Effects of milk yield genotype on immune, endocrine and metabolite interactions in dairy cows

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Abstract

Studies were designed to assess the impact of milk yield genotype on the innate immune response and its interactions with endocrine and metabolic components when growing heifers and lactating cows are challenged with lipopolysaccharide (LPS). A novel aspect of these studies was the comparison of unique Holsteins that have not been subjected to selection since 1964 (unselected Holsteins; UH) with contemporary Holsteins (CH). In addition, our animal models included heifers from contemporary Red-Black Angus cows (CA) and our experimental designs included the use of repeated LPS administration to assess the impact of genotype on the acute immune response and on the development of a refractory (endotoxin tolerance) state. Pro-inflammatory cytokine and glucose concentrations were greater and cortisol concentrations increased more rapidly in Holsteins than in Angus heifers which is consistent with results from our collaborators that indicate Holsteins have a more robust immune response than Angus cattle. Differences in plasma concentrations of pro-inflammatory cytokines, glucose and cortisol, and in expression of adhesion molecules and phagocytic activity of polymorphonuclear leukocytes (PMNL) after LPS administration indicates UH heifers and cows have a more robust immune response than CH heifers and cows. This was further supported by hepatic gene expression data, which indicated greater expression of genes in the TLR4 signaling pathway and of genes involved in the production of pro and anti-inflammatory mediators (IL6, TNF, IL1RN, TGFB1) in UH than in CH cows during the acute immune response. In addition, results during the second challenge indicated greater development of tolerance to immunotoxin stimulation in UH than CH cows.

Overall, our results demonstrate that TLR4 signaling pathways have been altered by five decades of selective breeding and these changes contribute to a less robust and less controlled innate immune response in CH cows. Thus, immune activation and the ability to minimize negative effects of prolonged inflammation are reduced in the CH cow. Although we did not assess the impact of selection, we assessed functionality of several anti-ADAM17 antibodies and inhibitors and for, the first time, characterized the presence and activity of ADAM17 (a disintegrin and metalloproteinase-17) protein in bovine PMNL.

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Finally, I would like to thank my family and friends for their constant motivation, support and love.

Dedication

This thesis is dedicated to the veterinarian and animal science community.

I would also dedicate this work to my family. To my parents Pablo and Margarita, to my brother Guillermo and to my uncles and cousins Cousillas, Boam and Bellenda.

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CHAPTER 1: Literature Review

1. Genetic Selection in Dairy

Selective breeding practices have truly transformed the phenotypic and genotypic character of Holstein cows (Sonstegard et al., 2009). Over the past decades, the focus of the dairy industry has been on maximizing milk yield (Sundrum, 2015) and this has tremendously increased milk and component yields per U.S. Holstein. Annual milk yield per cow increased over 4-fold in the last 75 years (Baumgard et al., 2017). These increases have undoubtedly benefitted producers and consumers in the United States and worldwide. It has improved production efficiency and profitability while also greatly reducing the impact of dairy production on the environment (Capper et al. 2009). However, these also has dramatically altered metabolic fluxes in the cow to support the increased demand for nutrients to synthesize milk (Bauman, 2000) and there is great concern that genetic selection practices have made the contemporary cow more susceptible to disease and metabolic disorders, especially during the transition from pregnancy to lactation (Pritchard et al. 2013; Egger-Danner et al. 2015).

Unfavorable genetic correlations have been estimated between milk yield and diseases such as mastitis, milk fever, ketosis and retained placenta (Jones et al., 1994; Pryce et al., 1997; Schnitzenlehner et al., 1998; Lucy, 2001; Heringstad et al., 2005). As milk yield per cow increases, endocrine and metabolic adaptations occur to enable the cow to meet the metabolic demands of increased milk synthesis (Crooker et al. 2001), and these adaptations can disrupt the immune system compromising cow health and well-being (Pritchard et al., 2013; Egger-Danner et al., 2015).

Despite focused research and scientific emphasis placed on the periparturient period over the past decades, the incidence of problems during this period has not decreased (Drackley, 1999; Oliver et al., 2005; LeBlanc, 2010) as 30% to 50% of dairy cows are affected by some form of metabolic disorder or infectious disease around the time of calving (Leblanc, 2010). Producers are continually challenged to modify management practices to meet the ever increasing needs of the dairy cow (Bjerre-Harpøth et al., 2012), and this trend is expected to continue as the productive ceiling of the dairy cow has not been reached (Bauman, 2000; Baumgard et al., 2017).

Interest in increasing selection indices to include functional traits such as reproduction and health has been growing during the past decades (Miglior et al., 2005), yet when compared to production traits, emphasis on health remains low. The relative emphasis on selection for production (milk yield, fat and protein yield and percentage), durability (longevity, body size, overall udder, and other conformation traits), and health and reproduction (udder health, calving ease, and milking speed) is 48, 27, and 19%, respectively (Miglior et al., 2012; Egger-Danner et al., 2015; Cole & VanRaden, 2018). Emphasis on genetic selection for health traits related to the most important diseases in dairy (such as mastitis and lameness) was not used until recently due to the low heritability estimated for these traits (ranges from 0.01 – 0.10; Pritchard et al., 2013). However, increased on-farm recording programs have augmented the amount of data on health traits and most of the developed countries are now giving more emphasis to health, fertility and longevity (Miglior et al., 2012). Genetic gain with selection on health is slow, however it is 'positive and cumulative' (Pritchard et al., 2013).

Genetic gain depends on the reliability of the trait, the intensity of selection, the genetic variability in the population, and the generational interval. With new technologies in reproduction and with genomic selection (Kasinathan et al., 2015), evaluation of genetic progress can be done more quickly (Wiggans et al., 2017). With genomic selection, new traits can be predicted in genotyped animals without the need for progeny records, shortening the generational interval (Cole & VanRaden, 2018). In addition, incorporation of 'omics' technologies such as metabolomics and proteomics can lead to better phenotype descriptions augmenting selection opportunities (Egger-Danner et al., 2015; Cole & VanRaden, 2018).

Health traits and selection for disease resistance is gaining increasing attention and in April 2018 six new health traits, selected for their significant incidence rates and costs to dairy farmers, were included in the official U.S. Holstein sire evaluations. These newly included traits are resistance to hypocalcemia, displaced abomasum, ketosis, mastitis, metritis and retained placenta (CDCB, 2018 https://www.uscdcb.com/cdcb-health-traits/). Genetic resistance for these low heritable traits is cumulative and permanent, therefore disease resistance can be built up over time (Bishop & Woolliams, 2014).

Growing emphasis on functional traits and health indicates their importance. It is recognized that there is a critical need to minimize the incidence of metabolic disorders and infectious diseases in dairy cows, especially during the periparturient period. Efforts to reverse the negative impact of past selection practices through new selection strategies and marker assisted selection programs are improving the situation (CDCB, 2017

https://www.uscdcb.com/eval/summary/trend.cfm), however, additional, more-focused gene-assisted selection efforts are still needed. A better understanding of how the previous focus on selection for milk yield has altered the immune system, its interactions with endocrine and metabolic components, and the molecular control mechanisms regulating the innate immune response is necessary. The latter will contribute to efforts to identify lost and new functional health traits that can be included in future selection practices to improve cow health and well-being and contribute to enhancing food safety and security.

1.1. Animal model

In 1964, Dr. Charles Young started a static, low-merit unselected line (unselected UMN Holsteins, **UH**) and a contemporary high-merit selected Holstein (**CH**; Young 1977; Weber et al. 2007). The original cows in 1964 were paired by genetic merit and assigned to either low merit (unselected) or high merit (contemporary) line. The high merit cows and their female descendants have been inseminated with semen from sires (n= 4) with the highest predicted transmitting ability for milk (PTA-milk) available each year. The low-merit cows and their female descendants were bred, from 1964 to 1991, with semen from sires (4 sires/yr in a 5-yr rotation; 20 total) that were breed average for PTA-milk in 1964. Since 1991, breeding the UH cows and their female descendants has continued as the original design described, but with semen from sons of the original 20 bulls and the unselected cows. The coefficients of inbreeding are not allowed to exceed 6.25% for both low- and high-merit lines (Weber et al., 2007).

Genetic merit for milk yield of the UH cows has remained stable, while it has continued to increase for the CH cows similar to that of U.S. Holsteins. Therefore the high-merit contemporary cows represent the contemporary U.S. Holsteins and the low-merit unselected cows represent average U.S. Holsteins in 1964 (Weber et al., 2007). Fifty years of selection led to cows that produce 4,000 kg more milk per 305 days of lactation than their ancestors (Figure 1). The current milk yield of unselected cows is about 55% of contemporary cows (6,700 vs. 12,000 kg/lactation; Crooker et al., 2001; Weber et al., 2007).

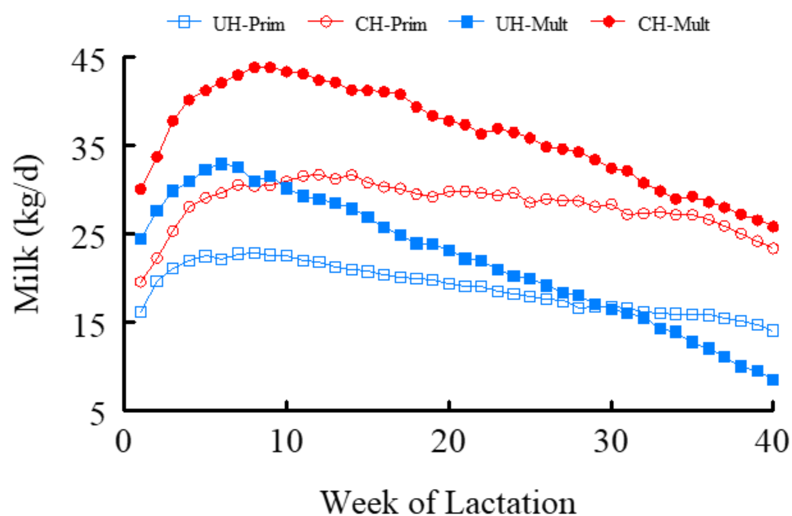

Figure 1.1. Effect of genetic merit and parity on daily milk yield. Data are from primiparous (Prim) and multiparous (Mult) unselected cows (UH) and contemporary cows (CH). Milk yield was greater ($P < 0.001$) for CH than UH cows (32.2 vs. 20.5 ± 0.8 kg/d) and for Mult than Prim cows (29.1 vs. 23.6 ± 0.8 kg/d; Weber et al. 2007).

This experimental model of unselected vs. contemporary Holstein genotypes managed under identical conditions and fed the same diets allows examination of immune, endocrine and metabolic alterations and regulatory components specifically due to genetic changes associated with selection for increased milk yield. These include

physiological changes required to meet the increased metabolic demands associated with increased milk synthesis.

2. Transition Period

The periparturient period, also referred to as the transition period, is defined as the period between three weeks before to three weeks after parturition (Grummer, 1995; Drackley, 1999). In relation to the dairy cow's health status, this is the most challenging and critical portion of the lactation cycle due to the tremendous adaptations that must occur and the ease at which these adaptations can be disrupted. Major physiological, nutritional, metabolic, and immunological changes occur within this time frame as the physiology of the cow transitions from a gestational non-lactating state to one of copious milk synthesis and secretion (Bell, 1995; Sordillo & Raphael, 2013). Disruptions to these adaptations make the cows prone to the development of metabolic disorders and disease.

During late gestation the nutritional demands of the fetus and uterus increase exponentially. Intake, however, is often reduced due to a reduction in rumen capacity (~20% reduction; Stanley et al., 1993) and to endocrine changes that induce parturition, as well as by parturition itself (Ingvartsen and Andersen, 2000). After parturition, the immediate onset of copious milk production requires a tremendous (3 to 4 fold) increase in nutrients to meet the metabolic demands of the mammary gland (Crooker et al., 2007). However, these increased requirements are not accompanied by an immediate or sufficient increase in feed intake, as the subsequent rate of increase in feed intake is not as rapid as the increase in milk yield (Bell & Bauman, 1997).

Despite being fed postpartum diets that contain more energy and nutrients, dairy cows experience significant negative energy balance (NEB) in early lactation due to the imbalance between nutritional needs and intake. This induces mobilization of body tissues to help meet these metabolic demands (Bell & Bauman, 1997; Moore et al., 2005). Severity of the energetic imbalance is often associated with adverse health events (Bell, 1995).

2.1. Metabolic adaptations during the Transition Period

Metabolic adaptations are needed to meet the dramatic increase in energy and nutrient requirements to support fetal demands and ensure milk production at the beginning of lactation. These adaptations include alterations in endocrine signaling and often are initiated by changes in gene expression (Bell, 1995). Bauman and Currie (1980) applied the concept of homeorhesis to the regulation of nutrient partitioning during lactation, and defined homeorhesis as "the orchestrated or coordinated changes in the metabolism of body tissues necessary to support a physiological state." Ingvartsen and Andersen (2000) described the direction of change in concentrations of homeorhetic hormones during mid- and late pregnancy and early lactation, and the assumed consequences of these changes on tissue sensitivity and responsiveness (Table 1).

