Differences in TLR2 pattern recognition receptor expression on blood mononuclear cells with and without ligand stimulation among dairy cattle classified by estimated breeding value (EBV) of adaptive immune response

by

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ABSTRACT

Differences in TLR2 Pattern Recognition Receptor Expression on Blood Mononuclear Cells with and without Ligand Stimulation Among Dairy Cattle Classified by Estimated Breeding Value (EBV) of Adaptive Immune Response

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University of Guelph, 2017

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This thesis investigated bovine toll-like receptor 2 (TLR2), an innate pattern recognition receptor (PRR), on bovine blood mononuclear cells (BMC). BMC were evaluated from dairy cattle classified into immune response (IR) groups based on their estimated breeding values (EBV) for antibody-mediated (AMIR) and cell-mediated (CMIR) IR. The first study investigated ex-vivo TLR2 expression by IR group for AMIR and CMIR traits separately. Cattle with High (H)-AMIR had a higher percentage of cells expressing TLR2 in contrast to Average (A)-AMIR and Low (L)-AMIR groups. The second study investigated TLR2 expression ex-vivo among cattle classified into 5 IR groups based on AMIR and CMIR traits combined. For both ex-vivo studies, CD14+ monocytes had the highest percentage of cells expressing TLR2 among BMC. Cows classified in the H-AMIR/H-CMIR group had the highest TLR2 median fluorescence intensity (MFI) of all IR groups and was significantly higher compared to A-AMIR/A-CMIR cows. The third study evaluated in-vitro TLR2 expression on BMC and subsets following culture with TLR2 ligands. After 18h of culture, the percentage of cells expressing TLR2 increased among all BMC and was highest for BAQ155A+ B cells compared to other subsets. Variation in TLR2 MFI expression induced by PAM3CSK4 and Man-LAM could be explained by pregnancy or parity fixed effects or their interaction. The fourth study evaluated in-vitro TLR2 expression and cytokine production in the context of IR group. H-AMIR/H-CMIR cows had significantly higher MFI for TLR2 expression compared to H-AMIR/L-CMIR cows following culture with PAM3CSK4 or LTA. For the CD14+ cell subset, MFI was highest for the H-AMIR/H-CMIR group compared to all other IR groups following culture with PAM3CSK4.
The pattern of TLR2 MFI expression for CD14+ monocytes was different for Man-LAM in that both the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups had significantly higher expression compared to other IR groups. IL-1β was negatively associated with TLR2 expression in that the highest IL-1β was observed with A-AMIR/A-CMIR cows and was lowest for H-AMIR/H-CMIR and L-AMIR/L-CMIR cows. The higher expression of TLR2 on BMC and associated cytokine expression among cattle that rank high for both AMIR and CMIR may contribute to enhanced adaptive IR and disease resistance.
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I declare that the work presented in this thesis was completed by me with periodic technical assistance from:

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for the TLR2 receptor studies
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LIST OF ABBREVIATIONS

AMIR - antibody-mediated immune response
cM - classical macrophage (CD14+CD16-)
Dectin-2 - transmembrane protein of the C-type lectin family
DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin, CD209
DTH - delayed type hypersensitivity
CMIR - cell-mediated immune response
EBV - estimated breeding value
ELISA - enzyme-linked immunosorbent assay
FACS - fluorescence-activated cell scan
FITC - fluorescein isothiocyanate
FSL-1 - fibroblast stimulating lipopeptide 1
HIR - high immune response
HIR™- high immune response technology
HuCaL - human combinatorial antibody library
intM - intermediate macrophage (CD14+CD16+)
IR - immune response
IR phenotype - immune response phenotype
KAB - knowledge, attitudes, and behaviour
LAM - lipoarabinomannan
LIR - lactational incidence risk
LPS - lipopolysaccharide purified from Escherichia coli J5 (0111:B4) or other Gram- organisms
LTA - lipoteichoic acid purified from S. aureus
MAN-LAM - mannose-capped- or mannosylated- lipoarabinomannan
MR - mannose receptor, member of C-type lectin family
MUT-LPS – lipopolysaccharides from Escherichia coli J5 (Rc mutant, rough strain)
cM - non-classical macrophage (CD14+CD16+)
pNPP - p-nitrophenyl phosphate substrate
PAMP - pathogen associated molecular pattern
PAM3CSK4 - Pam3CysSerLys4 - synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins
PBS - phosphate buffered saline
PE-TR - phycoerythrin-Texas Red
PRR - pattern recognition receptor
ROI - reactive oxygen intermediates
NOI - nitric oxide intermediates
TLR - toll-like receptor
TMB - 3,3',5,5'-tetramethylbenzidine substrate
TRIS - Tris(hydroxymethyl)aminomethane
INTRODUCTION

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PRRs) on the surface of epithelial cells and some leukocytes that bind to pathogen associated molecular patterns (PAMPS) of pathogens. A series of events occurs that transduces a signal to the nucleus of the cell, resulting in expression of proinflammatory cytokines that attract cells such as neutrophils, macrophages and dendritic cells that also express TLRs on their cell surface. Macrophages and dendritic cells, under the influence of TLR signaling and cytokines, act as antigen presenting cells, activating T helper cell sub-populations. T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune response (CMIR) to respond to intracellular pathogens such as *Mycobacterium avium* subsp. *paratuberculosis*, while T helper 2 (Th2) cells produce cytokines that tend to generate an antibody-mediated immune response (AMIR) towards extracellular pathogens such as *Escherichia coli*, one of the pathogens known to cause mastitis. Thompson-Crispi et al. (2013 and 2012) reported that dairy cattle classified by their estimated breeding value (EBV) for high antibody-mediated immune response (AMIR) and high cell-mediated immune response (CMIR) phenotype have less clinical mastitis, as well as less severe mastitis. That a reduced occurrence of disease is observed in animals classified as high for AMIR or CMIR immune response (IR), may relate to the nature of the innate response on first exposure to pathogens. As the innate response includes proteins such as enzymes and complement, various innate cells, and many different types of PRRs on and within cells, the exact combination of innate mechanisms required to signal and initiate an adaptive immune response is not yet known. Further, no studies to date have looked at PRR expression variation in the context of IR classification, pregnancy and parity. Therefore, a collection of studies was conducted to investigate the expression of bovine toll-like receptor 2 (TLR2) on bovine blood mononuclear cells (BMC) and cytokine expression before and after culture with ligands. This novel research on innate TLR2 expression was a logical exploration of one innate mechanism that facilitates pathogen recognition and therefore likely plays a role in enhancing or diminishing adaptive immune responses.

This research was also partially inspired by technology transfer activities to commercialize the High Immune Response (HIR) technology, a method developed at the University of Guelph to assess the immune response capacity of dairy cattle, and was a suggested focus of study by dairy industry stakeholders. The HIR™ technology allows cattle to be
classified based on their EBVs of AMIR and CMIR traits, and thus ties together the various components of this thesis in that all cattle investigated had known IR phenotypes. Cattle with the high IR phenotype have been shown to have less disease occurrence and as a result, require less therapeutic treatment with antibiotics. Reduced antibiotic use helps to reduce the risk of antibiotic-resistant pathogens in cows and their surroundings. HIR™ technology is a genetic tool to evaluate immune response that can be used for making management, culling and breeding decisions, to increase the number cows with high IR phenotypes, and thus are ‘greener cows’ for a ‘greener environment’. Technology transfer activities conducted as part of this PhD project are included in brief as an addendum to this thesis with links to more detailed information about HIR™ technology commercialization.
Note: This literature review is not an exhaustive literature review of host defense of the dairy cow, the pathogenic agents that cause infectious disease, nor the nutritional factors that cause metabolic disease. This review instead briefly describes the bovine immune system, discusses current challenges in dairy animal health, available data on the cost and incidence of disease, some current methods to diagnose and treat diseases of economic importance, as well as some current methods available to prevent disease. It addresses current knowledge transfer on how best to communicate research advances in dairy herd health management to dairy producers as this is critical to advancing herd health in a broader context. Additionally, this review provides a discussion about technology transfer, the challenges that exist within a normal high technology market, and what characteristics a new technology must have to break through and be adopted by that market, in this case, the dairy industry. These are all aspects that relate to my PhD research. 

This literature review is current as of August 2011 when it was due for the Graduate Seminar in Pathobiology PABI*6440 course. A more current literature review for research on TLR2 receptors on blood mononuclear cells (BMC) in Holstein dairy cattle is incorporated into the Introduction and Discussion of Chapters 1 through 4.

1.0 The Cost of Disease in Dairy Cattle

1.1 Current challenges in animal health

Infectious disease of livestock is one of the most costly problems facing the Agri-food industry. It is becoming more difficult to manage animal health due to the fact that infectious diseases, some of which are zoonotic, are emerging and re-emerging (Tomley and Shirley 2009). In addition, resistance to antibiotics by bacterial pathogens is a problem for both animals and humans. There is increasing pressure from consumers and the medical community for government to reduce antibiotic use (Torrence et al. 2001) and the situation is complicated by the sizeable costs associated with new drug development (Heldens et al. 2008). Consumer concern for both improved food-safety and animal well-being demands an alternative approach for disease prevention which does not rely on the extensive use of anti-microbials (Berge et al., 2009). Dairy cattle are challenged by diverse bacterial, viral, and other pathogens and may be subjected to many different infectious and metabolic diseases as young animals or as mature
animals during gestation, parturition and lactation. A superior immune system and optimal herd management are factors for improving health and disease resistance in dairy cattle.

1.2 The immune system of dairy cattle
The immune system of dairy cattle provides host defense via genetically and environmentally regulated cells and molecules that respond to disease-causing pathogens, as well as to other foreign antigens or altered self-molecules. The immune system can be characterized as having two interconnected systems – the innate system and the adaptive immune system.

The innate immune system includes host defense mechanisms like physical barriers (e.g. skin), proteins and enzymes that damage the pathogen membrane, and a number of different cell types including neutrophils, macrophages/monocytes, and eosinophils, that have phagocytic or cytolytic functions. Several types of soluble mediators such as proteins of the complement system, defensins, lactoferrin, lysozyme to name a few, can bind to structures on pathogens causing agglutination, interference with receptor binding, opsonization, neutralization, direct membrane damage, and recruitment of other soluble mediators and cells through inflammation (Firth et al. 2005). Some mechanisms of the innate immune system are constitutively expressed while others are activated once a host cell interacts with an invading microbe (Chaplin et al. 2010). Host cells recognize invading pathogens through PRRs like toll-like receptors (TLRs). These PRRs recognize conserved patterns on microorganisms and are able to distinguish self-antigens from non-self (Detilleux et al., 2001). Host cells can eliminate the invading pathogens by phagocytosis or the release of cytokines that recruit cells with phagocytic ability. This response is non-specific but fast acting and in general results in the rapid elimination of the pathogen. A common measurable indicator of innate host resistance in the mammary gland used by the dairy industry is somatic cell count (SCC). Cells found in normal bovine milk include neutrophils (1-10%), macrophages (66-88%), lymphocytes (10-27%) and epithelial cells (Ruegg 2002). The macrophages survey the bovine mammary gland for invaders and when bacteria infect the gland, they respond by initiating an inflammatory response that attracts neutrophils (PMNs) into the milk to destroy the bacteria. The SCC of an udder that is not infected is usually less than 200,000 cells/mL. Many cows maintain SCC values of less than 100,000 cells/mL (Ruegg, 2002).
The **adaptive** immune response requires presentation of an antigen or pathogen by cells of the innate immune system to effector cells of the adaptive system. It has the hallmark characteristics of memory, diversity, specificity and self/non-self recognition. Memory cells are generated that remain in the immune system for a long period of time. Memory cells play a major role in response to an invader on second exposure. They are able to recognize the pathogen with exquisite specificity and can respond more rapidly and efficiently to remove the pathogen. The adaptive immune system is further characterized by its ability to respond to different pathogens as a result of the activation of a variety of T lymphocytes in the blood and milk. These cells produce specialized profiles of cytokines that aid in generating antibody- or cell-mediated immune responses. For example, T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune response (CMIR) and T helper (Th2) cells produce cytokines that generate an antibody-mediated immune response (AMIR). Pathogens such as *Escherichia coli* that can cause mastitis, are in general categorized as extra-cellular. An extra-cellular pathogen is one that can grow and reproduce freely and may move extensively within the tissues of the body, but once ingested by phagocytic cells is typically digested and processed for antigen presentation. *E.coli* can be readily engulfed and broken down by macrophages, and peptides are presented by Major Histocompatibility (MHC) class II molecules to CD4+T cells to help stimulate an antibody response. Pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* (the causative agent of Johne’s disease in cattle) are intra-cellular microorganisms and the antigens are processed differently inside the host cell. In general, intra-cellular microorganisms can live inside the host cell, and processed peptides from these organisms presented to CD4+ T cells, then results in the generation of a type of CMIR response, called delayed-type hypersensitivity (DTH).

The bovine immune system has some unique features that are not common to other mammals, particularly humans and mice. Dairy cattle have a higher proportion of T-cells that express the γδ T cell receptor in comparison to other species. During the first 1-2 weeks following birth these cells constitute 60% of the total pool of T lymphocytes in blood in calves, and these decline and become similar in numbers to the αβ receptor T cell population with increasing age (Hein and Mackay, 1991). Gamma-delta cells are thought to play an important role in protection of epithelial surfaces, linking the innate and adaptive immune systems, and regulating immune
responses (Hein and Mackay 1991; Hoek et al., 2009). Regulatory T-cells (Tregs) play a role in maintaining homeostasis, but in contrast to humans and mice, CD4+CD25high/Foxp3 T cells in ruminants do not exhibit regulatory activity. This regulatory activity resides with the γδ population of T lymphocytes (Hoek et al. 2009). In addition, dairy cattle have some CD1 proteins to recognize lipids, but don’t have NKT cells due to lack of functional CD1d (Van Rhijn et al. 2006).

The adaptive immune system of the neonatal dairy calf is naive at birth and calves are born agammaglobulinemic. The calf is able to resist infection from pathogens if sufficient maternal antibodies are present in the colostrum from their dam. Colostrum contains a high concentration of immunoglobulins (Ig), which transfers passive immunity of the dam to the calf and enables it to resist infections. During the peripartum period concentrations of serum IgG1 in the dam decrease and relative concentrations of IgG2 increase (Detilleux et al. 1995) as a result of the active compartmentalization of IgG1 secretions during colostrogenesis. Colostrum must be ingested by calves within 24 hours of birth, for immunoglobulins to be absorbed, since the specialized cells in the small intestine of the calf that absorb immunoglobulin are replaced by 72 hours post calving thus inhibiting this mechanism for transfer of immunoglobulin into the lymph and blood circulation (Stott et al. 1979; Kung et al. 2011). This is referred to as gut closure. It is recommended that calves receive colostrum within 6 hours of birth to optimize absorption and minimize morbidity. Further, an adequate volume of colostrum of high quality (high concentration of immunoglobulin) would be of the greatest benefit to the calf (McGuirk and Ruegg 2000). Maternal antibodies however, are only present in the circulation of the calf for a short period and have a half-life of approximately 16 to 28 days (Fulton et al. 2008). Thus, the dairy calf in the very early stages of life must also rely on soluble protein mediators and cells of the innate system to resist infection and disease while the adaptive immune system develops (Firth et al. 2005). A high proportion of dairy calves (37% in US dairy herds) receive an inadequate amount of colostrum prior to gut closure resulting in failure of passive transfer or FPT making them susceptible to viruses and bacteria that cause diarrhea, respiratory diseases and septicemia (Nonnecke et al., 2009; National Health Monitoring System –NAHMS 2002). Prevention of disease in calves is complex and involves many factors including the combined expression of multiple genes that code host response, the virulence of pathogens in the
environment, and the management practices of the dairy producer.

The immune system of the dairy cow is strained during the peripartum period due to the stresses of gestation, parturition and lactation. This increases susceptibility to disease at the time of parturition and in the early stages of lactation. Many studies have demonstrated disease and impairment of host defenses at this time (Cai et al. 1994; Kehrli et al. 1989a; Kehrli et al., 1989b; Kehrli et al., 1994; Detilleux et al. 1995; and Shuster et al. 1996). Diseases such as mastitis, metritis and retained fetal membranes (retained placenta), and metabolic diseases like ketosis and displaced abomasum, occur within the first two to three weeks of lactation. According to Goff and Horst (1997) three physiological functions must be maintained during the periparturient period if disease is to be avoided: 1) adaptation of the rumen to lactation diets that are high in energy density, 2) maintenance of normocalcemia and 3) maintenance of a responsive immune system. The impairment of the host defense may be a consequence of nutritional and endocrine factors. A meta-analysis performed by Kelton et al. 1998, evaluating recommendations for recording incidence of disease, identified eight clinically important diseases: milk fever, retained placenta, metritis, ketosis, left displaced abomasum, cystic ovarian disease, lameness and clinical mastitis. These clinical diseases are of economic importance, resulting in substantial costs for treatment, veterinary services, lowered production quality and quantity, involuntary culling, and replacement if death occurs. Other diseases that are of economic interest include Johne’s disease, as well as calf respiratory disease and diarrhea. A number of diseases of cattle are reportable or communicable to other livestock and humans (Tomley and Shirley, 2009), but that is not the focus of this review. This review will be limited to a discussion of diseases of the greatest economic concern to the dairy industry.

1.3 Incidence and Treatment of Economically Important Diseases in Dairy Cattle

1.3.1 Mastitis

Mastitis is one of the most costly diseases for the dairy producer (Halasa et al. 2007). Cattle with mastitis have a higher than normal SCC in their milk, have swollen and painful quarters, increased heart rate, and small white flecks appear in the milk. It is caused by many different bacterial species that may be classified as major and minor pathogens. The major pathogens can be further subdivided into organisms that cause contagious intramammary infection, or are
considered environmental pathogens (Radostits et al. 2007). The common contagious pathogens are *Staphylococcus aureus, Streptococcus agalactiae* and *Mycoplasma bovis*. These organisms travel from infected quarters to susceptible quarters of other cows in the herd and can be spread by milking machine or by milker’s hands. Many herds also have a high incidence of infection with environmental pathogens that include *Escherichia coli, Klebsiella* sp., *Streptococcus uberis* and *Streptococcus dysgalactiae* (Radostits et al. 2007). Infection by these organisms is frequent but of short duration. It usually occurs in the summer months as a result of high temperature and humidity when more bacterial species are thriving, especially in the bedding where teat ends are exposed (Morse et al. 1988). The frequency of mastitis has been increasing over the years as a result of emphasis on production without consideration of factors that may affect health (Heringstad et al. 1999, Harmon et al. 1994). Recent research by Olde Riekerink 2008 examining pathogen specific mastitis incidence rates across regions of Canada estimated that the overall national incidence rate of clinical mastitis was 23 cases per 100-cow years. Ontario and Quebec have the highest incidence of clinical mastitis at 31.6 and 29.7 per 100 cow-years respectively. This number represents the number of cases of a disease to be expected in one year among 100 cows. Common treatments include infusion of antibiotics by the intramammary route and supportive therapies such as stripping the infected teats of mastitic milk, and the use of oxytocin to stimulate milk let down.

Fetrow (2004) has estimated the average cost of a case of clinical mastitis at $185 US per cow per year; for subclinical mastitis estimates have ranged from $140 to $340 CAD in losses (Gill et al., 1990). A 2009 report indicated that the average cost of mastitis, clinical and subclinical in Quebec herds was $200-250 CAD per cow per year (Carrier, 2009). A variety of diagnostic tools are available for use by dairy producers to detect clinical or subclinical mastitis. The California Mastitis Test or CMT is a cow-side test that may be used to evaluate milk quality. Milk from each quarter is squirted into a paddle with 4 sections and is mixed with a reagent and reactions are assessed. This test is limited in that it must be read within 15 seconds as some weak reactions will dissipate quickly, and the test does not indicate the type of pathogen involved in the infection. Other rapid tests for identifying mastitis include the use of bacterial culture (3M Petriplate, 3M, St. Paul, MN) and antibiotic sensitivity tests (MASTiK by Immucel, Portland, ME) that involve the collection of a milk sample. Results may be provided to producers within
24 hours to assess whether they should or should not treat a cow with antibiotics (Ruegg, 2002). Veterinarians are able to offer these tests in their clinics. One Ontario clinic advertised the cost of the 3M culture test at $8 per cow per sample and provided a cost-benefit analysis of how the test makes great economic sense to spend $8 to save $75 - $177 in antibiotics (depending upon the antibiotic used and duration of treatment) (Eldale Veterinary Clinic, Elmira, ON, June 2011). If a Gram+ bacterium is present, then the producer may proceed with antibiotic therapy and the money spent on treating with intramammary antibiotics is well worth it, but without this knowledge, a producer may waste money on the cost of the antibiotic and lost revenue from the withheld milk. The limitation of this test is that it cannot identify the type of Gram+, and that must be done in a laboratory such as the Animal Health Laboratory at the Ontario Veterinary College, in Guelph, ON. Producers in Ontario and Western Canada can also take advantage of contagious mastitis DNA testing (evaluated by PCR) offered by CanWestDHI in Guelph, ON.

Producers can request that their regular monthly milk sample be tested for 3 contagious mastitis pathogens in the laboratory, and then in consultation with their veterinarian can select the appropriate antibiotic to treat an infection. These tests offer a convenient method for producers to strategically treat cattle only infected with Gram+ bacteria that will respond to antibiotic therapy versus Gram- bacteria that are largely environmental pathogens and will not respond (Ruegg 2002). To be effective, it is important that the antibiotic is used properly and for the complete course of treatment or the producer runs the risk that an organism could develop antibiotic resistance. This may result in the development of mastitis cases in the future that cannot be successfully treated with antibiotics. Veterinary researchers are presently investigating the possibility that certain contagious pathogens like S. aureus may become more resistant to therapeutic treatment. They have started to use diagnostic tools like Sensititre (Trek Diagnostic systems, Cleveland, OH) an antibiotic susceptibility assay to evaluate the degree to which antibiotic resistance may be prevalent within culture isolates from cows (Saini et al., 2011). This particular susceptibility test has the ability to detect vancomycin resistant S. aureus (VRSA). These tools are useful for diagnosing mastitis, identifying the causative pathogen, and estimating its potential for resistance, but do not offer producers a test to reduce or prevent mastitis incidence.
1.3.2 Ketosis

Ketosis is a metabolic disorder where metabolism of fat and carbohydrates results in increased ketone bodies (beta-hydroxybutyrate -BHBA, acetoacetate and acetone) in the blood, milk, breath and urine of dairy cattle (Geishauser et al. 1998, Kelton et al. 1998). Ketosis typically occurs in the first six weeks following parturition. It occurs in dairy cattle because of an inability to intake enough nutrients to meet energy needs. This can lead to the pathologic state of hypoglycemia, a lower than normal level of glucose. Ketosis increases the odds of cattle developing a left displaced abomasum, metritis, cystic ovarian disease, and mastitis (Duffield et al. 1998). Ketosis may occur as a result of being fed inadequate feed for changing production demands from a dry to lactating state. The estimated incidence of ketosis in dairy cows in 1998 in Ontario was 12% (Green et al. 1999). Kelton et al., 1998 reported the lactational incidence risk (LIR) of developing ketosis from 1979 to 1995 ranged between 1.3% and 18.3%, with a median LIR of 4.8%. Treatment of ketosis to re-establish normoglycemia and reduce serum ketone body concentrations involves treatment with a bolus IV administration of 500 mL of 50% dextrose solution. Glucose therapy generally results in rapid recovery, especially in cases occurring near peak lactation. Administration of glucocorticoids including dexamethasone or isoflupredone acetate at 5-20 mg/dose, IM, generally results in a more sustained response. Propylene glycol (250-400 g/dose), acts as a glucose precursor and may be effective as ketosis therapy, especially in mild cases or in combination with other therapies.

Ketosis causes losses due to decreased milk production, reproductive efficiency, and increased culling and treatment costs. It is associated with a loss in body weight due to a declining appetite, as well with declining milk production because of a deficiency in glucose. Duffield et al. 2009 found that the greatest negative impact on yield occurred within the first two weeks of lactation. A concentration of BHBA in serum of 1400 micromol/L in week one after calving resulted in a milk yield loss of -1.88 kg/d. In week two, 2000 micromol/L of BHBA resulted in a loss of -3.3 kg/d. Dohoo and Martin (1984) estimated loss in milk yield because of ketosis is approximately 1.0-1.4 kg per day. Gillund et al. 2001 found that cattle with a previous ketosis event were less likely to conceive at first insemination thus requiring further inseminations for conception. Economic losses due to treatment costs, lost milk production, increased days open, and increased culling rates were estimated to total $145 US per case of ketosis (Guard, 1994).
Cow-side dipstick tests for the presence of ketone bodies in urine or milk are available to evaluate subclinical ketosis. Tests that measure BHBA in milk like PortaBHB (PortaCheck, Moorestown, NJ) are estimated to cost $1.75-2.00 US per strip. The cost of cow side tests and treatment of ketosis, in addition to lost production and reproductive fitness, contributes to the economic losses associated with this disease, and no test is available for sale towards enhancing its prevention.

1.3.3 Metritis

Metritis is defined as a disruption of the uterine epithelium with the presence of inflammatory cells. It is a postpartum uterine disease that is common in dairy cattle. Metritis occurs when cattle have difficulty with calving, retaining the placenta after calving or have excessive stretching of the uterus due to the calving of twins. These factors lead to an infection of the uterus caused largely by *Clostridium* sp., *Trueperella (Actinomyces) pyogenes*, and anaerobic Gram+ and Gram- organisms. Kelton et al. 1998 reported that among metritis studies from 1979 to 1995, the LIR rate ranged from 2.2% to 37.3% with a median incidence rate of 10.1% per 100-cow herd. Treatment of cows with postpartum metritis generally involves hormonal or antibiotic therapy alone or in combination. Antimicrobial agents assist in resolving bacterial infections; hormone therapy induces estrous, evacuating abnormal uterine contents, and increasing the production of mucus containing host defense mediators (Smith and Risco, 2009).

Economic losses associated with metritis include treatment costs, involuntary culling and reduced reproductive performance. The estimated cost of metritis is $236 US per case (Melendez et al. 2004). Research by Dubuc et al., 2010 found that metritis was associated with a 3.7 kg/day decline in milk yield at the first monthly DHI test, but normalized after consecutive monthly DHI tests. Dubuc et al. 2011, have provided parameters for diagnosis of metritis based on clinical signs and cytological findings. Clinical endometritis is characterized by a cervix >= 7.5 cm in diameter as assessed by transrectal palpation 20 days or more after parturition or by purulent discharge 26 days after parturition and may be measured using a Metricheck device (Sircrotech, Hamilton, NZ) and scoring vaginal discharge on a scale of 0 (clear) to 5 (foul smelling discharge). Cytological endometritis is characterized as an increased proportion of neutrophils in cytological samples obtained using an endometrial cytobrush or by low volume uterine lavage.
(Kasimanickam et al. 2004, Gilbert et al. 2005). Although diagnostic methods exist for metritis no predictive measure is available to producers to prevent metritis.

1.3.4 Retained Placenta

Retained placenta (RP) occurs when fetal membranes fail to separate and be discharged from the uterus within 24 hours after calving. It has been suggested that impaired function of neutrophilic leukocytes is responsible for retention of the placenta (Kimura et al. 2002). Kelton et al. 1998 reported that the LIR for retained placenta from 1979 to 1995 ranged from 1.3% to 39.2% with a median LIR of 8.6%. The most common treatment of RP is to allow the placenta to separate naturally with or without the use of medication. Hormones may be used to evacuate the uterine contents and may be helpful for the treatment of inflammatory cells. If hormone therapy is not successful, then treatment by palpation and irrigation with warm water followed by an intrauterine infusion of antibiotics is recommended (Manspeaker Dairy IRM 21). Economic losses from RP result from involuntary culling, treatment costs, lower body weight, decreased production and fertility (Manspeaker Dairy IRM 21) and have been estimated to total $285 US/case. Fourichon et al., (1999) found a loss in milk yield of 2kg/cow/d in cattle with retained placenta. Currently no predictive test is available to prevent retained placenta.

1.3.5 Other diseases of lower incidence

1.3.5.1 Cystic Ovarian Disease

Cystic ovarian disease (COD) is diagnosed when a smooth, rounded structure greater than 25 mm in diameter is present on one or both ovaries thus suppressing ovulation (Hooijer et al. 2001). COD is associated with a variety of factors (overconditioning, stress, concurrent ketosis or mastitis infection), but the most widely accepted explanation is a neuroendocrine imbalance, causing a decrease in gonadotropin releasing hormone (GnRH) and an increase in lutenizing hormone (LH) and estradiol 17-ß, resulting in the development of cystic ovaries. Cystic ovaries are associated with a period of infertility and a period of anestrous that lasts the duration of the cyst. Cattle with COD have impaired reproduction and require more inseminations to conceive. Kelton et al. 1998 reported that the LIR for COD from 1979 to 1995 was 1.1% to 16.1% with a median LIR of 8.0%. Although cattle with COD have higher milk production, the problems associated with reproduction are a major risk factor for increased culling of cattle, and a cost
benefit analysis yielded a net loss of $39 per cystic lactation (Bartlett et al. 1986). Diagnosis of COD may be done through serum testing of hormones or by ultrasound.

1.3.5.2 Left Displaced Abomasum
A left displaced abomasum (LDA) occurs due to a lack of motility in the abomasum. As a result of gas production, the partially gas-distended abomasum becomes displaced upward along the left abdominal wall lateral to the rumen (Merck Veterinary Manual, 2011). The LIR risk of LDA from 1982 to 1995 was 0.3 to 6.3% with a median LIR of 1.7%. Economic losses for LDA include lost milk production and the cost of surgery to treat LDA, and totaled $340 per case (Kelton et al. 1998). Milk loss has been estimated to range between 250 and 2000 kg per lactation (Martin et al., 1978).

1.3.5.3 Milk Fever
Milk fever or parturient paresis occurs when cows are not able to move a sufficient amount of calcium from their bones or diet to replace the calcium lost to their body due to milk production. Kelton et al. 1998 reported that the LIR of milk fever from 1975 to 1995 ranged from 0.3 to 22.3%, with a median LIR of 6.5%. Treatment of milk fever is directed toward restoring normal serum calcium levels as soon as possible to avoid muscular and nervous damage and recumbency. In addition to milk yield loss, additional losses are attributed to treatment costs, and increased days open, and have been estimated at $335 US per case (Guard, 1994). Cattle affected with milk fever experience a loss in milk yield ranging from 1.1 to 2.9 kg per day (Rajala-Schultz et al. 1999).

