difference in the number of DNA-methylated sites divided by the total number of clones for each sample (Figure 2.1b). Overall, there was a 9.0% increase (P = 0.010) in DNA methylation from the prepartum to the postpartum period for stimulated cells and a 15.5% increase for unstimulated cells (P = 0.017). Although the change in methylation was uniform for each promoter and each treatment for many of the CpG sites, 3 of the 6 sites were noteworthy: site 2 (−291 bp from the transcription start site (TSS)), site 3 (−220 bp from the TSS), and site 4 (−85 bp from the TSS) (Figure 2.1c). As opposed to the general decrease in methylation upon stimulation, these sites increased in methylation, site 2 increasing 10.7%
prepartum, site 3 increasing 5.3% prepartum, and site 4 increasing 2.8% postpartum (Figure 2.1c).

DNA methylation in the IL-4 promoter region increased upon ConA stimulation by 15.3% (P = 0.021) for prepartum samples and 12.6% (P = 0.017) for postpartum samples, as averaged for the 5 CpG sites in this location (Figure 2.2b). From the prepartum to the postpartum period there was an increase in methylation for both unstimulated (12.0%; P = 0.022) and stimulated (9.3%; P = 0.003) cells (Figure 2.2c). Unlike IFN-γ, IL-4 did not possess any sites in which methylation changed drastically. In addition, methylation increased more in the IFN-γ promoter region than in the IL-4 promoter region after parturition in both unstimulated and stimulated cells. Methylation differences were more marked in unstimulated cells for both promoters, with 15.5% and 12.0% increases for IFN-γ and IL-4, respectively, compared with 9.0% and 9.3%, respectively, in stimulated cells.

Effect of Dex on cytokine production

The unstimulated cells produced very little IFN-γ or IL-4 according to the results of ELISA of the supernatant from cultured cells. As expected, ConA stimulation caused a significant increase in cytokine production by cultured CD4+ T cells, to mean concentrations of 3351 pg/mL for IFN-γ and 1726 pg/mL for IL-4. Treatment with 10 μM of Dex completely abrogated this effect of ConA, reducing the cytokine concentrations of both IFN-γ (P < 0.001) and IL-4 (P = 0.23) to nearly zero (Figures 2.3a and 2.4a).

Effect of Dex on DNA methylation of cytokine gene promoters

The addition of Dex resulted in a 9.0% increase in IFN-γ promoter methylation and an 18.0% decrease in IL-4 promoter methylation (Figures 2.3b and 2.4b) compared with ConA stimulation.
Methylation patterns within the IFN-γ promoter region were consistent with the treatment effects on IFN-γ production, but this was not true for IL-4. The methylation profiles for IFN-γ and IL-4 were consistently inverse to one another.

2.5 Discussion

Despite substantial implications for dairy production, the peripartum period remains relatively uncharacterized, particularly in terms of endocrine-associated immune regulation. Previously, GCs were shown to have immunosuppressive capabilities, the levels of these major regulatory hormones fluctuating around the time of parturition (Burton et al., 2005; Preisler et al., 2000). Pregnancy is generally referred to as a “type 2 phenomenon,” as it is dominated by a type 2 IR bias through middle to late pregnancy in mice and humans (Aris et al., 2008; Raghupathy et al., 2001; Lim et al., 2000). At parturition there is a shift to a type 1 IR bias, and in dairy cattle there may be a switch back to type 2 dominance for a short period after parturition (Shafer-Weaver et al., 1999).

This was the first study, as far as the authors are aware, to initiate investigation of the possible epigenetic mechanism of DNA methylation on the function of cells of the bovine adaptive immune system, particularly during the peripartum period. IFN-γ and IL-4 production and DNA methylation of the gene promoters of these cytokines were used to investigate changes in the blood CD4+ T-cell populations of Holstein cows before and after parturition. IFN-γ and IL-4 were used as indicators of type 1 and type 2 IRs, respectively (Shafer-Weaver et al., 1999). Additionally, Dex treatment was administered to CD4+ T cells isolated from nonparturient cows to simulate the effects of GC regulatory hormones that can occur at parturition. The production of both IFN-γ and IL-4 increased upon ConA stimulation of CD4+ T cells isolated from PBMCs
in both prepartum and postpartum blood samples. The postpartum increase in IFN-γ production was not consistent with the previous observation in dairy cows of an apparent depression in IFN-γ concentrations of PBMCs and CD4+ T cells isolated from samples taken around parturition (Ishikawa et al., 2004; Shafer-Weaver et al., 1999). However, it was consistent with the previous findings in mice and humans of an overall type 1 bias around the time of parturition (Aris et al., 2008; Lim et al., 2000; Raghupathy, 2001). The IL-4 concentrations in our study were much lower than the IFN-γ concentrations, and no difference in cytokine levels between the prepartum and postpartum periods was observed in the CD4+ T cells. This contradicts the report of Shafer-Weaver et al., 1999 of an increase in IL-4 production on day 3 after calving, as determined by ELISA and reverse-transcription PCR. The discrepancy may be due to a difference in sampling periods: the previous investigators compared cows in mid- to late lactation with cows at day 3 after parturition, whereas the current study sampled cows 4 wk before calving (outside the peripartum period) and the same cows 4 d after parturition.

The results of the current study tend to strengthen the argument that pregnancy is dominated by a type 2 IR bias, with an increase in the production of a major type 1 cytokine (IFN-γ) at parturition and an increase in the production of a Th2 cytokine (IL-4) before calving, as in most mammalian species (Aris et al., 2008; Lim et al., 2000; Raghupathy, 2001).

DNA methylation plays a crucial role in the regulation of cytokine gene expression: changes in DNA methylation patterns can either enable or repress gene expression (Wilson et al., 2009). In this study changes in DNA methylation from the prepartum to the postpartum period were observed for the IFN-γ and IL-4 promoter regions. The regulator regions for bovine cytokine genes, specifically IFN-γ and IL-4, have not been established. ConA stimulation caused a general decrease in IFN-γ promoter methylation (3.0% prepartum, 9.5% postpartum), as was
hypothesized and as was consistent with the increased cytokine production, and an overall increase in IL-4 promoter methylation (15.3% prepartum, 12.6% postpartum), which was not consistent with the increase in cytokine production. Methylation increased in both the IFN-γ and the IL-4 promoter regions from the prepartum to the postpartum period, by 9.0% for ConA-stimulated cells and 15.5% for unstimulated cells for IFN-γ and by 9.3% for stimulated cells and 12% for unstimulated cells for IL-4; the percentages were averaged over all CpGs for the stimulated and unstimulated cells. This finding is consistent with previously reported epigenetic regulation of T-cells by immunoregulatory cytokines, including IFN-γ (Janson et al., 2009).

Interestingly, CpG site 3 (−220 bp from the TSS) and site 4 (−85 bp from the TSS) within the IFN-γ promoter region are conserved putative binding sites for Tbet and CREB, respectively, and may be important for transcriptional regulation relative to the other CpGs analyzed. T-bet is the master regulator for Th1-cell differentiation and is essential for Th1-cell commitment (Wilson et al., 2009). The conserved CREB binding site containing CpG 4 in dairy cows corresponds to the mouse IFN-γ promoter at −53 bp, which has been shown to determine IFN-γ transcriptional activity and the methylation of which inhibits CREB binding (Jones and Chen, 2006). Further investigation into the importance of these specific CpG sites and other enhancer regions to IFN-γ expression is needed for bovine CD4+ T-cells.

The results of this study were not necessarily consistent with production of both IFN-γ and IL-4 as hypothesized. This may be due to overriding ConA treatment effects or epigenetic changes in other DNA regulatory regions for these cytokines. Subsequent bioinformatic analysis of the IL-4 promoter region during the current study suggested that the CpG sites selected for study did not possess any putative transcription-factor-binding motifs known to regulate IL-4. No CpG islands were predicted in the IL-4 region used in this study, which may explain the discordance between
the IL-4 concentration data and the DNA methylation data for the IL-4 promoter. For example, Gata3, the master regulator for IL-4 locus transcription, does not bind specifically to the IL-4 promoter. According to results in other species, it binds to enhancers surrounding the IL-4 locus and is involved in regulating transcription of the IL-4, IL-5, and IL-13 loci (Lee and Rao, 2004; Ansel et al., 2006; Wilson et al., 2009; Zhu et al., 2010). For future studies this region should be explored for DNA methylation patterns of putative transcription-factor-binding sites and their influence on the expression of IL-4, as the promoter of the IL-4 gene does not appear to play a role in direct transcriptional regulation.

The increase in production of IFN-γ and IL-4 caused by ConA stimulation was completely abrogated by treatment with Dex in vitro. This finding was similar to that in studies in vivo of the immunosuppressive effects of Dex on leukocyte function in Holstein cows (Nonnecke et al., 1997; Burton et al., 2005). The expected Th2-promoting effect of Dex was not clearly demonstrated in the current study, as the secretion of IFN-γ was significantly reduced, as would be expected in Th2 response, there was also a reduction in IL-4 secretion, which was not expected in a Th2 promoting environment. In addition, treatment with Dex antagonized the effect of ConA stimulation and, as expected, increased IFN-γ promoter methylation by 9%. IL-4 promoter methylation decreased by 18% upon Dex treatment of ConA-stimulated CD4+ cells, which was inconsistent with the observed decrease in IL-4 concentration in the culture supernatant.

In the current study the IFN-γ and IL-4 methylation patterns were inversely related. An inverse epigenetic relationship between the IFN-γ and IL-4 loci has been reported for other species (Wilson et al., 2009). In general, the lack of correlation between DNA methylation and cytokine levels may be the result of numerous features, such as discordances between transcription of
cytokine mRNA and secreted cytokine levels, the passive and the time dependent nature of DNA demethylation at the cytokine loci in T-cells, and the impact of enhancer and other extragenic regions on transcription, in which the DNA methylation status may be more influential. When comparing real-time PCR with ELISA data, especially for periods longer than 24 h, it is evident with mammalian cytokine loci such as *IL-4* that mRNA levels may decrease rapidly, but secreted cytokine levels decrease much less or even increase, creating a discordance between transcriptional activity and secreted cytokine levels (Mena et al., 2002). Furthermore, demethylation at 5' *IL-4* loci is not required for early *IL-4* transcript production, as shown in other species, meaning that the changes in the *IL-4* promoter region may not be important for transcriptional activity (Lee et al., 2009). DNA demethylation, which is essential for T-cell development (Reiner, 2005; Sanderse et al., 2006; Jones and Chen, 2006; Wilson et al., 2009), is a passive process, relying on the semiconservative nature of DNA replication for the dispersal of hemimethylated DNA in dividing daughter cells (Jones and Chen, 2006; Lee et al., 2002). As such, cell division is important for lineage specificity, and at least 4 cell cycles may be required for CD4+ cell-line specificity in lineage-promoting conditions in vitro (Grogan et al., 2001). Discordances between promoter methylation and cytokine secretion under Dex stimulation may be due to chromosomal activity that precedes DNA methylation. Specifically, GCs inhibit T-lymphocyte differentiation by inhibiting the production of lineage-specific transcription factors, cytokines, and receptors, albeit more potently for Th1 than for Th2 lineages (Liberman, et al., 2007). These considerations may explain not only the individual variation but also the discrepancy between total and site-specific promoter methylation and cytokine concentration in Dex treatment of CD4+ T-cells in vitro. Interestingly, though, *IL-4* promoter methylation decreased in this study, whereas *IFN-γ* promoter methylation increased, a result consistent with a
Th2 bias, as hypothesized. More specifically, Dex treatment mirrored the effect of parturition on the \textit{IFN-\gamma} promoter, with an increase in methylation of 9.0\% in both cases, although this similarity was not observed for \textit{IL-4}.

This study has indicated that ConA and Dex treatment effects, and possibly parturition effects, on bovine CD4+ T cells may be partially controlled through epigenetic modifications, specifically DNA methylation. Further work needs to be done to determine the effects of an individual inherent bias in type 1 or type 2 IR and how this can influence Th-cell populations around the time of parturition. It is also important to establish the exact DNA methylation profiles of key cytokine genes of naive T-cells and of Th1- and Th2- cells of the bovine species through site-specific bisulfite sequencing (Jones and Chen, 2006; Winders et al., 2011) or genome-wide analysis (Laird, 2010) in order to establish reference populations. Additionally, this study has provided evidence, similar to that from other species, that prepartum and postpartum dairy cows show differential cytokine secretion responses consistent with pregnancy having a type 2 IR bias that changes toward type 1 at parturition. Finally, treatment with Dex was able to reproduce the observed immunosuppressive effects on stimulated cells, although further characterization of its epigenetic mechanism is needed. This opens the door for the study of other major regulatory hormones, such as progesterone, oxytocin, and estrogen, and how they may contribute to our growing understanding of genetically and epigenetically regulated peripartum immune responsiveness.
2.6 References


### 2.7 Tables and Figures

**Table 2.1.** Primers for promoters of the bovine genes for interferon gamma (IFN-γ) and interleukin 4 (IL-4) from Bos taurus UMD 3.1.1 assembly

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>5'-3' sequence</th>
<th>Band size (number of base pairs)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ-B</td>
<td>CCACAAAAAGAATGATCAATG</td>
<td>548</td>
<td>55</td>
</tr>
<tr>
<td>IFN-γ-Y, converted</td>
<td>TTTGGATGAGGAGTTAATAT</td>
<td>609</td>
<td>55</td>
</tr>
<tr>
<td>IL-4-B</td>
<td>GAAGCCAAGGTGAAATCTA</td>
<td>698</td>
<td>58</td>
</tr>
<tr>
<td>IL-4-E, converted</td>
<td>GGAAGAAGTTAAGGTGAAAT</td>
<td>684</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 2.1a

![Bar graph showing IFN-γ concentration (pg/mL) during prepartum and postpartum periods. The graph compares unstimulated and stimulated conditions.](image-url)
Figure 2.1b

-334 -291 -220 -85 +1 +57 +72
-3% -9.5% 10.3% 15.5%

Figure 2.1c

-334 -291 -220 -85 +1 +57 +72
-9% 15.5% 9% 14.3%
**Figure 2.1 a-c.** Data for cultured CD4+ T lymphocytes from enzyme-linked immunosorbent assay (ELISA) of the cytokine interferon gamma (IFN-γ) and from polymerase chain reaction and gel electrophoresis to determine DNA methylation of the cytokine’s gene promoter region. Data for cultured CD4+ T lymphocytes from enzyme-linked immunosorbent assay (ELISA) of the cytokine interferon gamma (IFN-γ) and from polymerase chain reaction and gel electrophoresis to determine DNA methylation of the cytokine’s gene promoter region before and after stimulation with the T-cell mitogen concanavalin A (ConA). Blood samples were collected from 5 dairy cows 4 wk before parturition (Prepartum) and 4 d after parturition (Postpartum). a) Concentration of IFN-γ in culture supernatant; significant differences between unstimulated and stimulated cells (P < 0.05) are indicated by different letters above the bars, and significant differences between the prepartum and postpartum periods (P < 0.001) are indicated by different numbers above the bars. b) Percentage change in methylation between the prepartum and postpartum periods. c) Percentage change in methylation after stimulation with ConA. For panels b and c, positive values represent a gain in methylation, whereas negative values represent a loss of methylation.
Figure 2.2a

![Bar chart showing IL-4 Concentration (pg/mL) for Prepartum (-4 wks) and Postpartum (+4 days) comparing unstimulated and stimulated conditions. Bars with different subscripts indicate statistically significant differences.](image-url)
Figure 2.2a-c Corresponding data for the cytokine interleukin 4 (IL-4) and DNA Methylation at IL-4 gene promoter region.
Figure 2.3a

![Graph showing IFN-γ concentration in ConA and ConA+DEX](image)

Figure 2.3b

![Bar graph showing % change of CpG methylation](image)
Figure 2.3 a,b. Corresponding data for IFN-γ and its gene promoter region in CD4+ T lymphocytes isolated from blood collected from 3 dairy cows in mid-lactation (~100 d in milk). a) Concentration of IFN-γ in culture supernatant after stimulation with ConA alone or ConA plus the synthetic glucocorticoid dexamethasone (Dex); significant differences between the 2 treatments ($P < 0.05$) are indicated by different letters above the bars. b) Percentage change in methylation when Dex stimulation was added to ConA stimulation.
Figure 2.4a

Figure 2.4b
Figure 2.4a,b. Corresponding data for IL-4 cytokine concentration and DNA CpG Methylation IL-4 gene promoter region.
CHAPTER 3

Type I and Type II Cytokine Production of CD4+ T-Cells in Immune Response Biased Dairy Cattle around Calving

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CHAPTER 3

Type I and Type II Cytokine Production of CD4+ T-Cells in Immune Response Biased Dairy Cattle around Calving

3.1 Abstract

The peripartum period is a period of high stress, transition and management changes for dairy cows. It is associated with higher incidence of both metabolic and pathogenic disease. Both antibody- (AMIR) and cell- (CMIR) mediated immune responses play a key role in the maintenance of health in mammals protecting against extracellular and intracellular pathogens, respectively. Generally, interferon gamma (IFN-γ) has been associated with CMIR, whereas interleukin 4 (IL-4) has been associated with AMIR bias. It has been previously demonstrated that cows can be classified as high (H), average (A), and low (L) immune responders based upon their AMIR and CMIR to test antigens, and that this classification is associated with disease occurrence throughout lactation. The mechanisms behind these differences in phenotype and the effects of the peripartum period have not been fully investigated. The aim of this study was to determine the effects of the peripartum period on cytokine production of CD4+ T-cells or T helper (Th) cells, key mediators of the adaptive immune response. Immune response phenotyped cows were selected based on H-AMIR/L-CMIR (H-AMIR; n=10) and H-CMIR/L-AMIR (H-CMIR; n=11) response to test antigens. Isolated CD4+ T-cells collected at 28 days prepartum, 4 days postpartum, and 21 days postpartum from these groups were stimulated with Concanavalin-A (ConA). Subsequently, interleukin-4 (IL-4), interferon-gamma (IFN-γ), and interleukin-17A (IL-17A) concentrations were quantified. Other cytokines that were investigated included IL-6, IL-13, and IL-10 were not significantly different from unstimulated controls and therefore were not considered further. Overall, there was no obvious decline in IL-4, IFN-γ or IL-17A close to
calving observed from CD4+ T-cells from each of these phenotypically distinct groups of cows. However, CD4+ T-cells isolated from H-CMIR secreted more IL-4, IL-17A, and IFN-γ than H-AMIR, and these differences were significant at various time points, particularly on day 21 post-calving. This study indicates a genetic predisposition based on immune response phenotype of cytokine production from CD4+ T-cells around calving.
3.2 Introduction

The peripartum period is a sensitive period for dairy cows, a majority of health incidents occur during this period which affects production and longevity of a herd. Dairy cows undergo a period of immunological dysfunction during the peripartum period, three weeks prior to and after calving (Mallard et al., 1998). The peripartum period is accompanied by an increase in metabolic and infectious diseases (Koeck et al., 2012), which can affect the profitability of cows during their lactation and subsequent lactations (McLaren et al., 2006).

Overall hormonal, physiological, and management changes influence immune system cell populations and function leading to an overall altered responsiveness to immune stimuli through this period (Mallard et al., 1997; Detilleux et al., 1995). This altered immune response throughout pregnancy has also been observed in other mammalian species (Krishnan et al., 1996, Denney et al., 2011). The immunological dysfunction experienced by a cow during the peripartum period include decreased cell-mediated immune response (CMIR), associated with decreased CD8+ cytotoxic cell numbers and cytotoxic function (Shafer-Weaver and Sordillo 1997), as well as neutrophil dysfunction (Contreras and Sordillo, 2011; Detilleux et al., 1995; Gilbert et al., 1994). During the peripartum period cows experience altered antibody production throughout pregnancy and into early lactation with increased antibody production in the weeks leading up to calving, predominantly IgG1 (Detilleux et al., 1995; Guidry et al., 1980; Mallard et al., 1994; Wagter et al., 1999). This effect is relatively short-lived after calving with increased IgG, IgA, and IgM declining soon after parturition (Guidry et al., 1980). Decreased CD4+ Th cell numbers (VanKampen and Mallard, 1997; Ohtsuka, et al., 2004), proliferation (Mehzad and Zhao, 2008), trafficking (Kehri et al., 1999) and altered cytokine production (Shafer-Weaver et al, 1999; Ohtsuka et al., 2006) occur in the weeks prior to and after calving due to systemic
changes in CD4+ lineages resulting from the alterations in physiological state (Ohtsuka et al., 2006; Shafer-Weaver et al., 1999; Harp et al., 2004). More specifically, Shafer-Weaver et al., 1999, observed that type I and type II cytokine production by CD4+ T-cells around this period for cows is biased more towards an anti-inflammatory or type II response with increased expression of IL-4 in isolated CD4+ T cells.

Type I and type II immune responses play a crucial role in controlling invading intracellular and extracellular pathogens and are key players in disease resistance. Balance between these two branches of the adaptive immune response is necessary to produce a broad-based disease resistance. Type I immune responses are typically associated with CMIR and are characterized by an inflammatory response typically mounted to intracellular pathogens, which are often predominated by the production of IFN-γ (Spellberg and Edwards, 2001). CMIR responses have been associated with the increased resistance to intracellular infections (Almeriá et al., 2009; Coussens, 2004). Conversely, type II immune responses are mainly associated with particular isotypes of antibody that can be anti-inflammatory in nature and typically mounted to extracellular pathogens. Type II responses are predominated by the production IL-4, IL-13, IL-5, and IL-10 cytokines in cattle (Spellberg and Edwards, 2001). Th17 CD4+ T-cell have been identified in cattle and are protective for infection with the protozon Neospora caninum (Peckham et al., 2014). CD4+ Th cells are important mediators of AMIR and CMIR responses, by the production of lineage specific cytokine profiles, which are both genetically and epigenetically regulated as described in dairy cattle (Paibomesai et al., 2013). The negative correlation between these two branches has been well characterized with CD4+ T-cells lineages representing a significant factor in cell differentiation and subsequent response.
Immune response phenotypes of cows are ranked as high (H), average (A) or low (L) following exposure to a type I and type II like antigen, as developed and patented by Mallard and Wagter, 2007 at the University of Guelph. For the purpose of this study, biased individuals defined as those cows that possess either a strong AMIR or strong CMIR response and weak CMIR or AMIR immune response that were examined are referred to as H-AMIR and H-CMIR. Some of the mechanisms responsible for differences in immune response phenotype in cattle have been investigated previously. It has been previously shown that H-CMIR cows have significantly more $\delta\gamma$ T-cells while the H-AMIR cows have a higher percentage of circulating B-cells (Hine et al., 2011). The influence of different physiological states, such as calving, on particular T-cell subsets has not been previously investigated in cows of these distinct IR phenotypes. Therefore, the purpose of this study was to investigate cytokine production of CD4+ T-cells during the peripartum period of cattle with known immune response phenotypes. Cows better suited to return to immune competency after calving are expected to have less incidences of postpartum disease, improved production and improved reproductive success.