The gravid uterus at 250 days of gestation and the lactating mammary gland at 4 days postpartum, demand approximately a threefold surge of glucose, a twofold increase in amino acids (AA), and approximately a fivefold increase in the demand of fatty acids (FA; Bell, 1995). Increased hepatic gluconeogenesis from endogenous substrates, decreased peripheral tissue glucose utilization, increased FA mobilization from adipose

tissue and increased AA mobilization from muscle should compensate for the differences in demands and intake (Bell, 1995). However, glucose utilization rate by the mammary gland at the onset of lactation for lactose synthesis (Overton & Waldron, 2004) cannot be compensated by tissue mobilization, and there is a net reduction in blood glucose concentrations (Ingvartsen & Andersen, 2000).

Table 1. Changes in some homeorhetic and homeostatic hormones, tissue sensitivity, and responsiveness and effect in selected tissues in pregnancy and lactation. Adapted from Ingvartsen and Andersen (2000).

	Mid-pregnancy	Late pregnancy	Lactogenesis Early lactation
Potential homeorhetic hormones[1]			
Progesterone	↑	(↓)	↓
Placental lactogen		↑	↓
Estrogens		↑	↓
Prolactin	-	(↓)	↑
Somatotropin	-	(↓)	↑
Leptin	?	?	?
Homeostatic hormones[1]			
Insulin		↑	↓
Glucagon	-	-	-
CCK and somatostatin	?	?	?
Tissue sensitivity			
Insulin	↑	↓	↓
Catecolamines		↑	↑
Tissue responsiveness			
Insulin		↓	↓
Catecolamines	↓	↑	↑
Liver[2]			
Gluconeogenesis			↑
Ketogenesis			↑
Adipose tissue[2]			
Lipogenesis	↑	↓	↓
FA esterification	↑	↓	↓
Lipolysis		↑	↑
Glucose utilization		↓	↓
Skeletal muscle[2]			
Protein synthesis		↓	↓
Protein degradation		↑	↑
Glucose utilization		↓	↓

↓: decreasing; ↑: increasing; ?: unknown in ruminants; -: no significant changes.
[1]Plasma hormone concentration changes.
[2]Changes in rate of metabolic processes

Glucose concentrations drops abruptly postpartum, reaching their minimum in the firsts 1-3 weeks of lactation (Ingvartsen & Andersen, 2000). Therefore, the primary homeorhetic adaptations, which must occur for a successful transition, include an increase in hepatic gluconeogenesis and a decrease in the glucose oxidation by peripheral tissues at the onset of lactation. These changes help direct glucose to the mammary gland (Reynolds et al., 2003; Overton & Waldron, 2004) and preempt development of common metabolic disorders such as hypoglycemia and ketosis. Both of these disorders are often observed during the onset of lactation when increased gluconeogenic capacity either does not occur or is insufficient to meet demands for glucose (Donkin, 2012).

In ruminants, the main substrates for glucose synthesis under normal circumstances are propionate, lactate and AA. During times of feed restriction and energy deficiency, glycerol from adipose tissue can be used for glucose synthesis (Donkin, 2012). The primary source of propionate for gluconeogenesis is rumen fermentation. During the transition period feed intake is depressed which reduces propionate supply. During these conditions there is a greater dependence on lactate, glycerol and AA as gluconeogenic precursors (Danfær et al., 1995). The transition to lactation is marked by adaptations in the gluconeogenic activity in the liver that enables greater use of lactate, glycerol and AA to support whole body glucose needs (Donkin, 2012).

Metabolizable AA come predominantly from the intestinal absorption. Absorbed AA originate from microbial protein synthesis in the rumen and from dietary AA sources that are not degraded in the rumen (Kung & Rode, 1996). During the transition period

however, mobilization of tissue AA must occur to meet overall metabolizable AA needs. Absorbed and mobilized AA from the muscles are partitioned to either be oxidized for energy, for gluconeogenesis or for milk protein (Sundrum, 2015). Although utilization of propionate for gluconeogenesis is extensive, AA have the potential to increase in importance as gluconeogenic substrates when glucose demand is substantially increased (Overton et al., 1999).

The reduction in blood glucose results in lower insulin levels, which triggers fat mobilization through lipolysis. Early in the postpartum period, there is not only decreased production of insulin (Drackley et al., 2001), but also a transient state of insulin resistance in peripheral tissues (Bell, 1995). Insulin resistance allow glucose sparing for lactogenesis in the mammary gland by decreasing glucose utilization by insulin-sensitive peripheral tissues and by maintaining a constant lipolysis, that remains even when insulin concentrations are increased (Ospina et al., 2013).

Elevated lipolysis and lipid mobilization increase blood concentrations of non-esterified fatty acids (NEFAs; Sordillo & Raphael, 2013). During the transition period, cows increase their use of lipids as a source of energy to support lactation (Ingvartsen & Andersen, 2000). NEFAs are transported from adipose to the blood by albumin, where they can not only be used as an alternative source of energy by peripheral tissues such as muscle, but can also initiate negative feedback loops necessary to regulate increased lipolysis levels (Sordillo & Raphael, 2013). Constant blood glucose concentrations needed for milk synthesis and secretion without an excessive NEFA accumulation in the blood should be the overall effect (Sordillo & Raphael, 2013). However, not all cows

successfully adapt to this interval of NEB. There is a positive correlation between the excessive release of lipids from adipose tissues (with a subsequent accumulation and greater blood NEFA concentrations) and the onset of metabolic problems during the transition period (Ospina et al., 2013).

The liver removes approximately 15–20% of NEFAs from the blood entering the liver (Drackley & Andersen, 2006). These NEFA can either be completely oxidized for the liver's own energy, partially oxidized to produce ketone bodies (particularly in times of NEB), converted into triacylglycerols (TAGs) and packaged into very low density lipoproteins for transport back to the adipose tissue, or stored as TAG (McArt et al., 2013). Ketone bodies (acetone, acetoacetic acid and beta-hydroxybutyric acid [BHBA]) released by the liver are used by muscle and nervous tissue as energy substrate (Herdt, 2000), but when in excess, ketone bodies can cause ketosis (Grummer, 1995; Drackley, 1999). The liver takes up NEFAs in proportion to blood concentrations (Herdt, 2000), however, the ruminants' capacity to export hepatic lipids is limited (Drackley, 1999). This leads to excessive lipid accumulation and the subsequent development of fatty liver (hepatic steatosis; Drackley, 1999; Grummer, 1995). Greater concentration of NEFAs and BHBA in the blood are part of a normal adaptation to NEB in early lactation. However, excessive concentrations of NEFAs (> ~500 µmol/L) or BHBA (> 1400 µmol/L) can indicate severe maladaptation to this interval of NEB which is associated with decreased health and reduced production (Oetzel, 2004; Drackley & Andersen, 2006; McArt et al., 2013).

2.2. Immune status during the Transition Period

Function and effectiveness of the bovine immune and acquired host defense mechanisms are decreased during the periparturient period. During calving in particular, key host defense mechanisms are impaired which render the cow more susceptible to metabolic and infectious diseases (Sordillo et al., 2007). The onset of lactogenesis and uterine involution, together with the hormonal and metabolic changes that reflect the homeorhetic adaptations during the transition period, create a unique set of adaptive changes which can be detrimental for the dairy cow (Bradford et al., 2015).

Changes in the concentration of plasma NEFA directly affect immune cell functions. For example, Scalia et al. (2006) described the ways in which bovine polymorphonuclear cell (PMNL) function and viability were affected by plasma NEFA levels. Neutrophils exhibited increased reactive oxygen species (ROS) production but decreased viability in a NEFA concentration-dependent manner (Scalia et al. 2006). However, the immunosuppression with neutrophil and lymphocyte impaired activity observable during this period is due not only to high concentrations of NEFAs. Impaired neutrophilic activity is characterized by reduced activation, chemotaxis, adherence, phagocytosis, respiratory burst and release of lytic enzymes (Rinaldi et al., 2008; Sordillo & Aitken, 2009). Impaired function of these cells is thought to be primarily due to the effects of the elevated concentrations of glucocorticoids at parturition (Preisler et al., 2000). The act of parturition is itself a stressful event that increases production and blood concentrations of glucocorticoids.

Glucocorticoids are known to attenuate signaling pathways downstream of many 'danger' signals, thereby suppressing the production of inflammatory mediators (Cain & Cidlowski, 2017). For instance, toll-like receptor signaling is subject to regulation by glucocorticoids. Ligand of glucocorticoid receptors tether and inhibit transcription factors downstream, including NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), AP1 (activator protein-1) and IRF3 (interferon-regulatory factor 3; Cain & Cidlowski, 2017). Studies have also demonstrated that circulating glucocorticoids down regulate expression of the adhesion molecules L-selectin and CD18 on the surface of neutrophils. This reduces surveillance activity and, as a result, reduces immune response capacity (Burton et al., 1995; Weber et al., 2004).

Major shifts in immune cell populations and function occur simultaneously during the transition period (Mallard et al., 1998). There is an increase in metabolic disorders with subsequent inflammation that alters the bovine immune system and can effect an overproduction of inflammatory mediators (Sordillo et al., 2009). This condition can manifest when the synthesis of eicosanoids, cytokines and other pro-inflammatory molecules is stimulated; either by high fatty acid concentration or by recognition of bacterial virulence factors such as lipopolysaccharide (LPS; Contreras et al., 2012; Sordillo et al., 2009). These potent mediators initially stimulate local inflammatory reactions, but can also induce a systemic response if they gain access to the circulatory system (Aitken et al., 2011). In addition, the greater energy and nutrient content of the postpartum dairy cow diet alters rumen fermentation towards increased acid production which increases the presence of Gram-negative bacteria as well as the release of LPS and

other endotoxins into the intestinal lumen and blood (Zebeli & Metzler-Zebeli, 2012). These events further increase the likelihood of the overproduction of inflammatory mediators.

The periparturient period, therefore sees a high incidence of metabolic and production-related diseases including milk fever, mastitis, fatty liver disease, ketosis, metritis, hypomagnesemia and abomasal displacements along with complications from dystocia and retained placenta (Aleri et al., 2016; Drackley, 1999; McArt et al., 2013; Ribeiro et al., 2013). The immune dysfunction during this period provides opportunistic bacteria a greater opportunity to establish infections (Burvenich et al., 1994). Mastitis alone, one of the most prevalent diseases in dairy, costs the U.S. dairy industry about $1.7-2 billion annually or 11% of total U.S. milk production (Jones & Bailey, 2009). Control and prevention of disorders during this period is therefore extremely important for avoiding the high costs of substantial milk loss, reduced productive efficiency, additional labor, drug costs and potential loss of animals.

3. Pathogen recognition and innate immune activation

The innate immune system constitutes the first line of defense during infection and therefore plays a vital role in the early recognition and subsequent activation of a pro-inflammatory response to invading pathogens (Medzhitov & Janeway, 2000). On the other hand, the adaptive immune system is responsible for the elimination of pathogens in the late phase of infection and in the generation of immunological memory (Mogensen, 2009). The innate immune response has been regarded as relatively nonspecific and it is

mediated primarily by phagocytic cells and antigen presenting cells (APCs), such as granulocytes, macrophages, and dendritic cells (Iwasaki & Medzhitov, 2004).

The innate immune response relies on the recognition of evolutionarily conserved motifs on pathogens, called pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs), of which the family of Toll-like receptors (TLRs) has been the most studied (Akira et al., 2006; Medzhitov & Janeway, 2000). PAMPs are characterized by their invariance among entire classes of pathogens, as well as by their critical role in the survival of the pathogen and their distinction from host molecules (Janeway, 1989). However, in certain cases, PRRs also recognize host factors as "danger" signals. This occurs when host-cell derived molecular complexes manifest in abnormal locations as a consequence of trauma, ischemia and/or tissue damage, either in the absence or presence of pathogenic infection (Matzinger, 2002; Tang et al., 2012). These "danger" molecules, also known as damage-associated molecular patterns (DAMPs), can directly activate PRRs in a manner similar to that of PAMPs (Liston & Masters, 2017).

Upon PAMP recognition, PRRs signal to the host the presence of infection triggering pro-inflammatory and antimicrobial responses by activating intracellular signaling pathways (Akira & Takeda, 2004). The induced transduction pathways result in both the activation of gene expression and the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules and immunoreceptors (Akira et al., 2006); together, these initiate the innate immune response (Mogensen, 2009).

3.1. Toll-like receptors

Ten different TLRs have been described in the bovine (McGuire et al., 2005), each with its own ligand and functional characteristics. TLRs are predominantly expressed in tissues involved in immune functions such as spleen and peripheral blood leukocytes, as well as in those exposed to the external environment such as lung and the gastrointestinal tract. Their expression profiles vary among tissues and cell types. With the exception of TLR3, 7 and 9 which are localized in the endosomal compartment, TLRs are located on the cell's plasma membrane (Nishiya and DeFranco, 2004).