1.3.5.4 Lameness
Lameness may be caused by injury, or by infectious agents (e.g. digital dermatitis or foot rot). The incidence of lameness differs regionally, and can be a significant problem for some herds. The major pathogen which causes foot rot is the bacterium *Fusobacterium necrophorum*. Kelton et al. 1998 reported the LIR of lameness from 1972 to 1995 as 1.8% to 30% with a median LIR of 7.0%. Adequate foot baths and hoof trimming help reduce the incidence of lameness caused by infection and injury to a minimum, but lameness as a result of injury is more sporadic and difficult to prevent. Treatment for cattle affected by lameness due to injury includes cleaning
wounds, the application of topical antiseptic spray and use of topical or injectible antibiotics. Recommended practices include clean and comfortable housing and bedding for cattle, and a reduction in time standing on concrete. Cows should not be rushed along abrasive surfaces that can cause injury (Stokka et al. 2011). Economic losses from lameness include treatment costs, decreased milk production, increased culling and increased days open, which were estimated to total $302 per case (Guard, 1994).

1.3.5.5 Johne’s Disease
Johne's disease is an infectious and chronic wasting disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This bacterium causes the intestinal walls to thicken hampering the absorption of nutrients for production and growth. This results in weight loss and chronic diarrhea even though cattle may have a good appetite. Cattle will remain infected for life, and infected cattle may be more susceptible to mastitis and reproductive disorders. Tiwari et al., 2008 reported that the incidence of Johne’s disease in Canada was about 3.1%, slightly higher than Vary et al. 1990 that reported an incidence rate of 2.9%. There is currently no treatment for Johne's infections and it is usually recommended that if an animal is infected that it be immediately culled. There is no preventive vaccine available to combat infection but there is a vaccine that can minimize clinical signs and shedding of the organisms, however, it does not prevent new infections. The most important control measure for Johne’s disease is culling, and the prevention of spread between cows and their calves (Tiwari et al. 2008). The cost of a case of Johne’s is estimated to cost between $245 US per cow (Ott et al. 1999), and $385 CAD per cow (Tiwari et al., 2008) as result of reduced milk production, premature culling, replacement costs, loss of body weight in slaughter cattle and poor reproductive performance. Ott et al. 1999 also reported an annualized economic loss of 700 kg of milk per cow as a result of Johne’s. A milk ELISA test is available (offered by CanWestDHI, Guelph, ON) for producers to identify which of their cattle are infected with MAP. The test costs approximately $9 CAD. This is useful for producers, so that they can identify cattle that shed the bacterium, and cull them to remove MAP from their herds.

1.3.6 Calf diseases – Diarrhea and Respiratory Disease
Diseases that affect calves from birth to weaning include septicemia, respiratory disease and
diarrhea (McGuirk and Ruegg, 2000). Nonnecke et al. 2009 reports that these infectious diseases have incurred significant economic losses to the US dairy cattle industry with a mortality rate of 8 to 11% and a morbidity rate of 37%. Diarrhea is the most common cause of death in young calves and the highest risk period is from birth to 1 month of age. Diarrhea is caused by bacteria, viruses, and/or parasites, (McGuirk and Ruegg, 2000). Economic loss estimates for bovine viral diarrhea (BVD) have been reported in the Netherlands, but none are currently available in the literature for North America. Wentink et al. 1990 found that economic losses include abortion, stillbirth, delivery of calves with lesions, mucosal disease and animals persistently infected with BVD virus, and was on average reported to be 136 Dfl. per cow in the Netherlands. Assuming this is the equivalent in Euros, this represents a cost of $201 CAD per cow.

Bovine respiratory disease (BRD) is caused by a number of different bacteria and viruses, and affected cattle are usually infected with more than one pathogen. A study on the incidence of BRD in Ontario dairy herds reported that 15% of heifer calves were treated for BRD before weaning with a fatality rate of 5% (Waltner-Toews et al., 1993). The direct cost of treatment of BRD has been estimated in the US at $14.71 per calf per year for milk fed calves, $1.95 US for replacement heifers and $9.08 US per cow per year (Kaneene and Hurd, 1990).

1.4 Prevention of Disease in Dairy Cattle

1.4.1 Management programs
Leblanc et al. (2006) state that a fundamental advance in disease prevention has been the recognition of the multifactorial nature of all diseases of importance in dairy cattle. Health management is characterized by an integrated, holistic, proactive, database- and economically framed- approach to prevention of disease and enhancement of performance. The most important specific advance in the last 30 years has been the focus of research on the management of the transition period and the close-up dry period of cattle. Approximately 75% of disease in dairy cows typically happens in the first month after calving. Special care and attention is required in ensuring that the diet of the dry cow meets the necessary energy demands at the time of calving and lactation. Nutrition plays a key role in preventing metabolic and infectious disease in cattle during the peripartum period. In addition, mastitis control programs have been
promoted by several researchers and advisors involved with the dairy industry. Significant progress has been made to control contagious pathogens through udder health programs that include pre- and post-milking teat disinfection, milking machine maintenance, dry cow therapy, culling chronically infected cows, and milking infected cattle last (Gill et al. 1990). Udder health management programs for environmental pathogens include recommendations on cleanliness and housing, management of cows and calves around calving, and pre-milking preparation to aid in reducing the frequency of environmental mastitis. As well, starting about 30 years ago, the use of bulk tank SCC, an innate indicator of host resistance, was an indirect indicator of intramammary infection at the herd level. This became the standard method of monitoring bulk milk tank quality. Udder health programs have had a great impact on reducing bulk milk tank somatic cell counts (BMSCC) and this has improved milk quality and production of dairy products like cheese that are affected by high SCC. Currently in Ontario BMSCC must not exceed 500,000 cells/mL and soon this threshold or penalty level will be brought down to 400,000 cells/mL in August, 2012. After the inception of BMSCC testing, DHI companies soon began to offer individual cow composite samples for SCC, milk, fat and protein and other measures on a monthly basis. These two pieces of information provided the producer with tools to monitor their progress in the innate resistance control of intramammary infections. Research by Barkema et al. 1999 indicates that it is important to know what pathogens are present within a particular herd in order to appropriately design a herd-specific udder management program that addresses the necessary practices to prevent contagious or environmental pathogens causing mastitis. Additional measures to manage disease include: 1) maintaining cleanliness of housing; 2) using bedding such as sand in bedding areas that minimizes bacterial growth; 3) using calf hutches to reduce the transmission of disease between calves; 4) feeding colostrum immediately after calving; 5) pasteurizing colostrum fed to calves; 6) preventive parasite control treatments and 7) the use of dewormers in cows and calves to prevent parasitic infections, and the use of foot baths and scrubs to reduce the transmission of pathogens that cause digital dermititis, foot rot and possibly other communicable diseases of cattle (LeBlanc et al., 2008). Dairy herd improvement companies now offer diagnostic services that include testing milk with an ELISA kit to help identify pathogens. These tests are used to identify Johne’s disease, and leukosis, a blood borne viral infection which can cause tumours in the tissues of dairy cattle.
These ELISA tests cost approximately $8 and $9 CAD respectively and results are available in 2-3 days. As previously mentioned, DNA testing is also available to identify one of 3 different types of contagious pathogens that are common causes of mastitis. This test costs $24 CAD/sample and results are usually available within a day. This is an additional cost to producers that subscribe to SCC and component testing for their herds, but allows producers to get a sense of the prevalence of disease in their herd, and gives them the information needed in decisions to cull infected cattle that are carriers of disease. Another important role in managing health is monitoring disease. Database software tools such as Dairy Comp 305 (Valley Agricultural Software, Tulare, CA) have been designed to record information on reproduction, health and production information. Some software tools that work with robotic and parlour milkers record information such as milk conductivity (an electrical measure of resistance of the milk) and changes in resistance may indicate the possibility of subclinical mastitis. These software tools may also be useful for the easy import and export of information from sources that include dairy herd improvement companies. The ability to manually add data is helpful for disease collection, and while some producers are diligent in recording this information, still others are not. Being able to consistently collect accurate disease information for making phenotypic and genotypic associations between disease and immune response parameters has been difficult. Despite this challenge, some improvement has been made recently in standardizing the collection of health data through the National Herd Health Project in Canada (VanDoormal 2009)

1.4.2 Vaccination programs

Prevention of specific diseases like bovine viral diarrhea (BVD Types I & II) and infectious bovine rhinotracheitis (IBR) and parainfluenza (PI3) and bovine respiratory syncytial virus (BRSV) are preventable through the successful implementation of vaccination programs. These vaccines are available in killed or modified live viral (MLV) vaccines (Dubovi et al. 2000) and are used in combination. As there is a risk of abortion or malformations of the fetus when vaccinating pregnant cattle with modified live viral (MLV) IBR and BVD vaccines, killed vaccines are a preferred method of preventive control, and herds may easily be vaccinated at the same time in the fall or spring. While using a killed vaccine is a safer approach, the disadvantages of using a killed vaccine over an MLV are the increased cost and a slower rate and
strength of response, as more than one dose is required to confer protection. Vaccination of cows against viruses such as BVD may also confer protection to the fetus thus reducing the incidence of viral diarrhea in the neonatal calf. Although protection from disease is a valuable outcome from vaccination, it is possible that the administration of vaccines may be at the expense of a transient short term drop in milk production. A study by Bergeron and Elsner 2008 evaluated milk production pre-and post-vaccination with 2 commercial 5 way vaccines, and found that cows receiving Cattlemaster Gold FP5 (Pfizer Animal Health, New York, NY) experienced a statistically significant drop in milk production @ 1.83 kg/cow/day in contrast to cows receiving Triangle 4 + Type 2 BVD (Fort Dodge Animal Health, Overland Park, KS) at 0.63 kg/cow/day. A transient fever resulting in reduced milk production has been proposed by Scott et al., 2001 as a reason for post-vaccinal milk drop. Killed vaccines are also available to prevent mastitis caused by E.coli. These include J VAC (Merial, Duluth, GA), and UpJohn-J5-BACTERIN (Pfizer Animal Health, New York, NY). These are given during the dry off period in two or three injections leading up to the peripartum period, and confer some cross protection against other Gram- pathogens causing mastitis. This is due to the core antigen technology and the adjuvant system of the killed vaccine. Wilson et al., 2009 found that vaccination with J5 was associated with protection against milk production loss and culling following mastitis. While there have been several attempts at developing vaccines against Gram+ organisms like S. aureus, these have been effective for reducing the severity of infection (Sordillo 1995), but currently there is no effective vaccine for preventing new infections. Despite the expense associated with the cost of vaccines, and a possible transient drop in milk production, the investment in vaccination is well worth the cost to control and avoid the spread of infectious disease.

2.0 Selection for Enhanced Immune Response & Disease Resistance
Genetic selection mainly to improve dairy milk production traits with minimal emphasis for health traits has gradually led to an increase in the incidence of disease among dairy animals. (Harmon et al. 1994). Recently, the dairy industry has been diligently working to provide genetic solutions for improved health in order to correct this problem, particularly in light of the increase in emerging disease and antibiotic resistance in livestock (Tomley and Shirley 2009). Genetic regulation of immune response and selection for disease resistance is well documented and considered a preventative method to improve animal health (Stear et al. 2001). A study by
Detilleux et al. 2009, indicated that there may be a polygenic method by which cattle could be selected for resistance to mastitis. There is solid evidence that selective breeding for high (H) or low (L) immune response influences resistance to infectious disease (Kelm et al. 2001) and heritability of AMIR and CMIR are stable and moderate to high ($h^2=0.2$ to $0.3$), indicating that genetic selection is feasible (Hernandez et al. 2006 Proc WCGALP). In fact, identification of H immune responders is associated with lower disease risk and improved response to vaccines in dairy cattle (Wagter L. et al. 2000, 2003 JDS; Hernandez, PhD thesis 2007; DeLaPaz, MSc thesis 2008). Work by Wagter et al., 2000 demonstrated that dairy cattle could be classified based on their antibody response profile to ovalbumin and when evaluated for disease occurrence, cows that had a H antibody response were found to have no mastitis in 2 of the 3 herds investigated. Recent work done by DeLaPaz et al., 2008, on a large Florida herd to evaluate HIR response profiles on approximately 875 cows and evaluate health data, indicated that cows that had a H immune response to antigens for both AMIR and CMIR had significantly lower odds ratio risk for developing mastitis, metritis, ketosis, and retained fetal membranes compared to those that had an average (A) or a L immune response. For selection of broad-based disease resistance to be effective, the individuals selected for breeding subsequent generations must demonstrate a favourable AMIR and CMIR response to both extra-and intra-cellular organisms respectively. Integrating quantitative genetic strategies such as estimated breeding value (EBV) of AMIR and CMIR to enhance the immune system is a potential solution to improve disease resistance of food-producing animals (Mallard et al. 2011). Breeding companies now offer semen from breeds like the Norwegian Red that have been documented to be associated with lower occurrence of mastitis (Mejdell et al., 1994) and a recent study comparing the IR between pure bred Holstein and cross-bred Holstein X Norwegian red cows, indicate that they have a higher AMIR and CMIR response in contrast to purebred Holsteins (Cartwright et al. 2011). Canadian sire proofs also now include information on the somatic cell (SCS) scores of their daughters. SCS is a logarithmic transformation of SCC, and is currently included as a trait of interest on bull proofs. The heritability of the SCS is $h^2=0.1$ to $0.12$ and while use of SCS is intended to help cause a decline in mastitis incidence, progress toward that end will be slow due to this low heritability (Dekkers and Burnside, 1994)

Another type of test that has become available for producers as a tool for health and production management is genetic profiling, through tests like the iGenity test offered by Merial (Duluth,
GA). Results from tests like these produce the genotype of the animal but do not provide information on the phenotypic expression of immune response genes that mediate host resistance to disease. The test costs approximately $30 CAD. While the test is available to dairy producers the market for this test has largely been the beef industry as dairy producers generally rely upon their dairy herd improvement reports to determine the overall health status of their herd (Merial, personal communication). While there may be some value in genomic evaluations of cattle, results on genotype should not be the sole deciding point for making health management decisions considering that innate and adaptive immune systems that respond to diverse types of pathogens are influenced by a very large number of genes. A phenotypic evaluation of the immune response capability of a dairy cow would much more useful towards evaluating the animals ability to respond to a variety of pathogens that cause disease (Mallard et al. 2011).

3.0 Marketing Better Health through Knowledge Transfer and Technology Transfer
3.1 Knowledge transfer and motivation to improve dairy animal health

Though many herd health management programs have been developed from years of herd health and production research, the benefits of this research cannot be fully realized unless they are adopted by dairy producers. Much research has gone into the advancement of health knowledge, but attention to the transfer of the developed knowledge has been limited. Knowledge transfer to dairy producers is not easy, and there are major challenges to getting producers to change their management behaviour in line with proven research. First, farmers that want to improve animal health still need to be convinced that it is worthwhile to make a change. Second, to improve herd health among all producers, all producers need to be motivated and educated to carry out herd health programs (Lam et al. 2008). Recent research in the Netherlands by Jansen et al. 2010a, has confirmed that producers differ in the way that they acquire and adopt information. If, when and how they take up this information and apply it depends upon their motivation, how they acquire information for learning, and the route of knowledge communication. Valeeva et al. 2007, evaluated the motivation of 100 producers in adopting best management practices for improved health, and found that motivation for improved management was driven by economics. The avoidance of penalties for low quality milk was more motivating than being rewarded with a premium price for high quality milk. The
drawback to being motivated by penalties, is that this type of motivation results in a compulsory behaviour that is facilitated by coercion (such as governmental regulations and restrictive provisions) and such behavioural change will only exist as long as coercion exists (Van Woerkum et al. 1999). Change that is voluntary is preferable, and more likely to persist compared to compulsory behaviour. An excellent example of voluntary behaviour towards improving health is evident by producers participating in the National Health Project in Canada. Following the implementation in 2007 program for recording 8 diseases affecting dairy cattle, 38% of producers enrolled in dairy herd improvement with CanWestDHI (Western provinces and Ontario) or Valacta (Quebec and the Maritimes) are voluntarily reporting health events on an on-going basis. This represents 41% of milk recorded cows in Canada. Progress needs to be made, but it is a great start. Additional participation may be dependent upon producer motivation to improve health, how producers acquire information and how their veterinarian presents information in an advisory capacity.

Leeuwis et al. 2006 developed a decision-making model based on a mindset in interaction with others, and indicates that what people do or not do depends on different factors, and are influenced by identity. A farmer’s mindset can then be seen as a combination of what farmers want, know, believe and perceive regarding cattle health. This mindset can support the farmers’ behaviour and the way it may be influenced. Lam et al. 2008 describes differences between farmers and discusses the importance of knowing how farmers would like to learn and which information sources they use. They have different learning styles and communication to producers likely should be adapted toward these different learning styles. Kolb theory indicates that each person’s ability to learn is enhanced by strategies that conform to the individual’s preferred learning style. Kolb differentiates 4 learning styles and these include the accommodator, the diverger, the converger and the assimilator. Accommodators are individuals that use concrete experience and active experimentation through trial and error. Lam et al. 2008 describes this type of producer as one who tries to get a machine running without looking at a manual and they learn by being able to try a new product to develop a concrete experience with it. Divergers tend toward concrete experience and reflective observation. A producer may be described as a diverger if they engage other perspectives on how to approach a problem through discussion with others. They appreciate excursions to model farms and open door days. Convergers are characterized by their ability to put theories into practice and appreciate step by
step instructions and treatment schedules put together for udder health programs. They are good at making practical applications of ideas and using deductive reasoning to solve problems. Assimilators learn by sorting information into concise logic. Lam et al. 2008 describes producers as assimilators as they gather information from multiple sources including websites, farming magazines, information from dairy shows, etc. They are like scientists and gather lots of information from more than one source, and gradually form an idea on the subject they are investigating.

Jansen et al. 2010b reported that producers tend to be reached by two routes of communication. First, the “central route” by which producers motivated to change their health management behavior, acquire and apply argument based knowledge (proven research concepts and some common sense) from the newspaper, internet or other media, and seek out information from their veterinarian and other producers. This is the formal learning style and is applied by farmers that fall into one of the four learning styles listed above. Producers that do not acquire information via the central route are considered “hard to reach”. They likely do not take maximum advantage of access to publication materials (newspapers or magazines) or the internet, do not regularly consult with their veterinarian or other producers, and therefore do not to stay up to date on local, national and international research that results in the development of guidelines regarding mastitis prevention and control. They are much less likely to apply best practices for preventing mastitis and thus are expected to have higher rates of mastitis and other diseases in their herds. As it turns out, these hard to reach producers are not a homogeneous group and some are motivated to improve animal health, and they implement management practices in differing ways. Knowledge transfer to this group may be obtained via a “peripheral route” using non-formal or informal methods, such as persuasive advertisement campaigns that may cause them to change their behaviour (Jansen et al., 2010b). One such campaign in the Netherlands was conducted to get producers to wear gloves during milking to prevent infection. This approach aimed at changing producers’ behaviour through peripheral cues such as the distribution of free glove samples, humorous postcards, and the launch of a website and campaign during an agricultural fair. Results from a telephone survey of hard to reach producers (as identified by their herd health veterinarians) before, during and after the campaign indicated an increase from 20.9% and 42% in use of gloves. This research indicated that both the central and peripheral routes are effective at transferring knowledge to a specific
target group of farmers and changing their behaviour.

Most Knowledge Attitude and Behaviour (KAB) research in the literature concerning North American farmers has focused on the sun prevention behaviors of farmers (wearing or not wearing a hat) and how this may cause an increased risk for skin cancer (Silk and Parrott 2006). Little or no research is available on how North American dairy farmers’ attitudes and knowledge affect how they acquire and apply animal health management tools. Barriers to applying knowledge may lie in the method of communication, or the inability of some veterinarians to effectively communicate knowledge because they do not understand how their individual clients learn. Further to that, the methods of communication used or even a lack of communication may be the reason why certain technologies or methods are not implemented effectively. Veterinarians and other advisors play a very significant role in informing their clients on best practices and if along the way there is any doubt or lack of confidence in protocols, this can reduce compliance. Understanding what barriers exist is necessary to enhance knowledge transfer to improve the prevention, rapid identification and swift treatment of emerging and re-emerging diseases.

3.2 Marketing technologies from academia

The process of bringing a new scientific technology from the bench to the marketplace is complex. Proper introduction of a new technology requires that: 1) the research behind it be sound; 2) it is effective at accomplishing its claim; 3) it is safe; 4) there is an infrastructure for production, distribution, and sales of the product or service; and finally 5) there are customers or a market to serve. Several considerations must be made about the technology, whether it will create a start-up business that operates in product markets, or a start-up that will operate in markets for technology (Pries and Guild, 2007). In addition, appropriate market research must be done through the collection of demographic and psychographic data on the proposed target market. With this information, market segments with different needs can be identified, allowing the inventor or company to appropriately target their new technology to those that will use it, and position it so that it is attractive and available for that target group (Strategic Agri-Marketing, 2011).
Although some great technologies have been developed and researchers at universities are doing great work, they are not doing well at getting these innovations into the marketplace. It is estimated that while only 12% of academic faculty are repeat commercializers and account for 80% of commercialized innovations (Hoye and Pries, 2009), 88% of academics fail at commercialization. It may be beneficial to work with a company that may be interested in the technology and would be willing to license the technology and develop markets around that technology as it has the sales and distribution and regulatory channels in order to make it a success. This is called a start-up market for technology and this will allow the technology to enter into the marketplace, and not be stalled in the technology gap between the bench and marketplace. Pries and Guild 2011 also identifies characteristics that aid in the decision of an appropriate business model for that technology to be a success. These include: 1) patent and other legal protection; 2) specialized complementary assets; 3) commercial uncertainty and 4) technological dynamism (life expectancy of the technology). Specialized complimentary assets to commercialize the technology include manufacturing capabilities, distribution channels, a knowledgeable sales force, and after sales support. Commercial uncertainty includes uncertainty that the technology is ready for market, and uncertainty that there is a market for additional services and products can be derived from the technology. Pries and Guild 2011 found that if a technology has patent or legal protection, there was greater success that the technology would be commercialized by transferring limited rights of the technology to existing firms. Further they found that greater commercial uncertainty was associated with a greater likelihood that the technology would be commercialized by the creation of a new firm or the transfer for the rights of the technology to an existing firm. With every technology there is a life expectancy attached to it with certain changes that must occur in order for it to be adopted by the market. Start-up firms, existing firms and researchers need be aware of the technology adoption cycle of high tech that has been described well by Geoffrey A. Moore in Crossing The Chasm. Once in the market, they will need to change their marketing strategy several times as the technology moves through this cycle. The technology, if successful, will evolve from attracting the innovator and early adopter, to crossing the chasm by adapting its technology for a segment called the early majority (the early market). It is comprised of pragmatists that want to make progress but without creating too much risk. Once well established in the early marketplace, the technology may then again be adapted for the late majority of individuals (the mainstream market) looking
for a reduction in price or a simplified version of the product or service.

An example of a new technology that may be successful to operate within the dairy marketplace to meet the challenges in animal health, is High Immune Response (HIR) (Mallard et al. 2011). It is a unique patented evaluation technology developed to identify dairy cattle with high adaptive immune response capabilities. The test includes a blood sample to evaluate antibody-mediated immune response (AMIR) and a skin thickness measurement to evaluate cell-mediated immune response (CMIR), and may be done on calves as young as 2 months of age. Dairy cattle with high immune response following immunization with specified test antigens are at a lower risk for developing disease as compared to animals that demonstrate an average or low immune response. Identification of high, average and low immune response dairy cattle may be useful to the producer as an effective health management tool for culling, grouping, breeding and the treatment of cattle. This technology has the potential to significantly improve the health and food quality of Canadian dairy cattle through the reduction of antibiotics and disease treatment costs, and through enhanced resistance to economically important diseases such as mastitis. Most dairy producers rely on the existing services provided by their veterinarians and other support industry to make informed decisions about the health status of their herd. Nonetheless, certain diseases are not well-controlled using traditional treatment or preventative strategies, such as vaccination or antibiotic therapy. Emerging and re-emerging diseases in livestock remains a major concern to both animal and human health, as well as food safety and food quality. Quantitative genetic approaches, such as HIR, that improve animal health and well-being and that do not use antibiotics, genetically-modified organisms or animal cloning, are considered most favourably by consumers. HIR gives the producer or breeding company a valuable glimpse into the future and allows them to make timely decisions to improve overall herd health.
CHAPTER I

Expression of TLR2 Pattern Recognition Receptor on Mononuclear Cells of Dairy Cattle
Ranked Using Estimated Breeding Values (EBV) of Adaptive Immune Response Traits

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ABSTRACT

Toll-like receptor 2 (TLR2) is a pattern recognition receptor expressed on the surface of epithelial cells and some leukocytes. Binding of bacterial ligands such as lipoproteins and lipopeptides to TLR2 induces proinflammatory innate responses and contributes to the development of adaptive immune responses. In the present study we investigate whether expression of TLR2 by blood mononuclear cells differs among Holstein dairy cows classified as high (H), average (A), and low (L) immune responders, based on their estimated breeding values (EBVs) for antibody-mediated (AMIR) and cell-mediated (CMIR) immune responses. Cells were stained using fluorochrome-labeled monoclonal antibodies, and characterized using flow cytometry. A significantly higher proportion of unstimulated mononuclear cells from H-AMIR cows expressed TLR2, compared to cells from L-AMIR, and H-CMIR cows. Expression of TLR2 may contribute to the enhanced antibody responses and reduced incidence of clinical disease reported for H-AMIR cows.

Keywords: dairy cattle, TLR2, innate
INTRODUCTION

Optimal host defense begins with the innate system and includes barriers such as the epithelium as well as enzymes, proteins and cells that have the ability to respond to a first encounter with a pathogen. Effective signaling by the innate system leads to the influx of intermediary cells such as neutrophils and macrophages, leading eventually to the development of an effective adaptive immune response. The adaptive immune system has the hallmarks of memory, specificity, diversity and self-nonself recognition. The innate and adaptive systems are interconnected and allow the host to respond to an invading pathogen with some general reactivity on first exposure, but with exquisite specificity on second and subsequent exposures.

The host is alerted to the threat of an invading pathogen through the activation of pattern recognition receptors (PRRs). Both vertebrates and invertebrates express PRRs. They can recognize the molecular patterns that are conserved among pathogens (Takeda et al., 2004). Toll-like receptors (TLR) are a group of PRRs expressed by epithelial cells and leukocytes, that bind to pathogen associated molecular patterns (PAMPS). When these receptors bind to PAMPS a series of events occurs that transduces a signal to the nucleus, resulting in expression of cytokines including type I interferons and chemokines (Kumar et al., 2009). These cytokines attract other innate cells such as neutrophils, macrophages and dendritic cells that also express TLRs on their cell surface. Macrophages and dendritic cells, under the influence of TLR signaling and cytokines, act as antigen presenting cells, activating T helper cell sub-populations. T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune response (CMIR) to respond to intracellular pathogens such as Mycobacterium avium ssp. paratuberculosis (the cause of Johne’s disease). In contrast, T helper 2 (Th2) cells produce cytokines that tend to generate an antibody-mediated immune response (AMIR) towards extracellular pathogens such as Escherichia coli known to cause mastitis.

Work by Wagter et al. (2000) has shown that dairy cows with a high AMIR have less disease, and a recent study of 58 dairy herds (part of the Canadian Bovine Mastitis Research Network) has shown that high AMIR responders have a lower incidence of mastitis compared to average and low responders and tend to have less severe mastitis (Thompson-Crispi et al., 2013).
A study in a large dairy in the USA, has also documented a lower incidence of mastitis, ketosis, metritis and retained placenta in cows with high AMIR and CMIR in comparison to average or low responders (Thompson-Crispi et al., 2012). Therefore the objective of this paper was to evaluate TLR2 expression in the context of High (H), Average (A) and Low (L) immune response for AMIR and CMIR immune response traits, and to compare TLR2 expression in blood leukocyte subsets between H-AMIR and H-CMIR groups.

**MATERIAL & METHODS**

**Animals.** Twenty-eight cows previously phenotyped for AMIR and CMIR were classified by their estimated breeding value (EBV) into High, Average, and Low groups based on a procedure described by Thompson-Crispi et al. (2013). Cows with an EBV of greater than one standard deviation above the mean were considered high immune responders, and cows with an EBV more than one standard deviation below the mean were considered low immune responders. Cows in the following groups were studied: H-AMIR/A-AMIR/L-AMIR and H-CMIR/A-CMIR/L-CMIR.

**Cell Isolation and Staining.** Mononuclear cells were isolated from whole blood using SepMate tubes and Histopaque 1077 density gradient solution. Cells were counted and re-suspended in PBS containing 0.5% BSA. Cells were assayed for viability. Cells were then added onto a Costar 96 well round bottom plate (Sigma, St. Louis, MO) at 1.0x10⁶ cells per well, for a total of 9 wells per row. The first well was an unstained negative control, the second an isotype control to detect non-specific binding of mouse IgG1 (AbDSerotec). The next four wells were single stained in this order: human monoclonal antibody (mAb) anti-bovine TLR2 (CD282) conjugated with Fluorescein isothiocyanate (FITC) (AbDSerotec), then mouse IgG1 anti-bovine CD14 (monocytes; VMRD) mAb, mouse IgG1 anti-bovine BAQ155A (a pan B cell marker; VMRD, Galeotti et al., 1993) mAb; and mouse IgG1 anti-bovine CD3 (a pan T cell marker; VMRD) mAb. The final three wells were double stained for TLR2 in combination with CD14, BAQ155A, or CD3. The secondary antibody used to label the mouse mAbs was goat anti-mouse IgG1 conjugated to phycoerythrin-Texas Red (Invitrogen, San Diego, CA). Cells were incubated for 30 minutes for primary and secondary antibody, were washed 3 times after each stain, and
were resuspended in 1% paraformaldehyde overnight and were examined by flow cytometry the next morning.

**Cell Scan and Analysis.** Cell marker expression was measured using two-colour detection on a Becton Dickinson FACScan. Raw data were analyzed using FlowJo (version 7) software. Cells were gated to include monocytes and lymphocytes together and results were expressed as a percentage.

**Statistical Methods.** Log-transformed TLR2 expression data were evaluated using the PROC GLM procedure of SAS using the following models:

Model 1  \[ Y = \mu + \text{AMIR group} + \text{parity} + \text{error} \]
Model 2  \[ Y = \mu + \text{CMIR group} + \text{parity} + \text{error} \]

Where \( Y \) = log percent of a cell population with the phenotype: TLR2+, TLR2+CD14+, TLR2+BAQ155A+, or TLR2+CD3+

Student’s t-test was used to compare H-AMIR and H-CMIR groups for the expression of TLR2, and also to compare Average and Low immune response groups.