3.3 Material and Methods

Animals

Holstein dairy cows used for this study were housed the University of Guelph Elora Dairy Research Center. Blood was taken from 21 cows ($n=21$) 28 days before calving (prepartum), 4 days after calving (early postpartum), and 21 days after calving (late postpartum). All samples were taken from the same cows for each time point for that analysis of group effect on peripartum period cytokine production. The dates for prepartum blood samples were based upon the predicted calving date. Cows were either first or second parity, between 2-4 years of age, and
previously identified as high immune responders, as described by Hine, et al. (2011). This study included H-AMIR/L-CMIR cows (n=10) which refers to individuals that possess the highest statistical residual values for antibody production and a low or average delayed type hypersensitivity (DTH) residual to a type I and type II antigens as previously described (Hine et al., 2011, Martin et al., 2015, Thompson-Crispi et al., 2012).

Also included were H-CMIR/L-AMIR cows (n=11). In this study, the H-CMIR/L-AMIR were individuals that possessed the highest residual values for DTH response and a low or average antibody response. The use of animals for this study was approved by the University of Guelph Animal Care Committee (AUP#04R063).

Blood Collection and Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood was collected from the caudal vein in 10 mL EDTA vacutainer tubes (BD, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque 1107 (Sigma, Oakville, ON) as per the manufacturer’s instructions. Whole blood (15 mL) was overlaid on 15 ml of Histopaque 1107 (1:1 ratio) in 50 mL Falcon conical tube and centrifuged (400 g, 30 minutes, room temperature (rt)). PBMC layer was aspirated and PBS was added to a total volume of 45 mL and centrifuged (250 g, 12 min, rt). Remaining red blood cells were lysed with 1-2 mL of sterile distilled water for 45 seconds. The PBMC pellet was washed twice more with 45 mL of phosphate-buffered saline PBS through centrifugation (250 g, 12 min, rt). Viable PBMC were counted with trypan blue exclusion dye (Sigma, Oakville, ON) and a hemocytometer.
**CD4+ T-cell Selection and Isolation**

Mini-magnetic activated cell sorting (MACS) system (Miltenyi Biotech, Auburn, CA) was used to isolate a CD4+ cell enriched population from the viable PBMC population. PBMC (1x10^7) were incubated with 100 µL of mouse IgG1 anti-bovine CD4+ antibody (ILA-11, VMRD) incubated for 30 min at 4°C and then washed with PBS (300 xg, 10 min, rt). The resulting cell pellet was then incubated with 20 µL goat anti-mouse IgG1 microbeads and 80 µL MiniMACS buffer (PBS, 2 mM EDTA, 0.5% BSA; degassed) per 1x10^7 cells for 15 min at 4°C. Cells were washed with 45ml of PBS and suspended in 1mL of MiniMACS buffer to a total maximum concentration of 2x10^8 cells/mL. The cell suspension was added to the MiniMACS magnet bound column and was washed three times with 500 µL of MiniMACS buffer. The MiniMACS column was removed from the magnet and 1 mL MiniMACS buffer was used to elute bound cells. This was process was repeated and selected samples were confirmed by flow cytometry (data not shown).

**CD4+ T-Cell Culture Conditions**

Isolated cells were cultured for 24 hours in 96-well round bottomed plates at 2.5x10^6 cells/mL in 200 µL of RPMI media (RPMI, 30 mg/L glutamine, 10% fetal calf serum, and penicillin/streptomycin). Half of the cells were plated with no mitogen stimulation (unstimulated cells) and the other half of the cells were plated with 2.5 µg/mL of Concanavalin-A (ConA), a known T-cell mitogen. At 24 hours, cell culture supernatant was collected, pooled and stored at -20°C for future ELISA analysis. Cells were collected and stored at -80°C for future DNA methylation analysis.
Cytokine ELISA

Cytokine concentrations were determined by ELISA for IL-4, IFN-γ, and IL17A for all samples. A bovine IFN-γ ELISA kit (MabTech, Cinnicanti, OH, USA) was used to determine IFN-γ from collected cell culture according to the manufacturer’s instructions. Bovine IL-4 ELISA kits (Thermo-Fischer Scientific, Kanata, Ontario) were used to determine IL-4 concentrations according to the manufacturer’s instructions. Lastly, the bovine IL17A kits (Kingfisher Scientific, USA) were used to determine IL-17A concentrations according to the manufacturer’s instructions.

Data Analysis

Data were analyzed independently using a general linear model that included repeated measures using PROC MIXED (SAS Version 9.2; SAS Institute, Cary, North Carolina, USA). The structure of the variance-covariance matrix of repeated measures was chosen based on the lowest Akaike information criterion (AICC). Normality was tested using the Shapiro-Wilks test statistic. Residuals were plotted against explanatory variables to determine outliers in the data and the need for data transformation. Data that were not normally distributed were log transformed. Least squares means (LSMeans) were estimated and back transformed to be reported as cytokine concentration. The statistical model used was to determine difference between time points of sampling and immune response groups (H-AMIR and H-CMIR):

\[ y_{ijkl} = \mu + r_i + t_j + c_k + p_l + r_i \times t_j + \varepsilon_{ijkl} \]

where: \( y \)=response vector of the observation; \( \mu \)= overall mean; \( r_i \)= immune response group (H-AMIR or H-CMIR); \( t_j \)= time from calving (-28 days, +4 days, or +21 days); \( c_k \)= type of cytokine concentration.
(IFN-γ, IL-4 or IL-17A); \( p \) = parity (0, 1 or 2); \( \varepsilon \) = residual error. For each measure of immune response, non-significant effects and interactions \( (P > 0.2) \) were removed from the model.

3.4 Results

Changes in CD4+ T-cells cytokine secretion observed around calving

Isolated CD4+ T-cells from cows during the peripartum period, collected at 4 days after calving had no obvious decrease in cytokine production compared to samples collected at 28 days before calving and 21 days after calving. IFN-γ significantly increased from prepartum samples to postpartum samples collected 3 weeks after calving only in cows classified as H-CMIR (Figure 3.2). IL-4 concentrations significantly decreased from prepartum samples to the late postpartum samples for H-AMIR cows, whereas IL-4 significantly increased from CD4+ T-cells that were isolated from H-CMIR cows (Figure 3.3). IL-17A did not show any change between prepartum, early postpartum and late postpartum samples for either CD4+ T-cells isolated from H-AMIR and H-CMIR cows (Figure 3.4).

Parturition effects differ between H-CMIR and H-AMIR CD4+ T-cells

Cows that are classified as H-CMIR and H-AMIR displayed differing cytokine production profiles depending on cytokine of interest and time point relative to calving, with the most notable differences in the late postpartum period (Figure 3.2-3.4). In particular, CD4+ T-cells isolated from H-CMIR tended to produce more IFN-\( \gamma \) \( (p=0.059) \) than cells isolated from H-AMIR cows at three weeks after calving. IL-4 concentrations were also greater in H-CMIR cows \( (p<0.05) \) at 21 days after calving. This trend of CD4+ T-cells isolated from H-CMIR producing more cytokine than their H-AMIR counterparts was also observed for IL-17A at both 28 days before \( (p<0.05) \) and 21 days after \( (p<0.05) \) calving.
3.5 Discussion

The identification of cattle that are inherently superior to withstand pathogen challenge within the peripartum period would benefit the dairy industry through a decrease in disease occurrences through this sensitive period and ensure a timely return to estrus. It has been previously shown that cattle identified as having a H-AMIR and H-CMIR phenotype have decreased disease occurrence on farm with limited effect on production (Thompson-Crispi et al., 2013; Thompson-Crispi et al. 2012). These cattle have the benefit of longevity within the herd, the production of high quality colostrum and decreased antibiotic use with a significant reduction in incident of mastitis (Thompson-Crispi et al., 2013). The specific immune mechanisms behind these IR phenotypes and how different physiological states, such as calving, affect cattle have not been fully investigated. Therefore, the purpose of this study was to investigate the effects of IR phenotype on CD4+ immune response during the peripartum period, specifically for those animals that possess either a H-AMIR/L-CMIR or a H-CMIR/L-AMIR phenotype. In this study, CD4+ T-cell functionality was determined by cytokine production when stimulated with general stimuli, ConA a T-cell mitogen, that was not used to determine immune response phenotype of these cows in vivo. It was expected that animals with CMIR or AMIR biased phenotypes would present different cytokine profiles after CD4+ T-cells stimulation. Previous results had shown that both IFN-γ and IL-4 were expressed and produced in response to ConA by isolated CD4+ T-cells at 24 hours after stimulation (Paibomesai et al., 2013). For the purpose of this study, cell culture supernatant was harvested at 24 hours to reduce the potential influence of culturing time on epigenetic marks contained within the CD4+ T-cell subset. Overall, CD4+ T-cells from H-CMIR cows produced more cytokine than H-AMIR cows at the 24 hr time point for all cytokines.
investigated (IFN-γ, IL17A, and IL-4). H-CMIR cows produced greater IL17A at 28 days before calving compared to H-AMIR cows CD4+ T-cells.

CD4+ cells have been coined as mediators of the adaptive immune response, through the production of different cytokine profiles which help direct adaptive immune response to specific pathogens. T-cells are one of the key cell types that translate and transmit pathogen specific recognition patterns, which elicit a strong AMIR or CMIR response and regulate the immune response (Bannerman, 2010; Gunther, et al., 2011). This study gives evidence of a systemic change in CD4+ T-cell immune responsiveness based on IR phenotype throughout the peripartum period. Genetic predisposition could leave certain cows susceptible to infection and extend days to conception in the next lactation. It has been previously shown that T-cells isolated from cows outside of peripartum period have different cytokine profiles than T-cells isolated four days after parturition (Shafer-Weaver et al.,1999). T-cells isolated from the IR biased cattle and stimulated with ConA showed different cytokine profiles based on the time from calving. Differences between H-AMIR and H-CMIR cattle cytokine profiles were observed only at 21 days after the calving. Previously, it has been shown that IR phenotype influences cytokine production of isolated PBMC in mid-lactation, outside of the peripartum period (Martin, et al., 2015). Martin et al., 2015 showed that H-AMIR cows produced significantly more IL-4 and H-CMIR cows produced significantly IFN-γ at mid-lactation. However, peripartum period effects influence cytokine production of H-AMIR and H-CMIR cows as IFN-γ, IL-4, and IL-7A are diminished in prepartum and early postpartum samples. These differences in cytokine production between H-AMIR and H-CMIR cows are most apparent during this period as the influence of the peripartum period fades and influence of IR phenotype becomes more apparent. Cows that were genetically predisposed toward H-CMIR returned to prepartum period cytokine production.
before H-AMIR cows. Therefore, a selection towards the H-CMIR trait could favour a decrease in immune suppression through this period and decrease peripartum period disease. However, environmental, metabolic, and physiological changes throughout the peripartum period may also influence the ability of the immune system to respond. Hormonally, progesterone decreases at the time of parturition and remains low until the return of estrus with follicles appearing as early as seven days after parturition (Mateus et al., 2002). This difference in time of follicular development could explain some of the variation observed between H-AMIR and H-CMIR CD4+ T-cell cytokine production in the late postpartum period.

Immune system cell populations, migration and functions vary throughout gestation. Stress and sex hormones influence bovine CD4+ T-cell cytokine profiles skewing the population towards Th2 lineage throughout pregnancy (Paibomesai et al., 2013; Burton et al., 1993; Shaver-Weaver, et al, 1999). Stress hormones, such as cortisol, are highly expressed at the time of parturition in most mammals and have immunomodulatory effects on both innate and adaptive immune cells (Dhabhar, 2014; Esposito et al., 2014). Prolonged exposure to cortisol induces a Th-2 dominance in CD4+ T-cells (Ramirez et al., 1996). In the cow, cortisol spikes at calving, but quickly resolves within a few days of parturition, influence on immune system cell functionality and populations may be inducing a more local change in immune response in the uterus close to parturition opposed to a systemic change. Glucocorticoids stimulate lactation and causes immune cells to traffic to the mammary gland. The results from this study show no preference in CD4+ T-cells towards either Th1 or Th2 lineage early after calving for either H-CMIR or H-AMIR cows. Cows of the H-CMIR secreted more IL-17A at two of the three time points measured. It is expected that these cattle would be more superior in responding and controlling intracellular pathogen and protozoan infection (Peckham et al., 2014). In fact, there is an indication that cows
with the H-CMIR phenotype are more resistant to intracellular pathogens (Pinedo et al., 2009). The highly expressed IL-17A in H-CMIR compared to H-AMIR cows supports this theory. Conversely, cows with the H-AMIR phenotype are more resistant to extracellular pathogens (Thompson-Crispi et al., 2013). It should be noted however, that in the current study only examined CD4+ T-cells were examined and thus does represent other immune cell types.

All cows experience bacterial contamination of the reproductive tract after parturition, which requires proper clearance before normal reproductive performance continues (LeBlanc, 2012). Uterine involution completes at different times in cows after calving from a few weeks to almost two months and influences the return to estrus as prostaglandin F2α remains high (Lindell et al., 1982). The ability to clear this bacterial infection is directly linked to the reproductive success of that cow in that lactation affecting both days to first service and conception rate by delaying uterine involution (Foldi et al., 2006; Mateus et al., 2006). LeBlanc et al., (2012) hypothesizes that inability for a cow to return to a normal immune state 3-4 weeks after parturition may be the cause of infertility in dairy cattle. In the current study, three weeks post calving H-AMIR and H-CMIR cows had different cytokine production after ConA stimulation of isolated CD4+ Th cells. The variation of cytokine patterns between these distinct genetic immune response phenotypes represents a genetic bias for the return of immune competency after calving and could explain the predisposition of cattle for fertility issues. Thompson et al., 2013 showed that cattle with enhanced immune response were less likely to develop reproductive disorders such as retained placenta and metritis. The current study gives evidence for other measures including cytokine production to determine immune competency throughout the peripartum period. Due to the limited population assessed in this study, no reproductive performance measures were assessed in these cattle. Future studies, should include an assessment of bacterial infection of the
reproductive tract after calving of H-AMIR and H-CMIR cattle and evaluation of reproduction performance in association with peripheral T-cell cytokine concentrations as a measure of immune competency in peripartum cattle.

3.6 Conclusion

In conclusion, cytokine profiles of CD4+ T-cells vary between cows having either H-AMIR versus H-CMIR phenotype with the greatest differences in IFN-γ, IL-4 and IL-17A at 21 days post-calving. Cows of the H-CMIR phenotype tended to produce higher concentrations of IFN-γ, IL-4, and IL17A cytokines at this time point compared to H-AMIR cows sampled in this population. H-CMIR cows returned to prepartum cytokine concentrations at 21 days after calving compared to H-AMIR cows, suggesting that peripartum period effects waned sooner in H-CMIR cows. Overall, cattle that were predisposed towards H-CMIR produced higher concentrations of IL-4, IFN-γ and IL-17A at 21 days after calving, which could enhance the cow’s ability to clear reproductive tract infection and potentially return to estrus sooner. This could affect overall reproductive success and productivity in the subsequent lactation, by delaying the return to estrous and increasing days open. Selecting for H-CMIR phenotype could increase the cow’s immune response ability to return to optimal function after calving. Evaluating cytokine differences in CD4+ T-cells from biased immune response groups gives further understanding into the mechanism that dictate these unique phenotypes.
3.7 References


3.8 Tables and Figures

Figure 3.1

![Graph showing AMIR and CMIR residuals for H-AMIR and H-CMIR cows. The x-axis represents CMIR residuals, and the y-axis represents AMIR residuals. The graph includes data points for both H-AMIR and H-CMIR cows, with different markers for each group.]
Figure 3.1. Phenotypic AMIR and CMIR residuals based on antibody and cell mediated immune response for cows classified as H-CMIR (n=11) and H-AMIR (n=12). Phenotypic residuals were used to determine H-AMIR (■) and H-CMIR (●) groups. Briefly, cows were ranked based upon the antibody responses to a type 2 antigen, while cell mediated responses were determined by delayed type hypersensitivity (DTH) to a type 1 antigen. Cows were selected based upon possession of the highest residual for antibody production and a low or average DTH residual representing H-AMIR cows. H-CMIR cows were selected based on the possession of the highest residual for DTH response and a low or average antibody response.
Figure 3.2

![Bar chart showing IFN-γ concentration (pg/mL) over days from calving for H-AMIR and H-CMIR cows.](chart)

- **Days from Calving**: -28 Days, +4 Days, +21 Days
- **IFN-γ Concentration (pg/mL)**: Y-axis ranges from 0 to 20,000
- **H-AMIR Cows** indicated by white bars
- **H-CMIR Cows** indicated by dark bars

Legend:
- □ H-AMIR Cows
- ■ H-CMIR Cows

Annotations:
- A for H-AMIR
- 1 for H-CMIR
- # for H-CMIR

Statistical significance indicated by lowercase letters and numbers.
Figure 3.2. IFN-γ secretion from isolated CD4+ T-cells from immune response biased cows around parturition. IFN-γ secretion from isolated CD4+ T-cells from immune response biased H-CMIR and H-AMIR cows during the peripartum period at -28 days before calving, 4 days after calving, and 21 days after calving. Isolated T-cells were stimulated with ConA (2.5 μg/mL) for 24 hours; cell culture supernatant was collected and IFN-γ was determined by ELISA. Bars represent Least Squares Means with confidence intervals. Different letters indicate significant differences between sample points within the H-AMIR phenotype. Different numbers represent differences between sample point within the H-CMIR phenotype. Symbols represent difference between H-CMIR and H-AMIR groups. # p<0.10.
Figure 3.3

IL-4 Concentration (pg/mL) vs Days from Calving

-28 Days  + 4 Days  + 21 Days

H-AMIR Cows  H-CMIR Cows

* Indicates significant difference.
**Figure 3.3.** IL-4 secretion from isolated CD4+ T-cells from immune response biased cows around parturition. IL-4 secretion from isolated CD4+ T-cells from immune response biased H-CMIR and H-AMIR cows during the peripartum period at -28 days before calving, 4 days after calving, and 21 days after calving. Isolated T-cells were stimulated with ConA (2.5 μg/mL) for 24 hours; cell culture supernatant was collected and IL-4 was determined by ELISA. Bars represent Least Squares Means with confidence intervals. Different letters indicate significant differences between sample points within the H-AMIR phenotype. Different numbers represent differences between sample point within the H-CMIR phenotype. Symbols represent difference between H-CMIR and H-AMIR groups. * p<0.05.
Figure 3.4

IL17A Concentration (ng/mL) vs Days from Calving for H-AMIR and H-CMIR Cows.

-28 Days:
- H-AMIR Cows: **a**
- H-CMIR Cows: 1

+ 4 Days:
- H-AMIR Cows: 1
- H-CMIR Cows: **a**

+ 21 Days:
- H-AMIR Cows: **a**
- H-CMIR Cows: 1

Legend:
- □ H-AMIR Cows
- ■ H-CMIR Cows
**Figure 3.4.** IL-17A secretion from isolated CD4+ T-cells from immune response biased cows around parturition. IL-17A secretion from isolated CD4+ T-cells from immune response biased H-CMIR and H-AMIR cows during the peripartum period at -28 days before calving, 4 days after calving, and 21 days after calving. Isolated T-cells were stimulated with ConA (2.5 μg/mL) for 24 hours; cell culture supernatant was collected and IL-17A was determined by ELISA. Bars represent Least Squares Means with confidence intervals. Different letters indicate significant differences between sample points within the H-AMIR phenotype. Different numbers represent differences between sample point within the H-CMIR phenotype. Symbols represent difference between H-CMIR and H-AMIR groups. * p<0.05.
CHAPTER 4

Characterization of DNA Methylation Profiles of Bovine IFN-γ and IL-4 Cytokine Genes

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CHAPTER 4

Characterization of DNA Methylation Profiles of Bovine \( IFN-\gamma \) and \( IL-4 \) Promoter Regions

4.1 Abstract

Epigenetic modifications play a pivotal role in gene expression in the mammalian genome and help define cellular phenotype. DNA methylation, for example has been reported to regulate immune cell differentiation (Deaton et al., 2014). The current study focused on DNA methylation and its role in T-helper 1 (Th1) and T-helper 2 (Th2) differentiation in \( Bos Taurus \), where limited literature exists. The objective of the current study was to define DNA methylation at the promoter regions of \( interferon \gamma \) (\( IFN-\gamma \)) and \( interleukin-4 \) (\( IL-4 \)) cytokine genes which are respectively expressed by Th1 and Th2 cells in cattle. CD4+ T-cell were cultured in Th1 and Th2 biased cell culture conditions and mitogen activated prior to DNA methylation assessment. The Th1 and Th2 cells displayed differential expression of \( IFN-\gamma \) and \( IL-4 \) genes after stimulation with ConA. Th1 predominantly expressed \( IFN-\gamma \), while Th2 predominantly expressed \( IL-4 \).

Bisulfite pyrosequencing was used to determine DNA methylation of \( IFN-\gamma \) and \( IL-4 \) promoter regions. The average DNA methylation across the promoter regions for \( IFN-\gamma \) and \( IL-4 \) was 43% and 51% respectively. Th1 clones had less DNA methylation across the \( IFN-\gamma \) promoter region than Th2 cells. Conversely, Th2 cells had less DNA methylation across \( IL-4 \) promoter than Th1 culture CD4+ T-cells. Specific CpG sites in both \( IFN-\gamma \) (-334 and -220 base pair from transcription start site) and \( IL-4 \) (-321 and +175 base pair from transcription start site) showed differential methylation between Th1 and Th2 cultured cells. The current study provides a characterization of DNA methylation of specific CpG sites in \( IFN-\gamma \) and \( IL-4 \) promoter regions in bovine CD4+ Th cells.
4.2 Introduction

T-helper (Th) cells are important mediators of the adaptive immune response. Different Th cell populations drive adaptive immune responses towards either cell- (CMIR) or antibody- immune response (AMIR), an important process that aids in the control of specific pathogens. Recent studies have shown that T-cell lineage differentiation during microbial infection is influenced by strength of pathogen signals from antigen presenting cells and cytokine microenvironment (review by Chang et al., 2014). Stimuli received by naïve Th cells drive unique cytokine profiles and invoke cell differentiation towards a Th1 or Th2 phenotype that support CMIR and AMIR, respectively (Geginat et al., 2014; Tubo et al., 2013). CMIR is generally elicited in response to intracellular pathogens such as viral infection, and is characterized by production of the cytokine interferon-γ (IFN-γ). AMIR in contrast, is generally elicited in response to extracellular pathogens, and is characterized by a production of cytokines including interleukin-4 (IL-4), interleukin-13 (IL13), and interleukin-5 (IL5) (Kanno et al., 2012).

The Th phenotype shown primarily in mice and human is controlled in part by genetic background and epigenetic modifications at key regions in the genome including gene promoter regions and regulatory regions (Deaton et al., 2014; Dong et al., 2013). Genetic variants in the bovine IFN-γ gene, for example, have been associated with resistance against numerous intracellular pathogens and is associated with relative IFN-γ gene expression (Verschoor et al. 2011; Pinedo et al, 2009). Overall, single polymorphisms (SNP) exist throughout the genome that is associated with AMIR and CMIR biased IR in cattle (Thompson-Crispi et al, 2014).