TLRs recognize molecular patterns characteristic of Gram-positive and negative bacteria, fungi and viruses (PAMPs). Cell walls and membranes of bacteria are composed of repetitive arrays of carbohydrates, proteins and lipids, of which many are not found in animal cells (Murphy et al., 2012). Among these PAMPs are the lipoteichoic acids (LTA) of Gram-positive bacterial cell walls and the lipopolysaccharides (LPS) of the outer membrane of Gram-negative bacteria, these are particularly important in the recognition of bacteria by TLRs and the subsequent activation of the innate immune system (Murphy et al., 2012).

The TLRs are type I integral transmembrane glycoproteins, and due to the homology in the cytoplasmic region, are members of a larger superfamily which includes the interleukin-1 receptors (IL-1Rs). However, the extracellular region of the TLRs and IL-1Rs are very different; the extracellular region of TLRs contains 18-25 copies of leucine-rich repeat (LRR) motifs, whereas that of IL-1Rs contains three immunoglobulin-like domains (Akira & Takeda, 2004; Murphy et al., 2012). The multiple LRRs in TLRs

create a horseshoe-shaped protein scaffold which is adaptable for ligand binding and recognition on both outer (convex) and inner (concave) surfaces (Murphy et al., 2012). The cytoplasmic tail of TLRs and IL-1Rs have a conserved region of ~200 AA known as the Toll/IL-1Rs (TIR) domain (Akira & Takeda, 2004; Murphy et al., 2012).

3.1.1. Leucine-rich repeats

Each LRR consists of a sequence of 24-29 AA and hydrophobic AA that contains a β-strand and an α-helix connected by loops (Akira & Takeda, 2004). Despite conservation among LRR domains, different TLRs can recognize several structurally disparate and unrelated ligands (Akira & Takeda, 2004; Janeway & Medzhitov, 2002). The TLRs' cellular localization is correlated somewhat with the molecular patterns of their ligands. TLR1, 2 and 4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. On the other hand, TLR3, 7 and 9, which are each involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface (Akira & Takeda, 2004).

To date, at least one ligand has been identified for each TLR, except for TLR10. Triacylated lipoprotein for TLR1; peptidoglycan and LTA, of Gram-positive bacteria, for TLR2; double-stranded RNA of viruses, for TLR3; LPS, of Gram-negative bacteria, for TLR4; flagellin, a component of bacterial flagella, for TLR5; diacylated lipoprotein for TLR6; viral RNA and single-stranded self-RNA for TLR7 and TLR8; and bacterial unmethylated CpG DNA for TLR9 (Yamamoto et al., 2004; Guiducci et al., 2013).

3.1.2. Toll/IL-1Rs (TIR) domain

The signaling cascade downstream of TLR is generated from its cytoplasmic TIR domain (Janeway & Medzhitov, 2002). Ligand binding through PAMP-TLR interaction induces oligomerization of the receptor, changing the conformation of the TIR domain tail and subsequently triggering intracellular signal transduction (Mogensen, 2009). The conformational change of the TIR domain recruits downstream signaling adaptor molecules, including the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), the TIR-domain-containing adapter-inducing interferon-β (TRIF), as well as the TRIF-related adaptor molecule (TRAM) and the TIR-domain-containing adapter-protein (TIRAP). Recruitment of these adaptor molecules culminates in pro-inflammatory cytokine production and its subsequent immune responses (Troutman et al., 2012; Yamamoto et al., 2004).

With the exception of TLR3, which signals through the adaptor TRIF, all TLRs utilize MyD88 for signal transduction upon ligand binding. TLR4 is the only TLR which can utilize both a MyD88-dependent and MyD88-independent pathway, whereby TRIF is the critical signaling adaptor for the MyD88-independent node. TIRAP and TRAM serve as shuttling adaptors for TLR2 and TLR4, with TIRAP mediating recruitment of MyD88 and TRAM mediating recruitment of TRIF (Troutman et al., 2012; Yamamoto et al., 2004).

3.2. LPS

LPS is the major component of the outer leaflet of Gram-negative bacteria's outer membrane (Nijland et al., 2014). One cell of *E. coli,* the model organism for Gram-

negative bacteria, contains approximately 3.5×10^6 LPS molecules (Rietschel et al., 1994). LPS consists of three different parts, a hydrophobic domain known as lipid A (or endotoxin), a non-repeating "core" oligosaccharide, and a distal polysaccharide (or O-antigen; Raetz & Whitfield, 2002). The lipid A is the hydrophobic anchor of LPS. It is a glucosamine-based phospholipid that constitutes the outer monolayer of the outer membranes of most Gram-negative bacteria (Rietschel et al., 1994).

LPS is widely used as an endotoxin to evaluate the immune response. Administration of endotoxins minimizes the potential for actual infection as well as the uncontrolled variation of bacterial growth and the loss of study animals. As defined bacterial PAMPs, endotoxins offer an additional advantage in the modelling of inflammation by targeting identified PRRs and accessory molecules for recognition and for the signaling cascade (Rainard et al., 2008). Challenges with LPS provides the opportunity to evaluate the inflammatory response to a given pathogenic agent without a live bacterial infection.

3.3. Immune recognition of LPS through the TLR4 pathway

The lipid A of LPS is not recognized by the host while anchored inside the bacterial outer membrane (Nijland et al., 2014). However, bacterial growth or lysis can effect the release of the LPS and expose lipid A. Once exposed, LPS can be detected at picomolar levels (Raetz & Whitfield, 2002) by TLR4 present on monocytes, macrophages, PMNL, and endothelial cells of the host (Nijland et al., 2014).

Recognition of LPS by TLR4 is facilitated by a glycosyl-phosphatidylinositol-linked receptor that lacks a transmembrane domain called CD14. This receptor is mainly

expressed on monocytes and macrophages, and to a lesser extent on neutrophils (Wright et al., 1990). Binding of LPS to CD14 is facilitated by the acute phase protein LPS-binding protein (LBP), and is the CD14-LPS-LBP complex which is recognized by TLR4 in complex with myeloid-differentiation protein 2 (MD2) interacting with CD14 (Nijland et al., 2014; Rainard and Riollet, 2006). Both TLR4 and MD2 are found to be essential for signaling (Fitzgerald et al., 2004). Upon recognition of LPS by the TLR4-MD2 complex, dimerization occurs, initiating signaling by the interaction of the intracellular TLR4-TIR domains (Nijland et al., 2014). Dimerization of the TIR domain leads to the recruitment of adapter molecules while the activated signaling cascade leads to the activation and expression of the transcription factors NFκB and AP1 as well as interferon regulatory factors (IRF). Activation of NFκB and AP1 leads to the transcription of genes involved in the activation of the innate defense, primarily to induce the expression of pro-inflammatory cytokines and chemotactic factors. IRF factors are likewise important for inducing antiviral type I interferons (Murphy, 2012). A schematic overview of the immune recognition of LPS is presented in Figure 2.

There are other accessory activation pathways involved when the TLR4 are activated (Netea et al., 2004). Activation of TLR4 can recruit the adaptor molecules TRIF and TRAM, which mediate unique signals and lead to the secretion of IFN-β, indirectly up-regulating IFN-dependent genes such as IP-10 and iNOS (Netea et al., 2004).

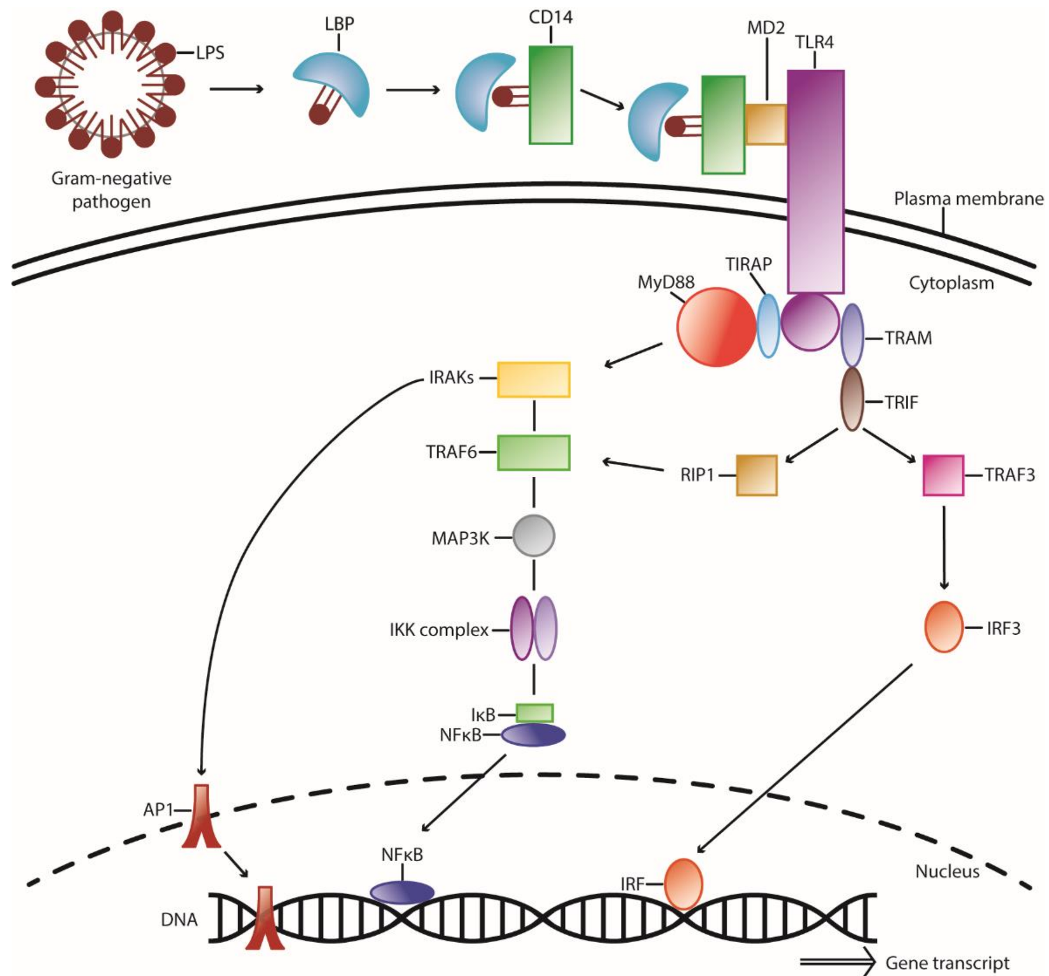

Figure 1. The TLR4 signaling pathway. When LPS is released from the bacterial membrane, it is bound by LBP and transferred to CD14. MD2 then binds the LPS and forms LPS-MD2-TLR4 complexes. Dimerization of TLR4 molecules occurs and leads to the recruitment of adapter molecules: TIRAP, MyD88, TRAM and TRIF. MyD88 is associated with the serine-threonine protein kinase interleukin-1 receptor–associated kinase (IRAK). IRAK is then phosphorylated and associated with the tumor necrosis factor–associated factor 6 (TRAF6) adapter protein. Oligomerization of TRAF6 activates a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, which leads to the activation of the IKKα and IKKβ kinases (IKK complex). These kinases phosphorylate IkB on serine residues, thus targeting IkB for degradation and releasing NFκB. NFκB then moves into the nucleus and starts transcription of pro-inflammatory cytokines, such as IL-6 and TNF-α. Via TRIF and TRAM, a different pathway, type I interferon genes are also transcribed, leading to the production of INF-β. (Chen, 2005; Lu et al., 2008; Kawasaki & Kawai, 2014).

4. Inflammatory response and pathogen clearance

Activation of the innate immune system triggers inflammation, which is necessary to remove infectious agents and to begin tissue healing. The clinical manifestations of inflammation are redness, heat, swelling and pain (Murphy et al., 2012). Therefore, the physiological response includes release of soluble mediators, vasodilatation, increased blood flow, increased endothelial cell permeability with extravasation of fluid, cellular influx (chemotaxis) and increased cellular metabolism (Sordillo et al., 2009). The result of an effective inflammatory response is the rapid elimination of pathogens or other insults that cause detrimental changes to host tissues. However, when regulation is inadequate, the inflammatory response can be excessive and result in inflammatory diseases of dairy cattle, such as coliform mastitis and septic shock (Burvenich et al., 2007). A properly controlled response is necessary to avoid excessive expression of the cytokines that lead to fever, edema, pain, tissue damage, systemic inflammation and possibly death (Tracey, 2007). The immune system's response must therefore be sufficient for the elimination of infection while yet controlled in order to avoid host tissue damage (Sordillo et al., 2009).

The inflammatory response begins with the pro-inflammatory signaling pathways induced by PRRs after the binding of PAMPs; and the transcription factors NFκB, AP1, and IRF3/7 play pivotal roles (Mogensen, 2009) due to their capacity to stimulate production of cytokines and IFNs. NFκB inducible genes, includes those for pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, as well as chemokines, including IL-8 and RANTES (Ghosh et al., 1998).

Furthermore, after activation there is upregulation in the expression of cell adhesion molecules which causes responding immune cells to localize sites of infection (Lippolis, 2008; Mogensen, 2009).