**RESULTS**

**AMIR classification.** The Proc GLM model significantly accounted for the variation in TLR2 expression (\( R^2 = 0.39, p < 0.04 \)). In addition, Type III sums of squares indicated that AMIR group was approaching significance (\( p < 0.06 \)), whereas increasing parity was significantly associated with increased TLR2 expression (\( p < 0.034 \)). The LS Mean for TLR2 expression on mononuclear cells of H-AMIR cows was significantly higher (\( p < 0.03 \)) than that for L-AMIR cows (Figure 1). **CMIR classification.** The Proc GLM model was not significant in accounting for the variation in TLR2 expression by CMIR group. Data not shown.

**Comparison of TLR2 expression between High, Average and Low AMIR and CMIR groups.** A Student’s t test of the percentage of mononuclear cells expressing TLR2 between H-AMIR and H-CMIR groups indicated that there was a significant difference (\( p < 0.05 \)) between H-AMIR and H-CMIR groups, but not significantly different between Average A-
AMIR and A-CMIR groups, or Low L-AMIR and L-CMIR groups (Figure 2). Additionally, TLR2 expression was significantly different between H-AMIR and H-CMIR on all white cells combined and monocytes (CD14+), but not T cells (CD3+) and B cells (Figure 3).

**DISCUSSION**

TLRs bind to different types of pathogens by dimerizing with themselves or other TLRs to alert the cell of an infection (Kumar et al., 2009). For example, TLR2 can dimerize with TLR6 to detect lipoteichoic acid (LTA) on Gram+ organisms like *Staphylococcus aureus* or *streptococci* known to cause mastitis. TLR2 can also dimerize with TLR1 to bind lipoarabinomannan (LAM), a component of mycobacteria such as *Mycobacteria avium* ssp. *paratuberculosis* (MAP) that causes Johne’s disease. Diseases including mastitis are costly for the dairy industry. The cost of mastitis in Canada is estimated to be $200 per case of mastitis, and one out of every five dairy quarters in Canada is infected with a mastitis-causing pathogen at any given point in time (Canadian Bovine Mastitis Research Network, “What’s New in the World of Mastitis Research?” [http://www.medvet.umontreal.ca/rcrmb/dynamiques/PDF_AN/Results/NewspaperWhatsNew.pdf](http://www.medvet.umontreal.ca/rcrmb/dynamiques/PDF_AN/Results/NewspaperWhatsNew.pdf) (2009). Recent work by Thompson-Crispi et al., 2013) has shown that H-AMIR animals have significantly less mastitis. Additionally H-AMIR cows tended to have less severe mastitis than L-AMIR cows. In the current study evaluating differences in TLR2 expression on cells among AMIR and CMIR phenotypic groups, cows with H-AMIR had more total mononuclear cells expressing TLR2 compared to L-AMIR cows. As TLR2 is responsible for binding PAMPs associated with pathogens causing mastitis and initiating protective immune responses this may be a possible explanation for better protection in cattle with a H-AMIR phenotype.

This study also looked at the percentage of monocytes, T, and B cells expressing TLR2 among cows that were biased for H-AMIR (N= 6) and or H-CMIR (N=9) responses. Work by Kwong et al., 2011 in cattle reported a greater proportion of CD14+ monocytes among peripheral blood mononuclear cells expressing TLR2. In the current study where animals were categorized
into H-AMIR or a H-CMIR phenotype, there was a significantly greater percentage of CD14+ monocytes expressing TLR2 among H-AMIR cows in contrast to H-CMIR cattle. That H-AMIR cows had a higher proportion of CD14+ monocytes expressing TLR2 may indicate that monocytes of H-AMIR cows are better at detecting pathogens and initiating an immune response.

Though not significant, T cells from H-AMIR cows had a higher expression of TLR2 compared to H-CMIR cows. It has been reported that the expression of TLR2 on bovine and murine γδ T cells can stimulate the production of cytokines that initiate an immune response independent of T-cell receptor (TcR) stimulation (Wesch et al., 2011). So while the population of T cells evaluated in this study was low and not further defined by their expression of γδ or αβ TcR, the fact that H-AMIR cows expressed more TLR2 may contribute to enhanced innate signaling and the development of a strong antibody response phenotype. Further study to define the T cell population using additional cell surface markers may better reveal differences in TLR2 expression between H-AMIR and H-CMIR groups.

Results from this study also showed no significant difference between the percentage of B cells expressing TLR2 between H-AMIR and H-CMIR groups. Given that B cells are involved in generating an antibody response, there was an expectation to identify a significant difference in the percentage of cells expressing TLR2 between these groups. In this study, the frequency of B cells was low, and cells were not defined by additional staining of surface receptors such as IgM or other markers. Further study to better define B cell populations may help to identify key differences in TLR2 expression between H-AMIR and H-CMIR groups.

This study examined baseline expression of TLR2 on mononuclear cells isolated from the blood without activation by pathogens or molecular structures (ligands) derived from pathogens. Future studies will evaluate differences between AMIR and CMIR response groups before and after stimulation with ligands derived from intracellular (e.g. MAP) and extracellular (e.g. E. coli) pathogens known to cause disease in dairy cattle. Research in this area will help to better elucidate innate mechanisms, such as TLR activation, involved in effectively signaling an adaptive immune response.
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LITERATURE CITED


Figure 1. LS Means of percentage of mononuclear cells expressing TLR2 for cows classified by antibody mediated immune response (AMIR). The x axis represents AMIR Group and the Y axis percent expression on all gated mononuclear cells. Significant differences are indicated by letters (*p<0.03). Means which have a letter in common do NOT differ significantly at the P < 0.05 level.

Figure 2. Percent of mononuclear cells expressing TLR2 among H-AMIR & H-CMIR, A-AMIR & A-CMIR, and L-AMIR and L-CMIR groups. Significant differences are indicated by letters based on Student’s t test (*p<0.05). Means which have a letter in common do NOT differ significantly at the P < 0.05 level.

Figure 3. Percent of mononuclear cells expressing TLR2 by subsets, for H-AMIR (N=6) and H-CMIR (N=9) cows. Cell subsets: all mononuclear cells; CD14+ monocytes; BAQ155A+ B cells; CD3+ T cells. Within a cell subset, means with different letters differ significantly (Student’s t test, *p<0.05). Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
CHAPTER II

*Ex-vivo* expression of TLR2 Pattern Recognition Receptor on Blood Mononuclear Cells of Dairy Cattle Ranked using Estimated Breeding Value (EBV) for Adaptive Immune Response Traits


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ABSTRACT

Toll-like receptor 2 (TLR2) is a pattern recognition receptor (PRR) expressed on epithelial cells and some leukocytes. Binding of bacterial ligands to TLR2 induces pro-inflammatory innate host defence responses that contribute to the development of adaptive immune responses. A study was conducted to evaluate the baseline expression of TLR2 on blood mononuclear cells (BMC) of cattle classified by their estimated breeding value (EBV) for adaptive immune responses. Eighteen cows were selected for study based on their antibody-mediated (AMIR) and cell-mediated (CMIR) immune response traits. Cattle that were greater than one standard deviation above the mean EBVs for AMIR or CMIR were classified as High (H), those more than one standard deviation below the mean were classified as Low (L), and responses in between were classified as Average (A). Phenotypic groups studied were as follows: L-AMIR/L-CMIR, L-AMIR/H-CMIR, A-AMIR/A-CMIR, H-AMIR/L-CMIR and H-AMIR/H-CMIR. Mononuclear cells were stained using monoclonal antibodies (clones HCA152F, MM61A, BAQ155A, MM1A specific for TLR2, CD14, B cells, and CD3 respectively), and characterized using flow cytometry. Ex-vivo, CD14+ mononuclear cells had the highest percentage of cells expressing TLR2 among total BMC. Among study groups, L-AMIR/L-CMIR (LL) cows had a significantly higher percentage of CD14+ monocytes expressing TLR2, whereas H-AMIR/H-CMIR cows had a significantly higher median fluorescent intensity (MFI) of TLR2 expression compared to A-AMIR/A-CMIR cows on CD14+ monocytes. The higher expression of TLR2 MFI on CD14+ monocytes among cattle that rank high for both AMIR and CMIR may contribute to their known enhanced adaptive immune responses and disease resistance. An alternate strategy to Pathogen Associated Molecular Pattern (PAMP) detection appears to be employed by cows of the LL phenotype that have a greater percent of CD14+ monocytes expressing TLR2. However, cows in the L-AMIR/L-CMIR immune response group have been previously shown to be a greater risk of disease which may suggest this is the inferior strategy for pathogen detection.
The host depends on both innate defense and adaptive immune responses to prevent or reduce the severity of disease by potential pathogens. The innate system provides rapid defense against first exposure to a pathogen, while the adaptive system, although exquisitely specific, requires time for the maturation and activation of T and B lymphocytes into effector and memory cells (Werling et al. 2006). Epithelial cells and some leukocytes have protein receptors known as pattern recognition receptors (PRRs) that are able to bind to pathogen associated molecular patterns (PAMPs) unique to pathogens. There are several classes of PRRs. These include toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Kumar, 2009) and C-lectin receptors that bind carbohydrate moieties (Malmuthuge et al. 2012, Booth et al. 2011).

TLRs are widely studied PRRs that have the ability to sense microbial components in contact with a cell surface as well as microbial components within a cell. They are highly conserved across species (Akira et al. 2006). To date, 13 TLRs have been identified in mammalian species: twelve TLRs in mice (TLR1-9,11-13; Beutler 2009), 11 TLRs in humans (TLR1-10,11; Beutler 2009), and 10 in cattle (TLR1-10; McGuire et al. 2006). TLRs have a large extracellular domain consisting of 20 leucine rich repeats of 20-30 amino acids that form a hook-like structure, a transmembrane domain, and an intracellular toll/interleukin-1 receptor (TIR) domain (Akira 2006). These receptors may dimerize with themselves or other TLRs in order to effectively bind a unique PAMP of a pathogen. After ligand binding, a series of phosphorylation events occurs, transducing a signal to the nucleus that results in the production of reactive oxygen and nitrogen intermediates, and upregulation of expression of pro-inflammatory cytokine genes including IL-1ß, IL-6, TNFα, and Type 1 interferons – (IFNα and IFNß), as well as co-stimulatory molecules (Kumar, 2009 and Werling et al. 2003). Constitutive expression of TLR2 has been shown in myeloid cell populations (Werling, 2006) but there is evidence that it is also found on the surface of lymphoid cell populations such as human B cells (Booth et al. 2011) and on bovine gamma-delta T cells (Jutila et al. 2008). PAMP binding to the extracellular domains of TLRs initiates signals that eventually lead to the development of an adaptive immune response by activating T helper cell sub-populations that drive a Th1 (cell-mediated immune response) or Th2 (antibody-mediated immune response).
In the dairy cow, PAMPs from pathogens can bind to epithelial cells of various mucosal tissues including the mammary gland, to generate a signal that attracts innate cells such as neutrophils, macrophages, and dendritic cells. A variety of organisms are implicated in the development of mastitis, and thus may bind to different PRRs resulting in activation via different signaling pathways (Schukken et al., 2011). The ability of the innate response to stimulate the development of a strong adaptive immune response is important in order to prevent disease and enhance resistance to disease. Thompson-Crispi et al. 2013 reported that dairy cattle classified by their EBV for high antibody mediated immune response (AMIR) and high cell-mediated immune response (CMIR) phenotype have less clinical mastitis, as well as less severe mastitis. A previous study by Wagter et al. (2000) reported that cattle ranked high for AMIR have less disease compared to average and low immune responders. Cattle with high AMIR have a higher expression of TLR2 on the surface of their blood mononuclear cells (Wagter et al. 2014), and this may be a possible explanation for why high AMIR cattle have less disease. Work by Hine et al. 2011, has also reported that pregnancy status may affect CMIR in relation to the age of the animal, but not AMIR. The study by Wagter-Lesperance et al. 2014 evaluated the independent effects of AMIR and CMIR EBV on TLR2. Therefore the objective of this study was to evaluate the effect of parity and pregnancy on TLR2 receptor expression on the surface of blood mononuclear cells (BMC) of dairy cattle ranked by EBV for both AMIR and CMIR traits.

**MATERIALS AND METHODS**

**Animals.**

Eighteen Holstein cows housed at the University of Guelph Elora Dairy Research Facility were previously phenotyped for AMIR and CMIR and classified by EBV according to a patented procedure described by Thompson-Crispi et al. (2013). Cows with EBVs greater than one standard deviation above the mean were classified as high (H) immune responders; cows with EBVs more than one standard deviation below the mean were classified as low (L) immune responders, and all others were classified as average responders (A). In the current work cows were non-randomly selected for study from the following 5 groups: 1) L-AMIR/L-CMIR (N=3); 2)L-AMIR/H-CMIR (N=3); 3)A-AMIR/A-CMIR (N=6); 4)H-AMIR/L-CMIR (N=3); and 5)H-AMIR/H-CMIR (N=3) (Fleming et al. 2016). Although cows with similar breeding values for
immune response are not inbred, they are more similar for the traits of interest than average. Therefore fewer animals are generally required to demonstrate statistical differences than when using unclassified outbred cattle. In addition, a power analysis indicated that a sample size of 3-6 animals was sufficient for evaluating differences between IR phenotypic groups. This study was conducted in accordance with animal utilization protocols approved by the Animal Care Committee at the University of Guelph (AUP 3555).

**Cell Isolation and Staining**

Mononuclear cells were isolated from whole blood by centrifugation at 1000 x g for 15 minutes, using SepMate tubes and Histopaque 1077 density gradient solution. Cells were re-suspended in PBS containing 0.5% bovine serum albumin (BSA). Cells were then added onto a Costar 96 well round bottom plate (Sigma, St. Louis, MO) at 1.0x10^6 cells per well, for a total of 9 wells per row. The first well was an unstained negative control, the second an isotype control to detect non-specific binding of mouse IgG1 (Bio-Rad, Raleigh, NC). The next four wells were single stained in this order: human (human combinatorial antibody library; Hu-Cal) monoclonal antibody (mAb) anti-bovine TLR2 (CD282; clone HCA152F, Bio-Rad, Raleigh, NC) conjugated with Fluorescein isothiocyanate (FITC), then mouse IgG1 anti-bovine CD14 (clone MM61A, VMRD) mAb, mouse IgG1 anti-bovine BAQ155A (a pan B cell marker; VMRD; Galeotti et al., 1993) mAb, and mouse IgG1 anti-bovine CD3 (clone MM1A; a pan T cell marker; VMRD) mAb (*Table 1*). The final three wells were double stained for TLR2 in combination with mAb specific for CD14 or B cells or CD3. The secondary antibody used to label the mouse mAbs was goat anti-mouse IgG1 conjugated to phycoerythrin-Texas Red (PE-TR; Invitrogen, San Diego, CA). Cells were incubated for 30 minutes for primary and secondary antibody, were washed 3 times after each stain, and were resuspended in 1% paraformaldehyde overnight and analyzed by flow cytometry the next morning.

**Cell Scan and Analysis.**

Cell marker expression was measured using two-colour detection on a Becton Dickinson FACScan equipped with a 488nm laser and two detection filters 533, 585 nm. Raw data were analyzed using FlowJo (version 10) software. Cells were gated by forward and side scatter to include monocytes and lymphocytes together and to exclude dead cells and debris (*Figure 1A*) and *ex-vivo* results were expressed as the percentage of cells with a positive stain for TLR2
(Figure 2A) and CD14 (Figure 3A). Unstained negative controls were used to identify the threshold for single stained cells. These controls are represented in Figure 1B-1E. Histograms for single stained cells were used to establish thresholds between negative (negative peak) and positively stained cells (positive peak). These single stain thresholds were then used as controls for double stained cells. In this study, only brightly stained cells (CD14+, BAQ155A+, and CD3+) with an identifiable peak were included in this study for comparison between EBV groups. Phenotypic group data were reported as the percentage of cells expressing TLR2, but also as the median fluorescence intensity (MFI) for TLR2 on cells expressing TLR2.

Statistical Methods

Normality. The Proc Univariate procedure of SAS was run and the Shapiro-Wilk test was used to assess the normality of the distribution of data.

Multiple Linear Regression. The PROC GLM procedure was performed to evaluate the contribution of IR phenotype or cell subset, pregnancy, parity, and dilution to the variability of log-transformed TLR2 expression data.

Model #1 – Effect of IR phenotype

Y = μ + parity + pregnancy + IR phenotype + dilution + error

Where,

Y = the log percent or median fluorescence intensity (MFI) of TLR2 expression of the total blood mononuclear cell (BMC) population expressing TLR2, OR the subset population expressing TLR2+CD14+, or TLR2+BAQ155A+ or TLR2+CD3+

μ = population mean;
parity = number of times previously pregnant,
pregnancy = pregnant 1 or not-pregnant 0
IR phenotype = group = high, average or low for AMIR or CMIR according to the following classifications: 1) LAMIR/L-CMIR; 2) L-AMIR/ H-CMIR; 3) A-AMIR/A-CMIR; 4) H-AMIR/ L-CMIR; 5) H-AMIR/H-CMIR

dilution = to allow GLM to correct for whether TLR2 antibody was used neat (1), or at a dilution of 1/5 depending on experiment (2).
Model #3 – Effect of cell subset
Y = μ + parity + pregnancy + cell subset + dilution + error,
where
Y = the log percent or median fluorescence intensity (MFI) of TLR2 expression
parity = number of times previously pregnant
pregnancy = pregnant 1 or not-pregnant 0
cell subset = CD14+, BAQ155A+ or CD3+ cell subsets
dilution = to allow GLM to correct for whether TLR2 antibody was used neat (1), or at a
dilution of 1/5 depending on experiment (2).

LS means were presented if models were deemed significant at the P < 0.05 level. Any
variables that were insignificant were removed from the model. Duncan’s test for multiple range
comparisons between cell subsets was used for output from Model #2.

RESULTS

Cell subsets
Duncan’s test for multiple comparisons indicated that the percent of cells expressing TLR2 was
significantly higher for CD14+ monocytes cells compared to B cells (BAQ155A+) and T cells
(CD3+) (Figure 4).

Phenotypic groups
Results from Proc GLM indicated that pregnancy did not significantly contribute to the variation
in the percentage or the median fluorescence intensity (MFI) of TLR2 on BMC, and it was
therefore removed from the model. Among phenotypic groups, there was no significant
difference in percentage or median MFI for TLR2 expression on total BMC. Among the CD14+
monocyte subset, phenotypic group (P < 0.02) and not parity significantly affected the variation
in the percentage of cells expressing TLR2 (Table 2). Cattle that were L-AMIR/L-CMIR, had
the highest percentage of cells with TLR2 compared to other IR groups (Figure 5). Phenotypic
group P < 0.11 and parity P < 0.09 tended to affect to the variation in MFI for TLR2 expression
on CD14+monocytes (Table 2) with a trend for MFI to be highest among heifers who had not
DISCUSSION

The ability of the host to mount an adaptive immune response is dependent upon how well innate receptors can signal its development. Epithelial cells and leukocytes constitutively express innate PRRs. These receptors may be located on the surface or internally on or in the endosomal compartment, but the mechanism is essentially the same. The receptor binds to the PAMPs, signals the recruitment of adapter proteins that then lead to the activation of transcription factors that result in the production of reactive oxygen intermediates (ROI) and nitric oxide intermediates (NOI), as well as cytokines that drive an increased innate response which eventually leads to an effective adaptive response to clear a pathogen (Werling et al. 2006). Cattle have been reported to have variation in adaptive immune response, a polygenic trait that is directly underpinned by about 2000 genes and approximately 6000 genes indirectly (De Vliegher et al. 2012 and Aitken et al. 2011). Immune response traits have been shown to have a moderately high heritability and cattle can be ranked as high, average or low immune responders using their EBV for immune response. Cattle that have a high immune response have been shown to have less disease compared to low immune responding animals (Thompson-Crispi 2013, Wagter et al. 2000). Mastitis, a common infection in dairy cattle, occurs with less severity among cattle classified as high immune responders in contrast to low immune responders (Thompson-Crispi, 2012). Previously it was reported that cattle that have a high antibody mediated immune response have a higher expression of TLR2 on the surface of blood mononuclear cells. (Wagter et al. 2014). The ability of cattle to mount an effective adaptive immune response and thus be resistant to disease may be associated in part with a higher expression of these innate receptors on the surface of cells. In the current study data was presented on the average expression of total cells and individual subsets, and was further evaluated based on EBV of immune response. Among cell subsets, those expressing CD14, a monocyte marker, had the highest expression of TLR2 which is consistent with previous reports (Kwong et al. 2011; Werling et al., 2006). Monocyte-derived cells are well known for their roles...
in antigen processing and presentation, the development of reactive oxygen species and the production of proinflammatory cytokines in the development of an adaptive immune response (Mirkovitch et al. 2006). A study by Hussen et al. 2014 reported that among CD14+ bovine monocytes, 89% are reported to be classical monocytes (cM; CD14+CD16-), 4.7% are intermediate monocytes (intM; CD14+CD16+), and 5.7% are non-classical monocytes (ncM; CD14+CD16+). In this study, monocyte cells that stained brightly for CD14 were evaluated and likely were mostly of the cM monocyte type. The higher expression of TLR2 on these brightly staining cells indicates the potential readiness of CD14+ monocytes to dimerize with other TLRs like TLR1 or TLR6, to bind pathogens that then lead to the development of a signal cascade, transcription activation and then cytokine translation and expression.

In this study, it was observed that for the CD14+ monocyte subset, where parity tended to contribute to the variation in TLR2 MFI, there was a reduced expression of TLR2 for 1st parity heifers compared to heifers that either had not yet calved (parity 0) and 2nd parity cows. This may be explained by the fact that these first parity heifers would have experienced parturition and lactation for the first time. Various peripartum stressors experienced for the first time may have contributed to a reduced expression of TLR2 on their CD14+ monocytes. Although outside the scope of this investigation, further examination of known peripartum stressors on the expression of TLR2 could be examined.

Although cattle with a L-AMIR and L-CMIR phenotype had a higher percentage of cells expressing TLR2 and cattle with H-AMIR and H-CMIR had a lower percentage of cells expressing TLR2, the MFI of expression was highest for the H-AMIR and H-CMIR group. MFI is a measure of the intensity of the fluorescence on these cells and a reflection of the number of receptors per cell. Having more receptors per cell likely would be more advantageous for mounting an appropriate proinflammatory response. Few cells with more receptors could be more easily regulated compared to many cells with relatively few receptors that become activated to produce proinflammatory cytokines to attract other cells of the immune system. In the current study the MFI was higher in the High IR phenotype (H-AMIR/H-CMIR) compared to other groups, and was significantly higher than in the A-AMIR/A-CMIR group, but not to the L-AMIR/L-CMIR group. This may offer the High responders an advantage but that would need to be further investigated.

It is important to note that CD14+ dim cells that are CD16+ were not evaluated in this study. These markers define intM macrophages in cattle, and whereas cM are the most
proinflammatory macrophage in mice and human, the intM population is the most proinflammatory in cattle (Hussen et al. 2013). This study did not evaluate dim CD14+ cells which might be useful for detecting differences between IR phenotypes. In addition, the sample size of each IR phenotype for this study was small and selected non-randomly and may have increased the probability of missing a difference between IR groups. The results from this study of bovine TLR2 expression are in alignment with an earlier study (Wagter-Lesperance et al 2014) and do suggest that cattle within the High IR phenotype are at an advantage to readily engage with PAMPS of pathogens to initiate the development of a beneficial adaptive immune response. That cattle with high AMIR and CMIR have less disease may be explained, at least in part, by this enhanced expression of TLR2 receptor on the surface of sentinel cells.

CONCLUSION

Cattle with low AMIR and CMIR phenotypes had the highest percentage of BMCs expressing TLR2, but cows with the high adaptive AMIR and CMIR immune response have a significantly higher baseline MFI of expression of TLR2 on the surface of BMCs compared to average immune responders. The ability of H-AMIR and H-CMIR cattle to express more TLR2 on the surface of CD14+ BMCs may relate to their ability to bind to pathogens, then develop high adaptive immune responses contributing to a reduced incidence of disease. However, the downstream functional differences between having a greater percentage of CD14+ monocytes expressing TLR2 versus a higher MFI of TLR2 were not examined in this study. It may be that these are different means of achieving PAMP detection in cows of diverse IR phenotypes.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Laura Wright and the staff of the University of Guelph Elora Dairy Research Facility for the collection of blood, providing reproduction and health data. This study was conducted with University of Guelph Animal Care Committee approval and was funded by a Natural Sciences and Engineering Research Council of Canada Discovery grant to B.A. Mallard.
REFERENCES


Table 1. Primary and secondary antibodies used for flow cytometric analysis of blood mononuclear cell populations.

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<th>Primary</th>
<th>Isotype</th>
<th>Secondary</th>
<th>Fluorochrome</th>
<th>Dilution</th>
<th>Vendor/Clone</th>
<th>Laser line (nm)</th>
<th>Emission Filter (nm)</th>
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<td>--</td>
<td>fluorescein isothiocyanate (FITC)</td>
<td>Neat</td>
<td>Bio-Rad/ HCA152F</td>
<td>488</td>
<td>533/30</td>
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<tr>
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<td>488</td>
<td>533/30</td>
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<tr>
<td>Goat Anti-Mouse IgG1</td>
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<td>Invitrogen/M32017</td>
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Table 2. GLM of Expression of TLR2 on blood mononuclear cells at 0hr *ex-vivo*.

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<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>--</td>
<td>NS</td>
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<td>% of CD14+TLR2+</td>
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<td>NS</td>
<td>--</td>
<td>NS</td>
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<td>MFI of BAQ155A+ TLR2+</td>
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</tr>
<tr>
<td>% of CD3+TLR2+</td>
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<td>MFI of CD3+TLR2+</td>
<td>NS</td>
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Figure 1. 1A) Gating strategy of *ex-vivo* blood mononuclear cells (BMC) of unstained controls with stained samples for 1B) TLR2-FITC(FLH1), 1C) CD14-PETR(FLH2), 1D) BAQ155A-PETR(FLH2), and 1E) CD3-PETR(FLH2).
Figure 2. A) Histogram of blood mononuclear cells (BMC) stained with human anti-bovine TLR2 conjugated with fluorescein isothiocyanate (FITC). First peak is negative and second peak is positive. B) Scatter plot of cells -/+ for TLR2.
Figure 3. A) Histogram of single CD14+ stained blood mononuclear cells (BMC) with phycoerythrin-Texas Red (PE-TR). First peak is negative and second peak is positive. B) Scatter plot of cells single CD14+ stained and C) Scatterplot of cells double CD14+TLR2+ stained BMC.
**Figure 4.** *Ex-vivo* LS mean of the percentage of blood mononuclear cells (BMC) expressing TLR2. Among subsets, CD14+ BMC have a significantly higher expression of TLR2 compared to BAQ155A+ (B cell) and CD3+ (T cell) BMC. Duncan’s test for multiple comparisons indicated significant differences between cell subsets by letters above the bars. Means which have a letter in common do NOT differ significantly at the P <0.05 level.
Figure 5. *Ex-vivo* LS mean of the percentage of TLR2 on CD14+ monocytes among IR groups classified by EBV for AMIR and CMIR. Significant differences between phenotypic groups are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the $P < 0.05$ level.
**Figure 6.** *Ex-vivo* LS means of the median fluorescence intensity (MFI) of TLR2 on CD14+ monocytes among IR groups classified by EBV for AMIR and CMIR. Significant differences between phenotypic groups are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Chapter III

Comparison of In-Vitro Expression of TLR2 Pattern Recognition Receptor on Bovine Blood Mononuclear Cell Subsets Cultured with TLR2 Ligands

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ABSTRACT

Toll-like receptor 2 (TLR2) is a pattern recognition receptor (PRR) expressed on epithelial cells and some cells of the immune system. Binding of bacterial ligands to TLR2 induces pro-inflammatory innate host defence mechanisms that contribute to the development of adaptive immune responses. A study was conducted on seventeen Holstein cows to compare expression of TLR2 on blood mononuclear cells (BMC) and cell subsets of cattle *ex-vivo* and *in-vitro* following ligand stimulation with lipopolysaccharide from *Escherichia coli* (LPS); lipopolysaccharide (rough strains) from *E. coli* J5 (Rc mutant; MUT-LPS), lipoteichoic acid (LTA), PAM3CSK4, and Mannose-capped lipoarabinomannan purified from H37Rv *Mycobacterium tuberculosis* (Man-LAM). Mononuclear cells were stained using monoclonal antibodies (clones HCA152F, MM61A, BAQ155A, MM1A specific for TLR2, CD14, B cells, and CD3 respectively), and examined using flow cytometry. *Ex-vivo*, CD14+ mononuclear cells had the highest percentage of cells expressing TLR2 among total BMC. *In-vitro* after 18 hours of culture, the percentage of cells expressing TLR2 increased among all BMC and was significantly higher for BAQ155A+ B cells compared to other subsets. Variation in TLR2 expression on all BMC and subsets following various treatments were analyzed by GLM. Variation in TLR2 expression induced by PAM3CSK4 and Man-LAM could be explained by pregnancy or parity fixed effects or their interaction, but this was not the case when cells were cultured *in-vitro* for 18 hours with LTA, LPS or MUT-LPS.
INTRODUCTION

Host defense requires both innate and adaptive immune responses to prevent or reduce the severity of disease. Effective host defense relates to the quality of the first-line innate response to a pathogen that can effectively initiate the development of an adaptive response. One critical component of the innate system is a group of protein receptors on epithelial cells and some leukocytes known as pattern recognition receptors (PRRs). PRRs bind to pathogen associated molecular patterns (PAMPs) unique to pathogens. Toll-like Receptors (TLRs) are an important class of PRRs that recognize microbial components of pathogens. These receptors may dimerize with themselves or other TLRs in order to effectively bind to many PAMPs on several pathogens. After a receptor binds to a PAMP, a signal is transduced to the nucleus resulting in the production of pro-inflammatory cytokine genes including IL-1β, IL-6, TNFα, and Type 1 interferons – (IFNα and IFNβ), as well as reactive oxygen and nitrogen intermediates (Kumar, 2009; Werling et al., 2003). TLR2 is expressed largely in myeloid cell populations (Werling, 2006) but has also been detected on bovine gamma-delta T cells (Jutila et al., 2008) and the surface of human B cells (Booth et al., 2011). PAMPs bind to epithelial cells of various mucosal tissues including the mammary gland, to generate a signal that attracts innate cells such as neutrophils, monocytes, and dendritic cells to generate signals that in turn lead to the development of an adaptive immune response by activating cell-mediated immune or antibody-mediated immune responses.