Epigenetic modifications control gene expression and influence overall cellular phenotype and individual phenotype by controlling gene expression in a cell- and tissue- specific manner. There
are numerous epigenetic mechanisms that control gene expression including histone modification, DNA methylation, as well as post-transcriptional modifications by microRNA (Feeney et al., 2014; Law and Jacobsen, 2010). In conjunction, these mechanisms manipulate chromatin structure and the relationship with transcription factors, DNA, and transcriptional machinery that influences gene expression. The influence of epigenetic modifications can be in close proximity or situated distantly from the affected gene with epigenetic modifications either located upstream or downstream from the transcription start site (TSS) (Law and Jacobsen, 2010). Cytosine-phosphate-Guanine (CpG) motifs exists across 1% of the eukaryotic genome with methylation occurring on 70-80% of CpG sites that vary according to specific tissue and cell type, species, gender, individual, age, environmental exposures and genomic regions (Bestor et al., 2014; Jones, 2012; Boks et al., 2009). DNA methylation occurs primarily within CpG motifs located throughout the genome (Jones, 2012), however non-CpG motifs may also be subject to DNA methylation, but to lesser extent (Arand et al., 2012). The occurrence of CpG motifs is not uniform throughout the genome, with increased numbers of CpGs found within CpG “islands” and CpG “shores”, as well as decreased numbers of CpGs found in intergenic regions and non-coding regions (Jones, 2012). The presence of DNA methylation in close proximity at transcription start sites, across the gene, and at distant regulatory regions has been associated with down-regulation of a gene expression. Contrary to this, the absence of DNA methylation has been associated with gene transcription and expression (Jones, 2012). DNA methylation is established on the genome by DNA methylation transferase (DMNT). Retention of DNA methylation on a DNA strand is maintained by DMNT through cellular replication, but can be lost through passive mechanisms and less often through active DNA methylation removal (Smith and Meissner, 2014). DNA methylation is a dynamic epigenetic mark that can be
influenced by environmental exposure. T-cell cytokine production has been shown to be under the control of epigenetic modifications including DNA methylation, histone modifications, and microRNA. Specifically, DNA methylation within the IFN-γ and IL-4 promoters has been associated with down-regulation of gene expression and protein production (Dong et al., 2013; Guan et al., 2011; Paibomesai et al., 2013; Falek et al., 2000).

In cattle, DNA methylation has recently been characterized for placental tissues (Su et al., 2013), peripheral blood mononuclear cell (PBMC) (Doherty et al, 2013), CD4+ Th cells (Paibomesai et al., 2013), fibroblast (Green et al., 2015; Green and Kerr, 2014), sperm (Saadi et al., 2016) and endometrial tissue (Walker et al., 2013) using genome-wide bisulfite conversion sequencing. To date, there are a limited number of studies that have investigated specific CpG DNA methylation sites within cytokine gene promoter regions. Therefore, the focus of this study was to assess DNA methylation using bisulfite pyrosequencing within the promoter regions of IFN-γ and IL-4 of in vitro skewed Th1 and Th2 bovine CD4+ T-cells. To deal with tissue- and cell-defined epigenetic modifications, CD4+ T-cells were isolated from blood from known average immune response cattle outside the peripartum period and were cultured in vitro. Similar to our previous study (Paibomesai et al., 2013) it is predicted that there would be an increase in methylation within the IFN-γ and IL-4 promoter regions and this would be associated with a decrease in gene expression. More specifically, it is hypothesized that, CD4+ T-cells that were cultured under Th1-skewing conditions would have increased methylation at IL-4 gene promoter and less DNA methylation within IFN-γ promoter region, and CD4+ T-cells cultured in Th2 skewed conditions would have greater DNA methylation within the IFN-γ promoter region and less DNA methylation within IL-4 promoter region.
4.3 Material and Methods

Animals

Holstein dairy cows were housed at the University of Guelph Elora Dairy Research Station. Blood was collected from 5 mid-lactation cows that were previously phenotyped as average immune responders according to protocols outlined in Hine et al. (2012) and Martin et al. (2015). Dairy cattle used in this study were previously categorized as high, average or low for AMIR and CMIR by assessing responses to type I and type II antigens using the High Immune Response Technology (US Pat. No. 7258858; Wagter and Mallard, 2007). Cows in this study were all in their third parity. Whole blood (80 to 100 mL) was collected from the caudal vein into 10-mL ethylene diamine tetraacetic acid (EDTA) Vacutainer tubes (BD, Franklin Lakes, New Jersey, USA). All experimental procedures and animal handling used in the study were approved by the University of Guelph Animal Care Committee under the guidelines of the Canadian Council on Animal Care (AUP#04R063).

In vitro Th1 and Th2 culture conditions

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation according to manufacturer’s instructions using Histopaque (Invitrogen Canada, Burlington, Ontario) from mid-lactation cows and used for subsequent Th-1 and Th-2 skewing cultures based on Fitch et al. (2006). PBMCs (1x10^6 cells/mL) were plated in 24-well NUNC plates in 1 mL of complete RPMI-10 medium, 30 mg/L glutamine, 10% fetal calf serum and penicillin/streptomycin (Invitrogen Canada, Burlington, Ontario). Th1 cell culture conditions consisted of RPMI supplemented with 10 ng/mL recombinant bovine IL2 (R&D Systems Inc., Minneapolis, USA), 20 µg/mL rabbit anti-bovine IL-4 (Thermo-Fischer, Neapan, ON), and
1µg/mL ConA. Th2 cell culture conditions consisted of 10 ng/mL recombinant bovine (IL2) (R&D Systems Inc., Minneapolis, USA), 20 µg/mL rabbit anti-bovine IFN-γ (Thermo-Fischer, Neapan, ON), 4 ng/mL recombinant bovine IL-4 (Thermo-Fischer, Neapan, ON), and 1 µg/mL ConA. PBMC were incubated in Th1 and Th2 cell culture conditions for 5 days, 37°C, 5% C2O. PBMC were then washed with 40 mL of PBS and plated at 1x10^6 cells/mL in complete RPMI-10 with 10 ng/mL recombinant bovine IL2 (R&D Systems Inc., Minneapolis, USA) for 3 days, 37°C, 5% C2O to expand the cell population. CD4+T-cells were then isolated using MACS, as described above and plated in Th1 and Th2 cell culture conditions for a further 4 days, 37°C, 5% C2O. CD4+ T-cells were washed, and plated in complete RPMI-10 media, 30 mg/L glutamine, 10% fetal calf serum and penicillin/streptomycin (Invitrogen Burlington, Canada) with 1 µg/mL ConA. CD4+ T-cells were harvested at 24 hours after stimulation and stored at -80°C for future RNA and DNA methylation analysis.

**CD4+ T-Cell Isolation**

The mini- Magnetic activated cell sorting (MACS) system (Milntyi Biotech, Auburn, CA) was used to isolate a CD4+ T-cell-enriched population from the viable PBMC population. First, PBMC (1x10^7) were incubated with 100 µL of mouse IgG1 anti-bovine CD4+ antibody (ILA-11, VMRD) incubated for 30 min at 4°C and then washed with PBS (300 xg, 10 min, rt). The resulting cell pellet was then incubated with 20 µL goat anti-Mouse IgG1 microbeads and 80 µL MiniMACS buffer (PBS, 2 mM EDTA, 0.5% BSA; degassed) per 1x10^7 cells for 15 minutes at 4°C. Cells were washed with 45 mL of PBS and resuspended in 1 mL of MiniMACS buffer to a total maximum concentration of 2x10^8 cells/mL. The cell suspension was added to the MiniMACS magnet bound column and was washed three times with 500 µL of MiniMACS buffer. The MiniMACS column was removed from the magnet and 1 mL MiniMACS buffer was
used to elute bound cells. This process was repeated, and selected samples were confirmed by flow cytometry to have 99% purity (data not shown).

RNA extraction

RNA was extracted using RNeasy Mini Kit from Qiagen as per manufacturer’s instruction (Qiagen, Mississauga, Ontario). Isolated RNA was treated with the Turbo-DNA free system (Ambion, Burlington, ON), as per the manufacturer’s instructions. cDNA was prepared from 500 ng RNA using the Superscript III First Strand cDNA synthesis system (Invitrogen, Burlington, ON), as per the manufacturer’s instructions. cDNA was stored at -20°C prior to quantitative real-time PCR (qPCR) analysis. Primers for qPCR (Table 4.1) were designed using Primer3 primer design (NCBI) and Secondary Structure Estimation software (Integrated DNA Technologies, Coralville, Iowa) as per Martin et al. (2015). qPCR was performed on the Roche Light Cycler480 II in 96-well plates (Roche, Mississauga, ON). Gene expression values were calculated using the PFAFFFL fold change equation (Pfaffl, 2001).

Gene expression was presented as the difference or fold change between stimulated and unstimulated samples. These values were normalized based on the expression of the internal reference gene, β-actin, which was determined to be stably expressed in bovine PBMCs in this study and others (Meade et al., 2006; Robinson et al., 2007; Spalenza et al., 2010).

Bisulfite Pyrosequencing

To evaluate DNA methylation, genomic DNA (gDNA) was extracted with DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON) from cultured Th1 and Th2 CD4+ T-cells. Multiple Alignment and Visualization Tool (MULAN; Ovcharenko et al., 2005) was performed to analyze the homology between human and bovine IFN-γ and IL-4 genes. Alignment of 7.5 kb of the
human and bovine IFN-γ/IFN-γ loci; Human chromosome position is ch12:66833573-66841031 and 11kb of human and bovine IL-4/IL-4 loci; human chromosome position ch5:132036908-132037919 was completed by MULAN through the tba method. Similarity between the DNA sequences with at least 50% similarity identity over at least 100 bp is shown in the histogram plot. Homologous regions are considered to be 75% similarity identity (Ovcharenko et al., 2005).

gDNA was bisulfite-treated with EpiTect Fast DNA Bisulfite Kit with 500 ng of starting DNA (Qiagen, Mississauga, ON) according to the manufacturer’s instructions. Bisulfite DNA (10ng) was then subjected to amplification by PCR for listed bisulfite pyrosequencing primers in Table 4.2 that were designed using PyroMark Assay Design 2.0 software (Qiagen, Mississauga, Ontario). PCR program: 95°C for 10 minutes; 45 cycles of (95°C for 30 sec, primer specific annealing temperature (Table 4.2) for 30 sec, and 72°C for 45 sec) and concluded with a final extension step at 72°C for 10 min. PyroMark PCR Kit (Qiagen, Mississauga, ON) was used in 20 µL reactions all samples with 1µL of bisulfite treated DNA (10 ng). All PCR products were run on 1% agarose gel for verification of PCR product size and amplification before subsequent pyrosequencing. PCR products were incubated with 2 µL Streptavidin Beads (Qiagen, Mississauga, ON) per 20 µL of sample in 40 µL of binding buffer (Qiagen, Mississauga, ON) for 10 min with shaking (300xg, rt.). Meanwhile, a 96-well PQA plate (Qiagen, Mississauga, ON) was loaded with 0.4 µM sequencing primer per well in 40µL of annealing buffer (Qiagen, Mississauga, ON); primers are listed in Table 4.2. A vacuum manifold was used to capture streptavidin-bound PCR products. Briefly, probes were applied to V-bottom 96-well plate for 15 secs to capture the streptavidin-bound PCR products, then removed and submerged in 70% ethanol for 5 sec, then 8µM NaOH for 5 sec and product was washed for 15 secs in wash buffer (Qiagen, Mississauga, ON). Vacuum was released and probes were submerged in PQA plate.
with sequencing primer. The PQA plate was heated for 2 minutes at 80°C and before analysis on PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON).

Bisulfite pyrosequencing was performed on a PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON). Negative controls and positive controls were included on the plate. Negative controls consisted of only sequencing primer, only pooled DNA from non-bisulfite converted DNA, pooled DNA bisulfite converted with and without the sequencing primer. Data was collected and analyzed for quality using Pyro Q-CpG software, with sample number varying from CpG site to site due to differences in sample quality and efficiency of the bisulfite conversion. Those samples that did not meet the quality standards were repeated.

Statistical Analysis

DNA methylation data were summarized using PROC TABULATE and reported in Table 4.3 according to CpG methylation site and overall average methylation across the promoter region. Data were analyzed independently using a General Linear Model that included repeated measures using PROC MIXED (SAS Version 9.2; SAS Institute, Cary, North Carolina, USA). Kolmogorov-Smirnov (K-S) test was used to determine normality of data (K-S test; p<0.05). Differences in gene expression data were analyzed using PROC ANOVA in SAS (SAS Version 9.2; SAS Institute, Cary, North Carolina, USA), with significance p≤0.05. The statistical model used was:

\[ y_{ijkl} = \mu + r_i + t_j + r_i x t_j + \epsilon_{ijkl} \]

where: \( y \) = response vector of the observation; \( \mu \) = overall mean; \( r_i \) = cell culture treatment (Th1 or Th2); \( t_j \) = site of methylation (IL-4 CpG -329,+12,+128,+175,+193 or IFN-\( \gamma \) CpG -334,-
291,+57,+72) ; \varepsilon = \text{residual error. Significant interactions were retained in the model } p < 0.10 \text{ and significance } p \leq 0.05.

4.4 Results

*In vitro* culture conditions result in Th1 and Th2 like cells from bovine CD4+ T-cells

PBMCs were cultured in Th1 and Th2 conditions for 8 days and CD4+ T-cells were isolated for gene expression and assessment of promoter region CpG sites. Th1 cultured bovine CD4+ T-cells had higher *IFN*-γ gene expression (p>0.05) than Th2 cultured CD4+ T-cells, as shown in Figure 4.2. Conversely, Th2 cultured CD4+ bovine T-cells had more *IL*-4 transcripts than the Th1 cultured CD4+ T-cells as shown in Figure 4.2 (P>0.05).

Characterization of DNA methylation *IFN*-γ and *IL*-4 promoter regions

Th1 and Th2 cultured CD4+ T-cells were used to validate DNA methylation signatures of *IFN*-γ and *IL*-4 promoters. The *IFN*-γ promoter region is located on chromosome 5, at chromosomal location of 5q22-q24 based on the *Bos taurus UMD_3.1.1* assembly (NCBI). *IFN*-γ is composed of 4 exons and spans from location 45830158..45834981 base pairs in length. Figure 4.1a shows the characterized *IFN*-γ promoter region, highlighting 6 CpG motifs and their relative distance from the transcription start site. Overall, 5 CpG sites located in the *IFN*-γ promoter (-334, -229, -220, +54, and +72 from the transcription start site [TSS]) had an average DNA methylation of 43% ± 16% as defined by bisulfite pyrosequencing, with a maximum methylation 69% and minimum methylation of 22% as reported in Table 4.3.

The *IL*-4 promoter region is located on chromosome 7 at chromosomal location 7q15-q21 which is composed of 4 exons that span base pairs 22993178..23001067 based on the
Bos_taurus_UMD_3.1.1 assembly (NCBI). Figure 4.1a shows a characterization of IL-4 promoter region, highlighting 5 CpG motifs and their relative distant from the transcription start site. Five CpG sites located in IL-4 promoter (-329, +12, +128, +175, and +195 from TSS) had an average DNA methylation of 51% ± 9% as defined by bisulfite pyrosequencing, with a maximum methylation of 64% and minimum methylation of 31% as reported in Table 4.3. Alignment of these bovine genes with human genes shows homology through IFN-γ and IL-4 promoter region for both genes, signified by >70% identity between the two sequences, as shown in Figure 4.1b.

In vitro skewed bovine Th1 and Th2 cells have different methylation signatures

Altered DNA methylation signatures were observed between the Th1 and Th2 skewed T-cells at the promoter region for both IFN-γ and IL-4. Th1 cells (42%) had 8% less methylation across the IFN-γ promoter compared to methylation status of Th2 T-cells (50%) which produced less IFN-γ transcript, see Figure 4.4. Conversely, Th2 T-cells (44%) had 7% less methylation than Th1 clones at the IL-4 promoter region (51%) which correlated with higher levels of IL-4 transcript for Th2 clones shown in Figure 4.4.

There were numerous differences in the percent of methylation observed at specific CpG sites in both IFN-γ and IL-4 promoter regions between Th1 and Th2 cultures (Figure 4.3c,d). Two CpG sites in the IFN-γ promoter showed differential methylation between Th1 and Th2 cultures. IFN-γ CpG -334 and -220 from the TSS had greater methylation for Th2 cultures compared to Th1 cultures with differences in DNA methylation of -11% and -21%, respectively. For IL-4, CpG -321 and +175 had greater DNA methylation for Th1 compared to Th2 skewed bovine CD4+ cells with differences of +14% and +13%, respectively. This demonstrates the variety of
DNA methylation profiles between regions, conditions and individuals as observed in Figure 4.3a,b.

4.5 Discussion

Th1 and Th2 skewed cultures were used to assess DNA methylation signatures at the bovine IFN-γ and IL-4 promoter regions. We adapted an in vitro protocol for skewing Th cells to provide insight into determining the specific bovine CpG sites in select bovine CD4+ T-cells subsets (Fitch et al., 2006). This study shows that Th1 and Th 2 cell expression of IFN-γ and IL-4 is related to the epigenetic modification, DNA methylation. Changes in DNA methylation were observed across the promoter region for in vitro bovine Th1 and Th2 skewed cultures for both IL-4 and IFN-γ. Genome-wide methylation studies using mice have identified numerous regions upstream from the TSS that are associated with IFN-γ activation (Komori et al., 2015). The bovine genome is composed of 5-10% of highly methylated regions (HMR) according to Su et al. (2014), who investigated global methylation of placental tissue from cattle. These authors concluded that a majority of HMR exist within introns (5’ untranslated regions (UTR), coding sequence, and 3’UTR) in the bovine genome and are composed of 5 to 25 CpG in a localized region. This confirms that regions close to the transcription start site (TSS) in cattle are highly populated with CpG motifs. DNA methylation is controlled by DNA methyltransferase (DMNT), which has previously been identified in bovine fibroblast cells and bovine macrophages giving evidence that cattle possess the machinery to maintain and add on DNA methylation to the genome (Green et al., 2013; Oh et al., 2015).

The inverse relationship between gene expression and DNA methylation of the promoter region infers that the promoter region is a regulatory region for IFN-γ. This study supports previous
results that suggest DNA methylation status of bovine \textit{IFN-\gamma} and \textit{IL-4} promoter regions is related to cytokine production from CD4+ Th cells (Paibomesai et al., 2013). The association of hypomethylation at the \textit{IFN-\gamma} promoter and \textit{IFN-\gamma} expression is not unique to cattle and has been reported in mice (Winders et al., 2004; Jones et al., 2006; Falek, et al., 2003) and humans (Dong et al., 2014). For instance, naïve T-cells isolated from mice possess hypermethylated regions across the \textit{IFN-\gamma} promoter region (Winders et al., 2004). \textit{IFN-\gamma} promoter regions plus other regulatory regions play a key part in the control of \textit{IFN-\gamma} gene expression. DNA methylation has been observed in human \textit{IFN-\gamma} promoter region and conserved non-coding sequence (CNS)-1 show a dynamic relationship with \textit{IFN-\gamma} expression (Dong et al., 2013; Komori et al., 2015), and is also present in cattle as shown in this study and previous studies (Paibomesai et al., 2013). It would be interesting to investigate the distal 3'\textit{IFN-\gamma} regulatory regions in detail in future studies to determine the relationship with bovine \textit{IFN-\gamma} expression.

Previously, Paibomesai et al. (2013), showed that in cattle there was limited association between the \textit{IL-4} promoter region and IL-4 protein secretion, but the bisulfite sequencing technique that was used has since been improved upon in the present study with the introduction of bisulfite pyrosequencing, which is currently considered the gold standard for specific CpG DNA methylation assessment. In the current study, the \textit{IL-4} promoter displayed the same relationship as \textit{IFN-\gamma} with less DNA methylation across the promoter region that was inversely correlated with gene expression. \textit{IL-4} promoter region was hyopmethylated in Th2 cell cultures compared to Th1 cell cultures. There is evidence from other studies that show changes in promoter DNA methylation are associated with changes in gene expression of \textit{IL-4} (Mi et al., 2008). \textit{IL-4} promoter methylation has been associated with a loss of expression of \textit{IL-4} in cattle (Paibomesai et al., 2013), mice (Li et al., 2103) and humans (Canani et al., 2015). In mice, transcription
binding sites in the promoter region could explain the association between *IL-4* promoter DNA methylation and *IL-4* gene expression (Li et al., 2013). *IL-4* is transcribed as part of the Th2 cytokine locus that includes *IL-5* and *IL-13* and is under the control of Th2 locus control region that contains DNA methylation hypersensitivity sites. DNA methylation at regions within the Th2 locus control region have been associated with Th2 cytokine gene expression (Kim et al., 2007; Williams et al., 2013). The Th2 control region locus is located in *RAD50* a gene upstream of the *IL-4*, *IL5*, and *IL13* loci. Upon investigation, sequence similarity between *RAD50* in cattle, mice, and humans was low (<70%) as determined by Multiple Sequence Local Alignment and Visualization tool (Ovcharenko, et al., 2005), data not shown. Therefore, future work could focus on investigating the relationship between promoter DNA methylation and the Th2 locus control region if proven to exist in cattle.

Caution should be taken in interpreting these results as there is mounting evidence that CD4+Th cells exhibit great plasticity and possess the ability to change cellular phenotype according to conditions and current infection (Li et al., 2013). CD4+ T-cell populations represent a mosaic of cellular phenotypes including naïve, effector, and memory cells that carry differing DNA methylation signatures (Komori et al., 2015; Li et al., 2013). In addition to the different states of CD4+ Th cells, there are numerous lineages that exist including Treg, Th17, Th9 and Th22. As expected DNA methylation patterns differ for *IFN-γ* and *IL-4* promoter regions in naïve T-cells compared to differentiated Th1 and Th2 cells (Li et al, 2013; Komori et al., 2015). In the current study, there was similar average DNA methylation across the promoter region for *IFN-γ* (43%) and *IL-4* (51%) in Th1 and Th2 cell culture conditions. The reported percent of DNA methylation for CD4+ Th cells in cattle is similar to what has been reported in human studies which focus on CD4+ Th DNA methylation (Komori et al., 2015). Future work could focus on
distinguishing global methylation for cattle in naïve compared to memory cells, and Th1 and Th2 cultured to identify other genomic regions to target for in-depth sequencing analysis. Furthermore, future studies could investigate the influence of in vivo stimuli versus in vitro stimuli of different isolated Th cell lineage and cytokine gene DNA methylation. Nonetheless, these results present new insight into the epigenetic regulation of the bovine adaptive immune system.

4.6 Conclusions

Epigenetic modifications, such as DNA methylation play a pivotal role in the initiation of gene expression and development of cellular phenotype. There has been previous data in mice and humans that show differential DNA methylation between naïve, Th1 and Th2 CD4+ T-cells. The current study demonstrates differences in DNA methylation of IFN-γ and IL-4 promoter regions in Th1/Th2 skewed bovine T-cells assessed by bisulfite pyrosequencing. A decrease in DNA methylation was observed at specific CpG sites and across the whole promoter region for both IL-4 and IFN-γ under these differential culturing conditions. Although the influence of individual CpG methylation has not been described, there is evidence that decreased average DNA methylation across the promoter region is associated with increase in gene expression. The current study validates that concept in cattle for IFN-γ and IL-4. Future work could focus on defining new regions of the genome for different T-cell phenotypes in cattle and use the identified regions in this study in an ex vivo model.
4.7 References


Green, B. B., S. D McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.