4.1. IL-6

An immediate and transient expression of IL-6 is generated in response to environmental stress factors such as infections and tissue injuries after PPR activation (Tanaka et al., 2014). Immune, mesenchymal and endothelial cells as well as fibroblasts and many other cells are involved in the production of IL-6 in response to various stimuli (Akira & Kishimoto, 1992). In addition, other cytokines, such as TNF-α and IL-1β, can also activate transcription factors to produce IL-6 (Tanaka et al., 2014).

Interleukin-6 is synthesized at the initiation of the acute phase response and is considered responsible for signaling hepatocytes to produce acute phase proteins (APP). Once synthesized in a local site of infection or lesion during the initial stage of inflammation, IL-6 moves to the liver in an endocrine fashion way through the bloodstream, inducing the synthesis of a wide range of APP such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen and haptoglobin (Hp), while inhibiting production of albumin (Heinrich et al., 1990). IL-6 also reaches the bone marrow where it promotes megakaryocyte maturation leading to platelets release (Ishibashi et al., 1989). Changes in APP and blood counts of white blood cells and platelets are used to evaluate inflammation severity (Tanaka et al., 2014).

IL-6 is a cytokine which features pleiotropic activity. It not only induces APP production, but also plays an important role in the adaptive immune response by

stimulating B and T-cell differentiation and activation as well as antibody synthesis (Akira & Kishimoto, 1992; Tanaka et al., 2014). Moreover, IL-6 can promote differentiation and proliferation of several non-immune cells (for example, via the STAT1/3 pathway it stimulates chondrocyte proliferation for bone development; Sims et al., 2004). Because of the pleiotropic activity, strict regulation of IL-6 production is critical, as persistent production of IL-6 can lead to the onset or development of autoimmune and chronic inflammatory diseases or even cancer (Tanaka et al., 2014).

IL-6 signals through the IL-6 receptor (IL-6R; a unique binding-receptor for IL-6). Whereas IL-6R uniquely binds IL-6, the signal-transducing chain, through glycoprotein 130 (gp130), is shared with other members of the IL-6 family of cytokines (such as IL-11, cardiotrophin 1, cardiotrophin-like cytokine, IL-27 and IL-35; Kishimoto et al., 1995). The downstream signaling activation of gp130 in turn activates the Janus kinase (JAK)-STAT3 pathway and the JAK-SHP-2-mitogen-activated protein (MAP) kinase pathway (Tanaka et al., 2014). The regulation of various sets of IL-6 responsive genes, including APP, is accounted for by the transcription factor STAT3, which also induces the suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Both, SOCS1 and SOCS3, induce negative feedback loops to stop IL-6 production (Tanaka et al., 2014). When the source of stress, such as infectious pathogens, are removed from the host, both the ligand-induced internalization and degradation of gp130 and the recruitment of SOCS (Naka et al., 1997) are activated to stop the IL-6-mediated activation signals. There is also degradation of IL-6 mRNA by regnase-1 which ends IL-6 production (Tanaka et al., 2014).

4.2. TNF-α

IL-1β and TNF-α production after PRR activation intensifies the inflammatory response because they also induce NFκB and MAPK activation (Newton and Dixit, 2012). TNF-α is usually undetectable in healthy animals; during inflammation, however, elevated blood and tissue concentrations are found which correlate with the severity of the infection (Nakajima et al., 1997; Sordillo & Peel, 1992). Many different cells can produce TNF-α, including mast cells, T and B lymphocytes, natural killer cells, neutrophils, endothelial cells and fibroblasts; however the main sources of TNF-α are from activated macrophages and T lymphocytes (Bradley, 2008).

TNF-α is produced as a pro-TNF protein expressed on the plasma membrane. From there it can be cleaved in the extracellular domain by a matrix metalloproteinase named TNF-α converting enzyme (TACE, also known as ADAM17) to release the soluble form (Black et al., 1997). ADAM17 mediates the release of TNF-α from the cell surface, but it is also involved in processing numerous cell-membrane associated proteins, including TNF-α receptors, which can then be released to produce soluble forms which neutralize TNF-α actions (Wang et al., 2003).

TNF-α mediates most of its pro-inflammatory effects by binding to TNF-α receptor I (TNFR1 also called TNFRSF1A) and TNFR2 (also known as TNFRSF1B) which are differentially regulated on various cell types (MacEwan, 2002; Bradley, 2008). The intracellular domains of the two receptors show no sequence homology and once activated, they induce distinct signal transduction pathways (Grell et al., 1994; MacEwan, 2002). The pro-inflammatory and programmed cell death pathways which are activated

by TNF-α are largely mediated through TNFR1, while signals which promote tissue repair and angiogenesis are mediated by TNFR2 (Bradley, 2008).

The complete details of the ways in which TNF-binding triggers NFκB transcriptional activity continue to be unraveled (Newton & Dixit, 2012). What is known, however, is that TNFR1 binding recruits TNF-associated-death-domain protein (TRADD; Jones et al., 1999). TRADD initiates signaling by recruiting the receptor interacting protein-1 (RIP1) and TNFR-associated factor-2 (TRAF2) as well as TRAF5 (Lotocki et al., 2004), forming the complex TRADD–RIP1–TRAF which is then released from the TNFR1 to be internalized (Jones et al., 1999). It is thought that the subsequent signaling events recruits and activates various mitogen activated protein kinase kinase kinases (MAP3Ks) and transforming growth factor-beta (TGFβ)-activated kinase (TAK)-1 through RIP1. This in turn activates the β-subunit of the IKK complex (Yang et al., 2001; Blonska et al., 2005), leading to the phosphorylation of IκB proteins and releasing of NFκB to enter the nucleus and initiate gene transcription (Chen, 2005; Bradley, 2008). TRAF2 also contributes to the activation of NFκB through IKK complex binding (Devin et al., 2001) and through recruitment of the inhibitor of cellular apoptosis proteins (cIAP)-1 and -2 which also participate in IκB degradation (Chen, 2005).

The signaling events of TNFR2 are even less well-characterized than TNFR1. Signaling through TNFR2 seems to have shared and opposing effects with TNFR1 (Bradley, 2008). Through TNFR2, TNF-α in endothelial cells can activate endothelial/epithelial tyrosine kinase (Etk), a cytosolic kinase involved in cell adhesion, migration, proliferation and survival (Bradley, 2008).

The pro-inflammatory effects of TNF are mainly due to its effects on vascular endothelium. In response to TNF-α, endothelial cells promote inflammation by displaying disparate combinations of adhesion molecules for leukocytes, including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1; Mackay et al., 1993; Pober et al., 1986). In addition, TNF-α induces expression of cyclooxygenase-2, which increases endothelial cells production of PGI_2 (a prostaglandin) promoting vasodilatation, increasing local blood flow and vessel extravasation of fluid and macromolecules (Mark et al., 2001; Young et al., 2000).

4.3. Leukocyte recruitment

Inflammatory mediators and adhesion molecules induce vasodilation and vascular permeability. Inflammatory mediators (such as histamine, prostaglandins and nitric oxide) act on vascular smooth muscles; increased levels of leukocyte adhesion molecules on endothelial cells (promoted by TNF-α and other cytokines such as IL-1β) increases blood flow, brings in circulating leukocytes, and allows the movement of leukocytes and plasma proteins out of the circulation into the site of infection (Newton & Dixit, 2012). Leukocyte recruitment is a complex process involving a series of interactions of cell adhesion molecules and integrins expressed on both the surface of leukocytes and on the vascular endothelium.

4.3.1. Cell adhesion molecules and Integrins

Cell adhesion molecules and integrins control the process of rolling, adhesion, and transendothelial migration of leukocytes into infected sites (Iwasaki & Medzhitov, 2004; Laudanna et al., 2002). Monocytes, dendritic cells, neutrophils and natural killer

(NK) cells can infiltrate the tissue, however, neutrophils and NK cells are the key innate effector cells because they protect the host by killing pathogenic microorganisms and infected cells, respectively (Iwasaki & Medzhitov, 2004).

The initial tethering of leukocytes to the vessel wall and subsequent rolling are dependent mainly upon the selectin family of adhesion molecules (Patel et al., 2002). The 3 members of this family include E-, P- and L-selectin. E-selectin is synthesized by endothelium following stimulation by TNF-α, IL-1 and other molecules including LPS. P-selectin is found in α-granules of platelets and Weibel-Palade bodies of endothelium and moves to the cell surface upon activation. L-selectin (also known as CD62L) is found on the surface of most leukocytes and is shed from the surface of leukocytes upon activation (Kishimoto et al., 1989).

Selectins are type I transmembrane glycoproteins which bind to sialylated carbohydrate moieties. The structure of E-, P- and L-selectin are very similar. Each has a lectin-like domain to provide their binding properties, followed by an EGF-like domain and a number of consensus repeat (CR) domains, the amount of which varies by selectin. In humans for example, E-selectin has six CRs; P-selectin has nine; and L-selectin has two (Patel et al., 2002).

Effective PMNL recruitment to the site of infection requires L-selectin expression on their surface. Activated PMNL subsequently shed L-selectin as a prerequisite for β_2 integrin-mediated tight adhesion (Kishimoto et al., 1989). L-selectin is proteolytically cleaved (or shed) from the extracellular membrane by ADAM17 (Smalley & Ley, 2005; Mishra et al., 2017). L-selectin slows down cells upon contact with its specific ligands

on the endothelial cells (GlyCAM-1, CD34 and MAdCAM-1; Miyasaka, 1998), allowing PMNL such as neutrophils to roll along the vascular wall. At this point, CD11b/CD18 (also known as Mac-1 and CR3), one of the most important β_2 integrin involved in PMNL recruitment into inflamed tissue, translocate from cytoplasmic granules onto the cell surface, causing PMNL to bind tightly to endothelial cells through interaction with ICAM-1, its specific ligand (Rambeaud & Pighetti, 2005).

Selectin-ligand binding and L-selectin shedding triggers a signaling pathway that also causes leukocyte β_2 integrins CD11a/CD18 (also known as LFA-1, lymphocyte function-associated antigen 1) and CD11b/CD18 expressed on the cell surface to adopt a more extended conformation, increasing their affinity for endothelial ICAM-1 (Newton & Dixit, 2012). Interactions between the β2 integrins and ICAM-1 slow leukocyte rolling even further (Newton & Dixit, 2012) allowing the firm adhesion necessary for transendothelial migration to the site of infection.

The CD11/CD18 β2 integrins are a family of heterodimeric glycoproteins expressed on leukocytes (Perera et al., 2001). CD11a is expressed by B- and T-lymphocytes, monocytes, macrophages, neutrophils, basophils, and eosinophils. CD11b is expressed by most granulocytes as well as monocytes/macrophages, NK cells and subsets of B- and T-cells. CD11c is highly expressed in monocytes/macrophages and in NK cells (Naeim, 2008). Although all three types of β2 integrins are detectable on macrophages, they express CD11b/CD18 heterodimers predominantly (Perera et al., 2001). Once activated, these integrins initiate signals which remodel the cytoskeleton

facilitating the next step in the process of pseudopod extension and migration through the vessel (Alon & Ley, 2008).

4.4. Phagocytosis and Oxidative Burst

Once the physical barriers are overcome or evaded by infectious agents, and the innate immune system is activated, phagocytic cells such as macrophages, neutrophils and dendritic cells can ingest and kill microbes by producing toxic chemicals and powerful degradative enzymes (Murphy, 2012). In cases of Gram-negative bacteria infection, engagement of CD14, TLR4 and CD11b/CD18 receptors allow an appropriate cell response to LPS. After interaction with LPS, phagocytes change their morphological and functional properties to increase surface receptors expression and synthesis of pro-inflammatory cytokines as well as their phagocytic activity (Brekke et al., 2007; Underhill & Gantner, 2004).

Phagocytic cells express a number of receptors on the cell surface which stimulate the phagocytosis and intracellular killing. These phagocytic receptors include several members of the C-type lectin-like family (such as dectin-1 and mannose receptor), scavenger receptors class A (such as SR-A I, SR-A II and macrophage receptor with collagenous structure, MARCO), scavenger receptors class B (such as CD36), and Fc-receptors which bind the Fc-portion of antibodies (Murphy, 2012). Another set of receptors known as complement receptors (such as CD11b/CD18 also known as complement receptor 3, CR3), play a crucial role in macrophage and neutrophil phagocytosis by binding complement-coated and β-glucans bearing microbes (Murphy, 2012).

The act of phagocytosis activates many signaling pathways which together promote actin cytoskeleton rearrangements, extension of the plasma membrane and engulfment (Underhill & Ozinsky, 2002). Engulfment is normally followed by the death of the microbe inside the phagocyte, which is stimulated by another set of receptors, G-protein-coupled receptors, that signal for antimicrobial killing (Murphy, 2012). A variety of toxic products are produced which help to kill the engulfed microorganism, but the most important of these are reactive nitrogen species such as nitric oxide (NO), and reactive oxygen species (ROS) such as the super oxide anion and hydrogen peroxide (Murphy, 2012).

The reactive superoxide ions are produced by assembly of the NADPH oxidase on the phagosomal membranes (DeLeo et al., 1999). Ligation of phagocytic receptors such as CD11b/CD18 activates NADPH oxidase in neutrophils (Löfgren et al., 1999; Serrander et al., 1999), and Fc-receptors activate the NADPH oxidase in neutrophils and macrophages (Sakata et al., 1987; Gresham et al., 1988). The NADPH oxidase transfers electrons across the wall of the phagocytic vacuole forming O_2^- promoting microbial killing (Segal, 2008).