Characterization of the host cell surface receptor profile prior to and following challenge with PAMPs (or ligands) of organisms known to cause disease in dairy cattle may provide clues to the strength of the signal involved in conferring an effective adaptive immune response. Several studies have focused on the effects of PAMPS including lipopolysaccharide (LPS) from *Escherichia coli*, lipoteichoic acid (LTA) from *S. aureus*, and the synthetic triacylated lipopeptide PAM3CSK4. LPS binds to the TLR4-MD2 (myeloid differentiating factor 2) complex, but can also bind to TLR2 through the assistance of LPS binding protein (LBP) and CD14 (Buetler et al., 2009; Netea et al., 2002). LTA has similar properties to LPS and can stimulate immune cells through TLR2 to produce TNFα and other inflammatory cytokines (Schwandner et al., 1999). PAM3CSK4 is a triacylated lipopeptide recognized by both TLR2 and TLR1 and is a potent activator of the proinflammatory transcription factor, NF-κB.
(Aliprantis et al., 1999; Schenk et al., 2009). Other ligands of interest include lipoarabinomannan (LAM) from mycobacteria, a cell wall component that is important for preventing phago-lysosomal fusion inside the macrophage thus inhibiting phagocytosis and antigen processing and presentation to T cells. Mannose-capped lipoarabinomannan (Man-LAM), is a macroamphiphilic lipoprotein on the surface of the *Mycobacteria tuberculosis* (*M.* *tb.*) cell envelope. Man-LAM is a PAMP recognized by *Mycobacteria tuberculosis* (*M.* *tb.*) cell envelope. Man-LAM is a PAMP recognized by TLR2 and by some other receptors on the surface of dendritic cells and macrophages, such as C-type lectins, mannose receptor (MR), DC-SIGN and Dectin-2. (Vergne et al., 2014)

The objective of this study was to determine if differences exist in expression of TLR2 among *in-vitro* subsets of blood mononuclear cells post-ligand stimulation with LPS, MUT-LPS, LTA, PAM3CSK4, and Man-LAM.

**MATERIALS AND METHODS**

**Animals**

This study was conducted using seventeen Holstein cows housed at the University of Guelph Elora Dairy Research Facility. Cows evaluated were in parity 0, 1, or 2 and included heifers and cows that were pregnant but not lactating up until 3 weeks prior to predicted calving dates or were lactating (DIM 21-366) for at least 3 weeks after calving to avoid testing cattle during the periparturient period. This study was conducted to compare expression of TLR2 on blood mononuclear cells (BMC) of cattle *ex vivo*, and *in-vitro* following treatment stimulation with purified and synthetic ligands associated with pathogens known to cause disease in dairy cattle. This study was in accordance with animal utilization protocols approved by the Animal Care Committee at the University of Guelph (AUP 3555).

**Cell Isolation and Ligand Culture**

Mononuclear cells were isolated from whole blood by centrifugation at 1000 x g for 15 minutes, using SepMate tubes (Stem Cell Technologies, Vancouver, BC) and Histopaque 1077 density gradient solution (Sigma, St. Louis, MO). Isolated cell counts and viability were measured using
a Moxi Z Mini Automated Cell Counter (Orflo Technologies, Ketchum, ID). A measure of cell viability called Moxi Production Index (MPI) ranged from 0.85-0.95 with an average of 0.90 MPI. Prior to cell culture, cells were resuspended in 15mL conical tubes with enriched cell culture media (consisting of RPMI 1640 phenol red-free, 10% fetal bovine serum, 1% L-Glutamine, 0.4% penicillin-streptomycin, and 1.4uL of 2-mercaptoethanol) to a concentration of 2.0 X 10^6 cells per mL at a volume of 3mL. Three microliters of individual ligands were then added individually to the 15mL conical and included: lipopolysaccharide (LPS) from E.coli O111:B4, Rc mutant of O111:B4 E.coli (MUT-LPS) (Sigma, St. Louis, MO), lipoteichoic acid (LTA) purified from S. aureus and PAM3CSK4 (Invitrogen, San Diego, CA). The following reagent was obtained through BEI Resources, NIAID, NIH: Mycobacterium tuberculosis, Strain H37Rv, Purified Lipoarabinomannan (LAM), NR-14848 (Man-LAM). One mL of cell suspension for each treatment (Unstim, LTA, LPS, MUT-LPS, PAM3CSK4 and Man-LAM) was added to two wells of a 24 well Costar flat bottom cell-culture plate (Sigma, St. Louis, MO). All ligands were optimized for concentration prior to inclusion in this study, and all ligands tested provided optimal results for comparison at a concentration of 1mg/mL (1µg/µL). Cells were cultured for 18 hours (18h). This time point was selected based on a previous time course experiment that provided optimal TLR2 expression results at 18h.

**Cell Harvesting**

Plates were removed from incubation and placed on ice and maintained under sterile conditions. Cells were checked visually for viability and contamination prior to harvest. Non-adherent cells were harvested from each well by pipette and transferred to two 1.5 ml microcentrifuge tubes. Cells were then centrifuged at 400 x g for 10 minutes at 4°C. Supernatant was carefully removed so as not to disturb the cell pellet, pooled, and was stored at -80°C for cytokine evaluation. Both tubes of non-adherent cells were resuspended in 150 uL PBS with 0.5% bovine serum albumin (BSA), transferred and pooled in one 5mL BD Falcon round-bottom polystyrene tube (Becton Dickinson, Mississauga, ON). Total volume was about 400-500uL. Immediately after non-adherent cells were harvested from each well, 150uL/well of TrypLE Select 1X (ThermoFisher Scientific, Rockford, IL) was added to adherent cells fixed to the plate. These cells were then incubated at 37°C and were checked using an inverted microscope until cells detached (between 30-60 min). Cells were removed by pipette for both wells and harvested into one 1.5 uL microcentrifuge tube, and each well was rinsed and harvested 3 times with 150 µL sterile 1X
PBS and all rinses were added to the tube. Adherent cell suspensions were then spun at 250 x g for 10 min at 20°C. The supernatant was discarded, and then cells were resuspended in 300 uL PBS with 0.5% BSA for a total volume of 300-400ul. Non-adherent cells (400-500 uL suspension) were combined with adherent cells (300-400uL) for a total of 700-800µL of cell suspension. All samples were kept on ice.

**Cell Plating and Staining**

*Ex-vivo* BMC were isolated from whole blood and added at 2.0x10^6 cells per well and *in-vitro* BMC harvested from 18h culture plates were divided equally per well, onto a Costar 96 well round bottom plate (Sigma, St. Louis, MO). Cells were re-suspended in 100µL PBS with 0.5% BSA. The first well was an unstained negative control, the second an isotype control to detect non-specific binding of mouse IgG1 (Bio-Rad, Raleigh, NC). The next four wells were single stained in this order: human (human combinatorial antibody library; Hu-Cal) monoclonal antibody (mAb) anti-bovine TLR2 (CD282; clone HCA152F, Bio-Rad, Raleigh, NC) conjugated with fluorescein isothiocyanate (FITC), then mouse IgG1 mAb anti-bovine CD14 (clone MM61A, VMRD), mouse IgG1 mAb anti-bovine BAQ155A (a pan B cell marker; VMRD; (Galeotti et al. (1993)), and mouse IgG1 mAb anti-bovine CD3 (clone MM1A; a pan T cell marker; VMRD, Table 1). The final three wells were double stained for TLR2 in combination with mAb specific for CD14 or B cells or CD3. The secondary antibody used to label the mouse mAbs was goat anti-mouse IgG1 conjugated to phycoerythrin-Texas Red (PE-TR; Invitrogen, San Diego, CA). Cells were incubated for 30 minutes for primary and secondary antibody, were washed 3 times after each stain, and were resuspended in 1% paraformaldehyde overnight and analyzed by flow cytometry the next morning.

**Cell Scan and Analysis**

Cell marker expression was measured using two-colour detection on a Becton Dickinson FACScan equipped with a 488nm laser and two detection filters 533 and 585 nm. Raw data were colour compensated and analyzed using FlowJo (version 10) software. Cells were gated by forward (FSC) and side scatter (SSC) to include both monocytes and lymphocytes (*Figure 1A and 2A*) and *in-vitro* results were expressed as the percentage of cells with a positive stain. Unstained negative controls were used to identify the threshold for single stained cells. These controls are represented in *Figure 1B-1E*. Histograms for single stained cells were
used to establish thresholds between negative (negative peak) and positively stained cells (positive peak) (Figures 2B, 3A & 3C, and 4A & 4C). These single stain thresholds were then used as controls for double stained cells. In this study, only brightly stained cells (CD14+, BAQ155A+ and CD3+) with an identifiable peak were included in this study for comparison between cell subsets. A representative sample of contour plots are presented in the figures to show the percent expression of TLR2 on CD14+ monocytes (Figures 3B and 3D), and BAQ155A+ B cells (Figure 4B and 4D) following culture with LTA or Man-LAM.

**Statistical Methods**

**Normality.** The PROC Univariate procedure of SAS was run and the Shapiro-Wilk test was used to assess the normality of the distribution of the data. All data were log transformed.

**Multiple Linear Regression.** The PROC GLM procedure was performed to evaluate the contribution of cell subset, pregnancy, parity, and dilution to the variability of log-transformed TLR2 expression data.

\[ Y = \mu + \text{parity} + \text{pregnancy} + \text{parity*pregnancy} + \text{cell subset} + \text{dilution} + \text{error}, \]

where

\[ Y = \text{the log percent or median fluorescence intensity (MFI) of TLR2 expression} \]

\[ \text{parity} = \text{number of times previously pregnant}; \text{ For LPS, Mut-LPS, LTA and PAM3CSK4 parity distribution was parity 0 (N=4), parity 1 (N=5), and parity 2 (N=8) for Man-LAM parity distribution was parity 0 (N=3), parity 1 (N=5) and parity 2 (N=9).} \]

\[ \text{pregnancy} = \text{pregnant 1 or not-pregnant 0} \]

\[ \text{cell subset} = \text{CD14+, BAQ155A+ or CD3+ cell subsets} \]

\[ \text{dilution} = \text{to allow GLM to correct for whether TLR2 antibody was used neat (1), or at a dilution of 1/5 depending on experiment (2).} \]

LS means were presented if models were deemed significant at the P < 0.05 level. Any variables that were insignificant were removed from the model. Duncan’s test for multiple range comparisons between cell subsets was used for output. Student's t-tests were performed to assess differences in the LS Means of percentage of cells in subsets expressing TLR2 between 18h unstimulated vs. stimulated BMC (LPS, MUT-LPS, LTA,
PAM3CSK4 or Man-LAM) and are presented in Figure 6. MFI or percent positive cells were both evaluated but only MFI results are presented in the ANOVA Table 2 since this is where the model was significant. Any variables that were not significant were removed from the model.

RESULTS

**TLR2 Receptor Expression - Ex-vivo (0h)**

CD14+ mononuclear cells had significantly higher percentage of cells expressing TLR2 among total BMC. (Figure 5A)

**TLR2 Receptor Expression - In-vitro (18h)**

*Across Timepoints.* There was a significant difference between ex-vivo (0h) and in-vitro (18h) unstimulated TLR2 receptor expression within BMC cell subsets. There was a significant increase in the percentage of total BMC cells expressing TLR2, as well as BAQ155A+ B cells expressing TLR2 post-culture. However, TLR2 expression on CD14+ monocytes decreased, and CD3+ T cell expression of TLR2 remained unchanged (Figure 5B).

*Across Subsets.* Across subsets but within time-point 18h in vitro, the percentage of BAQ155A+ B cells expressing TLR2 was significantly higher compared to CD14+ monocytes and CD3+ cells for all treatments (unstimulated, LPS, MUT-LPS, LTA, and PAM3CSK4). For Man-LAM, BMC subsets were significantly different from each other with highest expression for BAQ155A+ B cells, then CD3+ T cells, then CD14+ monocytes

*Across Treatments.* CD14+ monocytes expressing TLR2 decreased for treatments compared to unstimulated, but was significantly lower following culture with LPS, MUT-LPS, Man-LAM and PAM3CSK4 (Figures 6A, 6B, 6C, and 6D). In addition, the percentage of BAQ155A+ B cells expressing TLR2 decreased for most treatments, and this difference was significant after LPS culture, however no decrease was observed in BAQ155A+ B cells in response to LTA.

*Parity and Pregnancy Effects.* The effects of pregnancy (pregnant vs. not-pregnant) and parity (0, 1, 2) on variation in TLR2 median fluorescent intensity (MFI) expression on BMC were examined. GLM analysis of MFI indicated that variation in TLR2 expression on all BMC subsets stimulated with LPS and MUT-LPS could not be explained by pregnancy or parity effects, and therefore these ligands were not investigated further in this study.
Fixed effects that contributed to the variation of TLR2 expression among BMC cell subsets are presented in Table 2. Variation in TLR2 expression on CD14+ monocytes stimulated with PAM3CSK4 was explained by the interaction term pregnancy by parity (P <0.03). Variation in TLR2 expression on CD14+ monocytes stimulated with Man-LAM was explained by pregnancy (P <0.03), parity (P <0.02) and the interaction term pregnancy by parity (P < 0.09). For unstimulated CD14+ monocytes, there was a tendency for pregnant cows to have a lower MFI compared to non-pregnant cows. Pregnancy or parity did not significantly contribute to variation in TLR2 expression on B cells after culture for all ligands. Pregnancy however, did contribute to the variation in TLR2M MFI on CD3+ T cells, and pregnant cows had a lower expression of TLR2-MFI compared to non-pregnant cows.

DISCUSSION

Monocytes, which are derived from the myeloid lineage are the major blood leukocyte expressing TLR2 (Kwong et al., 2011). Among the monocytes, three types have been identified in humans and cattle. These monocytes possess different markers with a variable intensity of expression of CD14 amounts as high (++ ) or low (+) on their surface. These monocytes possess the classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical (CD14+CD16+) phenotypes (Hussen et al., 2014). The majority (89%) of monocytic cells are classical and as this study focused on cells with a bright positive stain and positive peak on a histogram, it is likely that the majority of classical cells would have been captured in this study. It is worth noting that in addition to monocytes, lymphocytes can also express TLR. Lymphocytes are the dominant white blood cell type in the bovine circulatory pool (Roland et al., 2014) and normal values can range from 1800-8100 cells/uL in contrast to the monocyte population which is approximately 1/10 that of the lymphocyte population at 100-700 cells/uL (Wood and Quiroz-Rocha, 2010). B lymphocytes can express both TLR2 and B cell receptors. Interaction with pathogens or ligands contributes to up-regulation of costimulatory molecules and other receptors (Booth et al., 2011) enhancing activation of B cells and development of antibody production by plasma cells.

In this study, ex-vivo expression of TLR2 was highest among CD14+ monocytes confirming an earlier study by Kwong et al. 2011, but after in-vitro culture with LPS, MUT-LPS,
LTA and PAM3CSK4, the expression of TLR2 was highest on B cells (BAQ155A+ cells). Following culture, cells increased in granularity as indicated by increased side scatter (SSC, Figure 1A), thus indicating that cells may have become activated, and increased the expression of cell surface molecules like TLR2. This shift in B cell population has been shown in mice and humans following culture (Browne et al., 2012 and Gururajan et al 2007). Work by Taraktsoglou et al. 2011 showed that following culture of monocyte-derived macrophages with purified protein derivative (PPD) from Mycobacterium bovis, there was an upregulation of TLR2 and TLR4 mRNA transcripts, and culture with LPS caused up-regulation of TLR4 expression in that study.

In contrast, the percentage of BMC expressing TLR2 decreased following ligand culture compared to an unstimulated control. This decrease was significant for CD14+ monocytes following treatment with LPS, Mut-LPS, PAM3CSK4 and Man-LAM. This result is similar to previous findings in human macrophage and dendritic cells where binding of Man-LAM to mannose receptor (MR) can have an inhibitory effect on cell activation (Vergne et al., 2014). Man-LAM is an important lipoprotein necessary for the prevention of phago-lysosomal fusion in the endocytic pathway. It plays an important role in allowing mycobacteria such as Mycobacterium tuberculosis or Mycobacterium avium subspecies paratuberculosis (MAP) to infect monocytic cells and evade phagocytic clearance and removal. In addition, there are multiple receptors on the surface of monocytes that can bind with Man-LAM. Dectin-1 and TLR2 receptor bind to Man-LAM and initiate the production of pro-inflammatory cytokines (IL-12, IL-6 and TNFα), however mannose receptor (CD206) and DC-SIGN (CD209) can have the opposite effect as inhibitory receptors on the surface of the monocyte that bind to Man-LAM, and increase the production of the anti-inflammatory cytokine IL-10. A study by Souza et al., 2013 additionally reported that incubation of macrophages for 16h with Man-LAM from MAP, prevented phagolyosomal acidification and fusion and reduced killing when MAP bacteria were added in culture. This reduced killing was reported to be associated with the inhibition of the TLR2-MAPK-p38 signaling pathway but was independent of the IL-10 regulation pathway, and thus provided additional evidence of Man-LAM as a virulence factor in MAP infection. In addition to the CD14+ cell subset, a significant decrease in the percentage of BAQ155A+ B cells expressing TLR2 was observed following treatment with LPS. In the current study, the decrease in expression of TLR2 on the cell surface may be related to the possible down-regulation that has been reported previously, but may also simply be due to the internalization of receptor-ligand
complexes inside the cell. Triantafliou et al. 2004 has reported that HEK cells transfected with TLR2 internalize GFP-labelled LTA via lipid rafts with subsequent transport to the Golgi apparatus. This was independent of cell signaling, and may simply be a mechanism whereby TLRs are recycled to the cell surface, and bound antigen is labelled and targeted for removal.

Some variation in MFI expression of TLR2 after stimulation with ligands Man-LAM or PAM3CSK4 could be explained by individual parity or pregnancy effects, as well as their interaction term (parity*pregnancy) particularly among the CD14+ subset. TLR2 MFI following Man-LAM culture was highest for heifers (parity 0) and parity 1 cows compared to parity 2 cows and was also lowest for pregnant versus non-pregnant cows. The effects of pregnancy for different categories of parity following Man-LAM treatment indicated that pregnancy was associated with reduced expression of TLR2 on the surface of CD14+ monocytes for heifers (parity 0) but not in subsequent pregnancies. For PAM3CSK4 treatment, there was a reduced expression associated pregnancy for parity 0 and 2. The differences in TLR2 might be explained first by the fact that macrophage numbers decline with age and parity (Roland et al. 2014). However, this may also be explained by the role that macrophages play during pregnancy. Oliviera et al., 2008 reported that CD68+CD14+ monocytes declined in the peripheral blood during pregnancy as early as 54 days in gestation up until late gestation while at the same time there was a large accumulation of CD68+CD14+ macrophages in the stroma of the uterus. The authors of that study proposed that these cells were recruited to the uterus and may: 1) assist in the detachment of the placenta from the uterus at parturition, 2) assist in the removal of tissue debris during uterine involution post-partum, and 3) may also be involved in promoting an anti-inflammatory state for maternal-fetal tolerance during gestation to ensure the survival of the fetus until time of parturition. While Man-LAM and PAM3CSK4 showed a similar pattern of expression by parity within pregnancy, PAM3CSK4 induced higher LS Means by parity and pregnancy for TLR2 MFI overall. This is the first report as far as we know that gives some indication that parity and pregnancy influence TLR2 expression on CD14+ monocytes following ligand stimulation with purified and synthetic triacylated lipopeptides. These effects combined with differences in the expression of TLR2 on BMC subsets may contribute to the strength of innate signaling after TLR2 receptor binding to PAMPs of a pathogen to confer a protective adaptive immune response.
CONCLUSIONS

While an overall decrease in TLR2 expression was observed for most BMC, following 18h of culture, the BAQ155A+ B cell subset was the subset that had the highest percentage of cells expressing TLR2. Stimulation of this cell subset as a result of in-vitro culture with ligands may contribute to an up-regulation of the expression of TLR2. However, following culture there was an overall decrease in the expression of TLR2 on BMC for most ligands relative to the unstimulated control. In addition, following in-vitro culture with LPS, PAM3CSK4, LTA and Man-LAM, there was a significant decrease in the percentage of CD14+ cells expressing TLR2. This was also observed for BAQ155A+ B cells after culture with LPS. This downregulation may be due to the engagement of inhibitory receptors such as that for Man-Lam that can exist on BMC. The parity of the animal, as well as the pregnancy status, was shown in this study to affect the expression of TLR2 particularly on CD14+ monocytes post treatment with Man-LAM and PAM3CSK4. These effects combined with differences in the expression of TLR2 on BMC subsets may contribute to the strength of innate signaling after TLR2 receptor binding to PAMPS of a pathogen to confer a protective adaptive immune response.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Primary and secondary antibodies used for flow cytometric analysis of peripheral blood mononuclear cell (BMC) populations

<table>
<thead>
<tr>
<th>Primary</th>
<th>Isotype</th>
<th>Secondary</th>
<th>Fluorochrome</th>
<th>Dilution</th>
<th>Vendor/Clone</th>
<th>Laser line (nm)</th>
<th>Emission Filter (nm)</th>
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<tbody>
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<td>--</td>
<td>fluorescein isothiocyanate (FITC)</td>
<td>Neat</td>
<td>Bio-Rad/ HCA152F</td>
<td>488</td>
<td>533/30</td>
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<td></td>
<td>1:400</td>
<td>VMRD/ MM61A</td>
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<tr>
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<td>533/30</td>
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<tr>
<td>Goat Anti-Mouse IgG1</td>
<td>Goate</td>
<td>anti-Mouse</td>
<td>phycoerythrin -Texas Red (PE-TR)</td>
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<td>Invitrogen/M32017</td>
<td>488</td>
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Table 2. ANOVA of the effects of pregnancy (preg), parity, pregnancy*parity on the variation in median fluorescence Intensity (MFI) of TLR2 expression after 18h culture with ligands *in-vitro*

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<th>Treatment</th>
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<th>P value</th>
<th>Preg</th>
<th>Parity</th>
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<td>NS</td>
<td>NS</td>
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<td>CD3 MFI</td>
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<td>NS</td>
<td>NS</td>
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</tbody>
</table>

1. Both MFI and percent cells expressing TLR2 were examined but for the ligands, only results for MFI are displayed since MFI was best able to explain the variation in TLR2 expression. Percent of unstimulated BAQ155A cells expressing TLR2 were significantly (P < 0.04) explained by the model.
Figure 1. 1A) Gating strategy of *in-vitro* blood mononuclear cells (BMC) of unstained controls with stained samples for 1B) TLR2-FITC(FLH1), 1C) CD14-PETR(FLH2), 1D) BAQ155A-PETR(FLH2), and 1E) CD3-PETR(FLH2).
Figure 2. Representative plots of blood mononuclear cells (BMC) after 18h in-vitro culture with lipoteichoic acid (LTA):  A) Forward (FSC) and side scatter (SSC) plot of BMC; B) Histogram of BMC stained with human anti-bovine CD282(TLR2) with fluorescein isothiocyanate (FITC). First peak is negative and second peak is positive; and C) Contour plot of TLR2+ BMC.
Figure 3. Representative plots of CD14+ blood mononuclear cells (BMC) or CD14+ monocytes stained with phycoerythrin-Texas Red (PE-TR). A) Histogram of BMC after 18h culture of with lipoteichoic acid (LTA). First peak is negative and second peak is positive for CD14; B) Contour plot of CD14+TLR2+ monocytes after 18h culture with LTA. BMC positive for TLR2 and CD14 are identified in the upper right quadrant; C) Histogram of BMC after 18h culture with mannosylated lipoarabinomannan (Man-LAM); D) Contour plot of cells CD14+TLR2+ monocytes after 18h culture with Man-LAM.
Figure 4. Representative plots of BAQ155A+ blood mononuclear cells (BMC) or B cells stained with phycoerythrin-Texas Red (PE-TR). A) Histogram of BMC cells after 18h culture of with lipoteichoic acid (LTA). First major peak is negative and second major peak is positive for BAQ155A; B) Contour plot of BAQ155A+TLR2+ B cells after 18h culture with LTA. BMC positive for TLR2 and BAQ155A are identified in the upper right quadrant; C) Histogram of BMC after 18h culture of with mannosylated lipoarabinomannan (Man-LAM); D) Contour plot of BAQ155A+TLR2+ B cells after 18h culture with Man-LAM.
Figure 5. Percentage of blood mononuclear cells (BMC) expressing TLR2. A) *Ex-vivo* LS means of the percentage of blood mononuclear cells expressing TLR2 at 0h. CD14+ monocytes have a significantly higher expression of TLR2 compared to BAQ155A+ B cells and CD3+ T cells. B) *In-vitro* LS means of the percentage of TLR2+ BMC after 18h of culture. BAQ155A+ B cells have a significantly higher expression of TLR2 compared to CD14+ monocytes and CD3+ T cells. Significant differences between phenotypic groups are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Figure 6. In-vitro differences in LS Means of percent of cells expressing TLR2 after 18h culture. Percent of unstimulated cells expressing TLR2 are shown in black bars, and stimulated results are shown in the grey bars. A) Unstimulated vs. LPS; B) Unstimulated vs. MUT-LPS; C) Unstimulated vs. Mannosylated lipoarabinomannan (Man-LAM); D) Unstimulated vs. lipoteichoic acid (LTA); and E) Unstimulated vs. PAM3CSK4. Significant differences between subsets are indicated by lines above bars at the P < 0.05 and < 0.10 level.
Figure 7. LS means of Median Fluorescence Intensity (MFI) on CD14+ monocytes by A) parity and B) pregnancy after stimulation with mannosylated lipoarabinomannan (Man-LAM). Differences between means are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Figure 8. LS means of Median Fluorescence Intensity (MFI) on CD14+ monocytes by parity within pregnancy after stimulation with A) Mannosylated lipoarabinomannan (Man-LAM) and B) PAM3CSK4. Differences between bars are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Chapter IV

Differences in expression of TLR2 pattern recognition receptor on blood mononuclear cells and cytokine expression of dairy cattle classified based on adaptive immune response

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ABSTRACT

This study was conducted to investigate differences in the expression of TLR2, an innate pattern recognition receptor (PRR), on bovine blood mononuclear cells (BMC) and cell subsets in-vitro following 18h culture with TLR2 ligands. BMC were evaluated using flow cytometry from dairy cattle (N=17) that were classified into phenotypic immune response (IR) groups based on their estimated breeding values (EBV) for antibody-mediated (AMIR) and cell-mediated (CMIR) immune response. Cattle that were greater than one standard deviation above the mean EBVs for each trait (AMIR or CMIR) were classified as High (H), those more than one standard deviation below were classified as Low (L), and responses in between were classified as Average (A). Ligands tested include lipopolysaccharide from Escherichia coli (LPS); lipopolysaccharide (rough strains) from E. coli J5 (Rc mutant; MUT-LPS), lipoteichoic acid (LTA), PAM3CSK4, and mannose-capped lipoarabinomannan purified from H37Rv Mycobacterium tuberculosis (Man-LAM). In-vitro, when BMC were combined, H-AMIR/H-CMIR cows had the highest expression of TLR2 compared to other IR groups. TLR2 expression was significantly higher for this group compared to H-AMIR/L-CMIR following culture with PAM3CSK4 or LTA. For the CD14+ cell subset, MFI was also highest for the H-AMIR/H-CMIR group compared to L-AMIR/H-CMIR, A-AMIR/A-CMIR, and H-AMIR/L-CMIR cows following culture with PAM3CSK4. The pattern of MFI expression for CD14+ monocytes was different for Man-LAM in that both the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups had significantly higher expression compared to other IR groups. Although IL-1ß, IL-6, and IL-17A were explored, IR group only contributed to the variation in IL-1ß to Man-LAM. In fact, IL-1ß was negatively associated with TLR2 expression in that the highest IL-1ß was observed with A-AMIR/A-CMIR cows and was lowest for H-AMIR/H-CMIR and L-AMIR/L-CMIR cows. Of note, the three cows in the H-AMIR/H-CMIR group had no measurable response for IL-1ß. Variation in IL-6 and IL-17A response was observed following Man-LAM culture but was not significantly explained by the effect of group, pregnancy or parity. Results of this study indicate that IR phenotype contributes to variation in TLR2 expression on bovine BMC. In the case of Man-Lam, IR group also contributed to IL-1ß expression.
INTRODUCTION

Toll-like receptors (TLRs) are widely studied pattern recognition receptors (PRRs) that have the ability to sense components of micro-organisms that come in contact with the surface of host epithelial cells and leukocytes or with receptors within intracellular endosome. TLRs have a large extracellular domain comprised of 20 leucine rich repeats that form a hook-like structure, in addition to a transmembrane domain, and an intracellular toll/interleukin-1 receptor (TIR) domain (Akira et al., 2006). These receptors may dimerize with themselves or other TLRs in order to effectively bind a unique pathogen associated molecular pattern (PAMP) of a pathogen. After ligand binding, a series of phosphorylation events occurs, transducing a signal to the nucleus that results in the production of reactive oxygen and nitrogen intermediates, and the upregulation of the expression of pro-inflammatory cytokine genes including IL-17A, IL-1β, IL-6, and TNFα (Bougarn et al., 2011; Roussel et al., 2015) or the production of Type 1 interferons – (IFNα and IFNβ), as well as co-stimulatory molecules (Kumar, 2009; Werling et al., 2003). Werling et al. (2007) reported two main TLR2 pathways for cell activation which aid in the development of a pro-inflammatory response. These include a myeloid differentiation factor (MyD88)-dependent-interferon regulatory factor (IRF-3)-independent pathway and the MyD88-independent-IRF3-dependent pathway. These both result in activation of nuclear factor (NF-κB) and the transcription of pro-inflammatory cytokines. PAMPs binding to the extracellular domains of TLRs thus initiate a signal that eventually leads to the development of an adaptive immune response. A higher TLR cell surface receptor profile on host BMC prior to and following challenge with purified PAMPs from organisms may be associated with a reduced risk for acquiring infections and disease.

Several studies have looked at the effects of purified ligands on mononuclear cells such as lipopolysaccharide (LPS) from Gram- organisms such as E.coli or Salmonella, or lipoteichoic acid (LTA) from Gram + organisms such as S. aureus (Schwandner et al., 1999; Netea et al., 2002). Some studies have also used synthetic lipopeptides to study the effects of TLR2 ligand binding, as these are free of endotoxins that can contaminate cell culture, thus targeting the direct effects of the ligand of interest. PAM3CSK4 is a triacylated lipopeptide recognized by both TLR2 and TLR1, and FSL-1 is a diacylated lipopeptide that is recognized by TLR2 and TLR6.
These both stimulate a MYD88 dependent signaling cascade and are potent activators of the proinflammatory transcription factor, NF-κB (Aliprantis et al., 1999; Schenk et al., 2009).