### 4.8 Tables and Figures

**Table 4.1** Primer design and reaction conditions for real time RT-PCR gene expression experiments

| Gene symbol | Gene name  | Sequence 5’-3’ | Amplicon length (bp) | $T_a$C° | PCR slope | PCR efficiency
|--------------|------------|----------------|----------------------|--------|----------|----------------|
| **IFN-γ**    | Interferon-γ | F:TATCTCAGGGGCAACTAGG  
R:CTGAAGCGCCAGGTATAAGG | 250 | 62 | -3.432 | 1.956 |
| **IL-4**     | Interleukin-4 | F:CAGTGCTGGTCTTACTG  
R:CAAGAGGTCTTTTCAGCGTAC | 338 | 62 | -3.428 | 1.958 |
| **BACT**     | β-actin | F:GTTTTAGGCTGGACTTAG  
R:ACTTGGGAATGCTCGATCC | 254 | 62 | -3.493 | 1.933 |

1Annealing Temperature

2Determined using quintet dilution series
Table 4.2. Primers and reaction conditions for Bisulfite Pyrosequencing experiments and location of CpG from TSS for IFN-γ and IL-4 gene promoter regions

<table>
<thead>
<tr>
<th>CpG</th>
<th>Primer</th>
<th>Biotinylated (No/Yes)</th>
<th>Primer Sequence (5'-3')</th>
<th>Tm, °C</th>
<th>Amplicon Length (bp)</th>
<th>Bisulfite Sequence to Analyze</th>
<th>No. of CpGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>No</td>
<td>TTTGATGAGGA GTTAATAT</td>
<td>51.8</td>
<td>127</td>
<td>TTAAYGAGGGT AAAAGGAGGA GGTTGATAAA AATTTTT</td>
<td>1</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>Yes</td>
<td>TTATACCCACCCATACCATCT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
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<td>TGGATGAGGA GTGAATTTT</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Forward</td>
<td>No</td>
<td>GAGGGTAAAGGGA GGTGTATAAA</td>
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<td>125</td>
<td>ATTTTTTAAATT TTGGAYGGT GGAAGTGA A AGTTTTT TA AAGGA</td>
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<tr>
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<td>Reverse</td>
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<td>ATACCCACCCATACCACTT</td>
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<td></td>
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<tr>
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<td>Sequencing</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>IFN-γ</td>
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<td>No</td>
<td>AGAAGATATTAGTATTTTTTTTGGGAT TTGA</td>
<td>59.8</td>
<td>177</td>
<td>TAGGAGTTAT YGATTTTAAT TATTTGATTTTAATT AAGTAAGA</td>
<td>2</td>
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<tr>
<td></td>
<td>Reverse</td>
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<td>No</td>
<td>TAGTTGATTTTTGTTGTTGTTGTTTA TTG</td>
<td>59.5</td>
<td>171</td>
<td>TAGTTATTTAATG TATTTGATTTAATG TATTTTGA GAGATTTTA AAAYGTTGA TATTTTTATA AYGAGAAAGG</td>
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<td>59.9</td>
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<tr>
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<tr>
<td>IL-4</td>
<td>Forward</td>
<td>No</td>
<td>GGAAGAAGGTAAAAGGTGAATTTAA TTAAG</td>
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<td>314</td>
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Table 4.3. Summary statistics of DNA methylation at IFN-γ and IL-4 promoter regions reported as base pairs (bp) from transcription start site (TSS).

<table>
<thead>
<tr>
<th>Gene</th>
<th>CpG Site (bp from TSS)</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
</tr>
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<td>-334</td>
<td>40.18</td>
<td>16.01</td>
<td>11.68</td>
<td>44.78</td>
<td>55.00</td>
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<td></td>
<td>-291</td>
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<td>6.27</td>
<td>19.00</td>
<td>33.00</td>
<td>38.00</td>
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<tr>
<td></td>
<td>-220</td>
<td>51.83</td>
<td>21.17</td>
<td>29.00</td>
<td>50.00</td>
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<td>+57</td>
<td>52.56</td>
<td>29.80</td>
<td>9.82</td>
<td>66.00</td>
<td>81.00</td>
</tr>
<tr>
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<td>16.55</td>
<td>68.00</td>
<td>88.00</td>
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<tr>
<td></td>
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<td>16.64</td>
<td>22.05</td>
<td>48.30</td>
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<tr>
<td>IL-4</td>
<td>-329</td>
<td>65.50</td>
<td>14.02</td>
<td>48.50</td>
<td>63.00</td>
<td>100.00</td>
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<td>+12</td>
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<td>7.57</td>
<td>13.00</td>
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<td>+128</td>
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<td>25.47</td>
<td>78.00</td>
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<td>+175</td>
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<td>19.38</td>
<td>29.33</td>
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<td>20.87</td>
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<td></td>
<td>Total Methylation</td>
<td>51.38</td>
<td>9.76</td>
<td>31.04</td>
<td>50.24</td>
<td>64.20</td>
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</tbody>
</table>
**Figure 4.1a**

*IFN-γ PROMOTER REGION*

-334  -291  -220  -85  **TSS**  +57  +72

*IL-4 PROMOTER REGION*

-329  **TSS**  +12  +128  +175  +193
Figure 4.1b

*IFN-γ*

*IL-4*
Figure 4.1a,b. a) Graphical representation of IFN-γ and IL-4 promoter region and assessed CpG sites across the promoter region. b) Alignment of 7.5 kb of the human and bovine IFN-γ/IFN-γ loci; Human chromosome position is ch12:66833573-66841031 and 11kb of human and bovine IL-4/IL-4 loci; human chromosome position ch5:132036908-132037919. Similar DNA sequence is represented in the histogram plot with at least 50% identity over at least 100 bp is shown in the histogram plot. Arrows indicate transcriptional direction, pink/red regions represent conserved non-coding sequences, yellow areas represent UTR, blue areas represent conserved exons and green areas represent conserved untranslated regions. Alignment was completed by Multiple Alignment and Visualization Tool (MULAN; Ovcharenko et al., 2005).
Figure 4.2

The bar chart shows the fold change in the expression of IFNG and IL4 in Th1 and Th2 skewed CD4+ T-cells. The chart indicates a significant difference in expression levels, with the Th2 skewed CD4+ T-cells showing a marked increase compared to Th1 skewed CD4+ T-cells. The asterisks denote statistically significant differences.
**Figure 4.2.** Fold change expression of *IL-4* and *IFN-γ* from Th1 and Th2 *in vitro* skewed CD4+ T-cells isolated from average immune responders outside the peripartum period. Th1 and Th2 cell cultures were stimulated with ConA for 24 hours before RNA extraction. Gene expression is reported as fold change means +/- SEM. Fold change expression is reported relative to unstimulated samples that were collected 24 hours after Th1 and Th2 cell culture were started, and normalized to the internal reference gene BACT. *p*<0.05.
Figure 4.3c

Figure 4.3b

* p≤0.05
Figure 4.3a-d. Heatmap representation of DNA methylation of Th1 and Th2 cultured CD4+ Th clones for IFN-γ (CpG -334, -291, +57, +72) and IL-4 (CpG -329, +128, +175, +193). Total methylation is the average methylation for that individual across all CpG sites. The dark blue represents hypermethylation and yellow represents hypomethylation. DNA Methylation of IFN-γ (c) and IL-4 (d) promoter region determined by bisulfite pyrosequencing of selected CpG methylation sites in close proximity to the transcription start site of Th1(■) and Th2(□) in vitro skewed cell cultures from average immune response phenotype cows (n=5) outside of the peripartum period. CpG DNA methylation is reported a Least Square Mean ± SEM. *p≤0.05
Figure 4.4. Average DNA methylation across IFN-γ and IL-4 promoter regions of Th1 (■) and Th2 (□) skewed bovine CD 4+ T-cells. Percent DNA methylation is reported as Least Square Mean ± SEM. # p≤0.10 *p≤0.05
CHAPTER 5

Biased Immune Response Phenotypes of Dairy Cows Associate with Differences in DNA Methylation at IFN-γ and IL-4 Promoter Regions

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CHAPTER 5

Biased Immune Response Phenotypes of Dairy Cows Associate with Differences in DNA Methylation at Type I and Type II Cytokine Promoter Regions

5.1 Abstract

Over the past decade epigenetics has begun to dominate the investigation of disease susceptibility. DNA methylation has been extensively studied in relation to the mouse CD4+ T-cell immune response (IR). It has been shown that specific DNA methylation sites within the promoter region of IFN-γ and IL-4 has an influence on the overall gene expression of these genes and cytokine production in CD4+ T-cells. Therefore, the objective of this study was to determine the role of DNA methylation at the promoters of these cytokines in cows ranked as high (H) antibody (AMIR) or cell-mediated (CMIR) immune responders. Blood was collected from H-AMIR (n=10) and H-CMIR; (n=11) cows 21 days after calving. Isolated CD4+ T-cells were collected and stimulated for 24 hrs with Concanavalin-A. Genomic DNA was collected for subsequent bisulfite pyrosequencing for the determination of DNA methylation patterns within the promoters of these genes. CD4+ T-helper (Th) cells from H-CMIR cows produced significantly more IL-4 and IFN-γ in collected samples than CD4+ Th cells from H-AMIR cows. There was a high correlation between unstimulated and stimulated CpG DNA methylation for IFN-γ (R=0.92; p<0.001) and IL-4 (R=0.83; p<0.001), therefore only stimulated samples were analyzed for difference in CpG DNA methylation. Total promoter DNA methylation was not significantly different between H-AMIR and H-CMIR cows for IFN-γ (-2%) and IL-4 (-1%) promoter regions. However, H-CMIR cows had lower CpG methylation as H-AMIR cows at IFN-γ promoter CpG +57 (-6.6%; p<0.05) and CpG +72 (-7.5%; p<0.05) from the transcription start site. H-CMIR displayed a decrease in CpG methylation compared to H-AMIR at IL-4 promoter CpG +128 (-9%; p<0.05) which was significantly different. The influence of individual
CpG methylation in cattle on a cellular, individual phenotype and transgenerational influence has not been determined; however, the results presented here show this now merits further investigation.
5.2 Introduction

Genetic improvement of disease resistance in dairy cattle has been slow due to the multi-factorial nature of diseases, non-standardized health records, and limited heritability of health traits (Parker-Gaddis et al., 2014; Koeck et al., 2012, Koeck et al., 2013). Previously, it has been shown that the adaptive immune response (IR) can be used as a tool for the improvement of health traits in dairy cattle (Thompson-Crispi et al., 2013). Ranking cattle based on estimated breeding values (EBVs) of antibody (AMIR) and cell (CMIR) mediated responses provides an independent, quantitative, measurable method that is associated with less disease occurrence, longer herd life, and improved colostrum quality (Thompson-Crispi, et al. 2012a; Thompson-Crispi, et al. 2012b; Thompson-Crispi et al., 2013; Fleming et al., 2014). AMIR and CMIR play a crucial role in mounting robust protective immune responses to invading pathogens, effective response to vaccines, and the maintenance of homeostasis. Balance between these two branches of the adaptive immune response is necessary to establish broad-based disease resistance as shown in chickens, pigs and cattle (Abdel-Azim et al., 2005; Mallard et al., 1992, Pinedo et al., 2009; Thompson-Crispi et al., 2012a). It has been previously shown that AMIR and CMIR immune responses traits are moderately heritable ($h^2=0.20$) in cattle (Thompson-Crispi et al., 2013; Heriazon et al., 2013).

Cell mediated immune responses, are typically characterized by cellular and cytokine responses required to clear intracellular pathogens, such as viruses or facultative intracellular bacteria including *Mycobacterium bovis*. Whereas, AMIR is associated with cytokines and antibodies typically required to clear extracellular pathogens. CD4+ T-helper (Th) cells are important mediators of AMIR and CMIR, that produce high levels of lineage-specific cytokines that help generate long living memory cells, which are both genetically and epigenetically regulated.
(Dong et al., 2013; Li et al., 2012; Paibomesai et al., 2013). Recently, our lab has shown that isolated peripheral blood mononuclear cells (PBMC) from cows with high AMIR or high CMIR traits have increased and reciprocal cytokine profiles when compared after mitogen stimulation (Martin et al., 2015; Appendix A). Although many CD4+ Th cell lineages exist, this study focuses on epigenetic mechanisms that influence Th1 lineage, which mediates CMIR through the production of the cytokine IFN-γ, and Th2 lineage, which produce substantial amounts of IL-4 and promotes AMIR (Zhu, et al., 2010).

In the past decade epigenetics, has dominated the field of molecular genetics in hopes of gaining an understanding of the mechanisms behind complex phenotypes such as disease susceptibility (Albert and Kruglyak, 2015; Lawson et al., 2013). DNA methylation, histone modification, microRNAs, chromatin packaging are some of epigenetic marks that have been investigated in recent epigenetics research (Smith and Meissner et al., 2013). DNA methylation has been extensively studied in Th cell lineage differentiation with a focus on both IFN-γ and IL-4 promoter and downstream regulatory regions (Dong et al., 2013; Williams et al., 2013). Generally, the presence of DNA methylation at promoter regions is associated with decrease transcription by encouraging chromatin compaction and discouraging recruitment of DNA binding proteins (Williams et al., 2013). Increased DNA methylation in the IFN-γ promoter region has been associated with decrease IFN-γ transcript and cytokine production (Jones and Chen, 2006; Mi et al., 2013; Dong et al, 2013) and has been characterized in Th2 polarized T-cells as hypermethylated (Williams et al., 2013). IL-4 cytokine regulatory regions are closely related to the expression of IL-13 and IL-5 cytokine that compose the Th2 locus that are close in proximity and are stabilized by epigenetic mechanisms (Ansel et al., 2006; Lee et al., 2002; Deaton, et al., 2014). The IL-4 gene, promoter region, as well the Gata3 response element, all
display low level of DNA methylation which are associated with Th2 lineage commitment and IL-4 production (Deaton et al., 2014).

Previously, 5 CpG sites in the bovine IFN-γ promoter region and 6 CpG sites in the IL-4 promoter region had altered DNA methylation when T-cells were cultured under either Th1 or Th2 conditions (Paibomesai et al., 2013; Chapter 4). These altered DNA methylation patterns were enhanced when cells were dually treated with the T-cell mitogen ConA and dexamethasone, a synthetic glucocorticoid (Paibomesai et al., 2013; Chapter 2). Therefore, the purpose of this study was to investigate the association between immune response traits, AMIR and CMIR, with DNA methylation status at IFN-γ and IL-4 promoters using bisulfite pyrosequencing.

5.3 Methods and Material

Animals

Dairy cows that were used in this study were previously identified as High, Average, and Low immune responders based on the patented immune response phenotyping protocol as described in Hine et al., 2011 (US Pat. No. 7258858; Wagter-Lesperance and Mallard, 2007). Briefly, on day 0 blood samples were taken in 10 mL red-top vacutainer tubes (BD, Franklin Lakes, New Jersey, USA) and cows were immunized with a 1 mL of antigen preparation which contains type I and type II antigens as previously described (Hine et al., 2011; Thompson-Crispi et al., 2011). Fourteen days after primary immunization another blood sample was collected from previously immunized cows to determine type II antigen antibody concentration by ELISA. AMIR was determined by: [Day 14 Antibody] – [Day 0 Antibody]. Previously immunized cows were also tested for their ability to elicit a delayed type hypersensitivity (DTH) reaction in response to type
I antigen on day 14 as an indicator of CMIR. Briefly, double skin-fold thickness measurements (DSFT) were taken at site of injection prior to an intradermal injection of 0.1mL of the type I antigen to which the cows had been immunized two weeks prior. After 48 hours skin-fold measurements were taken again at the marked injection site on the tail fold and DTH responses were determined by: Test site DSFT – Control DSFT.

Cows were ranked as high (H), average (A), and low (L) immune responders based on the AMIR and CMIR measurements as determined in a general linear model which accounts for age, pregnancy status and the interaction between these terms. Figure 5.1 shows representative data of immune response ranking based on standardized AMIR and CMIR phenotypic residuals. Those cows that had a response that was one standard deviation above the population mean were considered high immune responders. Those cows which ranked as one standard deviation below the population mean were considered low immune responders. See Hine et al. (2011) for a full description of the protocol and model used to rank animals for this study. Those animals that possessed a H-AMIR and L-CMIR ranking (H-AMIR cows; n=10) and H-CMIR and L-AMIR ranking (H-CMIR cows; n=11) were used for the subsequent study. All methods and animal use protocols were approved by the Animal Care Committee under Animal Utilization Protocol (AUP #04R063). Cows were housed at the University of Guelph Elora Research facility and were all first calf heifers upon enrolment in this study.

**CD4+ T-cell Isolation and Culture Conditions**

Blood was collected from H-CMIR (n=10) and H-AMIR (n=11) cows three weeks after calving in 10mL EDTA coated vacutainer blood tubes (BD, Franklin Lakes, New Jersey, USA) and inverted several times to prevent clotting. Peripheral Blood mononuclear cells (PBMC) were
isolated by overlaying blood over Histopaque 4011 (Sigma, Oakville, ON), a density gradient, and were centrifuged at 400 x g for 30 min at room temperature (r.t.). The cloudy mononuclear cell layer was aspirated from the Histopaque density gradient and collected in a 50-mL falcon tube. Cells were washed three times by adding approximately 40 mL of Phosphate Buffer Saline (PBS) to cell pellet and centrifuging for 10 min, 250 x g, rt. Cells were counted on a Hemocytometer with Tryptophan Blue Staining to distinguish live and dead cells. CD4+ T-cells were isolated by indirect magnetic activated cell sorting Mini-MACS system (Miltenyi Biotech, Auburn, California, USA) according to the manufacturer’s instructions. Briefly, isolated PBMC were incubated with Mouse IgG1 anti-bovine CD4 (ILA11; VMRD, diluted 500-fold) at 100 µl/10⁷ cells for 30 min at 4°C. PBMCs were washed once with 40 mL of PBS and centrifuged for 10 min, 300 x g, rt. The cell pellet was suspended in 2 mL of degassed MACS Wash Buffer (PBS, 1 mM EDTA, 5% BSA, degassed) and goat anti-mouse IgG microbeads (Miltenyi Biotech, Auburn, California, USA) and incubated for 15 min at 4°C. Cells were then passed through Mini-MACS MD columns twice and positive fraction was collected for FACS analysis (data not shown) and subsequent cell culture.

Isolated CD4+ T-cells were cultured for 24 hours in 96-wells round bottomed plates at 2.5x10⁶ cells/mL in 200 µL of complete RPMI-10 media – RPMI-10, 30 mg/L glutamines, 10% fetal calf serum, and penicillin/streptomycin (Invitrogen, Oakville, ON). Half of the cells were plated with no mitogen stimulation (unstimulated cells) and the other half of the cells were stimulated with 2.5µg/mL of Concanavalin-A (ConA) (Sigma, Oakville, ON), a known polyclonal T-cell stimulant. At 24 hours, cell culture supernatant was collected, pooled and stored at -20°C for future ELISA analysis. Cells were collected and stored at -80°C for future DNA methylation analysis.
Cytokine ELISA

Cytokine concentrations were determined by ELISA for IL-4 and IFN-γ for all samples. Bovine IFN-γ ELISA kit (MabTech, Cinnicanti, OH, USA) was used to determine IFN-γ from collected cell culture according to the manufacturer’s instructions. Bovine IL-4 ELISA kit (Thermo-Fischer Scientific, Kanata, Ontario) was used to determine IL-4 concentrations according to the manufacturer’s instructions. All samples were run in triplicate and only those plates with CV% less than 10% were kept for analysis.

Bisulfite Pyrosequencing

To evaluate DNA methylation, genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON) from culture Th1 and Th2 CD4+ T-cells. gDNA was bisulfite-treated with EpiTect Fast DNA Bisulfite Kit with 500ng of starting DNA (Qiagen, Mississauga, ON) according to the manufacturer’s instructions. Bisulfite DNA was then subject to amplification by PCR for listed bisulfite pyrosequencing primers in Table 5.1, as previously described in Paibomesai et al., 2016; Chapter 4, that were designed using PyroMark Assay Design 2.0 software (Qiagen, Mississauga, Ontario). Bisulfite DNA (10ng) was then subjected to amplification by PCR for listed bisulfite pyrosequencing primers in Table 5.2 that were designed using PyroMark Assay Design 2.0 software (Qiagen, Mississauga, Ontario). PCR program: 95°C for 10 minutes; 45 cycles of (95°C for 30 sec, primer specific annealing temperature (Table 5.2) for 30 sec, and 72°C for 45 sec) and concluded with a final extension step at 72°C for 10 min. PyroMark PCR Kit (Qiagen, Mississauga, ON) was used in 20 µL reactions all samples with 1µL of bisulfite treated DNA (10 ng). All PCR products were run on 1% agarose gel for verification of PCR product size and amplification before subsequent pyrosequencing. PCR
products were incubated with 2µL Streptavidin Beads (Qiagen, Mississauga, ON) /20µL of sample in 40µL of Binding Buffer (Qiagen, Mississauga, ON) for 10 minutes with shaking (300xg) in a V-bottom 96 well plate. Meanwhile, 96 well PQA plate (Qiagen, Mississauga, ON) was loaded with 0.4 µM sequencing primer/well in 40µL of annealing buffer (Qiagen, Mississauga, ON), primers are listed in Table 5.1. Vacuum manifold was used to capture streptavidin bound PCR products. Briefly, vacuum manifold probes were applied to V-bottom 96 well plate containing Streptavidin-bound PCR products for 15 seconds, the vacuum manifold probes were then removed and submerged in 70% ethanol for 5 seconds, then 8µM NaOH for another 5 seconds and products was washed for 15 seconds in Wash Buffer (Qiagen, Mississauga, ON). Vacuum was released and probes were submerged in PQA plate with applied sequencing primer. The PQA plate was heated for 2 minutes at 80°C and before analysis on PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON).

Bisulfite pyrosequencing was performed on PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON). Data was collected and analyzed for quality on Pyro Q-CpG software, sample number varies from CpG site to site due to differences in sample quality.

Data Analysis

Bisulfite pyrosequencing data was analyzed and corrected for by the PyroQ-CpG Software (Biotage) with 10% CV as an acceptable cut off for variation between duplicates. Mean methylation with standard error were determined within each CpG site (IFN-γ CpG -334, -291, +54, and +75; IL-4 CpG -329, +128, +175, +193) and also averaged across IFN-γ and IL-4 promoter regions. PROC CORR was used to determine the correlation between unstimulated
and ConA stimulated DNA methylation across the *IFN-γ* and *IL-4* promoter regions and reported by Spearmen’s correlation.

Significance between H-AMIR and H-CMIR for DNA methylation was determined by PROC MIXED in SAS (SAS Version 9.2; North Carolina, USA). The structure of the variance-covariance matrix of repeated measures was chosen based on the lowest Akaike information criterion (AICC) (Akaike, H., 1981.). Normality was tested using the Shapiro-Wilks test statistic. Least squares means (LSMeans) were estimated and t-test used to compare contrasts. The statistical model used was:

\[ y_{ijkl} = \mu + r_i + d_j + c_k + t_l + \varepsilon_{ijkl} \]

where: \( y \) = response vector of the observation; \( \mu \) = overall mean; \( r_i \) = immune response group (H-AMIR or H-CMIR); \( d_j \) = days to calving (-28 or +21); \( c_k \) = methylation CpG site; \( t_l \) = Treatment (unstimulated or stimulated); \( \varepsilon \) = residual error. For each measure of DNA methylation, non-significant effects and interactions \((P > 0.2)\) were removed from the model.