Collaborator Recognition

The work presented in this thesis could not have been conducted without my collaborators.

I recognize and appreciate the efforts of those individuals:

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CHAPTER 2: Effect of Holstein genotype on heifer response to endotoxin administration

1. SYNOPSIS

Heifers (n = 4/genotype) from unselected (stable genotype since 1964, UH) and contemporary (CH) Holsteins that differed in milk yield (6,200 and 11,100 kg milk/305 d) were used to assess the impact of selection on acute-phase response to endotoxin. Jugular catheters were implanted 24 h before LPS administration. Blood samples were collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, and 24 h relative to *iv* administration of 0.5 µg LPS/kg BW. Body temperatures (BT) were determined at these sampling times and at 5 and 7 h. Dermal biopsies were collected after the 24 h blood sample and processed to isolate fibroblasts. Plasma was analyzed for glucose, IGF-1, cortisol, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), serum amyloid A (SAA), xanthine oxidase (XO) and nitric oxide (NOx) content. Isolated fibroblasts were exposed to IL-1β or LPS and culture media IL-6 and IL-8 content determined. Exposure to LPS increased BT 0.73°C in both genotypes and increased concentrations of glucose, cortisol, SAA, TNF-α, IL-6 and XO. Concentrations of glucose, TNF-α, XO and NOx did not differ between the genotypes but IL-6 and SAA concentrations were greater in UH than CH while cortisol and IGF-1 concentrations tended to be greater in UH heifers. After 36 h exposure to LPS, concentrations of IL-6 were greater in culture media from incubations of CH than UH fibroblasts but concentrations of IL-8 did not differ between genotypes. There was a trend (P = 0.08) for IL-8 concentrations to be greater in media from UH fibroblasts exposed to IL-1β for 24 h but IL-6 concentrations did not differ between genotypes.

Results indicate the unselected heifers have a more balanced pro and anti-inflammatory functions during an acute response indicating selection has altered bovine response to LPS.

Keywords: Acute-phase response, milk genotype, Holstein, lipopolysaccharide

2. INTRODUCTION

Selective breeding of dairy cows historically focused on milk yield because greater milk yield per cow increased productive efficiency, increased dairy profitability, and greatly reduced the impact of dairy production on the environment (Capper et al., 2009). As milk yield per cow increases, endocrine and metabolic adaptations occur to enable the cow to meet the metabolic demands of increased milk synthesis (Crooker et al., 2001) and these adaptations are known to impact the immune system (Pritchard et al., 2013; Egger-Danner et al., 2015). Selection intensity for health components has increased in recent years but concerns remain that the contemporary cow is more susceptible to disease and metabolic disorders than her ancestors (Pritchard et al., 2013; Egger-Danner et al., 2015). Additional efforts are needed to better understand factors that regulate immune response in order to reduce the need to use antibiotics in food producing animals.

As part of a regional project, Dr. Charles Young initiated a breeding study designed to produce a herd of Holsteins with stable genetics (Young, 1977). The University of Minnesota has maintained this herd since then and these unique Holsteins provide opportunities to examine the impact of selection on the contemporary Holstein. A genome-wide single nucleotide polymorphism marker comparison of unselected and

contemporary Holsteins demonstrated that more than 40% of the Holstein genome has changed since 1964 and identified 67 immunity genes within 227 genomic regions affected by selection (Ma et al., submitted). These unique unselected Holsteins provide powerful opportunities to examine the impact of selection on functional properties and genetic regulation in cow. This study was conducted to assess the impact of selection on the innate immune response when heifers or dermal fibroblasts from the heifers were exposed to endotoxins. An improved understanding of how selection for milk yield has altered the immune system and its interactions with endocrine and metabolic components will contribute to efforts to improve cow health and enhance food safety and security.

3. MATERIAL AND METHODS

3.1. Animals

Heifers were from unselected (UH) or contemporary Holstein (CH) cows. The UH cows have not been subjected to selection since 1964 and their genetic potential and actual milk yield have remained stable (Weber et al., 2007; unpublished University of Minnesota (UMN) records). The 15 month old UH and CH heifers (4/genotype) were housed at the UMN Research and Outreach Center in Morris, MN where the animal study was conducted. All animal procedures were approved by the UMN Institutional Animal Care and Use Committee. All cell isolations and cell culture studies were conducted at the University of Vermont.

3.2. In Vivo LPS Challenges

Heifers were adapted to handling conditions for at least 2 weeks before they were challenged with 0.5 μg LPS (*Escherichia coli* O111:B4; Sigma-Aldrich L4391) per kg

BW. Heifer estrous cycles were synchronized (Bridges et al., 2008) so they would be at day 8 of their estrous cycle on the day of LPS administration. Jugular catheters were implanted at least 24 h before *iv* administration of LPS. During the challenge, heifers had access to water but not to feed. Blood (10 mL) was collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, and 24 h relative to LPS administration, mixed with heparin (20 µL of 10,000 IU/mL), placed on ice and centrifuged (1500 x g rpm, 15 min at 4°C) within 30 min to isolate plasma. Plasma was aliquoted and stored at -20°C. Rectal body temperatures (BT) were determined at the time of blood collection and at 5 and 7 h after LPS administration.

3.3. Dermal Biopsies, Fibroblast Isolation and *In Vitro* Challenges

Full thickness skin biopsies (Kandasamy et al., 2011) were collected from the shoulder region of all heifers and shipped overnight on ice packs to the University of Vermont in 1X Dulbecco's PBS (DPBS; Hyclone Laboratories, Logan, UT) with 1X antibiotic cocktail (100 U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin B; Hyclone Laboratories). Upon receipt, fibroblasts from individual biopsies were isolated in 0.5% collagenase type I solution (Life Technologies, Grand Island, NY) and seeded in 25 cm^2 flasks (Corning Inc., Corning, NY) in Dulbecco's Modified Eagle Medium (DMEM; Hyclone Laboratories) with 10% FBS (Hyclone Laboratories), 1X antibiotic cocktail, and 1X Insulin-Transferrin-Selenium (ITS; Mediatech Inc., Herndon, VA). Confluent cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded in a 75 cm^2 flask (Corning Inc.) in DMEM with 5% FBS, 1X antibiotic cocktail, and 1X ITS. After approximately four days, cells were expanded into three 75 cm^2 flasks. Confluent cells were detached with trypsin, diluted in

DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma- Aldrich, St. Louis, MO) and nine aliquots of third passage cells preserved in liquid nitrogen.

Cryopreserved fibroblasts were revived and cultured in 75 cm^2 flasks in DMEM supplemented with 5% FBS, 1X antibiotic cocktail, and 1X ITS. Confluent cells were detached with 0.25% trypsin, washed, counted with a cell counter (Bio Rad, Hercules, CA) and seeded into 6-well plates (Corning Inc.) at 1.25×10^5 cells/mL in a total volume of 2 mL. After 24 h, culture media was replaced with 2 mL of either fresh media (negative control), media containing 100 ng/mL of ultra-pure LPS isolated from Escherichia coli O111:B4 (Sigma-Aldrich), or media containing 1ng/mL of recombinant bovine IL-1β (AbD Serotech, Raleigh, NC). Media was collected 24 or 36 h later (from IL-1β or LPS treated fibroblasts, respectively), centrifuged (10,000 x g) for 1 minute to remove cell debris and stored at -20°C until assayed for cytokine concentrations.

3.4. Sample Analyses

Plasma progesterone was measured in a single assay using a solid-phase RIA (Coat-A-Count Progesterone, DPC, Los Angeles, CA) to verify estrous cycle in all heifers. The minimal detectable concentration was 0.02 ng/mL. All samples were assayed in one assay and the intra-assay coefficient of variation was < 15%. Plasma glucose (Autokit Glucose, Wako Life Sciences, Mountain View, CA) concentrations were determined by colorimetric assay with volumes modified for use in a 96-well plate. Samples were analyzed in duplicate. Intra-assay coefficient of variation of one assay was 4.9%. Plasma IGF-I concentrations were quantified by using a validated double-antibody RIA (Weber et al., 2007). Samples were analyzed in triplicate. The minimal detectable

concentration of IGF-I was 0.20 ng/mL. All samples were assayed in one assay; the intra-assay coefficient of variation was 6.6%. Plasma cortisol concentrations were measured by a radioimmunoassay kit (ImmunoChem Cortisol, MP Biomedicals, Orangeburg, NY). The kit was validated for recovery and linearity of dilution with bovine plasma. Samples were analyzed in duplicate. The minimal detectable concentration of cortisol was 0.17 µg/dL. All samples were assayed in one assay and the intra-assay coefficient of variation was 7.4%.

Plasma TNF-α concentrations were measured by a specific double antibody RIA (Kenison et al., 1990). The minimum detectable TNF-α concentration was 4.00 pg/assay tube. Samples were assayed in duplicate in a single assay with an intra-assay coefficient of variation of < 15%. Plasma IL-6 concentrations were determined by a sandwich ELISA (Kandasamy & Kerr, 2012). The minimum detectable concentration was 0.01 ng/mL. All samples were assayed in a single assay and the intra-assay coefficient of variation was < 10%. Plasma serum amyloid A (SAA) in the 0, 8 and 24 h was determined by a commercial ELISA kit (Phase™ Range, Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Samples were analyzed in duplicate and according to the manufacturer's instructions. The kit was validated for recovery and parallelism with bovine plasma. The minimum detectable concentration of SAA was 1.50 µg/mL. All samples were assayed in one assay, and the average coefficient of variation between duplicates in a single assay was 6.5%.

Plasma xanthine oxidase (XO) activity in the 0, 8 and 24 h samples was determined (Kahl & Elsasser, 2006). All samples were assayed in a single assay and the

intra-assay coefficient of variation was < 10%. Plasma NOx was determined (Kahl et al., 1997) in the 0, 4, 8 and 24 h samples in a single assay and the intra-assay coefficient of variation was < 10%.

3.5. Statistical Analyses

Data from fibroblast cultures were analyzed by the Student's t-test (Graph Pad Prism 6.0) to assess effects of genotype. Data from *in vivo* measurements were analyzed by repeated measures using PROC MIXED by SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC) with time as the repeated effect. The spatial power law for unequally spaced data was specified as the covariance structure. The model included genotype, time and their interactions as fixed effects, and heifer as a random effect. Results are reported as least squares means. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$.

Overall *in vivo* responses to LPS administration were also assessed as area under the concentration by time curve (AUC) after the pre-administration mean of corresponding -1, -0.5 and 0 h values were subtracted from post-administration values. These AUC data were analyzed as described above with genotype as the fixed effect and heifer as a random effect.

4. RESULTS

4.1. In Vivo Challenges

Transient signs of a mild systemic acute pro-inflammatory response were observed in all heifers after LPS administration and were accompanied by a mild increase in BT that did not differ between genotypes. Mean BT peaked 5 h after LPS

administration (0.73 ± 0.09 ºC greater than pre-administration; $P = 0.011$) and returned to pre-challenge temperatures between 8 and 24 h after LPS administration (Figure 1). Clinical signs of sickness behavior such as labored breathing, increased salivation and coughing were resolved within 6 to 7 h after LPS administration.

The classic biphasic glucose response to LPS administration was similar for both genotypes ($P = 0.259$; Figure 2A). Glucose concentrations peaked 1 h after LPS administration, were less than baseline concentrations by 3 h and returned to baseline concentrations between 8 and 24 h after LPS administration. Plasma IGF-1 concentration were not affected by LPS administration ($P = 0.421$) but tended to be greater in UH than CH heifers (100.3 vs. 85.7 ± 4.5 ng/mL; $P = 0.061$). Cortisol concentrations peaked at 3 h, returned to baseline concentrations between 8 and 24 h (Figure 2B) and tended to be greater in UH than in CH heifers (4.9 vs. 4.2 ± 0.3 µg/dL; $P = 0.070$).

Plasma TNF-α concentrations peaked 2 h after LPS administration, did not differ from baseline concentrations at 4 h and did not differ between the genotypes ($P = 0.391$; Figure 3A). Interleukin-6 concentrations were greater in UH than in CH heifers (5.8 vs. 4.3 ± 0.4 ng/mL; $P = 0.029$; Figure 3B). In both genotypes, IL-6 peaked at 4 h post-LPS and remained greater than the pre-challenge mean concentrations through 8 h.

Concentrations of the acute-phase protein SAA peaked at 24 h after LPS administration and were greater in UH than CH heifers (125.2 vs. 86.1 ± 10.1 µg/mL; $P = 0.02$; Figure 3C). Xanthine oxidase concentrations increased in response to LPS, remained greater than pre-challenge mean concentrations through 24 h and did not differ between genotypes ($P = 0.307$; Figure 3D). Plasma NOx concentrations were not affected

by LPS administration (*P* = 0.632) and did not differ between genotypes (5.0 and 5.5 ± 0.5 μM for UH and CH, respectively; *P* = 0.505).