Cell wall components of mycobacteria have been observed to be important ligands and can stimulate the secretion of TNFα and other cytokines including IL-1β from macrophages (Underhill et al., 2011). A potent component of the mycobacterial cell wall is lipoarabinomannan or LAM and is a virulence factor important for preventing phago-lysosomal fusion inside the macrophage thus inhibiting phagocytosis and antigen processing and presentation to T cells (Souza et al., 2013). Mannose-capped lipoarabinomannan (Man-LAM) is a PAMP recognized by TLR2 and by some other receptors on the surface of dendritic cells and macrophages, such as C-type lectins including mannose receptor (MR), DC-SIGN and Dectin-2. (Vergne et al., 2014). Ligands such as Man-LAM can both stimulate (via the TLR2-p38-MAPK pathway) or inhibit cell activation via other inhibitory signal transduction pathways that are associated with mannose receptor (MR), DC-SIGN and Dectin-2. Additionally mycobacteria can also be recognized by gamma-delta T cells stimulating the production of proinflammatory IL-17A resulting in the production of other cytokines (Bougarn et al., 2011; McGill et al., 2014). So the cell types involved and the ligands binding to a mixture of mononuclear cells may affect the resultant cytokine responses and result in the development or inhibition of protective adaptive immune responses.

In the dairy cow, PAMPs from pathogens can bind to epithelial cells of various mucosal tissues including the mammary gland, to generate a signal that attracts innate cells such as neutrophils, macrophages, and dendritic cells. A variety of organisms are implicated in the development of mastitis, and thus may bind to different PRRs resulting in activation via different signaling pathways (Schukken et al., 2011). The ability of the innate response to stimulate the development of a strong adaptive immune response is important in order to prevent disease and enhance resistance to disease. Thompson-Crispi et al. (2013) reported that dairy cattle classified by their EBV for high antibody mediated immune response (AMIR) and high cell-mediated immune response (CMIR) phenotype have less clinical mastitis, as well as less severe mastitis. A previous study by Wagter et al. (2000) also reported that cattle ranked high for AMIR have less disease compared to average and low immune responders. Cattle with high AMIR were found to have a higher expression of TLR2 on the surface of their BMC (Wagter-Lesperance et al., 2014),
and this may explain in part why high AMIR cattle have less disease. The study by Wagter-Lesperance et al. (2014) evaluated the independent effects of AMIR and CMIR on TLR2, but not their combined traits. Therefore, the objective of this study was to determine if differences exist in expression of bovine TLR2 among in-vitro subsets of BMC or their cytokine expression, after stimulation with ligands using dairy cattle ranked by their estimated breeding value (EBV) for both AMIR and CMIR traits.

MATERIALS AND METHODS

Animals

This study was conducted using seventeen Holstein cows housed at the University of Guelph Elora Dairy Research Facility previously phenotyped for AMIR and CMIR according to a patented procedure described by Thompson-Crispi et al. (2013) that classified cows by EBV into High (H), Average (A), and Low (L) immune responder (IR) groups. Cows with EBVs greater than one standard deviation above the mean were classified as high (H) immune responders; cows with EBVs more than one standard deviation below the mean were classified as low (L) immune responders, and all others were classified as average responders (A) as described previously (Fleming et al., 2016). Cows evaluated were in parity 0, 1, or 2, and included cows that were pregnant and not lactating up until 3 weeks prior to predicted calving dates or lactating (DIM 21-366) for at least 3 weeks after calving to avoid testing cattle during the periparturient period. In the current investigation, cows were non-randomly selected for study from the following 5 groups: L-AMIR/L-CMIR (n=3), L-AMIR/H-CMIR (n=2), A-AMIR/A-AMIR (n=6), H-AMIR/L-CMIR (n=3), H-AMIR/H-CMIR (n=3). Although cows with similar estimated breeding values for immune response are not inbred, they are much more similar for the traits of interest than other non-phenotyped populations. Therefore, fewer animals are required to demonstrate statistical differences than when using unclassified outbred cattle. In addition, a power analysis indicated a sample size of 3-6 animals was sufficient for evaluating statistical differences between IR phenotypic groups. TLR2 expression on blood BMC among cattle classified by their EBV for combined AMIR and CMIR traits was compared following treatment with purified and synthetic ligands in-vitro. This study was conducted in accordance with animal utilization protocols approved by the Animal Care Committee at the University of
Guelph (AUP 3555).

**Cell Isolation and Ligand Culture**

Mononuclear cells were isolated from whole blood by centrifugation at 1,000 x g for 15 minutes, using SepMate tubes (Stem Cell Technologies, Vancouver, BC) and Histopaque 1077 density gradient solution (Sigma, St. Louis, MO). Isolated cell counts and viability were measured using a Moxi Z Mini Automated Cell Counter (Orflo Technologies, Ketchum, ID). A measure of cell viability called Moxi Production Index (MPI) ranged from 0.85-0.95 with an average of 0.90 MPI. Prior to cell culture, cells were resuspended in 15mL conical tubes with enriched cell culture media (consisting of RPMI 1640 phenol red-free, 10% fetal bovine serum, 1% L-Glutamine, 0.4% penicillin-streptomycin, and 1.4uL of 2-mercaptoethanol) to a concentration of 2.0 x 10^6 cells per mL at a volume of 3mL. Three microliters of individual ligands were then added individually to the 15mL conical tube and included: lipopolysaccharide (LPS) from *E. coli* O111:B4, Rc mutant of O111:B4 *E. coli* (MUT-LPS) (Sigma, St. Louis, MO), lipoteichoic acid (LTA) purified from *S. aureus* and PAM3CSK4 (Invitrogen, San Diego, CA). The following reagent was obtained through BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, Purified Lipoarabinomannan (LAM), NR-14848 (Man-LAM). One mL of cell suspension for each treatment (Unstim, LTA, LPS, MUT-LPS, PAM3CSK4 and Man-LAM was added to two wells of a 24 well Costar flat bottom cell-culture plate (Sigma, St. Louis, MO). All ligands were optimized for concentration prior to inclusion in this study, and all ligands tested provided optimal results for comparison at a concentration of 1mg/mL (1μg/μL). Cells were cultured for 18 hours (18h). This time point was selected based on a previous time course experiment that provided optimal TLR2 expression results at 18h.

**Cell Harvesting**

Plates were removed from incubation and placed on ice and maintained under sterile conditions. Cells were checked visually for viability and contamination prior to harvest. Non-adherent cells were harvested from each well by pipette and transferred to two 1.5 ml microcentrifuge tubes. Cells were then centrifuged at 400 x g for 10 minutes at 4°C. Supernatant was carefully removed so as not to disturb the cell pellet, pooled, and was stored at -80°C for cytokine evaluation. Both tubes of non-adherent cells were resuspended in 150 uL PBS with 0.5% bovine serum albumin
(BSA), transferred and pooled in one 5mL BD Falcon round-bottom polystyrene tube (Becton Dickinson, Mississauga, ON). Total volume was about 400-500uL. Immediately after non-adherent cells were harvested from each well, 150uL/well of TrypLE Select 1X (ThermoFisher Scientific, Rockford, IL) was added to cells fixed to the plate. These cells were then incubated at 37ºC and were checked using an inverted microscope until cells detached (between 30-60 min). Cells were removed by pipette for both wells and harvested into one 1.5 µL microcentrifuge tube, and each well was rinsed and harvested 3 times with 150 µL sterile 1X PBS and all rinses were added to the tube. Adherent cell suspensions were then spun at 250 x g for 10 min at 20ºC. The supernatant was discarded, and then cells were resuspended in 300 uL PBS + 0.5% BSA for a total volume of 300-400µl. Non-adherent cells (400-500 uL suspension) were combined with adherent cells (300-400uL) for a total of 700-800µL of cell suspension. All samples were kept on ice.

**Cell Plating and Staining**

*Ex-vivo* BMC were isolated from whole blood and added at 2.0x10^6 cells per well and *in-vitro* BMC harvested from 18h culture plates were divided equally per well, onto a Costar 96 well round bottom plate (Sigma, St. Louis, MO). Cells were re-suspended in 100µL PBS with 0.5% BSA. The first well was an unstained negative control, the second an isotype control to detect non-specific binding of mouse IgG1 (Bio-Rad, Raleigh, NC). The next four wells were single stained in this order: human (human combinatorial antibody library; Hu-Cal), monoclonal antibody (mAb) anti-bovine TLR2 (CD282; clone HCA152F, Bio-Rad, Raleigh, NC) conjugated with fluorescein isothiocyanate (FITC) then mouse IgG1 mAb anti-bovine CD14 (clone MM61A, VMRD), mouse IgG1 mAb anti-bovine BAQ155A (a pan B cell marker; VMRD; Galeotti et al., 1993), and mouse IgG1 mAb anti-bovine CD3 (clone MM1A; a pan T cell marker; VMRD, Table 1).

The final three wells were double stained for TLR2 in combination with mAb specific for CD14 or B cells or CD3. The secondary antibody used to label the mouse mAbs was goat anti-mouse IgG1 conjugated to phycoerythrin-Texas Red (PE-TR; Invitrogen, San Diego, CA). Cells were incubated for 30m for primary and secondary antibody, were washed 3 times after each stain, and were resuspended in 1% paraformaldehyde overnight and analyzed by flow cytometry the next morning.
Cell Scan and Analysis

Cell marker expression was measured using two-colour detection on a Becton Dickinson FACScan equipped with a 488nm laser and two detection filters 533 and 585 nm. Raw data were colour compensated and analyzed using FlowJo (version 10) software. Cells were gated by forward (FSC) and side scatter (SSC) to include both monocytes and lymphocytes (Figure 1A and 2A) and in-vitro results were expressed as the percentage of cells with a positive stain. Unstained negative controls were used to identify the threshold for single stained cells. These controls are represented in Figures 1B-1E. Histograms for single stained cells were used to establish thresholds between negative (negative peak) and positively stained cells (positive peak) (Figures 2B, 3A & 3C, and 4A & 4C). These single stain thresholds were then used as controls for double stained cells. In this study, only brightly stained cells (CD14+, BAQ155A+ and CD3+) with an identifiable peak were included in this study for comparison between cell subsets. A representative sample of contour plots are presented in the figures to show the percent expression of TLR2 on CD14+ monocytes (Figures 3B and 3D), and BAQ155A+ B cells (Figure 4B and 4D) following culture with LTA or Man-LAM.

Cytokines

Pro-inflammatory cytokines from culture supernatants were evaluated from BMC cultured 18h without ligand stimulation versus BMC cultured with LPS, MUT-LPS, or Man-LAM.

Evaluation of IL-1ß and IL-6 in cell culture supernatant - Optimization of cytokine assay

Cell culture supernatant from all treatments from a subset of 3 cows was tested to determine: 1) if results registered within range of a standard curve and 2) if it were possible to dilute supernatant to measure the various cytokines. Among 3 cows evaluated for IL-1ß and IL-6, the expression was negligible for LTA and PAM3CSK4 cell culture supernatant, and thus LTA and PAM3CSK4 supernatant from the other 15 animals was not evaluated further. For all stimulated samples an unstimulated sample at 18hr was tested for comparison.

IL-1ß protocol

Cytokines were evaluated using the Bovine IL-1ß ELISA Reagent Kit (Thermo Scientific, Rockford, IL). Coating antibody was diluted 1:100 in carbonate-bicarbonate buffer
(0.2 M) and was added to each well and incubated overnight at room temperature (rt). The following day, well contents were aspirated, and 300µL of blocking buffer (PBS + 4% BSA + 5% sucrose) was added to each well and incubated for 1h at rt. Lyophilized recombinant bovine IL-1β standard was reconstituted in reagent diluent (PBS pH 7.4 + 4% BSA). A two-fold dilution series of the standard was prepared from 2000pg/mL down to 31.5 pg/mL following the manufacturer's instructions. Among the 3 cows evaluated for IL-1β, a comparison between diluted and undiluted samples had a CV of <10%, therefore it was reasonable to dilute sample to conserve supernatant for evaluation. Samples were diluted 1 in 2, and 100µl of standard and sample was then added in duplicate and incubated for 1h at rt. The contents of the wells were then aspirated and washed with 300 µL of wash buffer (PBS + 0.05% Tween 20) per well. Detection antibody (diluted 1:100 in reagent diluent) was prepared, 100µl was added to each well, and incubated for 1h at rt. Wells were then aspirated and washed three times with 300 µL of wash buffer per well. Streptavidin-HRP was diluted 1:400 in reagent diluent, and 100µl was added to each well, and incubated for 30m at rt. Wells were then aspirated and washed three times with 300µL of wash buffer per well. 100µL of substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated in the dark for 20m at rt. Wells were then aspirated and washed three times with 300µL of wash buffer per well. 100µL of stop solution (0.16M Sulfuric Acid) was added to each well. Colour absorbance at 450 and 550 nm was measured on a Powerwave XS2 ELISA plate reader (BioTek, Winooski VT) using Gen 5 software. The mean of the optical density (OD) of the standards was then used to establish a standard curve using a four parameter regression model. The curve was then used to determine the concentration of the diluted IL-1β sample. The concentration of the diluted samples was then multiplied by the inverse of the dilution to obtain the concentration of IL-1β in each supernatant sample.

**IL-6 protocol**

Cytokines were evaluated using the Bovine IL-6 ELISA Reagent Kit (Thermo Scientific, Rockford, IL). The protocol was the same as above for IL-1β, except for the following: 1) a two-fold dilution series of the standard for Il-6 was prepared from 5000 pg/mL to 78.1pg/mL; 2) a standard curve was generated using a log-log plot of optical density to concentration; and 3) samples were added to duplicate wells, undiluted.
**Evaluation of IL-17A (LAM treatment only) - Optimization of assay**

Cell culture supernatant from all treatments from a subset of 3 cows was tested to determine two factors: 1) if results registered within range of the standard curve and 2) if it were possible to dilute supernatant. Among 3 cows evaluated, IL-17A was negligible for LPS and MUT-LPS, and thus was not evaluated further. LAM stimulation provided a readable signal when samples were plated neat (undiluted). For all stimulated samples an unstimulated sample at 18hr was tested for comparison.

**IL-17A Protocol**

IL-17A cytokine was evaluated using the Bovine IL-17-A Vet Set (Kingfisher BioTech, St. Paul, MN). Briefly, a two-fold dilution series of the IL-17A standard was prepared from 10ng/mL to a final concentration of 156.25 pg/mL in diluent buffer (PBS +4% BSA). Standard and sample supernatants (undiluted) were added in duplicate to pre-coated IL-17A antibody plates at 100μL/well and incubated 1h at rt. Plates were then aspirated and washed four times with wash buffer (PBS + 0.05% Tween 20). Then 100μL of detection antibody solution (diluted 1:24) was added to each well and incubated 1h at rt. Wells were aspirated again and washed four times with wash buffer. Diluted streptavidin-horseradish peroxidase enzyme (1:24) was added at 100μL/well) and incubated 30m at rt. Plates were then washed again four times with wash buffer and 100μL of TMB substrate solution was added to each well. The plate was then incubated in the dark for 30m at rt. 100uL of 0.18M sulfuric acid was then added to stop the substrate-enzyme reaction. Absorbance was read at 450nm on a Powerwave XS2 ELISA plate reader (BioTek) using Gen 5 software. The mean of the optical density (OD) of the standards was then used to establish a standard curve using a four parameter regression model. The means of sample replicates were used in the 4 parameter regression equation to determine the concentration of IL-17A.

**Statistical Methods**

**Normality.** The PROC Univariate procedure of SAS was run and the Shapiro-Wilk test was used to assess the normality of the distribution of data. All data were log-transformed.

**Multiple Linear Regression.**

**TLR2 Expression Data.** The PROC GLM procedure was performed to evaluate the contribution of IR phenotype, pregnancy, parity, and dilution to the variability of log-
transformed TLR2 expression data.

\[ Y = \mu + \text{parity} + \text{pregnancy} + \text{parity} \times \text{pregnancy} + \text{IR phenotype} + \text{dilution} + \text{error} \]

Where,

\[ Y = \text{the log percent or median fluorescence intensity (MFI) of TLR2 expression of the total blood mononuclear cell (BMC) population expressing TLR2, OR the subset population expressing TLR2+CD14+, or TLR2+BAQ155A+or TLR2+CD3+} \]

\[ \mu = \text{population mean;} \]

\[ \text{parity} = \text{number of times previously pregnant; For LPS, Mut-LPS, LTA and PAM3CSK4 parity distribution was parity 0 (N=4), parity 1 (N=5), and parity 2 (N=8) for Man-LAM parity distribution was parity 0 (N=3), parity 1 (N=5) and parity 2 (N=9).} \]

\[ \text{pregnancy} = \text{pregnant 1 or not-pregnant 0} \]

\[ \text{IR phenotype = group = high, average or low for AMIR or CMIR according to the following classifications: 1) LAMIR/L-CMIR; 2) L-AMIR/ H-CMIR; 3) A-AMIR/A-CMIR; 4) H-AMIR/ L-CMIR; 5) H-AMIR/H-CMIR} \]

\[ \text{dilution = to allow GLM to correct for whether TLR2 antibody was used neat (1), or at a dilution of 1/5 depending on experiment (2).} \]

LS means were presented if models were deemed significant at the \( P < 0.05 \) level. Any variables that were insignificant were removed from the model.

**Cytokines.** The PROC GLM procedure was used to evaluate the contribution of IR phenotype, pregnancy and parity to the variability of log- or square-root- transformed cytokine expression data.

\[ Y = \mu + \text{parity} + \text{preg} + \text{preg} \times \text{parity} + \text{IR phenotype} + \text{error} \]

Where \( Y = \text{the natural logarithm of cytokine (IL-1\beta, IL-6 and IL-17A) concentration in pg/mL.} \)

LS means were presented if models were deemed significant at the \( P < 0.10 \) or \( P < 0.05 \) level. Any variables that were not significant were removed from models.
RESULTS

**TLR2 Receptor Expression - In-vitro (18h)**

*Group, Pregnancy and Parity Fixed Effects.* The fixed effects of IR group, pregnancy and parity that contributed to the variation of TLR2 expression among BMC cell subsets are presented in Table 2. GLM results indicated that variation in TLR2 MFI expression on all BMC subsets stimulated with LPS and MUT-LPS could not be explained by group and were not evaluated further in that context. Variation in MFI was observed for all subsets, but only unstimulated BAQ155A B cells had significant variation in response that was explained by effects in the GLM model.

*Across IR Groups.* For BMC combined, H-AMIR/H-CMIR cows had a significantly higher MFI of TLR2 expression compared to H-AMIR/L-CMIR following culture with PAM3CSK4 or LTA (Figure 5A and 5B). For the CD14+ cell subset alone, MFI was significantly higher for the H-AMIR/H-CMIR group compared to L-AMIR/H-CMIR, A-AMIR/A-CMIR, and H-AMIR/L-CMIR cows following culture with PAM3CSK4, and MFI was significantly higher for the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups compared to other IR groups following culture with Man-LAM (Figure 6A and 6B).

*Cytokines Evaluated*

**IL-1β.** IL-1β response was negligible for unstimulated samples (data not shown), and was measurable for LAM (Figure 8A), LPS (Figure 9A), and Mut-LPS (Figure 9B) treated samples. IL-1β expression was higher for cells cultured with LPS and Mut-LPS compared to Man-LAM. Stimulated samples that were at the lower limit of detection (31 pg/mL or less) were arbitrarily classified as having a response of 31 pg/mL for purposes of analysis. For LPS and Mut-LPS, variation in expression was not explained by group, pregnancy or parity, and was therefore not further investigated in that context. Variation in IL-1β expression post Man-LAM culture only was explained by both group and parity (Table 3). H-AMIR/H-CMIR and L-AMIR/L-CMIR cows had lower LS means for IL-1β compared to other groups (Figure 7), and it is important to note that all H-AMIR/H-CMIR cows were non-responders to IL-1β and they had been assigned a cut-off value of 31 pg/mL.

**IL-6.** IL-6 response was negligible for unstimulated samples (data not shown), and was
measurable for LAM (Figure 8B), LPS (Figure 9B), and Mut-LPS (Figure 9D) treated samples. IL-6 expression was higher for cells cultured with LPS and Mut-LPS compared to Man-LAM. Stimulated samples that were at the lower limit of detection (78 pg/mL or less) were arbitrarily classified as having a response of 78 pg/mL for purposes of analysis. For LPS and Mut-LPS, variation in expression was not explained by group, pregnancy or parity, and was not investigated further. Variation in IL-6 response following culture with Man-LAM tended to be explained by parity (P <0.08; Table 3).

**IL-17A.** IL-17A was negligible for unstimulated samples (data not shown), and was measurable only for samples cultured with Man-LAM (Figure 9E). Although all Man-LAM stimulated samples showed a strong response, none of the variation in IL-17A could be explained by group, pregnancy or parity.

**DISCUSSION**

TLRs bind to a unique pathogen associated molecular pattern (PAMP) of a pathogen, then transduce a signal to the nucleus to produce cytokines to attract other cells that drive adaptive responses. TLR2 can dimerize with TLR1 to recognize triacylated lipoproteins, Gram- bacteria and mycobacteria. TLR2 can also dimerize with TLR6 to recognize Gram+ organisms and mycoplasma (Schenk et al., 2009). TLR2 has the capacity to respond to a variety of pathogens, and thus detailed study of the characterization of TLR2 expression post stimulation with ligands of organisms known to cause disease in dairy cattle can provide clues to how some innate mechanisms can enhance the initiation of an adaptive immune response. Cattle have been reported to have variation in adaptive immune response traits for antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR). These traits have a moderate heritability and cattle can be ranked as high, average or low immune responders using their EBV for each trait (Thompson-Crispi et al., 2012). Previously it was reported that cattle that have a high AMIR response have a higher expression of TLR2 on the surface of BMC (Wagter-Lesperance et al. 2014; Wagter-Lesperance thesis, Chapters 1& 2). Cattle that have a high immune response have also been shown to have less disease compared to low immune responding animals (Wagter et al., 2000; Thompson-Crispi et al., 2013). Mastitis has been noted to occur with less severity among cattle classified as high immune responders in contrast to low
immune responders (Thompson-Crispi et al., 2012). The ability of cattle to mount an effective adaptive immune response and thus be resistant to disease may be associated in part with a higher expression of innate receptors on the surface of BMC (including B and T lymphocytes and monocytes) or on epithelial cells such as those located in the udder or intestine.

A higher percentage of BAQ155A+ B cells were observed to express the most TLR2 in vitro post 18h culture (Wagter-Lesperance Thesis, Chapter 3) in contrast with CD14+ monocytes that have been observed previously to have the highest percentage of cells expressing TLR2 ex-vivo (Kwong et al., 2011; Wagter-Lesperance Thesis, Chapter 1&2). Of note, for BMC combined, TLR2 MFI post ligand culture was significantly lower for cells from H-AMIR/L-CMIR cows and highest for H-AMIR/H-CMIR cows cultured with LTA and PAM3CSK4. LTA is a virulence factor for S. aureus, one of the pathogens known to cause mastitis in dairy cattle, and the synthetic lipopeptide PAM3CSK4 is used in-vitro as a representative lipoprotein for mycobacteria and Gram- organisms. A possible explanation for why there was a low MFI for H-AMIR cattle may relate to the fact that H-AMIR cattle have less mastitis and less severe mastitis (Thompson-Crispi et al., 2012). Perhaps TLR2 on the surface of all BMC including B and T lymphocytes and monocytes, but mostly B cells post-culture, are actively binding and signaling the nucleus to produce cytokines to attract other immune cells (such as neutrophils, macrophages, dendritic cells), and could be internalizing some of the antigen for processing for antigen presentation to T cells. Thus, they may be better able to develop a strong proinflammatory signal to generate an adaptive immune response to pathogens like S. aureus that cause mastitis. In addition, it was observed in this study that cattle that were in the H-AMIR/H-CMIR group, when BMC were combined, had a high MFI for TLR2 overall, compared to H-AMIR/L-CMIR cattle. This may be a function of the ability of cows with this phenotype to not only internalize TLR2 receptor bound ligand, but also effectively process it and recycle TLR2 back to the surface of the cell. Further studies tracking the movement of tagged TLR2 receptors from the cell surface and into cytoplasmic compartments would be informative. For PAM3CSK4, the overall MFI among IR groups was similar, and was highest for H-AMIR/H-CMIR cows. For Man-LAM, overall, the MFI among IR groups was similar, but the extreme phenotypes (L-AMIR/L-CMIR and H-AMIR/H-CMIR) had the highest MFI. This was not expected, and thus it may be at the total BMC level where TLR2 expression following ligand culture has its biggest impact on IR group as it pertains to health attributes.

Investigations of cytokines among high and low adaptive IR groups have been
investigated previously in the context of the classic Th1 - Th2 paradigm, where IFN-γ is associated with high cell-mediated immune responses and high EBV for CMIR, and IL-4 is associated with high antibody-mediated adaptive immune responses and high EBV for AMIR (Martin et al., 2016). In this study, pro-inflammatory cytokines (IL-1β, IL-6, and IL-17A) were selected that had not been investigated previously for innate response to bacterial ligands common to pathogens that cause disease in dairy cattle. While LPS and Mut-LPS stimulated a response in IL-1β and IL-6 expression, only the variation of IL-1β expression in response to Man-LAM could be explained by IR group. The LS mean for IL-1β was highest for cows with A-AMIR/A-CMIR immune response, and lowest for H-AMIR/H-CMIR cows who showed no measurable IL-1β. Therefore, IL-1β was negatively associated with TLR2 expression on CD14+ monocytes in that the highest IL-1β was observed with A-AMIR/A-CMIR cows and was lowest for L-AMIR/L-CMIR and H-AMIR/H-CMIR cows. Perhaps monocytes from L-AMIR/L-CMIR cows cannot effectively signal the nucleus to produce IL-1β whereas cattle with H-AMIR/H-CMIR group not only have a high expression of TLR2 on monocytes, but may not need to produce IL-1β. Instead, they can effectively kill and remove a pathogen via another mechanism like nitric oxide production that has been observed in other studies of bovine macrophages (Emam et al., 2016; Gibson et al., 2016). High CMIR cattle have also been observed to have a reduced risk of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) based on the IDEXX antibody test. For each category increase in CMIR (L, A, H), the odds of being a strong positive responder for MAP decreased by 68% (Pinedo et al., 2009). These cytokine responses may relate to an enhanced ability of a cow with the high CMIR phenotype to also mount an effective innate response to MAP.

It is important to note that not all cytokine responses evaluated could be explained in the context of IR group, despite the fact that there was obvious variation in IL-1β, IL-6 such as was observed with LPS and MUT-LPS culture, and IL-17A in response to Man-LAM. LPS is a virulent component of *E.coli* that can cause mastitis in dairy cattle. The interaction with *E.coli* in epithelial cells of the mammary gland can produce the expression of IL-17A, which is an important cytokine that initiates the production of IL-6 and IL-1β (Roussel et al., 2015). The variation in cytokine response will require further investigation using intracellular cell staining techniques to evaluate proinflammatory cytokines produced internally by specific cell subsets at multiple time points, following the addition of ligands. Additional cytokines such as TNFα, IL-
8, IFNα and IFNβ may also be assessed to better discern the downstream effects of ligand binding and cytokine expression.

In this study, TLR2 MFI expression on combined BMC was found to be significantly higher for cows of the H-AMIR/H-CMIR phenotype compared to H-AMIR/L-CMIR cows after culture with PAM3CSK4 and LTA. For the CD14+ cell subset, MFI was also significantly higher for the H-AMIR/H-CMIR group compared to L-AMIR/H-CMIR, A-AMIR/A-CMIR, and H-AMIR/L-CMIR cows following culture with PAM3CSK4. The pattern of MFI expression for CD14+ monocytes was different for Man-LAM in that both the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups had significantly higher expression compared to other IR groups. These data indicate that IR group contributes to the variation in TLR2 expression on BMC. As the H-AMIR/H-CMIR IR group has been previously associated with a lower risk for disease (Wagter et al., 2000; Thompson-Crispi et al., 2012; Thompson-Crispi et al., 2013), and have been previously shown to have greater baseline expression of TLR2 on BMC (Wagter-Lesperance 2014, 2017 previous chapters), and based on the known function of TLRs, it is likely that TLR2 plays a role in steering adaptive immune response in cattle of different IR phenotypes. IR group also contributed to the IL-1β expression following Man-LAM culture. Taken together, the variation in TLR2 and cytokine expression may suggest that these parameters could be considered for assessing host response to pathogens in addition to measures of adaptive immune response capacity such as AMIR and CMIR.

CONCLUSIONS

Cows with the H-AMIR/H-CMIR phenotype were observed to have the highest MFI expression of TLR2 on total BMCs compared to the H-AMIR biased group (H-AMIR/L-CMIR) following culture with PAM3CSK4 or LTA. Among the CD14+ monocyte subset, MFI was also highest for the H-AMIR/H-CMIR group compared to all other groups following culture with PAM3CSK4. This pattern was slightly different in response to Man-LAM such that among the CD14+ monocyte subset the MFI was highest for cows of both the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups compared to other IR groups. It was noteworthy that IR group also contributed to the variation in IL-1β response to Man-LAM in that IL-1β concentration was lowest for H-AMIR/H-CMIR cows, who in-fact showed no IL-1β. This may indicate that other
innate mechanisms such as nitric oxide production may be involved. Variation in IL-1β and IL-6 responses following treatment with LPS or MUT-LPS, or IL-17A after treatment with Man-LAM, could not be explained by group, pregnancy or parity. A more intensive time-course investigation of intracellular and extracellular cytokine production following ligand stimulation would be useful to fully discern differences in TLR2 expression and functionality after ligand binding. The higher expression of TLR2 on BMC among cattle and associated cytokine expression among cattle that rank high for both AMIR and CMIR may contribute to enhanced adaptive immune responses and disease resistance.

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REFERENCES


Table 1. Primary and secondary antibodies used for flow cytometric analysis of peripheral blood mononuclear cell (BMC) populations.