### 5.4 Results

Correlation of DNA Methylation between Stimulated and Unstimulated CD 4+ T-cells

DNA methylation of CpG sites in the *IFN-γ* promoter region and *IL-4* promoter region were assessed by bisulfite pyrosequencing of unstimulated and ConA stimulated CD4+ T-cells. Isolated CD4+ T-cells were harvested at 24 hrs after ConA stimulation. Four CpG sites were assessed in the *IFN-γ* promoter region; CpG sites are denoted as base pair from transcription start site (TSS) (CpG-334, CpG-291, CpG+57, CpG+72). CpG methylation at the *IFN-γ* ranged from 30-80% averaged across the four CpG sites assessed, as shown in Figure 5.3a. Unstimulated and
stimulated CD4+ T-cell CpG methylation were highly correlated (R=0.92; p<0.001) across the four CpG sites in the IFN-γ promoter region which was assessed in this study as shown in Figure 5.2a. Four CpG sites were assessed for DNA methylation in the IL-4 promoter region (CpG-329, CpG+128, CpG+175, CpG+198). IL-4 CpG methylation across the promoter region ranged from 20-100% methylated as shown in Figure 5.3b. Unstimulated and ConA stimulated DNA methylation were moderately correlated (R=0.83, p<0.001) across the IL-4 promoter region in this study as shown in Figure 5.2b. Overall, there were no significant differences in IFN-γ and IL-4 promoter CpG methylation between unstimulated and stimulated CD4+ T-cells after 24 hours of stimulation with ConA when comparing average DNA methylation.

IFN-γ promoter methylation is lower in cows with H-CMIR phenotype

Difference in CpG methylation was observed between the 4 CpG sites in the IFN-γ promoter assessed in this study. Figure 5.3a, b shows the difference of CpG methylation between CpG sites at IFN-γ and IL-4 promoter regions (across the x-axis) and individuals (down the y-axis) as represented by yellow (0% methylation) and dark blue (100% methylation). Overall, there was 2% less methylation across the IFN-γ promoter region in CD4+ T-cells isolated from cows classified as H-CMIR than H-AMIR cows, but this was not a significant, Figure 5.4a. Individual assessment of CpG sites at IFN-γ (CpG-334, CpG-291, CpG+57, CpG+72) shows that there is significant difference in DNA methylation at CpG+57 and CpG+72. H-CMIR cows had less CpG DNA methylation at CpG +57 (-6%) and CpG +72(-8%) as shown in Figure 5.5a.

IL-4 promoter methylation is lower in cows with H-CMIR phenotype

There was large individual variation of DNA methylation at the IL-4 promoter between cows as shown in Figure 5.3b. Overall, there was no significant difference between H-AMIR and H-
CMIR cows (-1%) in average methylation across CpG sites assessed at the \textit{IL-4} promoter, Figure 5.4b. Assessment of individual CpG sites (CpG-329, CpG+128, CpG+175, CpG+198) across the \textit{IL-4} promoter region showed significant differences in DNA methylation at CpG+128 between H-CMIR and H-AMIR cows (-9%), as shown in Figure 5.5b.

\textbf{5.5 Discussion}

Epigenetic mechanisms, such as DNA methylation, can control gene expression, cell lineage and overall phenotype. Epigenetics bridges the gap between environment and an individual’s genetics providing a mechanism through which expression of genes can be fine-tuned to current environmental stimuli (Funston and Summers, 2013). Influences experienced in utero and during early life play a critical role in the development of the epigenetic phenotype (Lawson et al., 2013). Previous studies have shown that cows that are ranked as H-AMIR or H-CMIR have different cytokine profiles which are associated with DNA methylation patterns at the \textit{IFN-γ} and \textit{IL-4} promoter regions in purified CD4+ T-cells. DNA methylation is found throughout the genome on CpG motifs, these can exist individually or as part of a CpG island (Jones, 2012). Previously, Th cells isolated from H-CMIR cows were shown to produce more IFN-γ and IL-4 as compared to Th cells isolated from H-AMIR cows at 28 days after calving (Paibomesai et al., 2016a; Chapter 3). Cellular environment plays an important role in the determination of Th cell lineages. Th cell signal transduction is influenced by the strength and type of antigen stimulation, the cytokine microenvironment, and co-stimulatory factors. The cytokine microenvironment is orchestrated by the interaction of innate immune cells and antigen presenting cells, such as dendritic cells, with cells of the adaptive immune response (Collins et al., 1999; Peters et al., 2010; Zhu et al., 2010). H-CMIR may already possess an inherent immune microenvironment that promotes a particular response in circulating CD4+ T-cells, such as increased cytokine
production of IFN-γ (Martin et al., 2015; Appendix B). The cytokine environment promoted in H-CMIR cows could provide them with the mechanisms necessary to promote superior DTH reactions, protecting these cows from intracellular infections (Pinedo et al., 2009; Heriazon et al., 2009). Additionally, it has been shown previously that H-CMIR cows inherently have a larger portion of δγ T-cells, which are important mediators of the inflammatory response in the bovine species (Hine et al., 2012; Jutila et al., 2008). Hine et al. (2012) also showed that cows that were ranked as H-AMIR had additional monocytes and B-cells, which are the main contributors to AMIR responses. In addition to this, PBMC samples outside of the peripartum period were shown to preferentially produce IL-4 cytokine (Martin et al., 2015; Appendix B). In a previous study, differences in IFN-γ and IL-4 cytokine production in H-CMIR and H-AMIR were significant at three weeks after calving and therefore were sampled for further DNA methylation analysis (Paibomesai et al., 2016a; Chapter 3). Overall, the immune cellular microenvironment that has been shown in biased immune response cows could influence the ability to elicit an appropriate response, making them susceptible to particular diseases.

Numerous studies have shown how epigenetic marks can influence cell phenotypes in both normal (Dong et al., 2013; Li et al., 2013) and altered immune states, such as allergies (Canani et al, 2015). However, few studies have shown how the epigenome can influence immune response quantitative genetic traits in dairy cattle (Benjamin et al., 2016; Green et al., 2015; Walker et al., 2013; Doherty et al. 2016). In this study, we utilized previously identified immune response phenotype cows as a means of assessing DNA methylation influence on CD4+ Th cellular phenotype and the association with overall immune response traits (H-AMIR and H-CMIR). Through the use of bisulfite pyrosequencing we assessed the DNA methylation status at promoter regions of Th1 and Th2 lineage specific cytokines; IFN-γ, which is associated with
CMIR, and IL-4, which is associated with AMIR. Paibomesai et al., 2013, previously investigated IFN-γ and IL-4 promoter regions in bovine species which is comprised of 6 CpG motifs for IFN-γ (-334, -291, -220, -85, +57, +72 base pair from the TSS) and 5 CpG for IL-4 (-329, +12, +128, +175, +193 base pair from the TSS). This study assessed 4 IFN-γ promoter CpG sites CpG-334, CpG-291, CpG+57, CpG+72 and 4 IL-4 promoter CpG sites CpG-329, CpG+128, CpG+175, CpG+193 for DNA methylation.

Previously, confirmation of DNA methylation influence on IFN-γ and IL-4 transcription was investigated by in vitro skewed Th1 and Th2 cell cultures (Paibomesai et al., 2016b; Chapter 4). Interestingly, bovine DNA methylation patterns differed between cows ranked as H-AMIR and H-CMIR at key cytokine loci. H-CMIR cows had produced more cytokine which decreased in methylation status at specific CpG sites in IFN-γ and IL-4 promoter regions, but did not influence overall promoter DNA methylation. There was limited change in DNA methylation upon ConA stimulation contrary to what was previously reported by direct bisulfite sequencing at these same sites (Paibomesai et al., 2013). This could be due to the type and duration of Th cell stimulation and CpG sites that were assessed in this study compared to other species studies that show a dramatic change by 16 hours after stimulation with PMA (Li et al., 2012). The current study also utilized bisulfite pyrosequencing which is more accurate than the previously used clone bisulfite sequencing (Paibomesai et al., 2013). The current study demonstrates a high correlation between unstimulated and Con-A stimulated DNA methylation at select promoter CpG sites in IFN-γ (R=0.83) and IL-4 (R=0.92) genes. This suggests that there was very little change in DNA methylation in response to ConA stimulation when harvested at 24hrs. In this study, Th cells were only selected based upon the presence the CD4 marker and no further isolation was performed. Therefore, there is potential mixture of memory, effector and naïve CD4+T-cells of
different lineages (Th1, Th2, Th17, etc.) which exist in the samples used for DNA methylation assessment. Previous studies have shown heavily methylated promoter regions in IFN-γ and IL-4 genes in naïve CD4+ T-cells, but would require confirmation in cattle (Komori et al., 2015). Further work should include more defined CD4+ T-cell populations to determine differences in CpG DNA methylation, as cellular phenotype can have influence on overall DNA methylation of CD4+ T-cells expressing IFN-γ, IL-4 and a combination of IFN-γ and IL-4 (Deaton et al., 2014). Highly correlated methylation status between stimulated and unstimulated samples provides evidence that DNA methylation can be assessed ex vivo in cattle and provides information about the Th1 and Th2 CD4+ T-cell populations and indicates a predisposition of DNA methylation signatures before stimulation.

There is mounting evidence that underlying genetic sequence can influence epigenetic states to some extent (Gertz et al., 2011; Gibbs et al., 2010). Previous studies have shown that AMIR and CMIR IR traits correlate with differences in genotype (Thompson-Crispi et al., 2013). There is a possibility that epigenetic modifications were due to genetic variants known to already exist in dairy cattle immune response phenotype. Due to the limited population size in this study there is limited ability for the incorporation of genomic sequence information for analysis and cattle were treated as IR phenotype groups. A further study would be required to determine this interaction of genomic sequence and DNA methylation as has been eluted to in other studies in humans (Castellani et al., 2015). In previous studies, H-CMIR cows have been shown to produce higher cellular responses at both the animal and at the molecular level (Martin et al., 2015; Appendix A; Horison et al, 2009). This study expands on these data by including an analysis of DNA methylation of promoter regions for IFN-γ and IL-4 of specific IR phenotypes.
DNA methylation between H-AMIR and H-CMIR cattle did not differ based on total average CpG methylation status across IFN-γ and IL-4 promoter region. However, there is evidence that specific CpG sites were influenced by immune response phenotype status for both IFN-γ and IL-4 promoter regions. Specific CpG sites at the IFN-γ and IL-4 promoter were significantly different between H-AMIR and H-CMIR cattle such as IFN-γ CpG +57 and IL-4 CpG +72 and IL-4 CpG+128. The overall mechanism of how one CpG site can have effects at the cellular and cow level phenotype are currently unknown. Further confirmation of phenotype influence on CpG sites and larger population assessment would strengthen these data. The difference in CpG at individual sites ranged from 6-10%. Previous studies in cattle show a relationship of lipopolysaccharides (LPS) stimulation and DNA methylation status in promoter regions, gene, and intergenic regions across the genome (Benjamin et al., 2016). Benjamin et al., 2016, indicates that little difference was observed between cattle breeds (Holstein and Angus) at global methylation level and suggested that site specific CpG should be utilized to determine breed differences in DNA methylation. The current study supports these findings by Benjamin et al. (2016). The current study was unable to assess the influence of gene body and intergenic regions with the methods used, limiting the analysis to a few CpG sites per gene that were located in the promoter. Despite the limited CpG sites assessed there were still differences detected in the promoter region of IFN-γ and IL-4 genes between IR phenotype biased cattle. There is evidence in other species including plants and other mammals that show a strong link between DNA methylation and select phenotypes including occurrence of disease (Grundberg et al., 2013), other complex traits (Heyn et al., 2013), and flowering time in plants (Yaish et al., 2011). The link between phenotype and DNA methylation patterns in the genome are strong, but need further study to determine the influence in different species and different phenotypes in various
tissues. Nonetheless, this study indicates important epigenetic influence on the bovine immune system.

### 5.6 Conclusions

In summary, it was shown in this study that DNA methylation, an epigenetic mark, is associated with bovine immune response phenotypes at specific CpG locations in cytokine promoter regions. Bisulfite pyrosequencing proved to be an appropriate assay of bovine DNA methylation, providing high resolution data on CpG methylation status at specific cytokine loci in the promoter region in a cost and time effective manner. Specifically, higher IFN-γ production with in H-CMIR cows correlated with a decrease in DNA methylation at the IFN-γ promoter region when sampled at three weeks after calving. Similarly, IL-4 promoter region CpG methylation corresponded with an increase in IL-4 production by H-CMIR cows at three weeks after calving.

Further studies should be done to determine the overall influence of epigenetic marks on individual cow phenotypes, and whether this can be transmitted through generations. One of the most intriguing areas for future research will involve determining how epigenetics can be used as a tool in animal breeding.
5.7 References


Green, B. B., S. D McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.


### 5.8 Tables and Figures

**Table 5.1.** Primers and reaction conditions for Bisulfite Pyrosequencing experiments and location of CpG from TSS for IFN-γ and IL-4 gene promoter

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<tr>
<th>CpG</th>
<th>Primer</th>
<th>Biotinylated (No/Yes)</th>
<th>Primer Sequence (5’-3’)</th>
<th>Tm, °C</th>
<th>Amplicon Length (bp)</th>
<th>Bisulfite Sequence to Analyze</th>
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167
Figure 5.1

- H-AMIR Cows
- H-CMIR Cows

AMIR Residuals vs. CMIR Residuals
Figure 5.1. Representative AMIR and CMIR Ranking of heifers (n=128) from University of Guelph Research Station evaluated based on type 1 and type 2 adaptive immune response. Briefly, AMIR phenotype is evaluated based on change in antibody concentration after a 14-day type 2 test antigen challenge and phenotypic residuals calculated and displayed on the x-axis. CMIR phenotype is evaluated on delayed hypersensitivity (DTH) response at the tail fold to type 1 test antigen calculated and displayed on the y-axis. Phenotypic residuals were used to determine H-AMIR/L-CMIR (●; lower right quadrant) and H-CMIR/L-AMIR (■; upper right quadrant) IR biased cows. H-AMIR cows are 1 STD above the population mean for AMIR and 1 STD below the population mean for CMIR. H-CMIR cows are 1 STD above the population mean for CMIR and 1 STD below the population mean for AMIR. Phenotypic residuals are standardized and the population mean is represented at 0 on the x and y axis.
Figure 5.2a, b

A)

B)
Figure 5.2a,b. Correlation between *IFN-γ* (a) and *IL-4* (b) promoter regions methylation between unstimulated and ConA stimulated CD4+ T-cells harvested at 24 hours after stimulation from H-AMIR and H-CMIR cows. Spearman’s correlation are reported for both *IFN-γ* and *IL-4* promoter region. There is a high degree of correlation between unstimulated and stimulated methylation across CpG sites in the promoter regions of *IFN-γ* (R=0.92; p<0.001) and *IL-4* (R=0.83; p<0.01).
Figure 5.3a

IFN-γ Promoter

H-AMIR Cows

H-CMIR Cows

21 days after calving
Figure 5.3b

IL-4 Promoter

H-AMIR Cows

Unstimulated

ConA Stimulated

-329  +128  +175  +193  TOTAL

H-CMIR Cows

Unstimulated

ConA Stimulated

-329  +128  +175  +193  Total

3 Weeks After Calving
Figure 5.3a,b. Heatmap of $IFN-\gamma$ promoter (a) and $IL-4$ promoter (b) for unstimulated and stimulated ConA isolated CD4+ T-cells from H-AMIR (n=10) and H-CMIR (n=11) cows at 3 weeks after calving. The scale on the heatmap is as follows: yellow indicates 0% DNA methylation and black indicates 100% DNA methylation. Sections across the top represent CpG sites assessed for $IFN-\gamma$ (CpG-334, CpG-291, CpG+57, CpG+72) and $IL-4$ (CpG-329, CpG+128, CpG+175, CpG+198). Each box on the y axis represent individual cows and are grouped according to IR status, white boxes represent results that could not be determined. This diagram shows the degree of variation of DNA methylation from cow to cow and the relative contribution of each CpG site to the overall total methylation across the $IFN-\gamma$ and $IL-4$ promoter region.
Figure 5.4a

Figure 5.4b
Figure 5.4a,b. Boxplots of average DNA methylation across promoter regions for IFN-γ (a) and IL-4 (b) for H-AMIR (n=10) and H-CMIR (n=11) cows. There were no significant differences in average DNA methylation between H-AMIR and H-CMIR cows for both IFN-γ and IL-4 promoter region. Lower and upper quartiles are represented by the box and the line in the box is the media. Median values for IFN-γ were H-AMIR (52.98%) and H-CMIR (48.89%) DNA methylation of IFN-γ region. Median values for IL-4 were H-AMIR (54.43%) and H-CMIR (53.50%) DNA methylation at the promoter region.
Figure 5.5a

![Bar graph showing DNA methylation levels at different basepairs from IFNG TSS for H-AMIR and H-CMIR cows.](image)

- -334
- -291
- +57
- +72

DNA Methylation

Basepair from IFNG TSS

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<tr>
<th>H-AMIR Cows</th>
<th>H-CMIR Cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>* p ≤ 0.05</td>
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</tr>
</tbody>
</table>

Figure 5.5b

![Bar graph showing DNA methylation levels at different basepairs from IL-4 TSS for H-AMIR and H-CMIR cows.](image)

- -329
- +128
- +175
- +193

DNA Methylation

Basepair from IL-4 TSS

<table>
<thead>
<tr>
<th>H-AMIR Cows</th>
<th>H-CMIR Cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>* p &lt; 0.05</td>
<td></td>
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</tbody>
</table>

* p < 0.05
**Figure 5. 5a,b.** *IFN-γ* and *IL-4* promoter CpG DNA methylation from CD4+ T-cells isolated stimulated with ConA for 24 hours from H-AMIR (□; n=10) and H-CMIR (■; n=11) cows 21 days after calving. *IFN-γ* promoter region includes CpG -334, -291, +57, +72 from TSS and *IL-4* promoter region includes CpG -329, +128, +175, +193 from TSS. Significant differences of DNA methylation at specific CpG sites existed for both *IFN-γ* (CpG +57, CpG+72) and *IL-4* (CpG +128) between H-AMIR and H-CMIR CD4+ T-cell samples. Significant differences in DNA methylation at individual CpG sites are indicated with* p≤0.05.
CHAPTER 6

DNA Methylation Differences at Bovine $IFN-\gamma$ and $IL-4$ Cytokine Promoter Regions of CD4+ T-helper Cells around Peripartum

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CHAPTER 6

DNA Methylation Differences at Bovine IFN-γ and IL-4 Cytokine Promoter Regions of CD4+ T-helper Cells around Peripartum

6.1 Abstract

Epigenetic modifications act on the genome to fine tune cellular and individual phenotypes. The cow undergoes many physiological changes throughout pregnancy and during the peripartum period. The focus of this study was to further investigate DNA methylation at promoter regions of interferon gamma (IFN-γ) and interleukin 4 (IL-4) cytokines in dairy cattle classified based on their adaptive immune response phenotype, specifically cell- or antibody- mediated immune responses. Samples were collected 28 days prior to and 21 days after the predicted calving date. DNA methylation analysis was performed via bisulfite pyrosequencing. Both overall and CpG site specific DNA methylation at IFN-γ (CpG-334, CpG-291, CpG+52, CpG+72) and IL-4 (CpG-329, CpG+128, CpG+175, CpG+193) promoter regions were considered in this study.

There was a significant decrease in overall DNA methylation at the IFN-γ promoter region between prepartum and postpartum samples for high (H) AMIR (-4%) and H- CMIR (-9%) phenotypes. There were also individual CpG site differences at IFN-γ CpG+52 (-13%) and CpG+72 (-17%) between prepartum and postpartum samples for H-CMIR; this was not observed in H-AMIR cows. Overall, DNA methylation at the IL-4 promoter region decreased from prepartum to postpartum for both H-CMIR (-12%) and H-AMIR (-13%) phenotype cows. CpG site specific DNA methylation in the IL-4 promoter region decreased from prepartum to postpartum for both H-AMIR (IL-4 CpG+128 (-15%), CpG+175 (-23%), and CpG+193 (-11%) and H-CMIR (IL-4 CpG+175 (-24%)) phenotypes. This study represents one of the first studies to analyze DNA methylation status of CD4+ T-cells in dairy cattle across the peripartum period.
It provides evidence that DNA methylation changes in CD4+ T-cells in accordance with time from calving.
6.2 Introduction

The peripartum period represents a time of transition and increased disease occurrence for the dairy cow. Throughout this period there are numerous changes to immune function (Singh et al., 2008; Mallard et al., 1998; Fair, 2015) and metabolic status (Esposito et al. 2014; LeBlanc, 2012). Disease incident increases throughout the peripartum period, leading to an increase risk of culling for sick cows (Heise et al., 2015). Three weeks before calving to about three weeks after calving is generally considered peripartum which presents a period of immune dysfunction with changes in innate and adaptive immune cell populations, cell trafficking, and cellular function (Weikard et al., 2015; Fair, 2015). The adaptive immune response is described in two branches, the antibody-mediated immune response (AMIR) and cell-mediated immune response (CMIR). These two branches work in synergy to provide protection from extracellular and intercellular pathogens respectively, and are mediated by various cell types, such as CD4+ T-cells. CD4+ T-cells are composed of numerous phenotypes including, but not limited to, T helper (h)1, Th2, Th17, T-regulatory cells, and others (Burchholz, et al., 2016; Vahedi, et al., 2013). Th1 is associated with protection against intracellular pathogens and is associated with interferon gamma (IFN-γ) production. In contrast, Th2 is associated with protection against extracellular pathogens and is associated with interleukin 4 (IL-4) productions. Throughout pregnancy there is influence on the CD4+ T-cell phenotypes in mammals; for example, Th2 and T-regulatory cells dominate the mid- to late- trimesters, creating a bias towards an anti-inflammatory state to create a pregnancy-favourable environment (Polese et al., 2014; Fair, 2015; Oliveria, et al., 2013). Commonly in the cow, CD4+ Th cells undergo transition from Th2 bias throughout pregnancy to Th1 bias in the event of calving (Shafer-Weaver et al., 1999; Oliveria, et al., 2013). This change in CD4+ Th cell immune response may influence overall ability for the adaptive immune
response to recognize an invading pathogen, leading to immune dysfunction. The imbalance of adaptive immune responses after calving may leave an individual cow susceptible to disease. It is unknown whether epigenetic modifications influence this peripartum alteration in immune response bias.

Cows can possess different phenotypes in regards to adaptive immune responses to selected type 1 and type 2 antigens. In fact, cows can be classified based on AMIR and CMIR and ranked according to their estimated breeding values as high (H), average (A), and low (L) (Thompson-Crispi, et al., 2012a; Thompson-Crispi, et al., 2012b; Thompson-Crispi et al., 2013, Heriazon et al., 2013). Thompson-Crispi, et al. (2013) demonstrated that cows ranked with an L-AMIR and L-CMIR were more susceptible to mastitis, reproductive disease, and metabolic diseases. Previous studies have shown that an inherent immune response bias towards AMIR (H-AMIR/L-CMIR) or CMIR (H-CMIR/L-AMIR) can influence peripheral blood mononuclear cell (PBMC) cytokine mRNA transcription and cytokine production during mid-lactation (Martin et al., 2015). In addition, the production of IFN-γ and IL-4 cytokines by CD4+ T-cells from H-AMIR and H-CMIR cows is influenced by the peripartum period (Paibomesai et al., 2016a, Chapter 3).