4.2. In Vitro Challenges

After 36 h exposure to LPS, concentrations of IL-6 were greater in incubation media from CH than from UH fibroblasts ($P < 0.05$) but concentrations of IL-8 did not differ between genotypes (Figure 4). There was a trend (*P* = 0.08) for IL-8 concentrations to be greater in media from UH fibroblasts exposed to IL-1β but IL-6 concentrations did not differ between genotypes.

5. DISCUSSION

Since the mid-1960s, genetic selection and improvements in ration formulations, management practices, and preventive medicine have increased production efficiency and profitability of contemporary dairy cows (Capper et al., 2009). Despite these improvements, there are concerns that contemporary cows are more susceptible to disease and metabolic disorders than their ancestors (Pritchard et al., 2013; Egger-Danner et al., 2015). The unselected Holsteins provide a unique opportunity to examine the impact of the last 50 years of selection on immune function in Holsteins. Here we describe an initial examination of the impact of selection on an endotoxin-induced acute-phase response (APR) and its interactions with other immune related components and with endocrine and metabolic components.

As expected, the LPS induced alterations in endocrine, metabolic and immune parameters of the UH and CH heifers were similar to those reported for other immune challenge studies with young and mature cattle (Werling et al., 1996; Steiger et al.,

1999; Waldron et al., 2003). Cortisol is release in response to a variety of stressors including exposure to endotoxins. Two primary functions of the increase in cortisol are to stimulate mobilization of body reserves to provide energy to mount the acute response (Kvidera et al., 2017) and to regulate the inflammatory response to minimize tissue damage (Fisher et al., 2002). The initial hyperglycemia in the UH and CH heifers reflects enhanced glycogenolysis and gluconeogenesis to provide additional glucose for metabolism by immune cells. The glucose responses were similar between the UH and CH heifer which indicates readily available energy for the immune response did not differ between the genotypes. However, cortisol concentrations after LPS administration tended to be greater in UH than in CH heifers which indicate the potential for genotype differences in the regulation of cortisol.

Activation of the innate immune system includes an acute-phase response characterized by local inflammation as the first response to an infection or injury and a systemic response which occurs when local defenses are overwhelmed (Gabay & Kushner, 1999). The acute-phase response is stimulated by the release of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 from macrophages and monocytes at the site of injury or infection (Carroll et al., 2009). Inflammation rapidly induces the APR which serves to neutralize pathogens and reduce further pathogen invasion. The APR consists of fever, an increase in vascular permeability and production of acute-phase proteins (APP) by hepatocytes (Mihara et al., 2012). Increased BT and duration of sickness behavior did not differ between the LPS treated UH and CH heifers. These responses are mediated by the pro-inflammatory

cytokines which are produced by activated macrophages and monocytes (Harden et al., 2006). The LPS-induced alterations in TNF-α concentrations did not differ between the genotypes but the IL-6 response was greater in UH heifers. In contrast, IL-6 concentrations were greater when fibroblast from CH heifers were stimulated with LPS. This inconsistency of *in-vivo* and *in-vitro* response does not agree with previous reports (Green et al., 2011). The reason(s) for these discrepancies are not known, however the *in-vivo* results represent the complex whole body response to the endotoxin. When stimulated with IL-1β, fibroblasts from UH heifers tended to produced more IL-8 than CH fibroblasts. Although a positive correlation has been reported for LPS and IL-1β response by dermal fibroblasts (Green et al., 2011), receptors (TLR4 for LPS and IL-1R for IL-1β) and their signaling pathways differ (Verstrepen et al., 2008). Differences in the relative presence of the components in the IL-1R pathway of the UH and CH heifers could contribute to IL-8 concentration differences between the genotypes after IL-1β stimulation.

Although primarily regarded as a pro-inflammatory cytokine, IL-6 is a pleiotropic cytokine that has pro and anti-inflammatory functions and can affect processes ranging from immunity to tissue repair and metabolism (Scheller et al., 2011; Arango Duque & Descoteaux, 2014). The UH heifers tended to have greater cortisol concentrations and had greater plasma concentrations of IL-6 than the CH heifers. These differences could mean the UH heifers have a more balanced pro and anti-inflammatory response which could serve to reduce tissue damage associated with excessive response to pro-inflammatory signals (Tanaka et al., 2014).

The greater IL-6 and trend for greater cortisol concentrations in UH than CH also likely contributes to why the UH heifers had greater concentrations of SAA. Glucocorticoids can enhance the immune system by causing up-regulation of cytokine receptors and by acting synergistically with IL-6 and other cytokines to induce production of APP (Wiegers & Reul, 1998). Data indicates IL-6 is the primary cytokine regulator of APP synthesis as the combined impact of IL-6 with IL-1β or TNF-α stimulates hepatic APP synthesis, specifically SAA1 and SAA2, but the combination of IL-1β and TNF-α does not (Castell et al., 1989; Hagihara et al., 2004). The greatest serum concentration of SAA occurred 24 h post-administration in both genotypes and although this agrees with previous reports (Kahl & Elsasser, 2006; Carroll et al., 2009), the results indicate a longer sampling interval is needed to characterize the entire SAA response to endotoxin exposure.

Immune system activation has been associated with reductions in circulating IGF-I concentrations (Elsasser et al., 2007) but LPS administration did not alter IGF-I in the UH or CH heifers. Although this lack of effect is likely related to magnitude of the activation, the reason for this discrepancy is not clear. In contrast to previous reports of similar plasma IGF-I concentrations in UH and CH heifers (Baumgard et al., 2002; Weber et al., 2005), IGF-I concentrations tended to be greater in UH than in CH heifers but this could be associated with the small number of animals used in this LPS challenge study.

Indirect assessment of reactive oxygen species via plasma concentrations of XO and NOx did not identify an impact of genotype on LPS response. Although plasma XO

concentrations were increased in both genotypes after LPS administration and similar to those previously reported (Kahl et al., 1997; Kahl & Elsasser, 2004), plasma NOx concentrations were not increased. These results indicate the metabolic disruptions induced by acute exposure to LPS in this study were not sufficient to elicit major metabolic disruptions in either genotype (Elsasser et al., 2007).

6. CONCLUSION

During the acute response to LPS several physiologic, biochemical, and nutritional changes occurred in the genotypes. Differences between the genotypes indicate that unselected heifers may have more balanced pro and anti-inflammatory functions during an acute response. These results indicate selection has altered bovine response to LPS. Additional studies are needed to elucidate the impact of selection on the innate immune response and its interactions with endocrine and metabolic components.

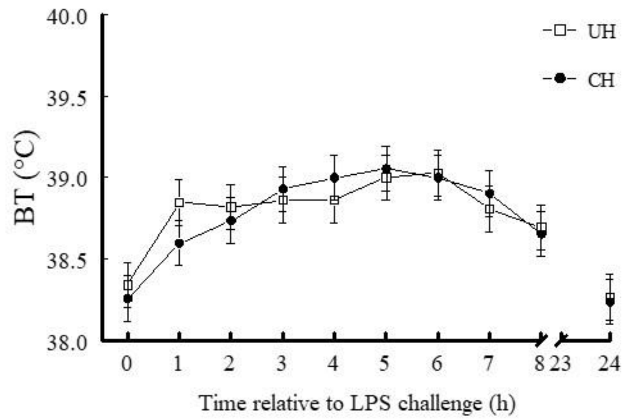

Figure 1. Body temperature (BT) in heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins after administration of 0.5 µg LPS/kg BW iv. Data represent least square means ± SEM. Rectal BT increased after LPS administration ($P <$ .0001), however there was no difference in BT response among genotypes ($P = 0.911$).

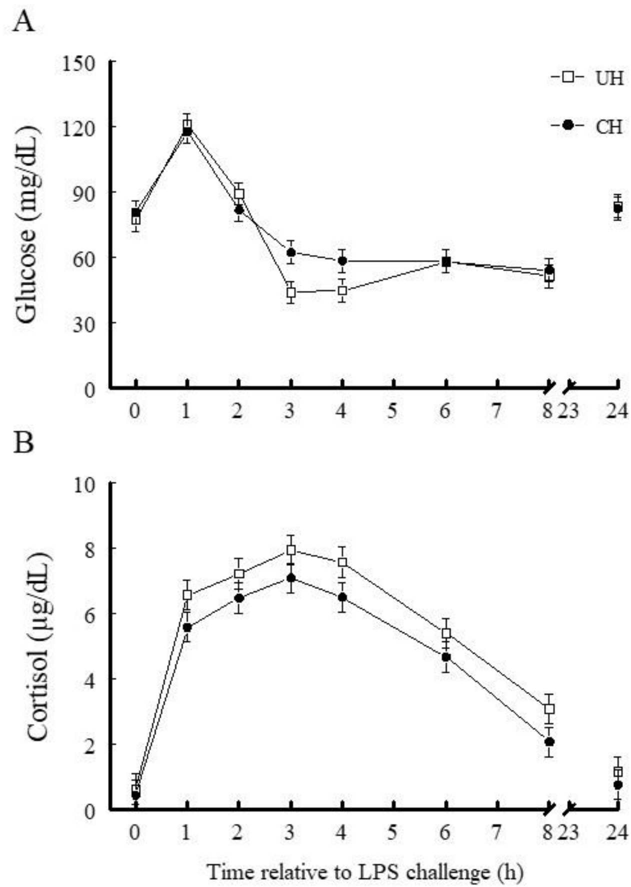

Figure 2. Plasma glucose (A) and cortisol (B) concentrations in heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins after administration of 0.5 µg LPS/kg BW iv. Data represent least square means ± SEM. Glucose presented a biphasic response to LPS that was no different between genotypes ($P = 0.259$). Cortisol concentration increased in response to LPS ($P < .0001$) and trended to be greater in UH than CH heifers ($P = 0.070$).

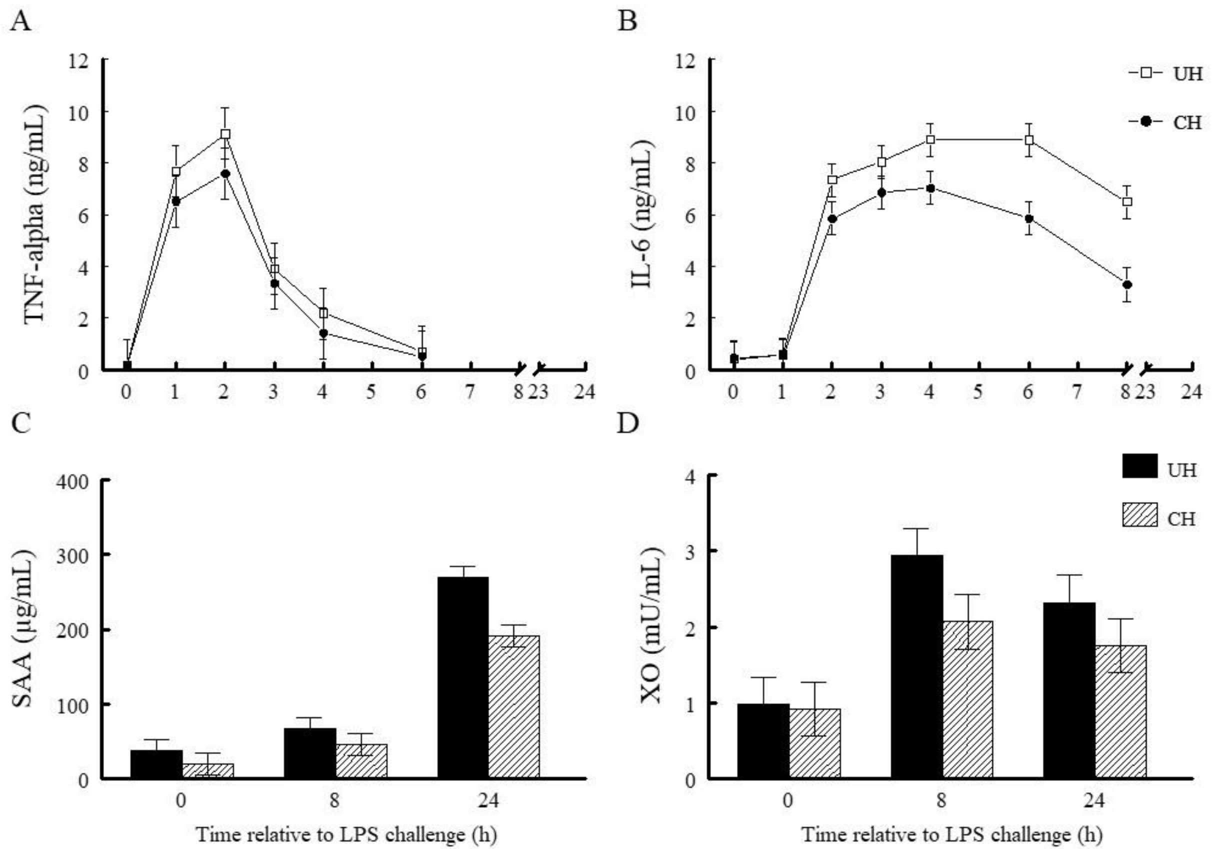

Figure 3. Concentrations of TNF-α (A), IL-6 (B) SAA (C) and XO (D) in heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins after administration of 0.5 μg LPS/kg BW iv. Data represent least square means ± SEM. There was time ($P <$ .0001) effect for all parameters due to increased concentrations after LPS administration. Concentrations of IL-6 and SAA were greater in UH than CH ($P < 0.042$), but there were no differences between genotypes for TNF-α and XO concentrations ($P > 0.306$).