<table>
<thead>
<tr>
<th>Primary</th>
<th>Isotype</th>
<th>Secondary</th>
<th>Fluorochrome</th>
<th>Dilution</th>
<th>Vendor/Clone</th>
<th>Laser line (nm)</th>
<th>Emission Filter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Anti- bovine CD282 (TLR2)</td>
<td>IgG1</td>
<td>--</td>
<td>fluorescein isothiocyanate (FITC)</td>
<td>Neat</td>
<td>Bio-Rad/ HCA152F</td>
<td>488</td>
<td>533/30</td>
</tr>
<tr>
<td>Mouse Anti- bovine CD14</td>
<td>IgG1</td>
<td>--</td>
<td>1:400 VMRD/ MM61A</td>
<td>488</td>
<td>533/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Anti- bovine B cell</td>
<td>IgG1</td>
<td>--</td>
<td>1:800 VMRD/ BAQ155A</td>
<td>488</td>
<td>533/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Anti- bovine CD3</td>
<td>IgG1</td>
<td>--</td>
<td>1:400 VMRD/MM1A</td>
<td>488</td>
<td>533/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG1</td>
<td></td>
<td></td>
<td>1:100 Invitrogen/M32017</td>
<td>488</td>
<td>533/30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Instrument: BD FACSscan
Table 2. ANOVA of the effects of group, pregnancy (preg), and parity on the variation in median fluorescence Intensity (MFI) of TLR2 expression after 18h culture with ligands \textit{in-vitro}.\textsuperscript{1}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dependent Variable</th>
<th>$R^2$</th>
<th>$P$ value</th>
<th>Group</th>
<th>Preg</th>
<th>Parity</th>
<th>Preg * Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>Total BMC MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>CD14 MFI</td>
<td>73.11</td>
<td>0.09</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>BAQ155A MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>CD3 MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td>LTA</td>
<td>Total BMC MFI</td>
<td>86.92</td>
<td>0.007</td>
<td>0.12</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>CD14 MFI</td>
<td>73.75</td>
<td>0.08</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>BAQ155A MFI</td>
<td>77.84</td>
<td>0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>CD3 MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td>PAM3CSK4</td>
<td>Total BMC MFI</td>
<td>98.75</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.04</td>
<td>NS</td>
<td>0.002</td>
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<tr>
<td></td>
<td>CD14 MFI</td>
<td>91.44</td>
<td>0.02</td>
<td>0.18</td>
<td>0.08</td>
<td>NS</td>
<td>0.09</td>
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<td>BAQ155A MFI</td>
<td>89.56</td>
<td>0.03</td>
<td>0.11</td>
<td>NS</td>
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<td>CD3 MFI</td>
<td>84.81</td>
<td>0.08</td>
<td>NS</td>
<td>0.08</td>
<td>NS</td>
<td>0.12</td>
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<tr>
<td>Man-LAM</td>
<td>Total BMC-MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CD14MFI</td>
<td>92.31</td>
<td>0.004</td>
<td>0.03</td>
<td>0.05</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>BAQMFII</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CD3MFI</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

\textsuperscript{1}. Both MFI and percent cells expressing TLR2 were examined but only MFI results are shown since they were best able to explain the variation in TLR2 expression.
Table 3. ANOVA of the effects of group, pregnancy (preg), and parity and preg*parity on the variation in cytokine concentrations in culture supernatant after 18h culture with Man-LAM\textsuperscript{2} in-vitro.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>R\textsuperscript{2}</th>
<th>P value</th>
<th>Group</th>
<th>Preg</th>
<th>Parity</th>
<th>Preg*Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1\beta.</td>
<td>86.88</td>
<td>0.02</td>
<td>0.008</td>
<td>NS</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6.</td>
<td>29.29</td>
<td>0.08</td>
<td>--\textsuperscript{1}</td>
<td>--</td>
<td>0.08</td>
<td>--</td>
</tr>
<tr>
<td>IL-17A</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>--</td>
</tr>
</tbody>
</table>

1. -- = removed from the model
2. Models for Mut-LPS and LPS were not significant.
Figure 1. 1A) Gating strategy of *in-vitro* blood mononuclear cells (BMC) of unstained controls with stained samples for 1B) TLR2-FITC(FLH1), 1C) CD14-PETR(FLH2), 1D) BAQ155A-PETR(FLH2), and 1E) CD3-PETR(FLH2).
Figure 2. Representative plots of blood mononuclear cells (BMC) after 18h *in-vitro* culture with lipoteichoic acid (LTA): A) Forward (FSC) and side scatter (SSC) plot of BMC; B) Histogram of BMC stained with human anti-bovine CD282(TLR2) with fluorescein isothiocyanate (FITC). First peak is negative and second peak is positive; and C) Contour plot of TLR2+ BMC.
Figure 3. Representative plots of CD14+ blood mononuclear cells (BMC) or CD14+ monocytes stained with phycoerythrin-Texas Red (PE-TR). A) Histogram of BMC after 18h culture of with lipoteichoic acid (LTA). First peak is negative and second peak is positive for CD14; B) Contour plot of CD14+TLR2+ monocytes after 18h culture with LTA. BMC positive for TLR2 and CD14 are identified in the upper right quadrant; C) Histogram of BMC after 18h culture with mannosylated lipoarabinomannan (Man-LAM); D) Contour plot of cells CD14+TLR2+ monocytes after 18h culture with Man-LAM.
Figure 4. Representative plots of BAQ155A+ blood mononuclear cells (BMC) or B cells stained with phycoerythrin-Texas Red (PE-TR). A) Histogram of BMC cells after 18h culture of with lipoteichoic acid (LTA). First major peak is negative and second major peak is positive for BAQ155A; B) Contour plot of BAQ155A+TLR2+ B cells after 18h culture with LTA. BMC positive for TLR2 and BAQ155A are identified in the upper right quadrant; C) Histogram of BMC after 18h culture of with mannosylated lipoarabinomannan (Man-LAM); D) Contour plot of BAQ155A+TLR2+ B cells after
Figure 5. LS Means of median fluorescence intensity (MFI) of TLR2 expression on total blood mononuclear cells (BMC) by immune response group. A) cultured 18h with PAM3CSK4 B) cultured 18h with lipoteichoic acid (LTA). Significant differences between phenotypic groups are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the $P < 0.05$ level.
Figure 6. LS Means of median fluorescence intensity (MFI) of TLR2 expression on CD14+ monocytes by immune response group. A) cultured 18h with PAM3CSK4; B) cultured 18h with mannosylated lipoarabinomannan (Man-LAM). Significant differences between phenotypic groups are indicated by letters above the bars. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Figure 7. LS means of IL-1β concentration by immune response group after stimulation with mannosylated lipoarabinomannan (Man-LAM). Differences between bars are indicated by letters at the P < 0.05 level of significance. Means which have a letter in common do NOT differ significantly at the P < 0.05 level.
Figure 8. Raw data for IL1-β (8A) and IL-6 (8B) concentration by immune response group after treatment with Man-LAM.
**Figure 9.** Raw data by cow for IL1-β and IL-6 expression after treatment with LPS (9A, 9B) and Mut-LPS (9C,9D), and IL17A after treatment with Man-Lam only (9E).
Chapter V

Synopsis
Chapter V – Synopsis

Effective signaling by the innate immune system leads to the development and steering of adaptive immune responses. Toll-like receptors (TLR) on antigen presenting cells (APCs) are a group of pattern recognition receptors (PRRs) that bind to PAMPs causing a series of events to occur that transduce various signals to the nucleus, resulting in expression of cytokines and other effector molecules. Cytokines attract various cells of the innate immune system such as neutrophils, macrophages and dendritic cells that also express TLRs on their cell surface. Macrophages and dendritic cells, under the influence of TLR signaling and cytokines, act as APCs, activating T helper cell sub-populations. T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune response (CMIR) to respond to intracellular pathogens such as viruses and facultative intracellular bacterium, including the bovine pathogens Mycobacterium avium ssp. paratuberculosis, while T helper 2 (Th2) cells produce cytokines that tend to generate an antibody-mediated immune response (AMIR) that help control extracellular pathogens such as Escherichia coli, one of the pathogens known to cause bovine mastitis. The host innate responses are varied and include multiple factors such as proteins (enzymes and complement), various innate cells (neutrophils and macrophages) and many different types of PRRs (TLRs, RIGs, NODs, C-type lectins) on and within leukocytes. The exact combination of innate defense mechanisms required to signal and confer an adaptive immune response are complex and still under investigation. In fact, to date there are scant publications investigating PRR expression in the context of IR classification, pregnancy and parity. The collection of studies presented here investigated the expression of bovine TLR2 on blood mononuclear cells (BMC) ex-vivo and in-vitro following culture with ligands similar to virulence factors of pathogens of dairy cattle.

In Chapter 1, the objective was to conduct a preliminary study of TLR2 expression on BMC in the context of High (H), Average (A) and Low (L) immune responders for AMIR and CMIR traits, and to compare TLR2 expression in blood leukocyte subsets between H-AMIR and H-CMIR biased groups. A significantly higher proportion of unstimulated BMC from H-AMIR cows expressed TLR2, compared to cells from L-AMIR, and H-CMIR cows. Expression of TLR2 may contribute to the enhanced antibody responses and reduced incidence of clinical mastitis reported for H-AMIR cows (Wagter et al., 2000; Thompson-Crispi et al., 2012;
Thompson-Crispi et al., 2013). The significance of this study was that it provided some of the first data on baseline TLR2 expression among cows classified by AMIR or CMIR traits. The limitation of this study was that relatively few cows were available in this experiment (n=28) and the expression of TLR2 was evaluated as separate traits, and not as combined traits. Some further statistical analysis of this data set indicated that when AMIR and CMIR were run as continuous traits, instead of categorical traits, an AMIR*CMIR interaction was observed. This is logical given that antibody responses are the product of cellular interactions where CD4+ helper T cells can activate B cells in the germinal centres of lymph nodes or spleen to initiate the development of plasma cells that produce antibody. Further studies of more animals from the 5 IR groups would be required to determine the combined interactive effects of the AMIR and CMIR traits.

In Chapter II, the objective was to evaluate ex-vivo expression of TLR2 on the surface of BMC among dairy cows ranked by EBV for both AMIR and CMIR traits. CD14+ mononuclear cells had the highest percentage of TLR2 among the total BMC population. Although cattle with a L-AMIR and L-CMIR phenotype had a higher percentage of cells expressing TLR2 and cattle with H-AMIR and H-CMIR had a lower percentage of cells expressing TLR2, the MFI of expression was highest for the H-AMIR and H-CMIR group. The MFI was higher compared to other IR groups, and was significantly higher than the A-AMIR/A-CMIR group. MFI is a measure of the intensity of the fluorescence on these cells and a reflection of the number of receptors per cell. The ability of H-AMIR and H-CMIR phenotypes to express more TLR2 on the surface of CD14+ BMCs measured ex-vivo likely relates to their ability to more readily bind pathogens, then develop high adaptive immune responses and subsequently resist disease. The significance of this study is that it provided some means to compare baseline levels of TLR2 expression among IR groups. However, the limitation of this study was that it did not provide an indication of how well the TLR2 receptors expressed would bind to ligands. Nor did this study evaluate cell functions associated with TLR2 expression.

In Chapter III, the objective was to determine if differences exist in expression of TLR2 among in-vitro subsets of BMC post-ligand stimulation. The ligands examined included LPS, MUT-LPS, LTA, PAM3CSK4, and Man-LAM and also investigated whether variation in expression was influenced by pregnancy or parity (irrespective of IR group). While an overall
A decrease in TLR2 expression was observed for most BMC following 18h culture, the BAQ155A+ B cell subset had the highest percentage of cells expressing TLR2 compared to other subsets. Stimulation of these cells as a result of in-vitro culture with ligands may be due to an up-regulation of the expression of TLR2. At 18h, CD14+ monocytes cultured with LPS, PAM3CSK4, LTA, and Man-LAM significantly had a lower TLR2 expression compared to the unstimulated control. Differences in the MFI of TLR2 expression after culture with PAM3CSK4 and Man-LAM could be explained by pregnancy or parity fixed effects, or the effect of being pregnant within a particular parity. The effects of pregnancy for different categories of parity following Man-LAM treatment indicated that pregnancy was associated with reduced expression of TLR2 on the surface of CD14+ monocytes for heifers (parity 0) but not in subsequent pregnancies. For PAM3CSK4 treatment, there was a reduced expression associated pregnancy for parity 0 and 2. Variation could not be explained by these effects when cells were cultured in-vitro for 18h with LTA, LPS or MUT-LPS. The significance of this study was that the parity of the animal, as well as the pregnancy status, was shown to affect the expression of TLR2, particularly on CD14+ monocytes post treatment with Man-LAM and PAM3CSK4. The limitation of the study was that binary data for pregnancy (pregnant versus non-pregnant) and parity groups (0, 1, and 2) were not evenly balanced for comparison. Although this was corrected for in the GLM statistical model, additional parity and pregnancy information may clarify the biological importance of these TLR2 effects.

In Chapter IV, the objective was to determine if differences exist in expression of bovine TLR2 among in-vitro subsets of BMC or their cytokine expression, after stimulation with ligands among dairy cattle ranked by their estimated breeding value (EBV) for both AMIR and CMIR traits. Cows with the H-AMIR/H-CMIR phenotype were observed to have the highest MFI of TLR2 expression compared to the H-AMIR biased group (H-AMIR/L-CMIR) following culture with PAM3CSK4 or LTA. Among the CD14+ monocytes, MFI was highest for the H-AMIR/H-CMIR group compared to all other groups following culture with PAM3CSK4. This pattern was slightly different in response to Man-LAM such that among the CD14+ monocyte subset the MFI was highest for cows of both the L-AMIR/L-CMIR and H-AMIR/H-CMIR groups compared to other IR groups. IL-1β was negatively associated with TLR2 expression in that the highest IL-1β was observed with A-AMIR/A-CMIR cows and was lowest for L-AMIR/L-CMIR and H-AMIR/H-CMIR cows. In fact, the three cows in the H-AMIR/H-CMIR were non-
responders for IL-1β. This may indicate that another innate mechanism such as nitric oxide production may be involved in driving or signaling an adaptive immune response for this IR group. Variation in IL-1β and IL-6 responses following treatment with LPS or MUT-LPS, or IL-17A after treatment with Man-LAM, could not be explained by group, pregnancy or parity. The significance of this study was the observation of distinct patterns of TLR2 and cytokine expression among IR groups on BMC post-culture. Further, the higher expression of TLR2 on BMC was observed for cows that rank high for both AMIR and CMIR, and this may contribute to enhanced innate and adaptive immune response and disease resistance for cattle with this IR phenotype. The limitation of this study was that cytokines were evaluated at only one time point (18h).

Future studies examining the association between TLR2 expressed on bovine BMC and how they signal the adaptive immune response should include:

- The exploration of other BMC cell subsets in peripheral blood – for example, as the majority of bovine BMC are lymphocytes, it would be valuable to more closely evaluate the expression of γδ T cells in the context of IR group. The literature shows that stimulation with mycobacteria such as Mycobacterium bovis, is stimulatory for TLR2 upregulation on γδ T cells and IL17A cytokine expression (McGill et al. 2014), and thus differences in response may be observed between cows in different IR groups. In addition, other BMC such as monocyte-derived macrophages (MDM), dendritic cells, and NK cells could be evaluated.
- Conducting flow cytometric triple staining to evaluate TLR2 expression on BMC cell subsets such as B, T(αβ, γδ), monocytes, MDM, dendritic cells and key intracellular cytokines (TNFα, IL-1β, IL-6, and IFNα and IFNβ, IL-10) in response to ligand binding, (provided the necessary bovine reagents are available)
- Time-course study - A more intensive time-course investigation of not only extracellular but intracellular cytokine production following ligand stimulation at multiple time points would be useful to fully discern differences in TLR2 expression and functionality immediately after ligand binding, up to and including 18h of ligand culture.
- Animals – the addition of more animals (N=>4) per IR group with balanced numbers in each parity and/ or IR group to increase the power for comparisons.
• Covariate and continuous data - The addition of more continuous data such as days pregnant and days in milk to the model may be useful for improving the statistical fit of the GLM models used to describe variation in TLR2 and cytokine expression
• Better reagents available – for example, at the time of this study, CD14+ directly conjugated monoclonal antibodies were not available, and thus the current availability of more directly conjugated monoclonal antibodies for bovine receptors like CD14+ conjugated to an offering of fluorochromes of choice should help to improve the design of a flow cytometric study, and the accuracy of detection of subsets expressing TLR2.
• Reagents under validation - the future availability of a TLR4 monoclonal antibody under validation at Washington State University will be useful to add to the findings on TLR2 receptor expression. TLR4 binds to LPS from pathogens like E. coli, a pathogen known to cause mastitis in dairy cows.
• Mammary epithelial tissue - TLR2 and cytokine expression of mammary epithelial tissue from cows of high and low IR cultured with TLR2 ligands, could also be evaluated

In conclusion, results from this collection of studies indicate that the parity of the animal, as well as the pregnancy status, affected the expression of bovine TLR2 after culture with biologically relevant ligands. In addition, a higher expression of TLR2 on BMC both ex-vivo and in-vitro following 18h ligand culture among cattle that rank high for both AMIR and CMIR was observed. This higher expression of TLR2 on total BMC may contribute to the lower incidence of disease among HIR cattle.
ADDENDUM
HIGH IMMUNE RESPONSE (HIR™) TECHNOLOGY TRANSFER

The High Immune Response (HIR™) technology is a patented test method that provides a way to identify animals with inherently superior immune responses and enhanced disease resistance. HIR can be used by livestock producers as a management and breeding tool to naturally improve the health of their herd. HIR provides benefits to the producer, the consumer and the animal through reduced use of antibiotics and other therapeutics resulting in a healthy sustainable food chain. The basic research detailing the benefits of HIR have been published in many scientific research journals and presented at various scientific conferences. As well, several extension publications have been written and presentations given throughout Canada, the USA, and Europe (delivered in English and French), to raise awareness of the benefits of HIR. These benefits are highlighted in a 2013 position paper written by L. Wagter-Lesperance for The Canadian Agri-Food Policy Institute (CAPI) entitled “A sustainable approach to livestock health”. This paper secured a 2nd place scholarship awarded by CAPI and Farm Credit Canada (FCC) (Appendix 1). In 2010, an Idea to Innovation (I2I) Phase 1 grant from NSERC was awarded to B. Mallard to conduct studies to validate original research and beta studies to demonstrate the value of HIR. Market research was conducted on interest and potential uptake of HIR by the dairy industry by L. Wagter-Lesperance, with the assistance of Drs. T. Funk and B. Mallard. As well, a Knowledge Translation and Transfer (KTT) grant was awarded to B. Mallard by the Ontario Ministry of Agriculture and Food and Rural Affairs (OMAFRA) to assist in raising awareness about HIR and its benefits among dairy producers, veterinarians, government and various dairy industry support stakeholders (breeding, pharmaceutical, nutrition, and herd improvement companies). From 2011-2013 the HIR team was involved in brand development producing a logo, a Facebook and Twitter page, banners for various dairy symposia, short informational videos on USB, a You Tube video on Farms.com (https://www.youtube.com/watch?v=4yjackdrQLI, produced with the University of Guelph SPARK program), and HIR™ technology training workshops for producers and veterinarians in Ontario and Quebec.

In October of 2011, results from the I2I and KTT grant were disseminated by L. Wagter-Lesperance at the Udder Health and Communications Conference in Utrecht, Netherlands (Appendix 2). Both qualitative (focus groups) and quantitative (market survey) market research highlighted a strong interest by the industry to uptake HIR to improve herd health. As well,
results revalidated the correlation between antibody in blood and colostrum/milk of cows and the blood of their calves at two days of age (Appendix 3). Beta studies on 4 herds were conducted and had noteworthy results but no economic values for HIR were derived. The application of HIR testing in mature sires from Semex as well as young bull calves, showed variability and did not interfere with federal health testing (Appendix 4). Once proven safe, additional testing by Semex of mature and young sires was conducted. In December 2012 the University of Guelph sublicensed the HIR™ technology to Semex to test all sires exclusively and market Immunity+ semen in Canada, the USA, and around the globe. Immunity+ was one of the top 10 “Innovation awards” at the World Dairy Expo in Madison, WI in October 2013. Over the past 3 years, progeny sired by Immunity+ bulls have been reported to have less morbidity and mortality compared to calves of non-Immunity+ sires (Larmer and Mallard, 2016). With semen sales of about $50M since January 2013, Immunity+ is a proven success for Semex, the University of Guelph, and dairy producers in Canada and abroad.
Addendum Appendix 1

A sustainable approach to improving the health of livestock

– published February 2013 on the CAPI website.
“Advancing a National Dialogue" Awards - Canadian Agri-Food Policy Institute

The Challenge - How can Canadian agri-food players produce more with less?

- This is about how the players can work differently together, and with other key stakeholders, to better manage the use of natural resources (e.g. water) and minimize impacts on the environment.
- It is about how agri-food players can anticipate and meet evolving global food demands and consumer expectations while maintaining a profitable agri-food sector.
- It is about providing practical operational ideas to do so, including how governments can support such change.

My Position

“Agri-food players can produce more with less by anticipating and meeting the evolving global food demands and consumer expectations while maintaining a profitable agri-food sector”.

The Canadian Agri-Food Sector may maintain profitability through the application of innovative sustainable agricultural practices that reduce wasteful inputs and involve careful management of existing resources over the long term. This is necessary for both plant and animal agriculture, but the focus in this paper will be on food-producing livestock where there are several emerging challenges that impact both human and animal health and well-being. These challenges include: 1) the increasing pressure to reduce antibiotic usage in order to curtail resistant pathogens, 2) the increasing costs to treat and prevent infection, and 3) the fact that diseases are both emerging and reemerging, and some of these diseases are zoonotic (transmissible to humans). The Canadian dairy industry generates $13.4 billion in economic revenue (Agriculture & Agri-Food Canada Statistics for the Canadian Dairy Industry 2011) and this information will be used to illustrate these challenges. Several services and products are available in animal agriculture to prevent, treat and diagnose specific diseases, but they do not provide an assessment of the animal’s immune response to a variety of organisms that cause different diseases. In keeping with the concept that prevention is more valuable than cure, a sustainable approach to address these challenges and increase productivity will be beneficial for the animal agriculture industry and will help support the production of food for a growing global population.

1. Consumer Concern about Antibiotic use in Animal Agriculture

As food-producing animals are managed and selectively bred to maximize production, they are also becoming more susceptible to certain diseases. Some of these diseases require treatment with antibiotics. Reports from the scientific community have identified organisms that have developed a resistance to antibiotics resulting from either the overuse of antibiotics, or inappropriate use of antibiotics to treat the organism causing infection (Berge et al. 2009, Sibergeld et al. 2008). The controlled use of antibiotics is relevant in most areas of animal agriculture, and it is strictly regulated, particularly in many European countries. In Canada, such regulations are less stringent. Nonetheless, important guidelines are in place within Canadian agriculture to ensure appropriate use of antibiotics. For example, dairy producers must withhold milk from treated cattle from bulk milk tanks until the withdrawal period indicated for that antibiotic have passed, or they risk a financial penalty (Dairy Farmer’s of Ontario 2012 – Raw
Milk Policy [http://www.milk.org/Corporate/pdf/Publications-RawMilkPolicyBook.pdf](http://www.milk.org/Corporate/pdf/Publications-RawMilkPolicyBook.pdf). This results in a loss in production for producers, but is a necessary measure to ensure that shipped milk doesn’t contain antibiotics or “inhibitors”. A high level of inhibitors, and/or somatic cells (cells of the innate immune system of the cow), is an indicator that the health of a particular herd may be compromised, either by poor environment, poor genetics, or a combination of both. These factors are continuously monitored to maintain the high-quality dairy food-chain that Canada is renowned for, but there is still room to improve dairy health while at the same time reducing traditional treatment interventions.

In several Scandinavian countries, governments are trying to ensure that the use of antibiotics is kept to a minimum (World Health Organization 2012 - [http://www.who.int/bulletin/volumes/89/3/11-030311/en/index.html](http://www.who.int/bulletin/volumes/89/3/11-030311/en/index.html)). In Denmark, the use of antibiotics is authorized and initiated by the herd health veterinarian and not the producer. The veterinarian is an independent professional and does not depend on the sale of drugs for income. So there is compliance by dairy producers to call on veterinarians, and this allows the veterinarian to accurately record disease occurrences. This information also allows the Danish government to monitor antibiotic use, and track antibiotic resistance (DANMAP 2012 - [http://www.danmap.org/About%20Danmap.aspx](http://www.danmap.org/About%20Danmap.aspx)). As additional antibiotic resistant organisms are identified in North America, such a policy involving government control and monitoring of antibiotic use could become the norm in countries like Canada and the United States.

In the past, commercial dairy cattle have been genetically selected for increased milk production, with little attention to the selection of health traits. This approach is associated with an increased rate of mastitis (an inflammation on the mammary gland), and other production diseases, particularly around the peripartum period. Other methods to achieve optimal milk production include the use of high energy nutritional diets, and in some countries like the United States, dairy cattle produce more milk when they are given injectable growth hormone or bovine somatotropin (BST). However, the use of BST in Canada is prohibited. In 1999, an expert panel of veterinarians appointed by the Canadian Veterinary Medical Association determined that such a practice presented a sufficient and unacceptable threat to the welfare of dairy cows, as it causes increased occurrence of disease (mastitis, lameness) and a higher rate of culling or removal from the herd. (Health Canada website: [http://www.hc-sc.gc.ca/dhp-mps/vet/issues-enjeux/rbst-stbr/rep_cvma-rap_acdy_tc-tm-eng.php](http://www.hc-sc.gc.ca/dhp-mps/vet/issues-enjeux/rbst-stbr/rep_cvma-rap_acdy_tc-tm-eng.php)).

**2. Disease Cost, Herd Life and Profitability of Healthy Cattle versus Disease Prone Cattle**

According to a recent report from the Canadian Bovine Mastitis Research Network, the cost of mastitis in Canada ranges from $110 to $320 per case per lactation (Canadian Bovine Mastitis Research Network - “What’s New in the World of Mastitis Research?” [http://www.medvet.umontreal.ca/rcrbm/dynamiques/PDF_AN/Results/NewspaperWhatsNew.pdf](http://www.medvet.umontreal.ca/rcrbm/dynamiques/PDF_AN/Results/NewspaperWhatsNew.pdf)). To reverse this trend a sustainable approach would be required to ensure that the increased production needed to feed a growing Canadian and global population does not compromise the health and well-being of cattle and the quality of the food that is produced. In an ideal world, animals that are selected for production should have less disease, require less antibiotics, and as such should have a longer herd life. These cows should produce enough milk per lactation such that their profitability per day of life far exceeds the value of a cow that, in contrast, has
extremely high production, but is always sick. The return on the investment of raising a young animal to breeding age is low if the amount of illness-related losses (veterinary bills, disease treatment, lost production, and recurrent infections) in the first or second lactation results in a short herd life for the animal.

3. Diseases are Emerging and Reemerging

Another concern is that there are diseases that are both emerging and re-emerging. This was particularly noted during the outbreak of bovine spongiform encephalopathy (BSE) in the UK in 1990 when thousands of animals, suspected to have consumed meat byproduct meal from sheep that had been infected with prions, were slaughtered. Also, another example of emerging disease in humans was the outbreak of H1NI influenza virus in 2009, which raised concerns about preparedness for the next influenza strain that might come (Tomley and Shirley 2009). Some of these diseases are zoonotic (transmissible between animals and humans), and thus animal agriculture, including the Canadian dairy industry, needs to prepare itself for diseases prevalent now and in the future.

Various dairy support industry companies provide services to diagnose, treat and prevent specific diseases in cattle. Examples include: 1) breeding companies that distribute sire proofs (breeding values) to improve mastitis that include Somatic Cell Score (SCS) as an indicator of udder health; 2) Dairy Herd Improvement Companies that provide information on Somatic Cell Count (SCC) & Bacterial Colony Forming Units in individual milk samples, and offer diagnostic Milk ELISA tests for S. aureus mastitis, Johne's Disease, Bovine Viral Diarrhea, and Bovine Leukosis Virus, and 3) Pharmaceutical companies that market and distribute vaccines that help prevent respiratory and gastrointestinal infections in cattle, and intra-mammary bacterial infections in lactating cows. However these methods do not deal with the natural immune response capability of the animal to combat a variety of organisms. The immune system is the body’s natural defense against invading pathogens and should provide clues to improve animal health. Therefore, a more broad-based and sustainable approach to reduce disease is required to ensure that livestock maintain both optimal health and production (Wilkie and Mallard 1999 and Mallard et al. 2011). These animals should have a robust immune system capable of resisting disease caused by a wide range of pathogens present in many different diseases.

A Sustainable Approach to Livestock Health that Meets Global Food Demands and Maintains Profitability

One sustainable approach to meet evolving global food demands and consumer expectation for healthy foods would be to adopt a system that incorporates efficient health management of livestock with superior inherent health genetics. A good example is dairy cattle with superior genes that have an enhanced ability to mount an effective immune response (Mallard et al. 2011). These animals have fewer diseases requiring less antibiotic treatment. In turn, this improves food quality and safety, as well as animal wellbeing.

The innovative High Immune Response Technology (HIR) illustrates this type of alternative strategy designed to naturally improve livestock health (de La Paz et al. 2008; Mallard et al. 2011). High Immune Response (HIR) is a unique patented evaluation technology developed at the University of
Various articles about HIR and a YouTube video are available for viewing in print and online (Ontario Milk Producer July 2011 “Identifying Healthier Cows”; YouTube video: http://ontag.farms.com/video/bonnie-mallard-high-immune-response-technology-farms-com). The HIR test system for dairy cattle identifies animals with high adaptive immune response capability. This technology has the potential to significantly improve the health and food quality of Canadian dairy cattle by reducing antibiotics and disease treatment costs, and enhancing resistance to major and costly diseases such as mastitis (Mallard et al. 2011). This system does not rely on genetically modified organisms (GMOs) or other synthetic manipulations, but takes advantage of the animal’s natural ability to mount a protective immune response. Dairy cattle with high immune response following immunization with specified inert test antigens are at a lower risk of developing disease in comparison to animals that demonstrate an average or low immune response. Identification of high, average and low immune response dairy cattle may be useful to the producer as an effective health management tool for culling, grouping, breeding and treatment of cattle. To understand how the HIR test works we need to provide a brief background on host response in dairy cattle.

### Host Response in Dairy Cattle

Dairy cattle, as well as other animals, are challenged by diverse bacterial, viral, and other pathogens. The immune system provides host defense via genetically and environmentally regulated cells and molecules that respond to pathogens. The immune system can be characterized as having two interconnected systems - the innate system which includes physical barriers and some enzymes and cells that have the ability to respond to a first encounter with a pathogen, and the adaptive immune system which has the hallmark characteristics of memory, diversity, specificity and self/non-self recognition. The adaptive immune system can be further broken down into two branches: first, the cell mediated immune response that is required against organisms like viruses and Mycobacteria, and second, the antibody mediated immune response that is required for response against bacteria like E.coli. This level of complexity within the immune system of mammals is necessary to combat a complex and diverse set of pathogens. The immune system has the ability to counter the threats of microbes and in many cases allows for harmonious relationships to be established. For selection of broad-based disease resistance to be effective, the animals selected for breeding subsequent generations must demonstrate favourable immune responses against several pathogens. This is one of the beneficial and unique features of the HIR technology.