Epigenetic mechanisms alter gene expression without a change in DNA sequence. Epigenetics represents the interface between environment and genetics, and plays a pivotal role in cell lineage, disease susceptibility and individual phenotype (Bergman and Cedar, 2012; Triantaphyllopoulos et al., 2016). There are numerous epigenetic mechanisms which contribute to control of gene expression including DNA methylation, histone modification, and microRNA. DNA methylation is a relatively stable epigenetic mark that plays a pivotal role in cellular phenotype across different tissues and cell types (Jones, 2012). DNA methylation can be maintained through generations of cells and passed down from parent cells to progeny cells, with
implications for the immune response. It has been shown the CD4+ T-cell phenotypes are influenced by epigenetic modifications, including DNA methylation (Vahedi, et al., 2013; Paibomesai et al., 2013; Paibomesai et al., 2016b, Chapter 4). Epigenetic modifications are affected by extrinsic (i.e. chemical exposure) and intrinsic signals (i.e. hormonal signaling), allowing plasticity to different environments (Triantaphyllopoulos et al., 2016, Kanno et al., 2012). DNA methylation is an epigenetic modification that has influence on DNA availability for transcription and subsequent gene expression. DNA methylation primarily occurs on cytosine-phosphate-guanine (CpG) motifs in the genome and possesses the ability to crosstalk with histone modifications that allows for methylation to influence histone modifications and histone modifications to influence methylation status (Schubeler, 2015; Du et al., 2015).

A previous study indicated that DNA methylation at specific CpG sites and average DNA methylation across at IL-4 and IFN-γ promoters differ between Th1 and Th2 cultured CD4+ T-cells. Assessment of DNA methylation in cattle that were previously immune response phenotype showed key CpG sites that differed between cattle that were biased towards either H-AMIR or H-CMIR. However, the average CpG DNA methylation across both the IL-4 and IFN-γ did not differ between cattle with different immune response phenotypes when sampled at 21 days after calving (Paibomesai et al., 2016b, Chapter 4). The focus of this study was to further investigate DNA methylation site specific changes at promoter regions of IFN-γ and IL-4 of dairy cattle classified based on their adaptive immune response phenotype, specifically H-AMIR and H-CMIR, at a prepartum time point (-28 days) and postpartum time point (+21 days). Samples were collected 28 days prior to the predicted calving date and 21 days after the calving date to compare time of sample between these immune responses classified animals. We hypothesize that DNA methylation will change from prepartum to postpartum samples with H-
CMIR cows having a greater decrease in DNA methylation for both *IL-4* and *IFN-γ* promoter regions.

### 6.3 Materials and Methods

**Animals**

Dairy cows that were used in this study were previously identified as high (H), average (A), and low (L) immune responders based on the patented immune response phenotyping protocol as described in Hine et al. (2011) (US Pat #7,258,858 Wagter-Lesperance and Mallard, 2007). Briefly, on day 0 blood samples were taken in 10mL red-top vacutainer tubes (BD, Franklin Lakes, New Jersey, USA) and cows were immunized with a 1mL of antigen preparation which contains type I and type II antigens as described previously Hine et al., 2011.

Cows with an H-AMIR and L-CMIR ranking (H-AMIR cows; n=11) and H-CMIR and L-AMIR ranking (H-CMIR cows; n=10) as identified in Paibomesai et al. (2016). All methods and animal use protocols were approved by the Animal Care Committee under Animal Utilization Protocol (AUP #04R063). Cows were housed at the University of Guelph Elora Research facility and were all first calf heifers upon study enrollment.

**CD4+ T-cell Isolation and Culture Conditions**

Blood was collected from H-CMIR (n=10) and H-AMIR (n=11) cows 28 days before and 21 days after calving in 10mL EDTA coated vacutainer blood tubes (BD, Franklin Lakes, New Jersey, USA) and inverted several times to prevent clotting. CD4+ T-cells were isolated by density gradient and MACs sorting as described in Paibomesai et al. (2016).
Isolated CD4+ T-cells were cultured for 24 hrs. in 96-wells round bottomed plates at 2.5x10^6 cells/mL in 200µL of complete RPMI-10 media – RPMI-10, 30 mg/L glutamine, 10% fetal calf serum, and penicillin/streptomycin (Invitrogen, Oakville, ON). Control cells were plated with no mitogen stimulation (unstimulated cells) and stimulated cells were plated with ConA (10mM) (Sigma, Oakville, ON). At 24 hrs. cell culture supernatant was collected, pooled and stored at -20°C for future ELISA analysis. Cells were collected and stored at -80°C for future DNA methylation analysis.

**Cytokine ELISA**

Cytokine concentrations were determined by ELISA for IL-4 and IFN-γ for all samples. Bovine IFN-γ ELISA kit (MabTech, Cinnicanti, OH, USA) was used to determine IFN-γ from collected cell culture supernatant according to the manufacturer’s instructions. Bovine IL-4 ELISA kit (Thermo-Fischer Scientific, Kanata, Ontario) was used to determine IL-4 concentrations according to the manufacturer’s instructions. All samples were run in triplicate and only those plates with CV% less than 10% were kept for final analysis.

**Bisulfite Pyrosequencing**

To evaluate DNA methylation, genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON) from cultured pre- and post-partum CD4+ T-cells isolated from with AMIR and CMIR biased cows at 28 days prior to calving and 21 days post calving, respectively. gDNA was bisulfite-treated with EpiTect Fast DNA Bisulfite Kit with 500ng of starting DNA (Qiagen, Mississauga, ON) according to the manufacturer’s instructions. Bisulfite DNA (10ng) was then subject to amplification by PCR for listed bisulfite pyrosequencing primers in Table 6.1 that were designed using PyroMark Assay Design 2.0 software, as previously described by
Paibomesai et al., 2016 (Qiagen, Mississauga, Ontario). PCR program: 95°C for 10 minutes; 45 cycles of (95°C for 30 sec, primer specific annealing temperature (Table 6.1) for 30 sec, and 72°C for 45 sec) and concluded with a final extension step at 72°C for 10 min. PyroMark PCR Kit (Qiagen, Mississauga, ON) was used in 20 µL reactions all samples with 1µL of bisulfite treated DNA (10 ng). All PCR products were run on 1% agarose gel for verification of PCR product size and amplification before subsequent pyrosequencing. PCR products was incubated with 2µL Streptavidin Beads (Qiagen, Mississauga, ON)/20µL of sample in 40µL of binding buffer (Qiagen, Mississauga, ON) for 10 minutes with shaking (300xg) in a V-bottom 96 well plate. Meanwhile, 96 well PQA plate (Qiagen, Mississauga, ON) was loaded with 0.4 µM sequencing primer/well in 40µL of annealing buffer (Qiagen, Mississauga, ON), primers are listed in Table 6.1. Vacuum manifold (Qiagen, Mississauga, ON) was used to capture streptavidin bound PCR products, briefly, vacuum manifold probes were applied to V-bottom 96 well plate containing Streptavidin bound PCR product beads for 15 seconds, vacuum manifold probes were then removed and submerged in 70% ethanol for 5 seconds, then 8µM NaOH for 5 seconds and products was washed for 15 seconds in Wash Buffer (Qiagen, Mississauga, ON). Vacuum was released and vacuum manifold probes were submerged in PQA plate with sequencing primer and binding buffer. The PQA plate was heated for 2 minutes at 80°C and before analysis on PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON).

Bisulfite pyrosequencing was performed on PyroMark ID 96 Pyrosequencer (Qiagen, Mississauga, ON). Samples were ran in triplicate and data was collected and analyzed for quality on Pyro Q-CpG software.
Data Analysis

Significant difference \((p \leq 0.05)\) between prepartum (28 days before calving) and postpartum (21 days after calving) for DNA methylation status was determined by PROC MIXED in SAS (SAS Version 9.2; North Carolina, USA). The structure of the variance-covariance matrix of repeated measures was chosen based on the lowest Akaike information criterion (AICC) (Akaike, 2014). Normality was tested using the Shapiro-Wilk test statistic. Least squares means (LSMeans) were estimated and t-test used to compare contrasts. The statistical model used was:

\[
y_{ijkl} = \mu + r_i + p_j + c_k + t_l + \varepsilon_{ijk}
\]

where: \(y\) = response vector of the observation; \(\mu\) = overall mean; \(r_i\) = immune response group (H-AMIR or H-CMIR); \(p_j\) = days to calving (-28 or +21); \(c_k\) = methylation CpG site; \(t_l\) = Treatment (unstimulated or stimulated); \(\varepsilon\) = residual error. For each measure of DNA methylation, non-significant effects and interactions \((P > 0.2)\) were removed from the model. Difference in DNA methylation between -28 days and +21 day samples was reported for H-AMIR and H-CMIR groups independently throughout the study. Only significant differences are reported \((p \leq 0.05)\) for the percent of DNA methylation for both total average methylation across the promoter regions for \(IFN-\gamma\) (CpG-329, CpG-291, CpG+57, CpG+72) and \(IL-4\) (CpG-329, CpG+128, CpG+175, CpG+193) and individual CpG sites. Only significant CpG sites are reported. \(IL-4\) and \(IFN-\gamma\) DNA methylation were analyzed separately.
6.4 Results

*IFN-γ* Promoter Region DNA Methylation Differences around the Peripartum Period

There were significant differences in total DNA methylation in samples that were collected from timepoints around the peripartum period. Due to the relationship between IR biased cows and DNA methylation status; the results will be reported according to H-AMIR and H-CMIR groups of cows. Average DNA methylation across the *IFN-γ* promoter region for both H-CMIR and H-AMIR cows was significantly less at postpartum, 21 days after calving, compared to prepartum, 28 days before calving, as shown in Figure 6.1a. Average DNA methylation across the *IFN-γ* promoter region decreased by H-AMIR (-4%) and H-CMIR (-9%) between prepartum and postpartum samples. Individual CpG sites significantly decreased DNA methylation from prepartum to postpartum for H-CMIR cows at sites *IFN-γ* CpG+57 (-13%) and CpG+72 (-17%), as shown in Figure 6.2a. In contrast, there were no significant differences in individual CpG sites of *IFN-γ* for H-AMIR cows between samples taken prepartum and postpartum, as shown in Figure 6.2a. Overall, there was substantial variation in the changes observed at individual CpG sites between individual cows as shown in Figure 6.3a.

*IL-4* Promoter Region DNA Methylation Differences around the Peripartum Period

Total average DNA methylation at the *IL-4* promoter region decreased from prepartum to postpartum for H-CMIR cows only (-11%). There was a significant decrease in DNA methylation observed in H-AMIR cows (-13%) as shown in Figure 6.1b. DNA methylation for individual CpG sites at the *IL-4* promoter differed for both H-AMIR and H-CMIR at varying sites across the *IL-4* promoter region. Individual CpG sites across the *IL-4* promoter region displayed a significant decrease in DNA methylation for H-AMIR cows at *IL-4* sites CpG+128...
(-15%), CpG+175 (-23%), and CpG+193 (-11%). H-CMIR cows had significantly less DNA methylation at \( IL-4 \) CpG+175 (-24%) in postpartum samples compared to prepartum samples, as shown in Figure 6.2b. The individual cow variation for DNA methylation at the \( IL-4 \) promoter region was diverse from cow to cow as shown in the heatmap in Figure 6.3b.

6.5 Discussion

Epigenetic markers help define cellular phenotype and can have influence on overall individual phenotype. Epigenetic modifications include, but are not limited to, DNA methylation, histone modification, and microRNA. Genetic background contributes to immune responsiveness of dairy cattle as demonstrated by Thompson-Crispi et al. 2014, but epigenetics allows the immune response to be flexible to pathogen invasion, pregnancy, aging, and environmental stressors. Previous studies have shown the relationship of epigenetic modifications with disease, phenotype, and environmental influences (Bergman and Cedar, 2012). In particular, DNA methylation represents an epigenetic mark with stability and strong inheritance from daughter cell to daughter cell, making it a target for genetic selection and for use as a biomarker (Jones, 2012).

There are limited studies of cattle on DNA methylation in CD4+ T-cells lineage, isolated from cattle. Our previous research demonstrated the association of mRNA concentration and DNA methylation status at \( IFN-\gamma \) and \( IL-4 \) promoter region, with a decrease in DNA methylation associated with increase in \( IFN-\gamma \) and \( IL-4 \) production by Th1 and Th2 cultured CD4+ Th cells (Paibomesai et al., 2016, Chapter 4). The next study in this series investigated the difference between cows that were ranked according to their adaptive IR. Paibomesai et al., 2016 (Chapter 3), demonstrated that when cattle possess either a bias towards AMIR or CMIR at 21 days after
calving there are significant differences at specific CpG DNA methylation sites in the *IFN-γ* and *IL-4* promoter region. In continuation of this line of investigation, this current study assessed DNA methylation from H-AMIR and H-CMIR around the peripartum period with samples at 28 days before calving and 21 days after calving. The objective of this study was determining differences in DNA methylation in response to the peripartum period in CD4+ Th cell populations in the dairy cow. DNA methylation may play a pivotal role in Th1 and Th2 biases through pregnancy, which is known to influence overall AMIR and CMIR function in cattle, and subsequent disease resistance (Thompson-Crispi et al., 2013).

DNA methylation was significantly different between prepartum and postpartum samples for H-CMIR cows. In contrast, there were no observed differences between prepartum and postpartum H-AMIR cows for IL-4 and IFN-γ cytokine. Previously, CD4+ Th cells from H-CMIR cows were reported to have an increase in both IFN-γ and IL-4 cytokine production at 21 days after calving when compared to their H-AMIR counterparts (Paibomesai et al. 2016, Chapter 3). This overall decrease in DNA methylation at *IFN-γ* promoter region CpG sites between prepartum and postpartum samples was associated with the increase in IFN-γ cytokine production that was observed at 21 days after calving for H-CMIR cows (Paibomesai et al., 2016, Chapter 3). This same relationship was observed for *IL-4* CpG sites in the promoter region with a decrease in DNA methylation for sites (Paibomesai et al., 2016, Chapter 3). The difference between prepartum and postpartum samples were staggering with difference reaching between 10-20% changes in DNA methylation between two samples from the same cow. This suggests that DNA methylation at the *IFN-γ* and *IL-4* promoter region may be playing a role in regulation of IFN-γ and IL-4 production and potentially overall CD4+ T-cell lineages across the peripartum period as the same relationship was observed in *IL-4* promoter region. In other species, the change in DNA...
methylation is also observed at the promoter region and is associated with differences in Th1 and Th2 cytokine production (Vahedi, et al., 2013). Temporal differences in DNA methylation in bovine fibroblasts has been investigated in the context of ageing by comparing between animals aged 5 months and at 16 months with little difference observed in total methylation of the methylome (Green et al., 2015). The current study showed temporal differences in DNA methylation of CD4+ T-cells around the peripartum period, which suggest future studies should consider the effect of stage of lactation and age on methylation data.

There are numerous mechanisms through which epigenetic modifications can be affected by both intrinsic and extrinsic factors. Changes in immune cell populations and trafficking are influenced by the endocrine system which plays a key role in pregnancy maintenance and initiation of parturition in the cow (Fair, 2015, Oliveria et al., 2013). Hormone concentrations vary throughout the peripartum period and have been shown to potentially influence immune cell phenotype (Tan et al., 2015; Fair, 2015). The most notable changes in hormone concentrations around parturition consist of estrogen, progesterone, prolactin and glucocorticoids (Fair, 2015). Paibomesai et al. (2013) investigated the influence of glucocorticoids on CD4+ T-cells isolated from mid-lactation Holsteins and showed that exposure to synthetic glucocorticoids decreased CD4+ T-cell cytokine production. For some of the H-AMIR group presented in the current study, there were profound decreases in CD4+ T-cell IL-4 production postpartum, which may indicate a sensitivity to glucocorticoids.

Progesterone has effects on the immune regulation for both the innate and adaptive immune response (Tan et al., 2015). Progesterone is produced by the corpus luteum throughout pregnancy which maintains feto-maternal tolerance and declines upon the initiation of parturition (Arck, et al., 2013). Cross-talk between the endocrine and immune systems plays a vital role in pregnancy
maintenance and successful parturition (Arck et al., 2013). Progesterone drives a Th2 basis throughout pregnancy to establish an anti-inflammatory state with an increase in CD4+ T-cell IL-4 production, decreased IFN-γ, increased Treg differentiation and decrease cell proliferation (Tan et al., 2015; Maeda, et al., 2012). Shafer-Weaver, et al. 1999, observed a shift in Th2 and Th1 effector cells throughout the postpartum period in cows, which could correspond with the change in hormonal concentration. There is evidence that hormones such as progesterone and estrogen can act through epigenetic mechanisms to regulate immune cell responses (Zhang and Ho, 2011; Stolzenberg and Champagne, 2016). Therefore, additional work should investigate the influence of hormone effects on epigenetic signatures of immune cells in the dairy cow. Metabolic status of the dairy cow can also have profound effects on immune dysfunction throughout the peripartum period, but was not monitored throughout this study (Aleri, et al., 2016; Ohtsuka, et al., 2010).

6.6 Conclusions

In conclusion, phenotype is largely defined by genetic background, but is fine tuned to the environment through epigenetic modifications. There is synergy between genetic differences and epigenetic modifications as they work in unison to establish the most appropriate response. This study provides further evidence for epigenetic differences between cows with inherent bias towards either AMIR or CMIR. The change in DNA methylation at key cytokine genes around peripartum may be associated with immune dysfunction through this transition period. CD4+ T-cells undergo numerous changes throughout pregnancy and peripartum, which may be the result of epigenetic changes due to the changing microenvironment to accommodate the pregnancy. Inherited differences in genetic background also have influence over epigenetic modifications. This study provides further insight into DNA methylation signatures in the dairy cow and how
different environmental changes from prepartum to postpartum influence CD4+ T-cells DNA methylation signatures. Further work should be done to establish DNA methylation status at other genes associated with immune response stimulation and regulation in the dairy cow and how it may fluctuate throughout the lifetime of the cow.
6.7 References


Green, B.B., S.D. McKay, D.E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics. 16(1):30


### 6.8 Tables and Figures

**Table 6.1** Primers and reaction conditions for Bisulfite Pyrosequencing experiments and location of CpG from TSS for IFN-γ and IL-4 gene promoter regions

<table>
<thead>
<tr>
<th>CpG</th>
<th>Primer</th>
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Figure 6.1a IFN-γ

![IFN-γ Bar Chart](image)

Figure 6.1b IL-4

![IL-4 Bar Chart](image)
Figure 6.1a,b. Average % CpG DNA Methylation across the IFN-γ (A) and IL-4 (B) promoter region of isolated CD 4+ T-cells from H-AMIR (□) and H-CMIR (■) cows at prepartum (-28 days) and postpartum (+21 days) time points. There were no significant differences in the average total % DNA methylation across the promoter regions of the IFN-γ promoter region decreased from prepartum samples to postpartum samples for both H-AMIR and H-CMIR. In comparison, H-AMIR and H-CMIR cows decreased DNA methylation from prepartum to postpartum samples at the IL-4 promoter. Significance differences are indicated by (---) and * (p≤0.05).
Figure 6.2a IFN-γ

% CpG DNA Methylation

-334  -291  +57  +72

H-AMIR PREPARTUM  H-AMIR POSTPARTUM  H-CMIR PREPARTUM  H-CMIR POSTPARTUM

Figure 6.2b IL-4

% DNA Methylation

-329  +128  +175  +193

H-AMIR PREPARTUM  H-AMIR POSTPARTUM  H-CMIR PREPARTUM  H-CMIR POSTPARTUM

*
Figure 6.2a,b. Percentage of CpG DNA Methylation at individual CpG motifs in IFN-γ (a) and IL-4 (b) promoter regions in isolated and ConA stimulated (24 hours) CD4+ T-cells from immune biased cows at timepoints across the peripartum period. H-AMIR (n=11) and H-CMIR (n=10) cows were selected based upon their possession of a biased adaptive immune response according to IR testing. Timepoints across the peripartum period were selected based upon the time of calving with prepartum samples collected at 28 days before the estimated calving date and postpartum samples collected at 21 days after the time of calving. DNA was collected from isolated CD4+ T-cells from H-AMIR prepartum (□), H-CMIR prepartum (■), H-AMIR postpartum (●), and H-CMIR postpartum samples (■) and assessed for DNA methylation through bisulfite pyrosequencing. IFN-γ CpG sites assessed in this study are located -334, -291, +57, and +72 base pairs (bp) from the transcription start site (TSS). IL-4 CpG assessed in this study are located -329, +128, +175, and +193 base pairs (bp) from the transcription start site (TSS). H-CMIR cows had significantly less DNA methylation than H-AMIR cows at IFN-γ CpG+72 for both prepartum and postpartum samples and IFN-γ CpG+57 in postpartum samples. There was significantly less methylation in postpartum DNA samples collected from H-CMIR cows than the prepartum samples at IFN-γ CpG+57 and CpG+72. H-CMIR cows also had significantly less CpG DNA methylation at IL-4 CpG+128 than H-AMIR cows in prepartum samples only. H-AMIR cows had significantly less DNA methylation at site IL-4 CpG+128, CpG+175, and CpG+193. H-CMIR cows had significantly less DNA methylation at IL-4 CpG+175. Significant difference between prepartum and postpartum samples by a solid line (▬). Significance is denoted by * p<0.05.
Figure 6.3a *IFN-γ*

Figure 6.3b *IL-4*
Figure 6.3a,b. Heatmap of percent DNA Methylation at CpG sites across the IFN-γ (a) and IL-4 (b) promoter region for H-AMIR (n=10) and H-CMIR (n=11) cows for the prepartum (-28 days calving) and postpartum (+21 days calving). The scale on the heatmap is as follows: yellow indicates 0% DNA methylation and black indicates 100% DNA methylation. Sections across the top represent CpG sites assessed for IFN-γ (CpG-334, CpG-291, CpG+57, CpG+72) and IL-4 (CpG-329, CpG+128, CpG+175, CpG+198). Each box on the y axis represent individual cows and are grouped according to IR status, white boxes represent results that could not be determined. This diagram shows the degree of variation of DNA methylation from cow to cow and the relative contribution of each CpG site to the overall total methylation across the IFN-γ and IL-4 promoter region.
CHAPTER 7
Synopsis and Future Directions

Epigenetics is a biological process that has gained much attention over the last decade. Epigenetics represents the intersection of environment and genetics which can have short term, lifelong and possible transgenerational influences. Epigenetics represents a large category of different mechanisms that do not directly change DNA sequence, but instead influence cellular and individual phenotype through regulation of gene expression (Petronis, 2010; Funston and Summer, 2014; Skinner et al, 2010; Heyn, et al., 2013). In recent years, there has been an invested interest researching epigenetic mechanisms as it relates to theories in human health sciences. However, there has been very limited research on epigenetics of dairy cattle health and production, with some interest in reproductive science (Su et al. 2013; Doherty et al., 2013 Walker et al., 2013; Saadi et al., 2016), milk production (Singh et al., 2010, Singh et al., 2012), and immune response (Doherty et al., 2013; Doherty et al., 2016; Green and Kerr, 2014, Paibomesai et al., 2013). Epigenetics has the potential to describe complex traits and act as the intersection of genetic sequence and environmental stimuli, and may play a key role in evolution, adaptation and disease status in mammalian species. This field is still at its infancy in livestock production science with preliminary work being completed in chickens, sheep, dairy and beef cattle (Triantaphyllopoulos et al., 2016; Feeny et al., 2014). The overall understanding of basic mechanisms and application in livestock production is still elusive, but warrants investigation as it could theoretically change how we raise and breed livestock species.