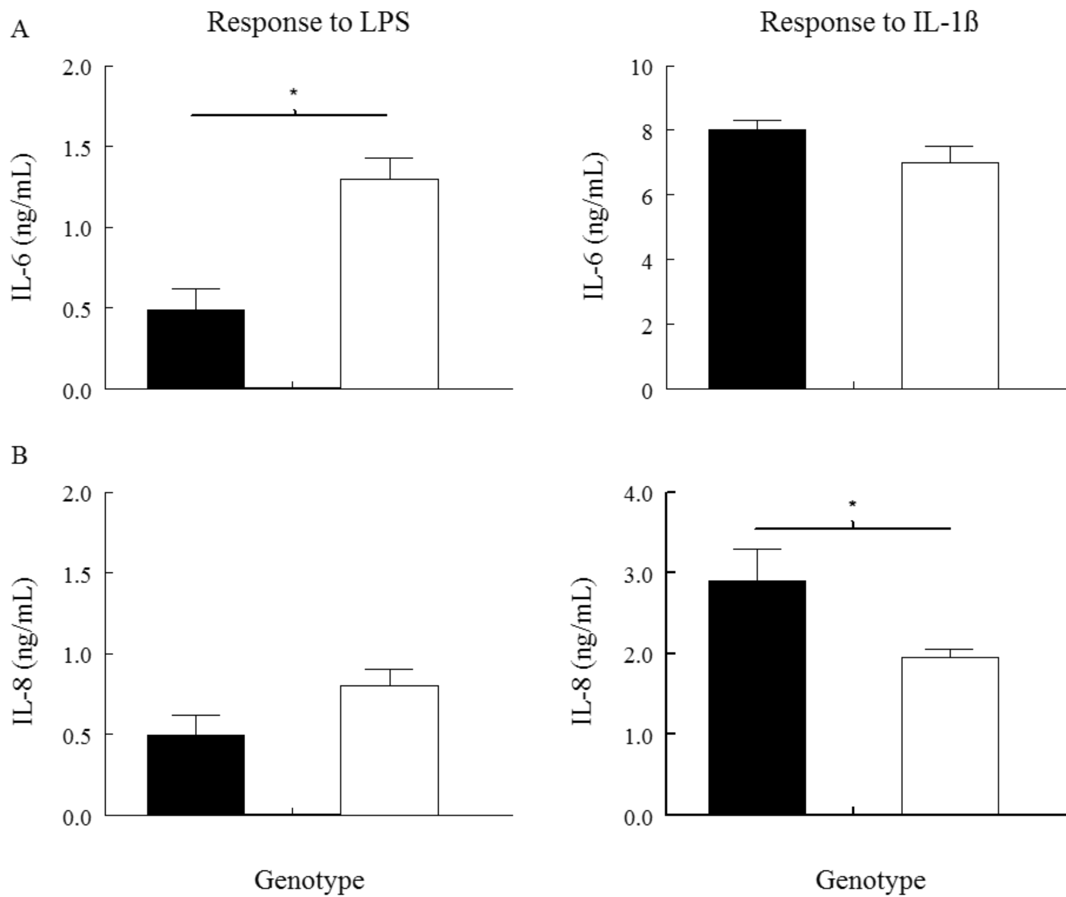

Figure 4. IL-6 (A) and IL-8 (B) protei production by cultured fibroblast in response to LPS (100 ng/mL) or IL-1β treatment for 36 and 24 h, respectively. Fibroblast were obtained from heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins 24 h after administration of 0.5 µg LPS/kg BW iv. Data represent least square means ± SEM. Concentrations of IL-6 were greater in incubation media from CH than from UH fibroblast exposed to LPS for 36 h ($P < 0.05$). There was a trend ($P = 0.08$) for IL-8 concentrations to be greater in media from UH fibroblast exposed to IL-1β.

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CHAPTER 3: Effect of bovine genotype on heifer response to repeated lipopolysaccharide (LPS) administration

1. SYNOPSIS

This study was designed to assess the impact of genotype on the innate immune system of growing heifers and its interactions with endocrine and metabolic components. Heifers (n = 4/genotype) from unselected (stable milk yield since 1964, UH) and contemporary (CH) Holstein cows that differed in milk yield (6,200 vs 11,100 kg milk/305 d) or from contemporary Black Angus (CA) cows bred to contemporary Red Angus bulls were challenged with iv administration of 0.5 μg LPS/kg BW on day 1 (challenge 1, C1) and d 5 (C2) of study. Plasma samples were analyzed for concentrations of glucose, insulin, IGF-I, NEFAs, cortisol, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), serum amyloid A (SAA), xanthine oxidase (XO) and nitric oxide (NOx). Rectal BT and concentrations of all plasma components were altered by time. There were genotype by time and challenge interactions for BT, glucose, insulin and TNF-α. During C1, BT increased earlier and peaked higher in CA than in UH or CH, glucose increased less in CA than UH or CH, and insulin and TNF-α increased more in CH than UH and CA. During C2, genotype had no impact on BT, glucose, insulin or TNF-α. There was an interaction of genotype and challenge for IL-6 as response in UH was greater than in CH or CA in C1 but there was no effect of genotype on IL-6 in C2. There was a genotype by time interaction for SAA as response at 24 h was greater in CA than UH and CH. There was a trend for a genotype by challenge interaction for XO as XO was greater in UH than in CH and CA in C1 but not in C2 and a trend for NOx to be

less in CA than in UH or CH. The more rapid increase in cortisol concentrations in Holsteins and the greater concentrations of TNF-α in CH and IL-6 in UH are consistent with Holstein heifers having a more robust innate immune response than Angus heifers. Differences between the UH and CH heifers indicates selection has altered aspects on the innate immune system in Holsteins. Although genotype affected the impact of LPS on the variables measured in this study, additional investigations are needed to determine specific reasons for these differences.

Keywords: Innate immunity, bovine genotype, Angus, Holstein, lipopolysaccharide

2. INTRODUCTION

Selective breeding of cattle during the past several decades focused primarily on increased production because greater production per animal can increase productive efficiency and profitability and reduce environmental impact of the industry (Capper and Bauman, 2013). This greater partitioning of available nutrients and energy towards production can decrease the amount available for other physiological functions (Bauman and Currie, 1980; Baumgard et al., 2017) and raises concerns that insufficient selection for health traits has rendered the contemporary cow more susceptible to disease and metabolic disorders than her ancestors (Pritchard et al., 2013; Egger-Danner et al., 2015). This is perhaps more apparent in dairy cattle where, in addition to historical records (AIP, https://aipl.arsusda.gov/), maintenance of unselected Holsteins has allowed comparisons with contemporary Holsteins and has demonstrated greater need to treat contemporary Holsteins for mastitis (Jones et al., 1994). This unselected vs. contemporary Holstein animal model has also demonstrated that selection practices have altered circulating

concentrations of endocrine and metabolic components (Weber et al., 2007). Although beef and dairy cattle are selected for some common traits including increased milk yield (American Angus Association, https://www.angus.org/Nce/GeneticTrends.aspx; AIP, https://aipl.arsusda.gov/), specific selection criteria for beef and dairy cattle differ so the impact on physiological functions could and likely does vary among cattle breeds. Indeed, breed-dependent genetic differences in immune responses have been identified in cattle (Bannerman et al., 2008; Carroll et al., 2011; Benjamin et al., 2016).

The innate immune system is the first line of defense against infectious diseases. The acute phase response of the innate immune system aids in pathogen neutralization which helps prevent further invasion and minimize tissue damage. This response involves multiple physiological changes including fever, increased vascular permeability, hepatic production of acute phase proteins (APP), and alterations in circulating concentrations of metabolic, endocrine and immune components (Carroll et al., 2009; Mihara et al., 2012). The immediate response is mediated largely by immune cells (primarily monocytes, macrophages, and neutrophils) that phagocytize and kill pathogens and simultaneously coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines (Arango Duqe and Descoteaux, 2014). There are multiple activation pathways, specific to particular organisms and/or compounds, which can elicit an immune response (Netea et al., 2004; Lachmandas et al., 2016). Gram-negative bacteria are responsible for many of the infections seen in cattle and clinical signs are mainly the result of host reactions to LPS after it is released from the bacterial cell wall and recognized by Toll-like receptor 4 (TLR4) on host immune cells (Steiger et al., 1999;

Netea et al., 2004). The host response to Gram-negative bacteria can be mimicked without causing an actual infection by administration of LPS (Steiger et al., 1999; Waldron et al., 2003). Repeated or prolonged exposure of the host to LPS, either from pathogens or from administration of the isolated compound, results in tolerance which is one of the primary mechanisms that regulate the magnitude and duration of the LPS-induced inflammatory response (Lopez-Collazo et al., 2013).

Several studies of the innate immune response of cattle to LPS or live *E. coli* administration have been published and most have focused on dairy cows or beef steers. However, assessment of immune response in growing heifers (Kahl et al., 2009; Green et al., 2011) and comparison of breeds or genotypes (Bannerman et al., 2008; Carroll et al., 2009; Ballou et al., 2012; Burdick Sanchez et al., 2013) are limited. Breed comparison studies have indicated differences in onset, cessation and duration of immune component responses to LPS (Bannerman et al., 2008; Carroll et al., 2009) but no overall difference in ability of the genotypes to eliminate (clear) the infection or resolve the inflammation (Bannerman et al., 2008). However, there is substantial evidence of differences in mastitis prevalence among breeds (Kelm et al., 2001) and recent *in vitro* work has demonstrated a more robust response to LPS in fibroblasts isolated from Holstein than from Angus heifers (Benjamin et al., 2016). Thus, the impact of bovine genotype on immune response warrants further investigation.

The primary objective of this study was to examine the impact of bovine genotype and breed on innate immune response. A novel aspect of this study is that we administered LPS to heifers from unique Holsteins that have not been subjected to

selection since 1964 (unselected Holsteins, UH; Weber et al., 2007), from contemporary Holsteins (CH), and from contemporary Red-Black Angus (CA) cows. In addition, identical LPS challenges were administered on day 1 and 5 of study to assess impact of genotype on the acute innate immune response and on the development of a refractory (endotoxin tolerance) state. This animal model provides the opportunity to assess the impact of 50 years of selection on the immune response of Holsteins and to compare immune response in prevalent, contemporary beef and dairy breeds.

3. MATERIALS AND METHODS

3.1. Animals, Experimental Design, and Treatments

Twelve heifers (n = 4/genotype; 19.6 ± 0.19 months of age) were fed the same diet ad lib between 1500 and 1700 daily and housed together for 47 d to adapt to facilities and handling procedures. Heifers received *iv* administration of 0.5 μg LPS (*Escherichia coli* O111:B4; Sigma-Aldrich L4391) per kg BW between 0700 and 0800 on day 1 (challenge 1, **C1**) and d 5 (**C2**) of study. Heifers were pregnant except for 2 CH heifers that were treated (Bridges et al., 2008) to be at day 8 of their estrous cycle on d 1. During each challenge, feed was withheld but heifers had ab libitum access to water. Jugular catheters were implanted at least 24 h before LPS administration and blood samples (10 mL) collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, and 24 h relative to LPS administration. Rectal body temperatures (BT) were determined at these times and at 5 and 7 h. Blood was mixed with heparin (20 μL of 10,000 IU/mL), placed on ice and centrifuged (1500 x g, 15 min at 4 °C) within 30 min. Plasma was aliquoted and stored at -20 °C until analyzed.

Heifers were observed daily throughout the study. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

3.2. Sample Analyses

Plasma glucose (Autokit Glucose, Wako Life Sciences, Mountain View, CA) and NEFA (NEFA-HR (2), Wako Life Sciences, Mountain View, CA) concentrations were determined by colorimetric assays with volumes modified for use in a 96-well plate. Samples were analyzed in duplicate. Intra and interassay coefficients of variation were 4.2 and 4.1% and 4.4 and 7.7% for glucose and NEFA, respectively. Plasma cortisol, insulin and progesterone concentrations were measured by RIA (ImmunoChem Cortisol, MP Biomedicals, Orangeburg, NY; ImmunoChem Insulin, MP Biomedicals, Solon, OH; Coat-A-Count Progesterone, DPC, Los Angeles, CA). The assays were validated for recovery and linearity of dilution with bovine plasma. Samples were analyzed in duplicate. The minimal detectable concentration of cortisol was 0.17 μg/dL and the intra and interassay coefficients of variation were 8.4 and 6.2%, respectively. The minimal detectable concentration of insulin was 4.60 μIU/mL and the intra and interassay coefficients of variation were 9.1 and 6.9%, respectively. The minimal detectable concentration of progesterone was 0.02 ng/mL. All samples were assayed in one assay and the intraassay coefficient of variation was < 15%. Plasma IGF-I concentrations were quantified with a validated double-antibody RIA (Weber et al. 2007). Samples were analyzed in triplicate. The minimal detectable concentration of IGF-I was 0.20 ng/mL. All samples were assayed in one assay and the intraassay coefficient of variation was 5.5%.