### The HIR testing procedure

HIR testing requires 3 visits on farm over a fifteen-day period to evaluate immune responsiveness. Since this is a test measuring the genetic capacity of the host to produce an effective immune response, animals need only be tested once in their lifetime. The test requires 5-10 min./per animal/per visit. Results provide direct information on each animal’s ability to mount an immune response that is associated with disease resistance. The testing procedure’s accuracy is based on 90-95% confidence intervals (Hernandez et al. 2007), and results are heritable ($h^2=0.22-0.25$), meaning that the beneficial genes underlying the trait can be passed on to future generations. Immune response scores are determined for each animal by adding together their Estimated Breeding Values for measured AMIR and CMIR (Mallard et al. 2011). Recent studies show that if a cow scores high for immune response, that cow is at less risk for
developing disease, compared to those that have a lower score. This reduced risk results in improved health, food quality and safety. Healthier animals should require less use of antibiotics thereby helping to minimize the prevalence of antibiotic-resistant pathogens.

**HIR Is a Novel Health Management Tool**

HIR is a tool that provides livestock producers with useful information for making health management decisions. This information can be used by producers as a culling or grouping tool for managing young stock and breeding stock in their herds. The producer can decide to cull or keep a low or average immune response cow or calf that is at a higher risk for developing mastitis and other diseases. This will therefore reduce or increase disease costs for the farmer, respectively. The expected economic gains from the application of HIR technology in the dairy industry will also create niche markets for dairy industry support organizations that will be able to provide tailored management services based on the immune profile of herds. This may include sire recommendations from breeding companies, optimized immunization schedules from pharmaceutical companies and adjusted ration formulations from feed companies. The additional validation of the use of milk whey in lieu of blood to rank lactating cattle based on their immune response to test antigens will result in the development of an innovative and convenient application of the HIR test that will add value to the technology.

**Canadian Dairy Industry & Supply Management**

In Canada the number of dairy farms has been declining, and so a smaller number of producers are meeting the domestic market demand for milk production (Agriculture & Agri-Food Canada Statistics of the Canadian Dairy Industry 2011). The Canadian dairy industry benefits from supply management such that producers, who purchase quota for the right to ship milk, are paid a fair price for their milk. This system allows the dairy market to always maintain a stable supply of milk, and while this system has been a benefit to the Canadian dairy industry it has been challenged recently by various groups including the Conference Board of Canada (Goldfarb 2009). According to this organization, supply management of fluid milk and tariffs on both imports of dairy and poultry products are a barrier for other trade by countries that oppose the tariffs. This has created a perceived negative trading environment for other sectors of the Canadian economy. While the Canadian dairy industry shows no indication that it will abandon a supply management system that runs well and is profitable, there still remains some outside pressure claiming that it is not as beneficial as claimed and that Canada and would be better off without it. If supply management was dismantled, producers would also have to produce a large volume of milk to meet both domestic and foreign market demand in order to be profitable. Imports from other countries would flood the market, and profitability would be diminished.

**HIR will Benefit the Canadian Dairy Industry and Agri-Food Sector**

The Canadian Dairy industry will benefit by being able to classify dairy cows provincially and nationally based on their immune response status. This data can be combined with information on all sires across Canada to create an HIR profile that may become a valuable part of a sire’s Lifetime Profitability Index (LPI) or bull proof. Information on immune response capability on both the dam and the sire side would significantly advance health traits in Canadian dairy cattle and thus bolster Canada’s status as a world class leader in exporting dairy genetics for superior immune response and disease resistance. The application of HIR technology will result in the
creation of a provincial, and potentially a national and international, cohort of herds accredited with “HIR and disease resistant” status. This is expected to increase the value of HIR livestock as a result of the overall reduction of disease and disease related costs, and increased profitability. More milk will be produced using fewer animals that have a longer herd-life. HIR technology will set the Canadian Dairy Industry apart in the International dairy community, and exports of milk and meat from HIR animals will generate a high demand globally, making those animals even more valuable and contributing significantly to the growth of the Canadian economy.

Summary

The application of the HIR response technology to improve livestock health will offer a natural and sustainable approach to help meet the food production required for the growing global population. For example in the dairy industry, the application of HIR will reduce the amount of antibiotic use at the level of the cow, as well as create a green benefit, by reducing antimicrobial resistance in the dairy cow’s environment. The creation of an HIR cohort of disease resistant cattle will identify Canada as a world leader in the production of milk that strives to diminish the use of antibiotics and enhances Canada’s reputation as a producer of safer and higher quality milk. It is absolutely critical that consumer expectation be at the forefront of any livestock health strategy that affects food quality, as it is the consumer that chooses to purchase or not particular food products. If there is any question as to the purity and nutritional value of the Canadian milk supply, real or perceived, the industry might be adversely affected. The consumer’s best interests must drive any motivation to conduct research or roll out products that alter the production of pure and nutritious foods, including milk. The HIR technology is a worthwhile innovation and a sustainable agricultural practice with the potential to naturally improve animal health genetics in Canada and around the globe. It draws from the growing knowledge in both genetics and immunology to improve animal health. In the dairy industry, the identification and selection of HIR calves, cows and sires could bring about a future dairy population that is at less risk for developing disease, requires little or no antibiotics, has a longer herd life, and greater profitability. This technology is a prized benefit to producers, consumers, the environment, the Canadian dairy industry, the Canadian dairy support industry, and the Canadian economy.
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Addendum Appendix 2

Feasibility of High Immune Response (HIR) technology as a health management tool to characterize immune response profiles of dairy cattle


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Abstract
High Immune Response (HIR) is a patented evaluation technology that has the potential to improve the health and food quality of dairy cattle through the reduction of antibiotics and enhanced resistance to economically important diseases such as mastitis. The test includes a blood sample to evaluate antibody-mediated immune response (AMIR) and a skin thickness measurement to evaluate cell-mediated immune response (CMIR). Dairy cattle with a high immune response to test antigens are at a lower risk for developing disease compared to average and low responding animals. Focus groups conducted in two Ontario dairy regions indicated significant interest in HIR (75% of producers) for culling, grouping, treating, and breeding animals. Pre-commercialization activities are underway to conduct: 1) a quantitative market assessment of interest in HIR throughout Ontario (N=128 producers, 3% of Ontario herds) to confirm qualitative focus group data; 2) HIR testing of Ontario AI cull-sires as an application of HIR and to demonstrate no cross-reactivity with governmental health testing, 3) a validation study of previous research to rank cattle based on antibody from milk in lieu of blood (N=21 cows) and 4) beta-testing of HIR on one to two Ontario dairy herds to demonstrate the economic value of HIR (N=250-350 animals per herd). Knowledge transfer and communication research of the HIR technology are also being conducted by attending dairy symposia throughout Ontario to speak to producers and veterinarians, to provide information media, and to recruit participants for educational workshops about HIR.

Keywords: disease, resistance, sustainability, knowledge transfer

Introduction and Background:
Genetic selection mainly to improve dairy milk production traits with minimal emphasis for health traits has gradually led to an increase in the incidence of disease among dairy animals. Recently, the dairy industry has been diligently working to provide genetic and management solutions for improved health in order to correct this problem, particularly in light of the increase in emerging disease and antibiotic resistance in livestock. Dairy cattle face challenge by diverse bacterial, viral, and other pathogens. The immune system provides host defense via genetically and environmentally regulated cells and molecules that respond to pathogens. The immune system can be characterized as having two interconnected systems - the innate system which includes physical barriers and some enzymes and cells that have the ability to respond to a first encounter with a pathogen, and the adaptive immune system which has the hallmark characteristics of memory, diversity, specificity and self/non-self recognition. These features allow the system to respond to an invader on second exposure more rapidly to remove that pathogen swiftly and effectively. The adaptive immune system can be further characterized by its ability to respond to different pathogens as a result of the activation of T helper cells. T helper 1 (Th1) cells predominately produce cytokines that generate a cell-mediated immune
response (CMIR) to respond to intra-cellular pathogens like *Mycobacterium avium paratuberculosis* that cause Johne’s disease, whereas T helper (Th2) cells produce cytokines that tend to generate an antibody-mediated immune response (AMIR) towards extra-cellular pathogens like *Escherichia coli* known to cause mastitis. For selection of broad-based disease resistance to be effective, the individuals selected for breeding subsequent generations must demonstrate a favourable AMIR and CMIR response to both extra-and intra-cellular organisms, respectively.

Genetic regulation of immune response and selection for disease resistance is well documented and considered a preventative method to improve animal health (Stear et al. 2001). There is solid evidence that selective breeding for high (H) or low (L) immune response influences resistance to infectious disease (Kelm et al. 2001) and heritability of AMIR and CMIR are stable and moderate to high, indicating that genetic selection is feasible (Hernandez et al. 2006). In fact, identification of H immune responders is associated with lower disease risk and improved response to vaccines in dairy cattle (Wagter et al. 2000; Hernandez, PhD thesis 2007; DeLaPaz, MSc thesis 2008). Work by Wagter et al. 2000 demonstrated that dairy cattle could be classified based on their antibody response profile to ovalbumin and when evaluated for disease occurrence, cows that had a H antibody response, were found to have no mastitis in 2 of the 3 herds investigated. Recent work done by DeLaPaz et al. 2008, on a large US Florida herd to evaluate HIR response profiles on approximately 875 cows and evaluate health data, indicated that cows that had a H immune response to antigens for both CMIR and AMIR had significantly lower odds ratio risk for developing mastitis, metritis, ketosis, and retained fetal membranes compared to those having an average (A) or a L immune response.

HIR technology may be useful as a management tool for the prevention or reduction of diseases like mastitis, which is the disease of most significant economic importance to the dairy industry. As well, the application of HIR may result in a reduction of a broad range of diseases. This will result in improved food quality, and a decreased dependence on antibiotics to treat disease. After several years of research, the HIR technology is now ready to be transferred to the dairy marketplace. Feasibility research must be conducted to test the hypothesis that the **High Immune Response (HIR)** technology is economically beneficial to the dairy industry and is ready for commercial application. Feasibility research will address the following sub-hypotheses: 1) The dairy industry is interested in HIR as management tool for disease control; 2a) Immune Response (IR) estimated breeding values (EBV) can be used to identify sires and their daughters with improved health, 2b) Sires can be IR tested without any adverse reactions or cross-reactivity with the required federal diagnostic tests; 3) Milk whey in lieu of sera can be used to rank cows for IR and milk samples are readily obtained through dairy herd improvement programs; 4) Beta-testing can help estimate the economic value of HIR; and 5) Knowledge transfer research is useful for evaluating awareness of HIR.

**Methods**

1) **Quantitative Market Assessment of HIR** – Over 1000 Ontario dairy producers were contacted to participate in an on-line survey to provide information on their dairy operation, management priorities and attitudes towards health management. They were presented with the HIR concept including the features of the test (Figure 1), and their interest and willingness to try HIR was evaluated. One hundred twenty eight producers (3% of Ontario
dairy herds) completed the survey on-line (n=117) and by letter mail (n=11). Results obtained with this sample size were accurate within +/-10% nineteen times out of twenty (95% CI).

2) **Sire Testing** – Ten proven sires scheduled for removal were HIR tested in 2 groups of 5 sires in October 2010 and January 2011. All sires were housed in a separate barn at the sire testing facility. Sires were immunized with specified antigens that stimulate immune response according to the patented test protocol, to evaluate AMIR by ELISA test, and CMIR by a skin thickness increase measurement (Figure 1). The HIR test was conducted after sires were determined to be negative for all Canadian Food Inspection Agency (CFIA) disease testing, including tuberculosis (Tb). They were then further tested by CFIA one week after HIR testing was complete.

**Figure 1.** Features of the High Immune Response Test in Dairy Cattle

3) **Validation Study** - Twenty one cows from the University of Guelph Elora Dairy Research station were immunized with antigens that stimulate AMIR and CMIR immune responses. Blood was collected 14 days before calving (day 0), and at calving blood and colostrum were collected. Both blood and milk were collected on days 1, 2, 3, 4, 5 after calving. Cows received another immunization on days 3, 4, or 5, and another blood and milk sample were collected 7 days later. A sub-sample of 9 cows were further immunized in late lactation, and two weeks later were evaluated for their AMIR responses in whey and sera. This study will validate previous research by Wagter et al. 2000, which demonstrated that cattle ranked high for serum antibody to ovalbumin in sera also have high antibody in colostrum and milk whey.

4) **Beta-Testing HIR** - Two Ontario dairy producers have had their herds tested using the HIR patented test protocol (Figure 1). After CMIR and AMIR data are compiled, animals will be ranked based on their IR EBV profile. Disease and health management costs will be collected and interpreted with the assistance of the producer to establish the value of the test relative to costs associated with treating and preventing disease.

5) **Development of communications media and a training workshop to raise awareness of HIR to assist with improving knowledge transfer of a health technology** – A USB drive containing
a video about HIR will be handed out to producers and veterinarians at various dairy and veterinary symposia throughout Ontario and the US. It will contain supporting documentation and contact information with a link to social media about HIR. Producers and bovine practitioners will be invited to participate in a workshop about HIR at a later date. A preliminary Knowledge Attitudes and Behaviour (KAB) survey given before and after the workshop to assess the knowledge transfer of HIR. Workshops will be organized in 2012, and results will be presented in late 2012.

Results

1) Quantitative Market Assessment of HIR in Ontario Dairy Herds

Producers were asked to respond by applying a level of importance to a list of the benefits and concerns cited by dairy producers that participated in qualitative HIR focus groups. The major benefits cited include the ability to cull animals early, HIR would lead to a more productive herd, and the reduced need for antibiotics. The major concerns include: expense, accuracy of test, and the inconvenience of a blood versus milk test. Eighty nine percent of producers surveyed indicated an interest in HIR (Figure 2). When asked how they would use HIR if they tried it and were satisfied with how it worked, the majority of producers (59%) indicated they would use it on their entire herd, 31% on their milking cows only, 5% on their calves, and 6% would try it on some of their animals. (Figure 3).

Figure 2. Producer Interest in HIR

Figure 3. Producer Uses of HIR
2) *Sire Testing*

Sires tested showed variation in AMIR and CMIR. There are sires that are high for CMIR and AMIR, some that are low for both, and some that are high for one or the other response. No adverse reactions were noted with the test and all sires were negative for CFIA health testing, including Tb, before the HIR test and following the HIR test. **Analysis data is expected to be ready for presentation in October 2011.**

3) **Validation Study**

Analysis of variance and correlation analysis are underway to validate that antibody response in sera ranks similarly in whey during early and late lactation. Visual inspection of individual graphs for each cow during the peripartum period indicates that animals, that are high or low for antibody in sera, have a high or low response in colostrum and whey, respectively. **Analysis data is expected to be ready for presentation in October 2011.**

4) **Beta-Testing HIR**

Both herds have completed HIR testing and AMIR responses are currently being assessed in the lab. General linear model analysis will be conducted, and estimated breeding values will be calculated. After the animals are ranked producers will assist in providing a perspective on the economic value of HIR. **Preliminary data on the economic value to be presented in October 2011.**

**Discussion**

The dairy industry is looking for sustainable approaches to minimize disease and therapeutic treatment, while at the same time optimize production in dairy cattle. Before the HIR concept and its features were presented in the market survey, producers were asked the following: 1) if they could identify disease prone animals within their herd, and were then asked to 2) express how valuable it would be to be able to confirm this using a diagnostic test. Producers indicated that it would be extremely (28%) or somewhat valuable (65%) to have a way of identifying a disease prone animal. Preliminary results of the quantitative market research of HIR indicated that 89% of Ontario producers surveyed are interested in the HIR technology and only 11% are not (Figure 2). Approximately 59% of 128 producers indicated that they would try it on their entire herd (Figure 3). These results are very promising for HIR as this indicates the majority surveyed would use the technology to test both their lactating cattle and young stock. Some producers (31%) indicated that they would only test their milking cows. Although there may be benefit in identifying IR of calves early to determine which animals will be the better investment for better health, few data points of information are available on calves in contrast to that collected on a lactating cow. With information on lactating cattle readily available from management software and dairy herd improvement reports, a producer can better evaluate the value of HIR to them. This group of producers may proceed to test calves after the milking herd is tested and are sufficiently satisfied with the technology and its claims. The quantitative market survey indicated that the top three major benefits of HIR include identifying animals for culling, increased productivity in herds, and the reduced use of antibiotics. The top 3 concerns include: expense, accuracy, and the inconvenience of a blood test over a milk test. The market assessment further indicated that producers understand HIR and its purpose. They see the value in the test and this evaluation has given the developers insight and direction on how to position HIR in the marketplace. The information provided by this market assessment was extremely
valuable to understand producer attitudes and potential willingness to try the HIR technology. It was instrumental in identifying concerns and while valid, some of these concerns are not supported by previous research on HIR. These concerns can be addressed through marketing campaigns at dairy and veterinary symposia, and technology transfer events. This knowledge transfer and communication strategy may help overcome some of the possible barriers to understanding and adopting HIR.

Phenotypic variation in HIR response in sires was evident in this feasibility study, and did not interfere with federal government health testing. While detailed statistical analysis is not yet available, variation in AMIR and CMIR results are expected to support that HIR will be useful for identifying sires and their daughters with improved health.

Validation data will be evaluated to confirm that cattle that are high for antibody response in sera, will also be high in colostral and milk whey in early and late lactation. A complete statistical analysis is required to confirm this correlation. This information will be extremely valuable to dairy herd improvement programs that currently provide management services through the regular collection of milk for component analysis and diagnostic ELISA testing for pathogens causing disease. As milk is more readily obtained than a blood sample, the evaluation of a milk sample in a herd improvement testing lab will offer a convenient option for assessing the AMIR part of the HIR test.

Beta-testing is a marketing application to test a product in the real world. It has allowed the developers to apply the procedure in different testing scenarios, to aid in understanding how best to apply the business model particularly in large herds. Although HIR test results for beta-testing for each herd are still being evaluated statistically and EBV calculated, we expect to complete these by the fall of 2011. Producers will give their opinion on the value of knowing which animals are H, A and L responders, and what it means to them in terms of savings or losses in their health management program. Taken together, beta-testing results and the market assessment will be very useful resources for developing a business model for HIR that will be feasible for the dairy marketplace.

Finally, a knowledge transfer communications study of HIR is expected to help dairy producers and veterinarians better understand and support HIR. The results of this knowledge transfer assessment will be evaluated and presented in late 2012.

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References


Addendum Appendix 3

Specific antibody in blood versus colostrum and milk in dairy cows and their calves

—to be formatted and submitted to Canadian Journal of Veterinary Research
Specific antibody in blood versus colostrum and milk in dairy cows and their calves.
by Wagter-Lesperance et al. (Page 000).
Calves require an adequate amount of colostrum (first milk) from their dams within 24 hours of birth. Colostrum contains antibodies transferred from the blood that have been produced following natural exposure or immunization to microorganisms. Cows with high concentrations of antibodies in blood also have high concentrations of antibodies in colostrum and milk. Calves given colostrum with high concentrations of antibodies have high concentrations of specific antibodies in their blood at two days of age. Passive immunity from high immune response cows provides enhanced quantities of antibodies to prevent infection and disease in calves.
Specific antibody in blood versus colostrum and milk in dairy cows and their calves


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ABSTRACT
A study was conducted to evaluate the correlation between antibody responses in serum and milk, and between first milk (colostrum) and calf sera. Twenty-one cows were immunized 2 wk before their predicted calving dates to stimulate an antibody-mediated immune response (AMIR) and received a secondary immunization on d 3, 4, or 5 after calving (d 0). A baseline blood sample was taken before primary immunization and blood was collected on d 0, 1, 2, 3, 4, 5 and again on d 10, 11 or 12 post-partum. Colostrum was collected within 12 hours post-calving; milk was collected at the morning milking on d 1, 2, 3, 4, 5 and again on d 10, 11, or 12. Calves were fed colostrum (from their own dams), and their blood was collected on d 2 to evaluate specific antibody transfer. Additionally, 9 of the 21 cows were evaluated in late lactation for antibody responses 4 wk following tertiary immunization to assess the relationship between serum and milk antibody. In early lactation, correlations between concentrations of antibodies in blood and colostrum/milk were positive (r = 0.48 to 0.76). The correlation between antibody concentrations in blood and milk in late lactation was positive for three wk after immunization (r = 0.67 to 0.84). The correlation between antibody concentrations in the blood of calves with those in colostrum of their dams was also positive (r = 0.74). Cows with high serum antibody concentrations in blood following immunization also had high concentrations in colostrum and milk indicating that it may be possible to tailor immunization programs against pathogens to improve the health of dairy cows and their calves.

**Keywords:** high immune responder, antibody-mediated immune response (AMIR), antibody transfer, colostrum, calves
INTRODUCTION

Dairy cattle are continually challenged by diverse bacterial, viral, and other pathogens. The immune system provides host defense via genetically- and environmentally-regulated cells and molecules that respond to pathogens. The immune system can be described as the interaction of two interconnected systems - the innate and adaptive immune systems. The innate system includes physical barriers such as skin, some enzymes, and cells that have the ability to respond and quickly react on first encounter with a pathogen (but do not generally exhibit enhanced responses on subsequent exposures). The adaptive system responds more slowly after initial exposure to foreign material, but has more exquisite specificity and can respond more rapidly on second and subsequent exposures to an invading pathogen or microbial challenge. The immune system is underpinned by as many as 2,000 genes (Thompson-Crispi et al., 2014; http://www.innatedb.com/redirect.do?go=resourcesGeneLists) and the expression of these genes makes up the immune response phenotype (Mallard et al., 2015). During the peripartum period (three wk before and three wk after calving), the nutritional and metabolic strains associated with calving and transition to high milk production can cause cows to become more susceptible to disease. Leblanc et al. (2006) estimated that 75% of disease in dairy cows occurs during the first month after calving, and there is increased interest in optimizing immune function over this time. Unfortunately, changes in functionality of the immune system during the peripartum period may adversely affect the ability of the host to eliminate pathogens (Ingvartsen et al., 2015). During this period the majority of cows show some degree of sub-optimal immune response, however despite these alterations in immune responsiveness, work by Wagter et al. (2000) has shown that there are cows that respond well during the peripartum period with a higher magnitude and quality of antibody response, compared to other cows. These individuals are described as high immune responders and have since been shown to have fewer disease events than average or low responders. In a recent study of 458 Canadian Holstein cows, high responders had a lower incidence and severity of mastitis due to *E. coli* and *S. aureus*, and other bacterial pathogens (Thompson-Crispi et al., 2013). Another study in a large US dairy herd involving 699 cows supports these findings, with reductions in the incidence of mastitis, ketosis, metritis and retained placenta in cows with high antibody mediated (AMIR) and cell-mediated (CMIR) immune responses compared to cows with average or low responses based on their estimated breeding values (EBV) for these traits (Thompson-Crispi et al., 2012).
Wagter et al. (2000) further reported that cattle with a high specific antibody response phenotype in sera also have a high antibody response in colostrum and milk. Colostrum (first milk) is a valuable resource and its careful administration is an important calf health management strategy. Calves at birth are essentially agammaglobulinemic. Maternal antibodies from the blood of the dam are transferred into mammary secretions in the last weeks before parturition to form colostrum. Ingestion and absorption of colostral antibodies into the circulation of the calf occurs during the first 12 to 24 hours of life and conveys passive immunity from the dam contributing to disease prevention in the neonatal calf. The timely delivery of a sufficient volume of high quality colostrum is necessary to ensure calf survival. In the study by Wagter et al. (2000), cows with a high antibody response to a test antigen in serum also had a high antibody response in colostrum and milk at d 0 (calving), and at wk 2, 3, 4, and 6 after calving. In addition, cows were evaluated for their serum antibody responses to a commercially available *E. coli J5* (O111:B4) mastitis vaccine; cows that had high serum antibody responses to the test antigen also had high antibody responses to *E. coli J5* (r = 0.66; *P* ≤ 0.001). This study demonstrated that high immune responders had an ability to provide enhanced antibody responses in milk, against a mastitis-causing pathogen during the peripartum period. Higher concentrations of antibodies in colostrum and milk can also benefit the calf. Antibodies secreted into the milk have been reported to enhance protection against enteric pathogens such as *E. coli* (Valente et al., 1988) and rotavirus (Fernandez et al., 1998; Parreño et al., 2004), and a recent study by Smith et al. (2014) reported that dams vaccinated at dry off and again 4 wk later against *Salmonella* Newport (a pathogen causing diarrhea in calves) successfully transferred specific antibodies via the colostrum to the serum of their calves.

While the study by Wagter et al. (2000) evaluated the amount of specific antibody in serum versus colostrum and milk post-partum, it did not investigate this relationship in very early and late lactation, nor the transfer of specific colostral antibody to calves. Therefore, the objectives of the current study were: 1) to assess the relationship between antibody response in serum and colostral whey and milk whey in very early lactation; 2) to describe the correlation between amount of antibodies in colostrum with serum antibodies of calves fed this colostrum; and 3) to assess the relationship between antibody response in serum and milk whey in late lactation.
MATERIALS AND METHODS

Overall Experimental Design
Cows were immunized using a standard immunization protocol to induce antibody before and after calving. Antibody was assessed in serum, colostrum and milk to determine correlations between antibody amounts in each source. Subsequently, calves were fed their mother’s colostrum and serum antibody was assessed to determine the correlation between antibody in colostrum and that transferred to her calf.

Cows and Sample Collection

Early Lactation. Twenty-one cows from the University of Guelph Elora Dairy Research Station (Elora, ON) were immunized two wk before their predicted calving dates (wk -2), with an inert type 2 antigen to stimulate AMIR as previously described (Thompson-Crispi, 2012), and were re-immunized on either d 3, 4, or 5, after calving to initiate a secondary response. Blood was collected at wk-2, d 0, 1, 2, 3, 4, 5 post-calving, and again at d 10-12 post calving (referred to as d 12 throughout the rest of this article), 7 d after secondary immunization. Colostrum and milk were collected by staff at the University of Guelph Elora Dairy Research Station. A composite sample of colostrum was collected using a bucket milker after first milking, and milk was collected from a meter jar during milking. Colostrum was collected and administered within 12 hours post-calving; milk was collected at the morning milking on d 1, 2, 3, 4, 5 and again on d 10, 11 or 12. Calves were fed colostrum from their own dams, in accordance with the standard protocol at the research station. The volume of colostrum fed in liters (L) and the time of feeding was recorded, and calf blood was collected on d 2 to evaluate colostral transfer of specific antibodies (Figure 1).

Late Lactation. Of the 21 cows in the study, 9 cows were available for follow-up in late lactation. The other 12 cows were either dried off (N = 6), had died/were euthanized (N = 5) or were sold (N = 1). These cows were immunized a third time at an average of 284 DIM (range 251-330 DIM). Blood and milk were sampled at immunization and for an additional four wk to compare serum and milk antibodies in late lactation (Figure 1).

Calves and Sample Collection
Blood samples were collected from 10 live calves by jugular venipuncture 2 d post-administration of colostrum from their own dam. The exact time of calving was confirmed for 8 of the calves. The ninth calf was born overnight and the time elapsed before feeding colostrum could not be exactly determined. The 10th calf was also born overnight and the volume of colostrum consumed was not recorded. Data from these calves were therefore excluded from analysis.

**Antibody Response in Serum and Whey**

Serum was separated from clotted peripheral blood by centrifugation (700 x g, 15 min) and stored frozen (-20°C) until time of assay. Milk samples were centrifuged twice (11,000 x g, 15 min) to remove fat. Whey was defined in this study as defatted milk including casein, and was stored frozen at -20°C. Antibodies were detected by ELISA according to the procedure described by Burton et al. (1993). Briefly, flat bottom 96-well polystyrene plates (Immulon 2 HB; Fisher Scientific, Ottawa, ON) were coated with a 3.11 x 10^{-5} M solution of type 2 antigen dissolved in carbonate-bicarbonate coating buffer (pH 9.6). Plates were incubated (4°C, 48h) then washed with wash buffer (PBS, pH 7.1 containing 0.05% Tween 20 [Fisher Scientific]) using a ELEX405 autoplate washer (BioTek, Winooski, VT). Plates were then blocked with PBS with 3% Tween 20 solution and incubated at room temperature (rt) for 1h. Plates were washed and diluted test sera (1/50 and 1/200) or whey (1/10 and 1/400) and controls were added using the quadrant system described by Wright (1987). After blocking, serum samples were added in duplicate and milk whey samples were added in quadruplicate and plates incubated at rt for 1h. Subsequently, plates were washed, alkaline phosphatase-conjugated rabbit anti-bovine IgG (whole molecule) (Sigma-Aldrich, Oakville, ON) diluted in wash buffer, was added to plates, and incubated (rt, 2h). Using the SigmaFast substrate system (Sigma-Aldrich), p nitrophenyl phosphate (pNPP) substrate and TRIS tablets were dissolved in ddH2O. Plates were washed and substrate was added to the plates and incubated (rt, 30 min). Plates were read on a Powerwave XS2 ELISA plate reader (BioTek) using Gen 5 software, and the optical density (OD) was recorded at 405 and 630 nanometers (nm) when the positive control reached an OD of ≥ 1.0. Optical densities of sample replicates were corrected by multiplying by the reciprocal of the mean of the positive control. Corrected means of each sample dilution were then added together to give an additive OD value (1/50 + 1/200 for sera or 1/10 + 1/400 for whey). Positive and negative control sera were used from two previous independent trials. Positive control sera
were pooled from a group of 10 high responder cows on d 7 after receiving a tertiary immunization with the type 2 antigen. Negative control sera were pooled from 10 cows on the d of primary immunization (d 0).

**Radial Immunodiffusion Assay (RID)**

Calf sera was also evaluated for total IgG by using an RID kit with a commercially prepared radial immunodiffusion plate and a IgG standard (Triple J farms, Bellingham, WA). Five uL of standard or sample sera was added to each well and the precipitate zone diameter was measured 24 h later. The linear range for detection of IgG was 200 to 3000 mg/dl and all samples were within this range.

**Milk Weights**

Milk weights in kg were recorded for morning (AM milk) and afternoon (PM milk) milking to evaluate the association of milk weight with specific whey antibody response. Correlations between whey antibody responses and milk weight were evaluated for AM milk and combined AM and PM milk weights (AMPM).

**Statistical Methods**

*Pearson Correlations.* Correlations of AMIR measurements between cow serum and whey, calf sera and colostral whey, and milk weights with whey were conducted using the Proc CORR procedure of SAS (SAS Institute Inc., Cary, NC)

*Multiple Linear Regression.* The Proc Univariate procedure of SAS was run and the Shapiro-Wilk test was used to assess the normality of the distribution of data prior to inclusion in a general linear model (GLM). The Proc GLM procedure of SAS was run to investigate: 1) the effect of d from immunization to calving, baseline antibody prior to immunization, and milk weight, on antibody response in colostrum and milk, and 2) the effect of volume, time to feeding and colostral specific antibody on the same specific antibody in calf sera.