Epigenetic mechanisms, such as DNA methylation and histone modifications, have major influence on immune system cell populations and immune processes in the body. Epigenetics
plays an important part in memory and activation of response (Komori et al., 2015; Kano et al., 2014). More specifically, CD4+ T-helper (Th) cell lineages are defined and propagated by DNA methylation at cytokine specific regions (Rodriguez et al., 2015; Kanno et al; 2014). However, there is debate as to how epigenetic modifications, such as DNA methylation and histone modifications, impacts transcription and overall cell phenotype (Bestor et al., 2014).

In this thesis, the focus was on CD4+ T-cell lineages around the peripartum period in cattle. Previous studies indicate that physiological influences through pregnancy and parturition affect CD4+ Th cells lineage decision with basis towards Th1 and Th2 (Maeda et al., 2012; Shafer-Weaver et al., 1999; Ohtsuka, et al., 2010; Oliveira et al., 2013). This phenomenon has been well documented in women with preeclampsia, where changes in CD4+ Th cell populations are an indicator for pregnancy complications and preterm birth (Chaouat et al, 2003). Pregnancy is associated with a bias toward Th2 phenotype in women, which contributes to feto-maternal tolerance. In cows, there is evidence of immune dysfunction through the peripartum period with changes in cell populations, trafficking, and responsiveness (reviewed by Fair, 2015, Aleri et al., 2016). These alterations in immune function have been identified in neutrophils, CD8+ T-cells, B-cells, and CD4+ Th cells (Mallard et al., 1998; Aleri et al., 2016). CD4+ Th cell lineage bias is not as clear in the cows as has been identified in women and warrants further investigation.

There has been debate as to what influence Th cell lineage has on the health of the cow during pregnancy and after parturition, or if other immune cells have a greater effect on health throughout the peripartum period (Fair et al., 2015). Therefore, this thesis is focused on identifying those differences in cows that have inherent biases in immune response throughout this sensitive peripartum period.
The adaptive immune response is defined by two branches – CMIR and AMIR. These two branches work in synergy to provide protection from a variety of pathogens. Adaptive immune response phenotype can be determined by stimulating the system with type 1 and type 2 antigens which stimulate a CMIR and AMIR response, respectively. These measurable responses, as determined by changes in antibody concentrations and delayed type hypersensitivity response, are used to determine the estimated breeding values of individuals for these traits (US Patent #7,258,858 Wagter and Mallard 2007; Thompson-Crispi et al 2012; Heriazon et al., 2013). These traits are used to classify cattle as high, average, and low immune responders for AMIR and CMIR, based on standard deviation from the population mean and used to predict the estimated breeding value of IR. Overall, immune response phenotype is moderately heritable at \( h^2 \sim 0.2 \) (Thompson-Crispi et al 2012a; Heriazon et al., 2013; Hernandez et al., 2005). A high immune response phenotype is associated with less disease occurrence (Thompson-Crispi et al., 2012b; Thompson-Crispi et al., 2013; Pinedo et al., 2009) and improved colostrum quality (Wagter et al., 2000; Fleming et al.; 2016). Immune response phenotyping based on the HIR® technology has recently been implemented in the dairy industry to improve disease resistance to common health issue, such as mastitis, metabolic disease, pneumonia, and infection with *Mycobacterium paratuberculosis*. Overall, immune response phenotypes are moderately heritable, however AMIR and CMIR are independent traits that in some studies show slight negative correlation, meaning that selection for one IR trait may inadvertently select against the other trait (Mallard et al. 2015; Mallard and Wilkie, 1999). Therefore, breeding for IR requires a selection of both H-CMIR and H-AMIR.

The objectives of this thesis were to, 1) assess CD4+ T-cell lineage, mainly Th1 and Th2, in AMIR and CMIR biased cows around the peripartum period, 2) determine DNA methylation
patterns at *IFN-γ* and *IL-4* promoter regions in controlled *in vitro* settings, 3) determine DNA methylation patterns at *IFN-γ* and *IL-4* promoter regions for cattle classified as H-AMIR and H-CMIR, and 4) determine the temporal differences in *IFN-γ* and *IL-4* DNA methylation patterns around the peripartum period for AMIR and CMIR biased cows.

**Experimental Design**

Cows for these studies were identified as High, Average, and Low immune responders for AMIR and CMIR traits based on the patented High Immune Response (HIR™) testing system (Patent #7,258,858; Wagter-Lesperance and Mallard, 2007). Briefly, cows were immunized with known type I and type II antigens, and evaluated for antibody (IgG) concentration to the type II antigen at Day 21 after immune challenge and delayed type hypersensitivity as determined by a change in skin fold thickness at after an intradermal challenge with the type I antigen measured 24 hours after. Cows that possessed an H-AMIR/L-CMIR and H-CMIR/L-AMIR were used for the assessment of influence of immune response genetic bias of CD4+ T-cell. CD4+ T-cells were isolated by MACS sorting at -28 days, +4 days, and +21 days from calving and stimulated with ConA for 24 hours. Cell culture supernatant was harvested and ELISA was used to assess *IFN-γ*, *IL-4* and *IL-17A* concentration. DNA was isolated from CD4+ T-cell and DNA methylation was assessed by bisulfite direct sequencing and bisulfite pyrosequencing at *IL-4* and *IFN-γ* promoter regions.

CD4+ T-cells were isolated from average AMIR and CMIR cows and cultured under Th1 and Th2 biased culture conditions and were used to develop the primers for bisulfite pyrosequencing. DNA methylation results were compared to mRNA concentrations for *IL-4* and *IFN-γ* from Th1 and Th2 biased cell cultures. CD4+ T-cells isolated from H-AMIR and H-CMIR samples
collected at +21 days from calving were used to determine DNA methylation difference between IR biased groups by bisulfite pyrosequencing and cytokine concentration after ConA stimulation by ELISA. Lastly, temporal differences in DNA methylation was determined by applying bisulfite pyrosequencing to CD4+ T-cells samples from H-AMIR and H-CMIR at -28 days and +21 days from calving.

**Synopsis of Results**

Objective 1 - Results indicated that H-AMIR and H-CMIR cows had differences in CD4+ T-cell cytokine production which was most notable at 21 days after calving. Th1, Th2, and Th17 cytokine production (IFN-γ, IL-4 and IL17A) varied between 28 days pre-calving to 21 days post-calving. These results confirmed previous results that showed changes in bovine peripartum immune response, more specifically CD4+ T-cell responses (Shafer-Weaver et al., 2009; Shafer-Weaver et al., 1999; Oliveria et al., 2013; Ohtsuka et al. 2010). In general, CD4+ T-cells from H-CMIR cows produced more cytokine in the late postpartum, 21 days after calving, compared to H-AMIR cows enrolled in this study. Overall H-CMIR cows had a significant increase in both IFN-γ and IL-4 from prepartum to late postpartum period, while H-AMIR showed no change (IFN-γ) or a decrease in cytokine production (IL-4). Difference in cytokine production of CD4+ T-cells around the peripartum period was expected, but it was unexpected to find that H-AMIR cows had decreased IL-4 production in the late postpartum.

Martin et al., 2015 had previously shown that the same H-AMIR cows that were sampled in this study had produced significantly more IL-4 at mid-lactation than H-CMIR cows. The difference being that the study by Martin et al 2015, did not use purified CD4+ T-cells but rather evaluated cytokines from blood mononuclear cells.
Objectives 2- Bisulfite pyrosequencing was used to determine DNA methylation in CD4+ T-cells of IFN-γ and IL-4 both in a controlled setting, in relationship to IR phenotype, and around the peripartum period. The first study used CD4+ T-cell cultured in Th1 and Th2 skewing conditions to assess IFN-γ and IL-4 promoter region DNA methylation. This study showed the impact of specific culture conditions on IFN-γ and IL-4 transcription as well DNA methylation signatures at promoter regions. There was a relationship in DNA methylation at IFN-γ and IL-4 promoter and transcription of IFN-γ and IL-4. Specifically, IFN-γ promoter DNA methylation of CD4+ T-cells was decreased in Th1 culture conditions and increased Th2 culture conditions, while the opposite was observed for IL-4 promoter region DNA methylation. Noteworthy was the CpG site specific differences in both the IFN-γ (CpG -334 (-11%) and CpG-220 (-21%)) and IL-4 (CpG -321 (+14%) and CpG +175 (+13%)) promoter when comparing Th1 culture conditions to Th2 culture conditions. This is the first account of CpG site specific differences of DNA methylation in bovine IFN-γ and IL-4 promoter. This supports the idea that DNA methylation at promoter regions is associated with transcription of the IFN-γ and IL-4 genes.

Objective 3 - DNA methylation at CpG sites of IFN-γ and IL-4 was influenced by IR phenotype as determined by ranking animals as H, A, and L for both AMIR and CMIR traits. Additionally, there was a strong to moderate correlation of DNA methylation of IFN-γ (R=0.92) and IL-4 (R=0.83) promoter regions between unstimulated and stimulated cells. These data suggest that there was little influence of ConA stimulation on cytokine production of CD4+ T-cells. There was relatively no change in promoter methylation for IFN-γ and IL-4 in response to ConA stimulation. There was a strong correlation between ConA stimulated and unstimulated CD4+ DNA methylation of IFN-γ and IL-4 genes. Total methylation of the IFN-γ and IL-4 promoter regions did not differ between H-AMIR and H-CMIR cows. However, there were specific CpG
site differences at IFN-γ CpG +57 (6%) and +72 (17%) and IL-4 CpG +128 (+9%) as compared between H-CMIR and H-AMIR. These data indicate a relationship between DNA methylation and immune response phenotype in a CpG site specific manner rather than as a change in total methylation across the IFN-γ and IL-4 promoter gene.

Objective 4 - Assessment of DNA methylation by direct bisulfite sequencing was successful in determining differences between non-phenotyped cows sampled at 28 days prepartum and 4 days post-partum. IFN-γ and IL-4 promoter CpG sites showed differential DNA methylation based on the time of sampling. Briefly, IFN-γ increased by 9% from pre- to post-partum samples of CD4+ T-cells, alternatively IL-4 promoter region decreased by 10% in methylation. There was indication of differences in methylation in a CpG site specific manner with IFN-γ CpG -291 increasing by 10%; however, nothing was as notable in the IL-4 promoter region. Treatment of CD4+T-cell stimulated cultures with Dexamethasone (Dex), a glucocorticoid, abrogated cytokine production completely and had significant impact on IFN-γ and IL-4 promoter regions. In response to treatment, the IFN-γ promoters decreased by 18% methylation while the IL-4 promoter increased by 9%. These drastic changes in DNA methylation could be observed as either a response to the treatment of Dex and the cause of the abrogation of protein production or a consequence of transcriptional silencing.

The most substantial change in DNA methylation was observed temporally between prepartum and postpartum samples that were collected at 28 days before calving and 21 days after calving. Total DNA methylation decreased for both IFN-γ and IL-4 promoter regions by -4% and -9% for H-AMIR and H-CMIR cows respectively at 21 days after calving. DNA methylation also decreased, by -13% and -12% for H-AMIR and H-CMIR cows from prepartum to postpartum, respectively, in isolated CD4+ T-cells. There were also CpG site specific changes in IFN-γ and
IL-4 promoter regions between prepartum and postpartum samples. From prepartum to postpartum IFN-γ CpG+52 (-13%) and CpG +72 (-17%) decreased in H-CMIR cows compared to H-AMIR cows. H-CMIR cows which produced more IFN-γ in the postpartum period as compared to H-AMIR cows which had no change in IFN-γ from prepartum to postpartum.

For IL-4, there were site specific differences in cells from both H-AMIR (IL-4 CpG +128 (-15%); IL-4 CpG+175 (-23%); IL-4 CpG +193 (-11%)) and H-CMIR (IL-4 CpG+175 -24%) cows. This decrease in methylation correlated with H-CMIR cows increased IL-4 production from prepartum to post-partum, but contradicted the H-AMIR cow decrease in IL-4 production from prepartum to postpartum. Also, DNA methylation is not the only marker that can predict transcription, since there is a need for transcription factors to initiate transcription, especially in the IL-4 gene which is a part of the Th2 locus (Komori et al., 2013).

Limitations

The research presented in this thesis contributes new information to the field of bovine immunoenepigenetics, which is a field in its infancy. However, there are numerous limitations and changes that should be considered when considering this work. First, cell selection is an important aspect of epigenetic studies that should be at the forefront of any study design. In this thesis, the method of magnetic-activated cell sorting was utilized to isolate CD4+ T-cells. The efficiency of this cell sorting method was determined through flow cytometry and produced relatively pure (>99%) isolated CD4+ T-cells. The issue that arises is that these CD4+ T-cells were not analyzed any further for functional capabilities other than cytokine secretion. Further characterization of the isolated cell population is needed to look at the proportion of CD4+ memory, effector, naïve cells, Th1 and Th2 sub populations before stimulation and after stimulation. Newer methods of
Flow cytometry would have presented the opportunity to further characterize these cells as memory, effector or naïve cells, which are known to have influence on DNA methylation signatures (Deaton et al., 2014). Flow cytometry also offers the opportunity to sort out cells that were either favouring Th1 or Th2 at the time of collection. This would have strengthened the study in that the characterization of bovine Th1 and Th2 from the H-AMIR and H-CMIR cows relates to genetic predisposition *ex vivo*.

In addition to the protein data, RNA collection from the H-AMIR and H-CMIR groups may have shown a more direct relationship between methylation and transcription. At the time of sample collection, the interest laid mostly in secreted cytokines as opposed to transcription of the selected cytokine genes and how methylation relates to cell cytokine secretion. Transcription data would have presented another layer of detail to the current experimentation and should be included in future studies.

Furthermore, CD4+ T-cell stimulation was also a limitation in this study. ConA, which is a known T-cell mitogen, was purposefully selected to stimulate CD4+ T-cell to determine cell bias cytokine secretion bias within this population. Although ConA is a potent mitogen that works well to stimulate T-cells, it does not represent those pathogens that may be found in the environment experienced by the cow. ConA has been used in the characterization of transcription and protein production in H-AMIR and H-CMIR groups and outperformed phorbol ester (PMA) in the stimulation of both type 1 and type 2 IR (Martin et al., 2015) and therefore was purposed as the method to stimulate CD4+ T-cells. Therefore, the bias in cytokine concentration between H-AMIR and H-CMIR in Chapter 3 should only be consider within the cytokine (IFN-γ, IL-4, and IL-17A) being analyzed and not compared to concentrations across the cytokines examined. Furthermore, additional type 1 cytokines (IL-10, Transforming Growth Factor-β, IL-12,
Tumor Necrosis Factor-beta,) and type 2 cytokines (IL-5, IL-6, IL-10, IL-13) should be considered in future studies to further define peripartum period influence on T-cell lineage, either using RNA or secreted cytokine. The current data presented in Chapter 3 of this thesis only explains the changes around the peripartum period for IFN-γ, IL-4 and IL-17A.

Additionally, the current studies used one population of cows (n=128) that were housed at a research farm. The reasons for the limited population were convenience and to reduce the influence of environment as the purpose of this thesis was to assess peripartum influences on the cow as opposed to management differences between farms. However, this choice limited the number of animals that were available for use as H-AMIR and H-CMIR responders, particularly since these phenotypes represent a relatively small proportion of the population (17%), and therefore decreased the power of this study. Due to the new techniques used in DNA methylation analysis during this study a limited population was chosen as the researchers developed a better understanding of the methods. In retrospect, analyzing all cows that calved through the period of this study would have offered greater statistical power to the study and the ability to relate DNA methylation at the chosen promoter regions with estimated breeding values for IR traits. At least, the inclusion of average AMIR and CMIR ranked cows would have answered some of the questions around the relationship of DNA methylation and IR traits. For now, this thesis lays the groundwork for a high throughput method of looking at specific CpG methylation status and could be utilized in future studies.

Finally, DNA methylation analysis methods are ever changing in the field of epigenetics. Bisulfite pyrosequencing was chosen as the method for analysis at specific CpG sites in the genome. However, the number of CpG analyzed by this method is relatively limited as new primers need to be developed for each CpG depending on the number of base pairs between the
two sites as there is a read length maximum of 100bp. In retrospect, pairing the site-specific DNA methylation analysis with a global DNA methylation analysis would have strengthened the thesis and offered a great assessment of DNA methylation sites associated with IR phenotypes. An example of genome-wide methods for DNA methylation analysis include Reduced Representation Bisulfite Sequencing that allows for identification of differentially methylated regions in the genome, as described by Doherty et al., 2016. This paired with the quantification of methylation by bisulfite pyrosequencing would help indicate regions that are outside of the promoter region which are influencing IR phenotype. For this reason, the current studies are limited in nature as they only investigate CpG sites that are in close proximity to the promoter region and thus has limited scope in application to other areas in the genome. The studies presented in this thesis also only applies to those gene promoters that were investigated (IFN-γ and IL-4). In addition, even though the greatest of care and quality control was exercised, DNA sample quality varied between animals and time points. No doubt this had impact on the resulting bisulfite pyrosequencing quality. Additionally, DNA methylation could be influenced by culturing time and therefore not fully represent the differences that exist between H-AMIR and H-CMIR phenotypes, but rather the influences of collection, transportation, cell sorting, and culture. Nonetheless, these influences are expected to be similar among phenotypes since all cells were treated the same in these studies. CD4+ T-cells could have been collected, cell sorted and kept for subsequent DNA methylation analysis to rule out the influence of culture on DNA methylation.

**Future Directions**

There are numerous gaps that exist in the field of bovine epigenetics and how it relates to immune function in cattle. Future experiments should investigate the distribution of CpG sites in
the bovine genome and identify regions that have enhancers and how this relates to CpG methylation. Future studies should also include an assessment of transcription factors at the promoter regions and enhancers of specific cytokine genes and how this relates to immune cell differentiation and lineage decisions. Studies that evaluate how DNA methylation changes with age, environmental effects and response to antigen stimulation are also warranted in dairy cattle. Previous studies in humans and other species have shown that DNA methylation increases with age. These changes in DNA methylation can lead to difference in disease occurrence and outcome (Zampieri, et al., 2015). DNA methylation changes are not only effected by environmental stimuli, but also change according to cell lineage and tissues that are influenced by sampling technique. Therefore, future works should assess numerous cell and tissue types to start building the epigenomic roadmap for cattle of different ages and breeds. Future studies could also use methods that assess global DNA methylation to investigate differences between cell types and tissues (Reduced Representation Bisulfite Sequencing, as described by Doherty et al., 2016) as well as site specific methylation assessment by bisulfite pyrosequencing of identified sites of interest.

DNA methyltransferase (DNMT) is an important protein in the maintenance of DNA methylation, and therefore future studies could include analysis of DNA methyltransferase interaction with DNA binding proteins, histone modifications and its presence in different immune cell populations and tissues. Future studies should further investigate DNA methylation distribution across the bovine genome. It is unclear how CpG methylation status and region of the methylation can impact overall transcription of a gene. There are many correlative studies in the field of human and mouse epigenetics with very few cause and effect studies that show the direct role of DNA methylation on gene expression (Bestor et al., 2014). Future experiments in
CpG DNA methylation should consider the influence of individual methylation compared to CpG DNA methylation across the region (i.e. promoter region) on transcription and cell phenotype. Future research should also investigate the impact of other epigenetics marks (i.e. histone modifications) on bovine transcriptional regulation particularly in immune cells. Additionally, epigenetic studies could work on relating DNA methylation status with phenotype and genotype.

In conclusion, collectively the experiments conducted in this thesis will contribute new information on the influence of IR phenotype on bovine CD4+ cells during the peripartum period. These data reveal that differences in immune cell function around the peripartum period is not only influenced by periparturient effects, but is also influenced by IR phenotype and is associated with DNA methylation differences at IFN-γ and IL-4 promoter regions. A better understanding of epigenetics should improve selection of immune superior cattle and could help eliminate the influence of the peripartum period on the bovine immune system, potentially reducing disease occurrence.

Additionally, this thesis contributes to the field of bovine epigenetics and represents some of the first studies to utilize bisulfite pyrosequencing in the assessment of DNA methylation at IFN-γ and IL-4 promoter regions in the context of IR traits across the peripartum period. Although DNA methylation as it relates to IR phenotype did not have a significant impact on total DNA methylation level, site specific differences were significantly different between cows with different IR phenotypes. This may contribute in the same manner as a SNP would in genome wide association study. This has yet to be confirmed. There were notable differences in DNA methylation across the peripartum period, suggesting that temporal changes in environment will have the greatest influence on the epigenome of bovine CD4+ cells. The challenge will come in
identifying epigenetic modifications that are transient, span the lifetime of animal, and those that are transgenerational and what contribution they each have in shaping an animal’s phenotype. Anticipation exists in the livestock industry as they await the introduction of epigenetic information for use in animal breeding, management and health. There is a strong need for further research to be done in the realm of epigenetics in livestock production systems. Epigenetic markers could prove to be a powerful tool in determining cell lineage, but needs to be thoroughly investigated in terms of regulation and functionality to be useful. In regards to animal breeding, epigenetics has potential to further the accuracies and describe the “missing heritability” in livestock breeding programs.
References


Green, B. B., S. D McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.


**APPENDIX A**

**Table S1.** Descriptive statistics of average CpG DNA Methylation across the IFN-γ promoter region.

<table>
<thead>
<tr>
<th>Days from Calving</th>
<th>IR Status</th>
<th>Treatment</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>H-AMIR</td>
<td>Stimulated</td>
<td>57.88</td>
<td>4.87</td>
<td>51.36</td>
<td>57.32</td>
<td>67.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unstimulated</td>
<td>57.14</td>
<td>5.02</td>
<td>49.00</td>
<td>57.06</td>
<td>64.12</td>
</tr>
<tr>
<td></td>
<td>H-CMIR</td>
<td>Stimulated</td>
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<td>5.26</td>
<td>49.93</td>
<td>52.33</td>
<td>64.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unstimulated</td>
<td>58.90</td>
<td>4.71</td>
<td>49.51</td>
<td>59.80</td>
<td>64.72</td>
</tr>
<tr>
<td>+ 21 Days</td>
<td>H-AMIR</td>
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<td>33.46</td>
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<tr>
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<td>Unstimulated</td>
<td>53.90</td>
<td>9.36</td>
<td>37.44</td>
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<td>68.32</td>
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<td>Stimulated</td>
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</table>
Table S2. Summary statistics of DNA methylation at the IFN-γ promoter region for immune response biased groups at 3 weeks after calving from isolated CD4+ T-cells stimulated with ConA for 24 hours.

<table>
<thead>
<tr>
<th>Immune Response Phenotype</th>
<th>CpG Site (bp from TSS)</th>
<th>Treatment</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-AMIR</td>
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<tr>
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<td></td>
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<td>5.00</td>
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<td>40.61</td>
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<td>84.46</td>
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<td></td>
<td>Unstimulated</td>
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<td>64.72</td>
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Table S3. Summary statistics of DNA methylation at the IL-4 promoter region for immune response biased groups at 3 weeks after calving from isolated CD4+ T-cells stimulated with ConA for 24 hours.

<table>
<thead>
<tr>
<th>Immune Response Phenotype</th>
<th>CpG Site (bp from TSS)</th>
<th>Treatment</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
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<td>ConA</td>
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<td>6.32</td>
<td>44.33</td>
<td>51.44</td>
<td>61.94</td>
</tr>
</tbody>
</table>
APPENDIX B

Short Communication: Cytokine profiles from blood mononuclear cells of dairy cows classified with divergent immune response phenotypes

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9.1 Abstract

Genetic selection for enhanced immune response has been shown to decrease disease occurrence in dairy cattle. Cows can be classified as high (H), average (A) or low (L) responders based on antibody-mediated (AMIR), predominated by type-2 cytokine production, and cell-mediated immune response (CMIR) through estimated breeding values for these traits. The purpose of this study was to identify in vitro tests that correlate with in vivo immune response phenotyping in dairy cattle. Blood mononuclear cells (BMC) isolated from cows classified as H-AMIR and H-CMIR through estimated breeding values for immune response traits were stimulated with Concanavalin A (ConA) and gene expression, cytokine production and cell proliferation was determined at multiple time points. A repeated measures model which included the effects of immune response group, parity, and stage of lactation, was used to compare differences between immune response phenotype groups. H-AMIR cows produced more IL-4 protein than H-CMIR cows at 48 hours, however there was no difference in gene expression of type-2 transcription factor gata3 or IL-4. BMCs from H-CMIR cows had increased production of IFN-γ protein at 48, 72, and 96 hours compared to H-AMIR animals. Further, H-CMIR cows had increased expression of the IFN-γ gene at 16, 24, and 48 hours post-treatment with ConA, although expression of the type-1 transcription factor gene tbx21 did not differ between immune response groups. Although proliferation of BMCs increased from 24-72 hours post ConA stimulation, no differences were found between the immune response groups. Overall, stimulation of H-AMIR and H-CMIR bovine BMCs with ConA resulted in distinct cytokine production profiles according to genetically defined groups. These distinct cytokine profiles could be used to define disease resistance phenotypes in dairy cows.
according to stimulation *in vitro*, however other immune response phenotypes should be assessed.

**9.2 Short Communication**

Genetic selection using measurable phenotypic immune response (IR) traits has been proposed as a candidate for improving the overall health of livestock (Mallard et al., 2015, 2011; Thompson-Crispi et al., 2012a). In dairy cattle, the selection for increased milk production traits has been associated with increased occurrence of both metabolic and infectious diseases (Fleischer et al., 2001; Hagiya et al., 2014; Koeck et al., 2013; Pritchard et al. 2013; Van Dorp et al., 1998) while also having a negative effect on reproduction (Pryce et al., 2004). Recent research indicates that dairy cattle can be classified as high (H), average (A) or low (L) responders based on their type-1 cell-mediated immune response (CMIR) and type-2 antibody-mediated immune response (AMIR) through estimated breeding values (EBV) (Heriazon et al., 2011; Hine et al., 2012; Thompson-Crispi et al., 2013). AMIR and CMIR have been demonstrated to be heritable, indicating it is possible to select for improved immune responsiveness (Heriazon et al., 2013; Thompson-Crispi et al., 2012b). Cows possessing a robust or H-immune response have a decrease in disease occurrences for both metabolic and infectious diseases (Thompson-Crispi et al., 2012a; Thompson-Crispi et al., 2013). Therefore, since these branches of the immune response provide protection against diverse pathogens, selection for robust and balanced CMIR and AMIR is expected to confer broad-based disease resistance to a range of pathogens (Abdel-Azim et al., 2005; Pinedo et al., 2009; Thompson-Crispi et al., 2012a). In mammals, AMIR and CMIR are both genetically (Chaudhri et al., 2004, Filbey et al., 2014, Thompson-Crispi et al., 2012b, Crean et al., 2005) and epigenetically regulated (Paibomesai et al., 2013, Scharer et al., 2013, Martino et al., 2011).
CMIR are typically mounted to protect against intracellular pathogens. Representing the cellular responses, CMIR is predominated by the expression of inflammatory cytokine such as IFN-γ and IL-12, which is controlled by the transcription factor Tbet. AMIR typically control and protect the host from extracellular pathogens (Estes and Brown, 2002; Zhu et al., 2010). GATA binding protein 3 (GATA3) is a T\(_H\)2 transcription factor which promotes production of AMIR cytokines, such as interleukins 4 (IL-4), 5 (IL-5) and 13 (IL-13). AMIR and CMIR are negatively correlated and there is evidence that these mechanisms are antagonistic to one another (Kanno et al., 2012, Edwards, 2014). Thus, the mechanisms of disease susceptibility of an individual could be explained, at least in part, through differences in cytokine profiles and transcription factor expression of T\(_H\) cells and BMC.

The purpose of this study was to investigate if there is a correlation between genetic parameters for immune response using \textit{in vitro} testing methods to develop a rapid immune response test. In the current study, cows are classified by assessment of overall AMIR and CMIR to a representative of an immune challenge and ranked based on IR-EBVs. The groups are defined as having a H-AMIR and a L-CMIR (H-AMIR) or H-CMIR and L-AMIR (H-CMIR) as contrasting phenotypes. Individuals expressing these phenotypes were then used to investigate potential of using in vitro challenge of BMC to determine adaptive immune response differences based on genetically defined groups. Immune responses were assessed by evaluating cytokine production by BMC and ability of BMC to proliferate.

Dairy cattle used in this study were previously categorized as H, A or L for AMIR and CMIR by assessing responses to type-1 and type-2 test antigens using the HIR technology (US Pat. No. 7258858) (Hine et al., 2012; Wagter and Mallard, 2007). Briefly, 128 cows were immunized intramuscularly at Day 0 with both a type-1 and a type-2 test antigen. Blood samples were
collected at Day 0 and Day 14 to evaluate antibody response to the type 2 test antigen by ELISA. To assess CMIR, a DTH test using the type-1 test antigen was performed in the caudal fold of the tail. Estimated breeding values were determined for both AMIR and CMIR. Cows were ranked based on distance from the population mean; cows are considered H-IR at 1 standard deviation (STD) above the population mean while L-IR are 1 STD below, see Figure S.1. All experimental procedures used in the study were approved by the University of Guelph Animal Care Committee under the guidelines of the Canadian Council on Animal Care. All animals showed no signs of clinical disease at the time of classification and at the time of sampling.

Blood (100ml) was collected from the coccygeal vein into EDTA containing tubes (BD Vacutainer, Franklin Lakes, NJ) and overlaid on Histopaque 1077 (Sigma-Aldrich, Oakville, ON) as per the manufacturer’s instructions. Following centrifugation (400 × g, 30 minutes), BMCs were collected and washed twice with PBS (300 × g, 13 minutes). BMC pellets were then re-suspended in supplemented RPMI 1640 (Pathobiology Media Supply, Guelph, ON) containing 1% penicillin streptomycin (Pathobiology Media Supply, Guelph, ON), 10% fetal bovine serum (Gibco, Burlington, ON), 2mM L-glutamine and 50uM B-mercaptoethanol (Sigma-Aldrich, Oakville, ON). Viable cells were stained with tryphan blue (Sigma- Aldrich, Oakville, ON) and quantified using a hemocytometer. Cells were diluted in supplemented RPMI to a final concentration of 2.5 x 10⁶ cells/mL. For mRNA and cytokine protein quantification, 1mL of BMCs were seeded onto Costar 24-well flat-bottom plates (Corning, New York, USA) in quadruplet for each time point (pooled after harvesting to show average gene expression and protein production). For BMC proliferation, 200uL of cells were plated onto Costar 96-well flat-bottom plates (Corning, New York, USA) in sextet. BMCs were either treated with 5ug/mL ConA (Sigma-Aldrich, Oakville, ON) or remained untreated to act as controls. Cell culture
supernatants were collected at 24, 48, 72 and 96 hours post-stimulation and stored at -20°C until analyzed for cytokine production. Cell proliferation was measured at 24, 48, 72 and 96 hours post-stimulation.

IFN-γ and IL-4 protein concentrations in cell culture supernatants were determined using the bovine IFN-γ ELISA (Mabtech, Cincinnati, OH) and bovine IL-4 ELISA (Thermo Fischer Scientific, Nepean, ON) kits according to the manufacturer’s instructions. The IFN-γ ELISA (Mabtech, Cincinnati, OH) detection range was 5-500 pg/mL and the IL-4 ELISA detection range was 16-1000 pg/mL. Triplicates from all time points for each individual animal were run on the same plate. Numbers of samples from H-AMIR and H-CMIR animals were balanced on each plate. Plates were read using a scanning multi-well plate reader (Biotex Powerwave XS2, Winooski, VT, USA). Samples were repeated if the coefficient of variation between replicates were greater than 10%. Cellular RNA was extracted from freshly cultured BMCs at 4, 16, 24 and 48 hours post-stimulation using 1mL TRIzol per well (Invitrogen, Burlington, ON) and stored at -80°C until analyzed for gene expression. RNA was extracted from TRIzol as per manufacturer’s protocol. Isolated RNA was treated with the Turbo-DNase free system (Ambion, Burlington, ON), as per the manufacturer’s instructions. cDNA was prepared from 500 ng RNA using the Superscript III First Strand cDNA synthesis system (Invitrogen, Burlington, ON), as per the manufacturer’s instructions. cDNA was stored at -20°C prior to quantitative real-time PCR (qPCR) analysis. Primers for qPCR (Table S2.1) were designed using Primer3 primer design (NCBI) and Secondary Structure Estimation software (Integrated DNA Technologies, Coralville, Iowa) and target product sizes validated by running on a 2% agarose gel (data not shown). qPCR was performed on the Roche Light Cycler 480 II in 384-well plates (Roche, Mississauga, ON). Gene expression values were calculated using the PFAFFL fold change.
equation (Pfaffl, 2001). Expression is represented as the difference or fold change between stimulated and unstimulated samples. These values are normalized based on the expression of the internal reference gene, β-actin, which was determined to be stably expressed in bovine BMCs in this study and others (Meade et al., 2006; Robinson et al., 2007; Spalenza et al., 2010).

Proliferation was quantified using the WST-1 cell proliferation assay kit (Roche, Mississauga, ON) according to the manufacturer’s instructions. OD values were read on the BioTex Powerwave XS2 multi-well plate reader (Winooski, VT, USA). The proliferative stimulation index was calculated by subtracting the average of the media controlled wells from the average of the six ConA stimulated and dividing by the average of the six unstimulated wells at each time point for each animal. Data were analyzed independently using a General Linear Model that included repeated measures using PROC MIXED (SAS Version 9.2; SAS Institute, Cary, North Carolina, USA). The statistical model used was:

\[ y_{ijkl} = \mu + r_i + t_j + p_k + s_l + \varepsilon_{ijkl} \]

where: \( y \) = response vector of the observation; \( \mu \) = overall mean; \( r_i \) = immune response group (H-AMIR or H-CMIR); \( t_j \) = time post stimulation (24, 48, 72, and 96 hours); \( p_k \) = parity (0, 1 or 2); \( s_l \) = stage of lactation (1 = not lactating, 2 = 1-80 DIM, 3 = 81-235 DIM, 4 = >235 DIM) \( \varepsilon \) = residual error. For each measure of immune response, non-significant effects and interactions (\( P > 0.2 \)) were removed from the model.

The current study revealed different patterns of production of type-1 and type-2 immune response mediators between genetically defined H-AMIR and H-CMIR cows. H-CMIR cows had greater gene expression and protein production of the type-1 cytokine IFN-γ, while H-AMIRs produced more IL-4 protein, a type-2 cytokine, in response to in vitro lymphocyte
simulation by ConA. Although this study does not investigate direct cause and effect; susceptibility to disease has been related to cytokine profiles in other species (Chaudhri et al., 2004; Flibey et al., 2014; Lehmann et al., 2000; Mortenson et al., 2004; Wang et al., 2014, Ottenhoff et al., 2002). Therefore, it can be speculated from the current findings that cows that display a biased response to type 1 and type 2 specific antigens and possess differing cytokine profiles may also differ in responses to intracellular and extracellular pathogen infections. Potentially, the selected in vitro immune response challenge tests could be used to identify immune response phenotypes.

H-CMIR cows had significantly greater IFN-γ protein production at 48 (p<0.05), 72 (p<0.05) and 96 (p<0.01) hours post-stimulation compared to H-AMIR cows (Figure S.2A), with highest production at the 72 hours. Expression of the type-1 cytokine IFN-γ was greater in the H-CMIR group at 16, 24 and 48 (p<0.05) hours than H-AMIR cows, with expression of the gene increasing before detection of the cytokine in cell culture supernatant (Figure S.3C). In contrast, expression of IFN-γ in H-AMIR cows decreased from 4 to 16 hours post-stimulation (p<0.05), but remained constant in H-CMIR group at the same peak time points. The expression of tx21 was increased relative to control samples at the 4-48 hour time points, peaking at 4 hours post-stimulation (Figure S.3A), however there was no significant differences in tx21 expression between the groups (Figure S.3A). Mice with aberrant IFN-γ cytokine production are more susceptible to viral infections with increased morbidity and mortality with infection rates twice that of their resistant counterparts which had normal IFN-γ production (Chaudhri, et al., 2004).

Conversely, H-AMIR individuals had greater production of IL-4 protein than H-CMIR cows at 24, 48 and 72 hours post-stimulation, although the difference observed was only significant (p<0.05) at 48 hours (Figure S.2B). No difference in expression of the type-2 cytokine gene IL-4
was observed between H-AMIR and H-CMIR. Expression of the type-2 transcription factor gata3 was unaltered in response to ConA stimulation in both H-AMIR and H-CMIR individuals (Figure S.3B). In comparison to type 1 cytokines, altered production of type 2 cytokines can increase susceptibility to extracellular pathogens, such as helminth or bacterial infections (Filbey, et al, 2014; Bruno et al., 2010). The elevated production of IL-4 in H-AMIR cows may help to provide protection of those cows to extracellular infections, such as those that commonly cause mastitis, as they would potentially possess a greater antibody mediated immune response. Thompson-Cripsi et al., (2013), reported that cows with L-AMIR have higher incidence of mastitis caused by Escherichia coli, Staphylococcus aureus, Streptococcus spp., and other bacterial species compared to H-AMIR cows, while no significant difference were found with the CMIR trait.

Tbet and GATA3 transcription factors are known to inhibit the expression of each other through negative regulatory feedback pathways, leading to the differentiation of T cells into divergent lineages (Kanhere et al., 2012). Here, stimulation with ConA led to increased expression of tbx21 (Figure S1), which may have contributed to the inhibition of gata3 expression and suggests that ConA is a mitogen that preferentially activates type-1 responses in BMCs. Regardless of this inhibition, and a subsequent lack of IL-4 mRNA accumulation, H-AMIR individuals produced more IL-4 protein than their H-CMIR counterparts (Figure S.3D). Tbet could be playing a pivotal role in inhibiting Th2 differentiation through molecular competition that would not be detected with the techniques used in the current study. Downstream events following gata3 and IL-4 expression may lead to the divergent AMIR phenotypes, although little research thus far has focused on the mechanisms of IL-4 mRNA stability and subsequent production and secretion of IL-4. Given the diverse cell population found in BMC samples, it is possible that the
difference between H-CMIR and H-AMIR was too minor to detect. Isolation of specific CD8+ and CD4+ T-cells from the BMC population would offer higher resolution of *Tbet* and *gata3* expression.

There is evidence in various species that the ability of the host to produce appropriate AMIR and CMIR cytokine profiles can cause differences in disease susceptibility in connection to their cellular responses. Alteration in the hosts ability to produce, secrete and recognize IFN-γ is associated with disease susceptibility (Ottenhoff et al, 2002) and disease outcome as shown in mice (Lehmann et al., 2000) and cattle (Bannerman et al., 2004; McGill et al., 2014). In cattle a decrease in IFN-γ production has been associated with increase susceptibility to *Mycobacterium bovis* (McGill et al., 2014), *Mycobacterium avium paratuberulosis* (Pinedo et al. 2009), intramammary infections of *S.aureus* (Bannerman et al. 2004, Kim et al., 2011) and *E.coli* (Bannerman et al., 2004). These differences in disease incidence could at least partly be due to genetic or epigenetic factors that control type 1 and 2 cytokine production either through difference in protein production or transcription factor binding. The divergent cytokine profiles observed in this study suggest that distinct type 1 and type 2 responses are important in regulating adaptive immune responses in bovine BMCs from H-AMIR and H-CMIR dairy cows and could be used as a method for identifying immune response biases.

Leukocyte populations differed between the H-AMIR and H-CMIR cows and are influenced by age and pregnancy status (Hine et al, 2012). H-AMIR animals have proportionally higher B-cells both before and after immunization. In contrast, H-CMIR cows had greater proportion of γδ T-cells than did H-AMIR animals prior to immunization, possibly influencing polarization of responses. On the basis of different leukocyte populations in these diverse H-AMIR and H-CMIR phenotypes, it was hypothesized that proliferation of subsets of BMCs may vary between
immune response groups. Proliferation of total BMCs in response to ConA treatment did not
differ between H-AMIR and H-CMIR individuals, suggesting that immune response phenotype
is largely not influenced by the ability of BMCs to expand in response to immune stimulation
with a mitogen. Proliferation of specific subsets of BMCs in response to ConA stimulation was
not examined in the current study.

Collectively, these data indicate that upon in vitro mitogen stimulation, lymphocytes from the H-
AMIR and H-CMIR phenotype cows have different type-1 and type-2 cytokine production
profiles. However, differential proliferation of BMCs in response to stimulation was not
observed between the immune response groups. H-AMIR animals had increased production of
the type-2 cytokine IL-4, while H-CMIR individuals exhibited both increased production of the
type-1 cytokine IFN-γ transcripts as well as IFN-γ protein in response to stimulation, indicating
that the H-AMIR and H-CMIR phenotype animals may have inherent biases in their cytokine
production responses to a general stimuli, such as a mitogen. Differences between IR groups are
most apparent at the cytokine protein level and could play a role in IR phenotype and disease
susceptibility in dairy cattle. This study provides evidence for methods in determining immune
competency which can be completed within 24 hours of sampling based on peak expression of
cytokine genes and cytokine secretion. The use of a mitogen in this study stimulated cell
proliferation and cytokine production within 24 hours of stimulation, but the tested mitogen
could be eliciting a bias in the response, therefore other antigens and mitogen should be tested to
confirm results. In vitro immune response testing is appealing when determining immune
response phenotypes as it is more rapid than the current two week test, less labor intensive on
farm, and less invasive. The current study only tested cows with extreme immune response
biases to determine the feasibility of using an in vitro test for immune response ranking. Future
studies would include a larger population which include dairy cows which possess an average immune response phenotype for both AMIR and CMIR. Nonetheless, it is likely that differentiating between closely related IR phenotypes of dairy cows solely with these in vitro methods would be difficult and still require cow side IR phenotyping.
9.3 References


Heriazen, A., K. A. Thompson, B. N. Wilkie, W. Mathes-Sears, M. Quinton, and B. A. Mallard. 2009. Antibody to ovalbumin and delayed-type hypersensitivity to Candida albicans and


### Table S2.1 Primer design and reaction conditions for real time RT-PCR gene expression experiments

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Sequence 5’-3’</th>
<th>Amplicon length (bp)</th>
<th>T_aC°1</th>
<th>PCR slope</th>
<th>PCR efficiency²</th>
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<tr>
<td><em>tbx21</em></td>
<td>T-box 21</td>
<td>F:CGAGGACTATATATACTGCCGC</td>
<td>133</td>
<td>61</td>
<td>-3.480</td>
<td>1.938</td>
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<tr>
<td></td>
<td></td>
<td>R:CAAGACCACGTCACATAC</td>
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<tr>
<td><em>gata3</em></td>
<td>GATA binding protein 3</td>
<td>F:CCAGACCAGAAACCGAAAAA</td>
<td>234</td>
<td>62</td>
<td>-3.134</td>
<td>2.085</td>
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<tr>
<td></td>
<td></td>
<td>R:ACCATACTGGAAGGGGTGG</td>
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<tr>
<td><em>IFN-γ</em></td>
<td>Interferon-γ</td>
<td>F:TATCTCAGGGGCCAACTAGG</td>
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<td>-3.432</td>
<td>1.956</td>
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<td></td>
<td></td>
<td>R:CTGAAGCGCCAGGTATAAAGG</td>
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<tr>
<td><em>IL-4</em></td>
<td>Interleukin-4</td>
<td>F:CAGTGCTGGTCTTTACTG</td>
<td>338</td>
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<td>-3.428</td>
<td>1.958</td>
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<td>R:CAAGAGGTCTTTTCAGCGTAC</td>
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<td><em>actb</em></td>
<td>β-actin</td>
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<td>R:ACTTGGGAATGCTCGATCC</td>
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¹Annealing Temperature

²Determined using quintet dilution series
AMIR and CMIR phenotypic residuals of cows (n=128) evaluated based on *in vivo* immune response to type 1 and type 2 antigens. Briefly, AMIR phenotype was based on increase in antibody concentrations from day 0, time of inoculation, to day 14 after administration of type 2 test antigens. CMIR phenotype was based on delayed hypersensitivity (DTH) response at the tail fold to type 1 test antigen compared to a PBS control. Phenotypic residuals were used to determine H-AMIR/L-CMIR (▲) and H-CMIR/L-AMIR (■) groups for this study; the other immune response groups (○) are represented in this diagram but were not included in the molecular analysis. H-AMIR cows are 1 STD above the population mean for AMIR and 1 STD below the population mean for CMIR. H-CMIR cows are 1 STD above the population mean for CMIR and 1 STD below the population mean for AMIR. Phenotypic residuals are standardized and the population mean is represented at 0 on the x and y axis.
Figure S2. IFN-γ (A) and IL-4 (B) cytokine concentration (pg/mL) in supernatant from cultured H-AMIR (□, n=8) and H-CMIR (■, n=7) BMC stimulated with ConA (5µg/mL). Concentrations were determined by sandwich ELISA and are reported as least squared means +/- SEM. * p<0.05. ** p≤0.01.
Figure S2.3a-d. Fold change expression of *tbx21* (A), *gata3* (B), *IFN-γ* (C), and *IL-4* (D) genes in H-CMIR (□, n=7) and H-AMIR(Δ, n=8) groups post ConA (5µg/mL) stimulation reported as least squared means +/- SEM at 4, 16, 24 and 48 hours. Fold change expression is reported relative to unstimulated samples, normalized to the internal reference gene *βact*. * p<0.05. ** p≤0.01.
Figure S2.4. H-AMIR (□, n=7) and H-CMIR (Δ, n=8) BMC proliferative stimulation index (stimulated absorbance/ unstimulated absorbance) post-ConA treatment (5µg/mL) as determined by the WST-1 assay.