**Model#1 Variation in Specific Antibody Response in Early Lactation**

1a) Antibody response in serum, colostral whey and milk whey in early lactation

\[ Y_{ijk} = \mu + S\text{Dayneg14}_i + D\text{aysItoC}_j + \text{parity}_k + e_{ijk}. \]
$Y_{ijk}$ is the antibody response in serum or colostrum/milk for early lactation d 0, 1, 2, 3, 4, 5, 12 where

$\mu$= overall mean

SDaysneg14$_i$= serum antibody (OD) prior to immunization as covariate

DaysItocj$_j$ = d from immunization to calving (range 8 to 19 d)

parity$_k$ = the number of previous pregnancies (1, 2, 3, ≥4)

e$_{ijk}$ is the error term

1b) Effect of milk weight on antibody response in colostrum and milk in early lactation

$Y_{ij}$= $\mu$ + parity$_i$ + AMmilk$_j$ + e$_{ij}$.

$Y_{ij}$ is specific antibody OD in colostrum and milk on d 0, 1, 2, 3, 4, 5, 12 where

$\mu$ = the overall mean

parity$_i$ = number of previous pregnancies (1, 2, 3, ≥4)

AMmilk$_j$ = the weight in kg of milk collected at AM milking

e$_{ij}$= error term

Similar models were run for AMPM milking using the combined milk weights from the AM and PM milking.

**Model #2 – Calf Serum Specific Antibody Variation**

$Y_{ijk}$= $\mu$ + specific antibody in colostrum$_i$ + volume of colostrum fed$_j$ + hours from calving to first feeding$_k$ + total IgG + e$_{ijk}$.

$Y_{ijk}$ is specific antibody in calf sera where

$\mu$ = the overall mean

specific antibody in colostrum$_i$ = antibody OD in colostrum at the time of feeding

volume$_j$ = volume of colostrum in liters (L) from dam fed to her calf, and

hours$_k$ = length of time from calving to first feeding

total IgG = total serum IgG as measured by radial immunodiffusion (RID) assay

e$_{ijk}$ = error term

Any calves that had incomplete data for milk volume or hours to first feeding after calving were excluded from the GLM analysis.
**Model #3 – Variation in Antibody Response in Late Lactation**

3a) Antibody response in serum and milk whey in late lactation

\[ Y_{ij} = \mu + \text{SLLWeek0}_i \text{ (or WLLWeek0}_i) + \text{parity}_j + e_{ij}. \]

*Y*<sub>ij</sub> is the antibody response in serum or colostrum/milk for late lactation wk 0, 1,2,3,4.

where

- \( \mu \) = the overall mean
- \( \text{SLLWeek0}_i \) = baseline value of sera antibody OD in late lactation prior to tertiary immunization, as covariate
- \( \text{WLLWeek0}_i \) = baseline value of whey antibody OD in late lactation prior to tertiary immunization, as covariate
- \( \text{parity}_j \) = the number of previous pregnancies (1, 2, 3, ≥4)
- \( e_{ij} \) is the error term

3b) Effect of milk weight on antibody response in milk in late lactation

\[ Y_{ij} = \mu + \text{parity}_i + \text{AMmilk}_j + e_{ij}. \]

*Y*<sub>ij</sub> is specific antibody OD in colostrum and milk on wk 0, 1, 2, 3, 4

where

- \( \mu \) = the overall mean
- \( \text{parity}_i \) = number of previous pregnancies (1, 2, 3, ≥4)
- \( \text{AMmilk}_j \) = the weight in kg of milk collected at morning milking
- \( e_{ij} \) = error term

Similar models were run for AMPM milking using the combined milk weights from the AM and PM milking.

Results were considered to be statistically significant if the \( P \) value was \( P < 0.10 \). For all models, variables were removed from the model if they did not significantly contribute to variation in the antibody response.
RESULTS

Variation in Specific Antibody Response in Early Lactation

Correlation between Antibody in Cow Sera, Colostral Whey and Milk Whey. Pearson correlations of antibody in sera and whey for early lactation are shown in Table 1. In early lactation, antibody in serum and whey were positively and significantly correlated from d 0 to d 12. Three cows had a higher than expected additive OD prior to immunization, suggesting a previous exposure to antigen which was accounted for in the GLM.

Multiple Linear Regression Analysis of Antibody in Cow Sera, Colostral Whey and Milk Whey. The potential contribution of background or baseline serum antibody OD to the variation in post-immunization OD was accounted for using Model 1a. Results from GLM Model 1a suggest that neither parity nor baseline antibody OD prior to immunization contributed to the variation in specific antibody in sera or whey in early lactation. However, the variable d from immunization to calving (DaysItoC) was associated ($P = 0.03$) with ODs for specific antibodies in whey from colostral whey on d 0, with higher OD values at calving associated with longest intervals from immunization to calving (Table 2).

Effect of Milk Weight on Antibody Response in Colostrum and Milk Whey in Early Lactation

Correlation between Milk Weights, Antibody in Colostral Whey and Milk Whey. Pearson correlations of milk weights (AM milk and AMPM) and whey antibody OD for early lactation are reported in Table 3. AM milk weight correlated positively with whey antibody response on d 2 ($r = 0.41; P = 0.06$) and d 4 ($r = 0.41; P = 0.06$) of early lactation. AMPM milk weight also correlated positively on d 2 ($r = 0.41; P = 0.06$) and d 4 ($r = 0.42; P = 0.06$).

Multiple Linear Regression Analysis of Milk Weights on Antibody in Colostral Whey and Milk Whey. Results from the GLM Model 1b indicated that AM milk ($P = 0.004$) on d 4 contributed to variation in whey antibody OD. As well, AMPM milk on d 4 ($P = 0.007$) contributed to variation in whey antibody OD (Table 4).

Maternal Antibody in Sera of Calves

Correlation Analysis of Antibody in Calf Sera with Colostral Whey of Dam. Ten calves were evaluated for serum antibody, but only 8 were included in the analysis due to missing data
(such as time from calving to feeding, or milk volume not recorded). There was a positive Pearson correlation \((r = 0.74, P = 0.04)\) between antibody in colostrum on d 0 and in sera of calves two d later, after ingesting colostrum from their own dams (Table 1).

**Multiple Regression Analysis of Effect of Antibody in Colostral Whey on Antibody in Calf Sera.** Results from GLM Model 2 indicated that neither colostral volume fed, nor the length of time from calving to first feeding, nor total serum IgG, contributed to the amount of specific antibody in calf sera. However, antibody OD in colostrum, significantly contributed \((P = 0.04)\) to the variation in specific antibody OD in calf sera (Table 5).

**Variation in Specific Antibody Response in Late Lactation**

**Correlation between Antibody in Cow Sera and Milk Whey.** Pearson correlations for antibody in sera and whey for late lactation are shown in Table 6. In late lactation, antibody in serum and whey were positively and significantly correlated prior to tertiary immunization \((r = 0.84, P = 0.004)\). Correlation coefficients for three wk following immunization were positive, but were negative at 4 wk post immunization.

**Multiple Linear Regression Analysis of Antibody in Cow Sera and Milk Whey.** Results from GLM Model 3a indicated that neither parity nor baseline serum antibody OD contributed to the variation in antibody response in sera or whey in late lactation (data not shown).

**Effect of Milk Weight on Antibody Response in Colostrum and Whey in Late Lactation**

**Correlation between Milk Weights, Antibody in Milk Whey.** Pearson correlations of milk weights (AM milk and AMPM) and whey antibody OD for late lactation are reported in Table 7. AM milk weight correlated negatively and significantly at wk 1 \((r = -0.69, P = 0.04)\) and wk 2 \((r = -0.65, P = 0.05)\) in late lactation, post tertiary immunization. AMPM milk weight also correlated negatively \((r = -0.62, P = 0.08)\) at wk 1 post tertiary immunization.

**Multiple Linear Regression Analysis of Milk Weights on Antibody in Milk Whey in Late Lactation.** Results of GLM Model 3b indicated that neither parity nor milk weight (AM milk or AMPM) contributed significantly to variation in whey antibody OD (data not shown).
Antibodies are a subset of immunoglobulins (Ig) specific for a pathogen or antigen of interest and can be measured by ELISA. Antibodies bind specifically to epitopes of pathogens to help clear a pathogen or its secreted toxins. Antibodies may: 1) opsonize to enhance phagocytosis; 2) fix complement, 3) neutralize toxins and 4) prevent adherence of pathogen to host cell receptors thus preventing colonization and/or invasion. Ig represent the total of all antibodies (of known and unknown specificities) and are critical to the host for survival. Butler (1981) reported that Ig circulating in the blood in the dairy cow includes IgG1 (~10.06 mg/mL on average), IgG2 (~9.04 mg/mL), IgM (~3.69 mg/mL), and IgA (~0.34 mg/mL). In the wk leading up to parturition, circulating IgG1 is transported from the serum to the colostrum. The majority of Ig in colostrum is of the IgG1 class (~64.9 mg/mL). This is followed by IgM (~8.7 mg/mL), IgA (~3.5 mg/mL) and IgG2 (~2.2 mg/mL) (Butler 1981). During the last two to three wk of gestation, IgG1 is transferred to the colostrum and is accompanied by a reduction in circulating serum concentrations of IgG1. It is estimated that as much as 500g of IgG1 per wk are transferred from the blood to the colostrum in the final 2-3 wk leading up to parturition (Brandon et al., 1971). In milk, the concentration of Ig is much less than in colostrum, but IgG1 remains the major subclass (0.64 mg/mL), followed by IgA (0.13 mg/mL) with very little IgG2 (0.05 mg/mL) and IgM (0.04 mg/mL) (Butler, 1981). In cattle, the transfer of Ig into the colostrum and milk is postulated to occur through neonatal Fc receptor (FcRn), a heterodimer of an MHC class I homolog (FCGRT) and beta 2-microglobulin (B2M). The efficiency of Ig transfer may be a reflection of the haplotypes of the FCGRT gene located on chromosome 18 (Zhang et al., 2009) and the beta 2-microglobulin B2M gene on chromosome 10 (Clawson et al., 2004), as well as the amount of antibody available for transfer. In dairy cows, an enhanced ability to transfer Ig effectively and efficiently into colostrum and milk may offer better protection to the dam against pathogens that can cause mastitis in the mammary gland, as well as for the calf against pathogens causing diarrhea, pneumonia and septicemia. In the present study, cows with high antibody responses in their sera also had higher antibody in colostrum and milk in early lactation and in late lactation. In addition to higher amounts of antibody in blood and colostrum, these cows may also possess a favorable FCRGT haplotype or B2M haplotype, or their FcRn receptors possess binding affinities that are more favorable for transfer compared to average or low immune responding cows.
Further investigation of this efficiency of transfer would be required to confirm this hypothesis. In this study, a positive correlation was found between antibody concentration in colostrum and antibody in the sera of calves that had ingested colostrum from their dams (r = 0.74, P = 0.04). As colostrum is essential for transfer of passive immunity from the dam to the calf, the timing of collection and administration, as well as the quality of this colostrum can have considerable effects on calf health (Conneely et al., 2014). When colostrum is fed to a calf, IgG are passively absorbed across the gut epithelium and pass into the lymphatics and into the circulation. This absorption is optimal within the first 6 hours, decreases from 6 to 12 hours as the gut epithelium of the calf becomes increasingly impermeable to large proteins including IgG, and is negligible by 48 hours (Chase et al., 2008). Results from the current study indicate that the amount of specific antibody in colostrum is significantly associated with the amount of the same specific antibody in serum of calves fed this colostrum. The effects of volume fed and time of feeding in this study were not found to significantly affect calf serum specific antibody. This study was large enough to identify the effect of immunization on antibody in colostrum, but too small to show the effects of volume and timing in the delivery of maternal antibodies to the calf. Although not cogent to this study, in a separate GLM model (data not shown) volume fed was found to contribute to the variation in total IgG, but not time of feeding. The importance of feeding colostrum with high IgG concentration in adequate volume in a timely fashion to maximize total IgG in the serum of the calf has been well established previously (Besser et al., 1991; Weaver et al., 2000). High total IgG in calf serum is associated with reduced disease in calves especially diarrhea and pneumonia. It is possible that a cow may have an adequate concentration of serum Ig (including antibodies against a wide array of pathogens) but may not have antibodies against a particular pathogen of interest. Such a cow could produce colostrum with a high concentration of total Ig but fail to provide protection against the particular pathogen of interest. In contrast, a cow may produce colostrum with a very low concentration of total Ig but have an exceptionally high concentration of antibodies to a particular pathogen because of natural exposure or vaccination. Both total Ig concentration and concentration of pathogen-specific antibodies need to be considered in disease management. In the current study, colostrum quality was assessed based on antibody amount, but colostrum quality must also be assessed on the basis of total IgG. Colostrum quality on farm is commonly estimated using a hydrometer or refractometer to provide a measure of total protein. These measures have been found to correlate with Ig concentration r^2=0.699, P <0.001 (Fleenor and Stott, 1980). The current study shows
that high AMIR cows respond to immunization with higher antibody concentrations in sera, and have a correlated level of antibody in colostrum. These results underscore the value of colostrum from these cattle, and the potential benefit of banking colostrum for administration to calves of dams that have a lower concentration of total protein, to provide enhanced protection against disease. It also demonstrates the value of immunising dams to enrich specific antibody in colostrum and milk.

Through the years, dairy cattle have been genetically selected for increased milk production, with little attention to selection for health traits (Miglior et al., 2005). Additionally, an increased rate of mastitis, and other diseases, have been reported to occur around the peripartum period (Leblanc et al., 2006). This may be particularly problematic if there is a potential risk of exposure to antibiotic-resistant pathogens during this period of susceptibility. There is increasing awareness in agriculture that the use of antibiotics should be minimized, and while blanket antibiotic therapy given during the dry period may help prevent mastitis, continuing to use this practice could contribute to increased antibiotic resistance. Thus, the dairy industry has been working diligently to develop management practices to maintain and improve health, that are not dependent on antibiotics (Berge et al., 2009; Saini et al., 2011). A sustainable approach would be to identify cattle with superior immune response traits and select these cattle for breeding to improve disease resistance (Mallard et al., 2015). Wagter et al. (2000) showed that periparturient cows classified as high responders for AMIR to a test antigen respond better to immunization with a commercial *E.coli* J5 mastitis vaccine in comparison to average and low immune responders. In the present study there was a positive and significant correlation between antibody in serum and in colostrum (and milk) during early lactation (0 to 5, and 12 d post calving) and during late lactation. The results of both studies demonstrate that high immune responder cows have the potential to have enhanced protection of the udder against mastitis during lactation and into the dry period, and a greater ability to provide protection against disease in their calves. This indicates the potential to improve immune responses among cows through vaccination programs tailored to meet individual herd needs. For example, vaccines given in late lactation and in the dry period against pathogens prevalent in the local herd can increase the amount of antibody in serum and colostrum, thus providing enhanced protection to both cows and their calves. Further, genetic selection to improve colostrum quality may also be possible. Antibody response has been shown to be heritable (h²~0.30, Thompson-Crispi et al., 2012) and
work by Fleming et al. (2016) has shown that cattle with high EBV for AMIR have higher concentrations of Ig in colostrum, compared to average and low AMIR responders. Thus, selectively breeding cows could be a good strategy to improve immune responses, colostrum quality and calf health at the herd level over time.

Milk weight was also found to be associated with antibody in milk. Milk weight was positively correlated with whey antibody amount in early lactation on d 2 and d 4 after calving, but was negatively correlated in late lactation (wk 1 and wk 2 post tertiary immunization). The positive correlation in early lactation reflects the increase in milk production post-partum within the first 60 d of lactation, and the concomitant increase in antibody synthesis following immunization, with transfer of these antibodies into milk. The negative correlation of milk weight with whey antibody response in late lactation is related to the decrease in milk production in late lactation, with greater dilution of antibodies post immunization in the cows producing high volumes of milk. In this study, cattle boosted with an antigen in late lactation produced an antibody response similar to what is observed in early lactation. The arithmetic mean of antibody (additive OD values) for the 9 late lactation cows, when evaluated in early lactation, was 1.826 for d 0 (~2 wk post-primary immunization) and 2.568 on d 12 (1 wk post-secondary immunization). In late lactation, the mean OD value was 2.674 (1 wk post tertiary immunization), which was not statistically different from 2.568 on d 12 (data not shown). Thus, an increase in the amount of antibody per unit of milk weight can be produced in late lactation and may provide additional protection against infection and disease. Further study with more animals is needed to clarify the association of milk weight data with antibody amount in milk from early to late lactation and its effect on disease outcomes.

CONCLUSIONS

This study supports previous findings that cattle with higher serum antibody responses also have higher amounts of antibody in colostrum and milk. This study also showed that the timing of immunization in late lactation can affect the variation in antibody in colostrum, and the amount of antibody in colostrum fed to calves contributes significantly to the amount of specific antibody in calf sera after ingestion of colostrum. Successful vaccination programs aimed at
improving herd health will need to take into account the optimal time to immunize cows prior to calving in order to maximize transfer of antibody into the colostrum. In summary, this study indicates that a combined strategy of vaccination against pathogens important in a particular herd, breeding for greater antibody response, and monitoring of colostrum for total Ig content, may be a reasonable approach to enhance immune response in dairy cows and enhance passive immunity in young calves.

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REFERENCES


Table 1. Pearson correlations (r) between specific antibody in sera of cows versus colostrum and milk during early lactation. Correlations for d of calving (d 0) to d 5, and d 12 post-calving are shown. N = 21.

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<td>0.71</td>
<td>0.0003</td>
</tr>
<tr>
<td>12</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**In addition, it should be noted that the correlation between antibody amount of d 0 colostrum with d 2 calf sera was r = 0.74, P = 0.04, N = 8.**
Table 2. GLM of the effects of baseline antibody response (SDaysNeg14), interval from immunization to calving (DaysItoC), and parity on specific antibody response in colostrum, milk and sera post primary immunization in early lactation. N = 21.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>d</th>
<th>R^2</th>
<th>P value</th>
<th>SDaysNeg14</th>
<th>DaysItoC</th>
<th>Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody in whey</td>
<td>0</td>
<td>73.18</td>
<td>0.03</td>
<td>0.104</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Antibody in sera</td>
<td>0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 3. Pearson correlations (r) between milk weight (AM milk = morning milking and AMPM = combined morning and afternoon milking) with specific antibody in milk during early lactation. Correlations for d of calving (d 0) to d 5, and d 12 post calving are shown. N = 21.

<table>
<thead>
<tr>
<th>d</th>
<th>AM milk (r)</th>
<th>P value</th>
<th>AMPM (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.29</td>
<td>NS</td>
<td>0.31</td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>0.35</td>
<td>NS</td>
<td>0.34</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>0.41</td>
<td>0.06</td>
<td>0.41</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.28</td>
<td>NS</td>
<td>0.32</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>0.41</td>
<td>0.06</td>
<td>0.42</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>0.16</td>
<td>NS</td>
<td>0.24</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>-0.31</td>
<td>NS</td>
<td>-0.30</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4. GLM of the effects of milk weight (AM milk = morning milking and AMPM = combined morning and afternoon milking) and parity on specific antibody response in milk in early lactation. N = 21.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>d</th>
<th>R²</th>
<th>P value</th>
<th>Milk weight</th>
<th>Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey antibody for AM milk (colostrum)</td>
<td>0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>42.95</td>
<td>0.05</td>
<td>0.004</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Whey antibody for AMPM milk (colostrum)</td>
<td>0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>38.01</td>
<td>0.09</td>
<td>0.007</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 5. GLM of the effects of specific antibody in colostrum, volume of colostrum fed, and time from calving to feeding on serum antibody in calves. $N = 8$; -- = not significant therefore removed from the model.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>$R^2$</th>
<th>P value</th>
<th>Colostrum Ab</th>
<th>Volume fed</th>
<th>Time to feeding</th>
<th>Total IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum antibody in calves</td>
<td>54.86</td>
<td>0.04</td>
<td>0.04</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 6. Pearson correlations (r) between specific antibody in cow sera versus milk during late lactation. Correlations from wk 0 [average d in milk (DIM)=284] to wk 4 post tertiary immunization are shown. N = 9 for wk 0, 1, and 2; N = 8 for wk 3; and N = 6 for wk 4.

<table>
<thead>
<tr>
<th>wk Post-Immunization</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Avg. 284 DIM)</td>
<td>0.84</td>
<td>0.004</td>
</tr>
<tr>
<td>1</td>
<td>0.69</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>0.72</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>-0.30</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 7. Pearson correlations (r) between milk weight (AM milk = morning milking and AMPM = combined morning and afternoon milking) with whey antibody in milk during late lactation. Correlations from wk 0 (average DIM=284) to wk 4 post tertiary immunization are shown. N = 9 for wk 0, 1, and 2; N = 8 for wk 3; and N = 6 for wk 4.

<table>
<thead>
<tr>
<th>wk</th>
<th>AM milk (r)</th>
<th>P value</th>
<th>AMPM (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.41</td>
<td>NS</td>
<td>-0.51</td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>-0.69</td>
<td>0.04</td>
<td>-0.62</td>
<td>0.08</td>
</tr>
<tr>
<td>2</td>
<td>-0.65</td>
<td>0.05</td>
<td>-0.47</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>-0.52</td>
<td>NS</td>
<td>-0.44</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>-0.33</td>
<td>NS</td>
<td>-0.72</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 1. Immunization schedule and collection schedule for blood, colostrum and milk in early and late lactation. Blood was collected at each time point. Colostrum was collected at calving (d 0) and milk was collected on d 1-5, 10, 11 or 12, and for four wk in late lactation. Blood was collected from calves at two d of age. N = 21 cows followed in early lactation; N=9 cows followed in late lactation.

Experimental Design

Early Lactation

- Primary Immunization
- Secondary Immunization
- Day 3-5

Late Lactation

- Tertiary Immunization
- DIM=394 days

- Blood collected at each time point
- Colostrum collected at calving (day 0)
- Milk collected on day 1-5, 10-12, & Late Lactation
Addendum Appendix 4

Sires and Bull Calf Testing Report
Part A

Testing bulls for high immune response (HIR) – Proven sires

Rationale: In the dairy industry, sires are proven based on daughter records for production and type. Proven sire semen is a valuable export commodity that requires careful management. However, there are few if any measures of health that are reported on these sires or their daughters. Therefore in this study, proven sires owned by the Semex Alliance were tested for the first time to assess their capacity to make robust and balanced immune responses, using the HIR protocol.

Hypothesis: It is possible to safely and efficiently assess immune response capacity in elite dairy sires using the HIR method to evaluate both antibody and cell-mediated immunity.

Subsequently, genetic variation in immune response of dairy sires will be shown as heritable such that sires with highest AMIR and CMIR will have daughters with less disease.

Objective: To evaluate AMIR & CMIR profiles of commercial Semex sires to determine safety and efficacy of the HIR test method.

Methods: Sires (n=15) scheduled for regular culling were identified by Semex (East-Gen Partner, then Gencor) that were also negative for all standard CFIA health diagnostic tests including tuberculosis (Tb) (Table 1). All cull sires tested were housed in a separate barn at the Semex sire testing facility (Guelph, Ontario). Sires were immunized with specified antigens that stimulate immune response according to the patented test protocol (Figure 1).

Figure 1- Sire testing protocol for HIR and CFIA testing

<table>
<thead>
<tr>
<th>HIR test protocol for Sires and CFIA testing N=15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
</tr>
<tr>
<td>- Collect blood for ELISA</td>
</tr>
<tr>
<td>- Immunize with type 1 &amp; type 2 antigens</td>
</tr>
<tr>
<td>- CFIA sera &amp; TB testing prior to immunization</td>
</tr>
<tr>
<td>TEST NEGATIVE</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
</tr>
<tr>
<td>- AMIR - Collect blood</td>
</tr>
<tr>
<td>- CMIR - Initiate skin thickness test and take background skin-fold measurements</td>
</tr>
<tr>
<td><strong>Day 15</strong></td>
</tr>
<tr>
<td>- Final skin-fold measurements 24 hours</td>
</tr>
<tr>
<td><strong>Day 22 &amp; 25</strong></td>
</tr>
<tr>
<td>- CFIA sera and TB testing in opposite tailfold</td>
</tr>
<tr>
<td>- Day 25 Read test</td>
</tr>
<tr>
<td>TEST NEGATIVE</td>
</tr>
</tbody>
</table>
Table 1 - Semi-annual and annual Canadian federal testing schedule for cattle diseases both on-farm and in isolation. Table includes name of laboratory where the test is performed.

<table>
<thead>
<tr>
<th></th>
<th>On-Farm</th>
<th>Isolation</th>
<th>SemiAnnual</th>
<th>Annual</th>
<th>Lab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculosis ID Caudal fold</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucellosis TAT</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>ADRI Nepean</td>
</tr>
<tr>
<td>Brucellosis CF</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>ADRI Nepean</td>
</tr>
<tr>
<td>Paratuberculosis ELISA</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>ADRI Nepean</td>
</tr>
<tr>
<td>Bovine Leukosis ELISA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>St Hyacinthe</td>
</tr>
<tr>
<td>Bluetongue ELISA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>NCFAD-Winnipeg</td>
</tr>
<tr>
<td>EHD AGID</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>NCFAD-Winnipeg</td>
</tr>
<tr>
<td>EHD SN</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>NCFAD-Winnipeg</td>
</tr>
<tr>
<td>IBR ELISA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>ADRI Lethbridge</td>
</tr>
<tr>
<td>Leptospirosis MAT</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>ADRI Lethbridge</td>
</tr>
<tr>
<td>BVD Immunoperoxidase</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>ADRI Lethbridge</td>
</tr>
<tr>
<td>BVD SN</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>ADRI Lethbridge</td>
</tr>
<tr>
<td>Trichomoniasis Culture</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>Saskatoon</td>
</tr>
<tr>
<td>Campylobacter Culture</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>ADRI Nepean</td>
</tr>
</tbody>
</table>

The bull sires then were further evaluated by CFIA 7 days after HIR testing was complete (Day 22) and 72 hours later (Day 25) for all required federal tests (listed in Table 1). This was done to determine if any cross reactions or associations existed between the HIR test and required CFIA testing for cattle.

Results

**AMIR and CMIR testing.** Variation in AMIR and CMIR traits was evident for sires such that sires could be classified as high (H), average (A) or low (L) immune responders in a way similar to that demonstrated previously for cows. Although the majority of sires were average responders, there were sires H for AMIR and CMIR (e.g. Sire 4, Figure 2), some that were L for both traits (e.g. Sire 14, Figure 2), and some that are H or L for one or the other response (e.g. Sire 2, Figure 2). Importantly, no adverse reactions were noted with the test (Figure 2).

**CFIA Federal Testing.** All sires were negative for CFIA health testing, including Tb, before the HIR test and following the HIR test. No associations or cross reactions were evident.
Conclusion: It is possible to safely, efficiently and effectively measure immune response capacity in commercial bull sires using the HIR patented test method. There was variation in AMIR and CMIR such that sires could be classified as H, A, or L immune responders. HIR testing did not interfere with CFIA federal testing results.

Technology Transfer & Commercialization: After these results were first released in 2011, an additional ~N=200 elite sires at 3 Semex bull test stations in Ontario were tested over a 1.5 year period. In December 2012 the Semex Alliance, Canada’s largest dairy genetics company, purchased an exclusive 10 year sublicense from the University of Guelph to test all their sires using the HIR testing method. Since that time approximately ~N=2000 sires have been tested. Semen from high immune response sires is marketed globally as Immunity+. Semex collects and monitors daughter morbidity and mortality data in large herds to validate the value of Immunity+ sire semen. Results demonstrate that Immunity+ daughters are less likely to get disease than non-Immunity+ daughters (Larmer and Mallard, 2016). Between January 2012-June 2014 over $50M of Immunity+ semen has been distributed to dairy herds worldwide.

Future Direction: Next steps are to complete validation of the genomics test for HIR (Thompson-Crispi et al. 2014). Preliminary data indicates that genome wide associations exist with AMIR and CMIR using the HIR test method. In addition, adding genomic estimated breeding values (gEBVs) for immune response to the existing EBVs for sire immune responses increases the accuracy of these estimates by about 30% (Semex Alliance Internal Report; Mehdi Sargolzaei, personal communication).

A genomics test for Immunity+ will enhance global access to HIR testing of cattle anywhere in
the world by the simple submission of a hair sample (follicle as source of DNA). This is expected to further enhance the opportunity to genetically improve dairy health genetics.

Next steps are to conduct a formalized statistical analysis of the association between disease and production data from daughters with immune response phenotype of all sires tested using the HIR testing method.

**Part B**

**Testing bulls for high immune response (HIR) – Bull calves**

**Rationale:** The immune system of neonatal calves is naïve and underdeveloped until approximately 2 months of age. Therefore the calf relies on maternal antibodies absorbed from the colostrum of the dam to protect against infection during this time. Given the naivety of the calf immune system, young bull calves may demonstrate different immune response variation profiles compared to older proven sires. Therefore 6 bull calves ~2-3 months of age were tested for immune response capacity using the HIR method. Additionally, it would be relevant to determine whether the HIR test interfered with CFIA Tuberculosis (Tb) testing of dairy calves.

**Hypothesis:** Variation in adaptive immune responses may be identified in very young dairy bull calves using the HIR patented test method.

**Objectives:** To evaluate variation in AMIR and CMIR profiles of bull calves using the HIR test method, and determine if CMIR DTH testing affects or interferes in any way with CFIA Tb testing.

**Methods:** Six Holstein bull calves in 2 groups (Group I & II), ~2-3 months of age at were evaluated for immune response using the patented HIR protocol. Group I was evaluated from October 4-November 1, 2011, and Group II was evaluated from November 7 to November 28, 2011. Calves were housed in the nursery at the University of Guelph Elora Research Station Ponsonby site. Calves were immunized with specified antigens that stimulate immune response according to the patented test protocol. CFIA Tb testing was conducted 7 days (Group I) and 14 days (Group II) after HIR testing by the CFIA assigned veterinarian.

**Results:**

**AMIR and CMIR testing.** Variation in AMIR and CMIR traits was evident among the 6 bull calves. Similar to mature Bulls, there were calves H for AMIR and CMIR, some that were L, and some that are H or L for one or the other response. No adverse reactions were noted with the test (Figure 3).

**CFIA Federal Testing.** Bull calves from both groups were negative for CFIA Tb testing following the HIR test indicating no adverse or cross reactions between these two tests.

**Conclusion:** Variation in AMIR and CMIR response among calves is similar to proven sires and demonstrates the possibility of testing young bull calves for HIR without any negative effects on
CFIA Federal Tb testing.

**Figure 3** Variation in AMIR (Antibody response at Day 14 over Day 0 baseline) and CMIR (DTH skin thickness) responses in N=6 Holstein bull calves.
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