Plasma TNF-α concentrations were measured by a specific double antibody RIA (Kenison et al., 1990). The minimum detectable TNF-α concentration was 4.00 pg/assay tube. Samples were assayed in duplicate, in a single assay with an intra-assay coefficient of variation less than < 15%. Plasma IL-6 concentrations were determined by a sandwich ELISA (Kandasamy and Kerr, 2012). The minimum detectable concentration was 0.1 ng/mL. Intra and interassay coefficients of variation were < 10%, respectively. Plasma serum amyloid A (SAA) was determined by an ELISA (Phase™ Range, Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Samples were analyzed in duplicate and according to the manufacturer's instructions. The ELISA was validated for recovery and parallelism with bovine plasma. The minimum detectable concentration of SAA was 1.50 µg/mL and the interassay coefficient of variation was 3.2%.

Plasma xanthine oxidase (XO) activity (Kahl and Elsasser, 2006) and nitrate+nitrite (NOx) concentration (Kahl et al., 1997) were determined in a single assay and the intra-assay coefficient of variation was < 10%.

3.3. Calculations and Statistical Analyses

Plasma profile data were analyzed by repeated measures using PROC MIXED by SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC) with time as the repeated effect. The spatial power law for unequally spaced data was specified as the covariance structure. The model included genotype, time, challenge and their interactions as fixed effects and heifer as a random effect. Overall response for each heifer was calculated as area under the time by concentration curve (AUC) after the concentrations were corrected by subtracting the heifer's pre-challenge mean from each post-administration value. Time

to peak or nadir concentration and time of an alteration in the concentration profile, such as a change in direction (point of inflection) of a response, were determined by visual appraisal. When required, the initial positive and subsequent negative AUC responses were calculated to and from the point of inflection, respectively. These response data were analyzed as described above with genotype, challenge and their interactions as fixed effects and heifer as a random effect. Effects of genotype, challenge and their interaction from the repeated measures and AUC analyses were comparable for all components except glucose which had a substantial inflection in the response profile. Results are reported as least squares means. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$.

4. RESULTS

Administration of LPS resulted in transient characteristic signs of mild systemic innate immune responses during C1 and C2. Labored breathing accompanied by increased salivation, nasal discharge and coughing as well as mild diarrhea and lethargy were observed in all heifers within 1 h of LPS administration. These clinical signs completely resolved within 6 to 7 h after each LPS administration. On day 1, plasma progesterone concentration in each heifer exceeded 3.4 ng/mL and confirmed that none of the heifers were in estrus.

Rectal BT peaked 4 h after LPS administration ($P < .001$) and increased 1.05 and 1.17 ± 0.09 ºC in C1 and C2, respectively (Figure 1). Rectal BT was elevated through 7 h during C1 and beyond 8 h during C2. There was an interaction ($P < 0.008$) of genotype, time and challenge for BT (Table 1) because BT increased earlier (BT > 0 h pre-

challenge mean at 2, 3 and 4 h) and peaked higher (40.2, 39.2 and 38.8 ± 0.2 ºC) in CA than in UH or CH heifers, respectively during C1. There was a trend ($P < 0.062$) for UH to have higher BT at 5 and 6 h than CH heifers during C1. Neither time of increase (1 h, $P > 0.363$) nor peak BT (40.0 ± 0.2 ºC, $P > 0.599$) differed among genotypes during C2 but peak BT of the Holstein heifers was greater during C2 than C1.

There was an interaction of genotype, time and challenge ($P = 0.011$) for glucose because concentrations increased less at 1 and 2 h in CA than UH or CH during C1, but did not differ among genotypes at any time during C2 (Figure 2A). Glucose concentrations at1 h tended to be greater ($P = 0.061$) in CH than in UH heifers. There was a biphasic glucose response with initial concentrations greater and later concentrations less than the pre-challenge concentrations. This inflection tended to occur later (2.7 vs. 2.5 h, $P = 0.056$) in C1 than in C2 but was not affected by genotype ($P = 0.387$) or the interaction of genotype and challenge ($P = 0.283$). Although there was no effect of challenge ($P = 0.116$) on overall glucose concentration (Table 1), the AUC response during the initial positive phase was greater ($P = 0.003$) in C1 than C2. There was a trend for a genotype by challenge interaction ($P = 0.086$) for the initial positive glucose response as AUC was greater ($P < 0.019$) in UH and CH than in CA during C1 (129.7, 145.6 and 29.1 ± 27.3 mg•h/dL, respectively) but did not differ ($P > 0.798$) among genotypes during C2 (29.6, 25.4 and 19.5 ± 27.3 mg•h/dL, respectively). There was no impact ($P > 0.159$) of genotype, challenge or their interaction on the negative phase of the glucose response during C1 (-99.9, -99.6 and -105.2 ± 17.5 mg•h/dL) or C2 (-91.2, -64.1, and -86.7 ± 17.5 mg•h/dL) for UH, CH and CA heifers, respectively.

There was a genotype, time and challenge interaction ($P = 0.012$) for plasma insulin as peak concentration was greater ($P < .0001$) in CH than in UH and CA during C1 but there were no differences ($P = 0.711$) among genotypes during C2 (Table 1, Figure 2B). During C1, insulin concentrations in CH and UH heifers did not differ ($P = 0.699$) at 3 h but both were greater ($P = 0.049$) than those in CA heifers.

Concentrations of IGF-I tended ($P = 0.088$) to be greater in CH and UH than in CA heifers (Table 1). There was an effect ($P = 0.039$) of time of sampling on IGF-I as concentrations were less than pre-challenge concentrations at 8 h (181.0 vs. 202.9 ± 9.6 ng/mL, respectively) but not at 24 h ($P = 0.123$). There were no interactions of genotype with time ($P = 0.523$) or with challenge (P = 0.239). There was a time by challenge interaction ($P < 0.001$) for IGF-I as concentrations during C1 were less ($P < 0.036$) than those during C2 at all but the 8 h sampling time.

There was an interaction of time and challenge ($P < 0.001$) for NEFA concentrations as concentrations at 0, 1, 6, 8 and 24 h during C2 were greater than those from the corresponding times in C1. There was a genotype and time interaction ($P = 0.001$) for NEFA as concentrations either did not differ ($P > 0.102$) among genotypes or were greater ($P < 0.052$) in CA than in CH heifers at 0, 6, 8, and 24 h. There was an overall biphasic response which was more pronounced in C2. At 8 h and at 24 h NEFA concentrations were greater ($P < 0.022$) than pre-challenge means for each heifer genotype.

There was a genotype and time interaction ($P < 0.001$) for cortisol (Table 1, Figure 2C) as UH and CH cortisol concentrations increased 1 h post-LPS (8.3 and 8.8 ± 0.5

ug/dL, respectively) and remained high through 4 h, while cortisol concentration in CA heifers did not peak (7.9 ± 0.5 ug/dL) until 3 h and had begun to decrease (5.5 ± 0.5 ug/dL) by 4 h (3 h vs. 4 h, $P < 0.001$). There was a time by challenge interaction ($P < 0.001$) for cortisol as concentrations after LPS administration were greater in C1 than C2 for all time points.

There was a genotype, time and challenge interaction ($P = 0.025$) for TNF-α as concentrations during C1 increased more (P < 0.004) in CH than UH or CA heifers but did not differ ($P = 0.506$) among genotypes during C2 (Table 1, Figure 3A). During C1, concentrations of TNF-α peaked at 1 h in UH and CA (4.3 and 6.0 ± 0.6 ng/mL) and at 2 h in CH (8.9 ± 0.6 ng/mL). Concentrations of TNF-α did not differ ($P > 0.601$) from the pre-administration concentration by 6 h in C1 and by 4 h in C2.

There was a genotype by challenge interaction ($P = 0.002$) for IL-6 as concentrations were greater in UH and CH heifers during C1 than C2 while response by CA heifers did not differ between challenges (Table 1, Figure 3B). There was an interaction ($P = 0.011$) of genotype and time for IL-6 as overall concentrations did not differ among genotypes at 1 or 8 h ($P > 0.579$) but tended to be greater ($P < 0.100$) in UH than in CH and/or CA heifers from 2 to 6 h.

There was a genotype by time interaction ($P = 0.049$; Table 1) for SAA as SAA concentrations did not differ ($P > 0.100$) among genotypes until 24 h when concentrations were greater in CA than in UH or CH (Table 1; Figure 3C). There was an interaction of time and challenge ($P < 0.001$) for SAA as concentrations during C2

tended to be greater ($P = 0.066$) at 0 h, were greater ($P = 0.013$) at 8 h, and were less ($P < 0.001$) at 24 h than the corresponding concentrations in C1.

Plasma XO concentrations were greater ($P < 0.001$) in C1 than C2 (2.7 vs. 1.8 ± 0.2 mU/mL, respectively). There was a trend ($P = 0.097$) for a genotype by challenge interaction, as XO concentration was greater in UH than in CH or CA (Table 1) during C1 but did not differ among genotypes during C2. There was a time by challenge interaction for XO ($P < 0.001$) as concentrations during C1 were greater at 8 and 24 h than the corresponding times in C2. Although XO concentrations decreased between 8 and 24 h, the 24 h concentrations (2.2 ± 0.2 mU/mL) were still greater ($P < 0.001$) than the 0 h concentrations (1.5 ± 0.2 mU/mL). Plasma NOx concentrations increased in C1 and C2 for all 3 genotypes. There were trends ($P = 0.100$, Table 1) for NOx concentrations to be less in CA than in UH or CH heifers (3.6, 5.1 and 4.3 ± 0.5 µM, respectively) and for a time by challenge interaction ($P = 0.092$) as the greatest NOx concentrations occurred at 8 h in C1 but at 4 h in C2.

5. DISCUSSION

Dairy and beef industries have emphasized selection for traits associated with greater production (O'Neill et al., 2010; Egger-Danner et al., 2015). However, selection for health and other components (functional traits) has been insufficient and there is concern that genetic selection practices have made the contemporary cow, especially the periparturient dairy cow, more susceptible to disease and metabolic disorders (Pritchard et al. 2013; Egger-Danner et al., 2015). Specific selection criteria for Holstein and Angus cattle differ and these differences could impact how selection has altered immune

response to pathogens. In this study, heifers from unique Holsteins that have not been selected for milk yield since 1964, heifers from contemporary Holsteins, and Red-Black heifers from contemporary Black Angus cows were used to examine the impact of genotype on innate immune response to LPS and its interactions with endocrine and metabolic components.

Pathogen recognition activates macrophages, monocytes and neutrophils to synthesize and release pro-inflammatory cytokines, such as IL-6 and TNF-α. These pro-inflammatory cytokines are important mediators of fever and sickness behavior and stimulate the APP response and inflammation (Klasing and Krover, 1997; Mihara et al., 2012). They can also modify the growth process by provoking homeorhetic responses that direct nutrients away from tissue growth to support the immune and inflammatory process (Klasing and Krover, 1997; Spurlock, 1997). A more robust but controlled pro-inflammatory response to endotoxin exposure may be beneficial for pathogen clearance (Rettew et al., 2010; Carroll et al., 2011), but enhanced production of pro-inflammatory cytokines during stressful periods can promote systemic inflammation (Sordillo et al., 1995). Indeed, a fast, short-lasting and regulated inflammatory reaction is generally considered beneficial for early resolution of infection (Burvenich et al., 2003) while a prolonged inflammatory response can damage tissue (Tracey, 2007).

Significant changes in BT, endocrine, metabolic and immune components were induced in all three genotypes by the repeated LPS challenge approach and the overall responses were similar to those observed in other studies. Febrile response to an endotoxin challenge is due to prostaglandin-mediated action of pro-inflammatory

cytokines on the hypothalamus (Kozak et al., 1998; Harden et al., 2006). Although our LPS dose (0.5 μg/kg BW) was less, the overall increase in body temperature was similar to increases (0.80 to 1.07 °C) observed when dairy and beef heifers (Steiger et al., 1999; Kahl et al., 2009) were infused or challenged with 2 to 2.5 μg/kg LPS. Akarsu and Mamuk (2007) demonstrated that the *E. coli* O111:B4 LPS serotype we used has a more potent hyperthermic activity than the *E. coli* O55:B5 serotype used by Kahl et al. (2009) which could contribute to the similar BT response despite a reduced dose of LPS.

Interestingly, BT was the only immune component measured in this study that increased during C2 and this increase only occurred in the Holstein heifer genotypes. Responses of all other immune components except SAA and NOx decreased during C2. Although overall SAA concentrations were not reduced, the 24 h concentrations in C2 were reduced compared to C1. The lack of a decrease in NOx concentrations during C2 does not agree with previous LPS-cattle studies (Kahl and Elsasser, 2006). Our results indicate the refractory or tolerance period for the primary inflammatory cytokines persisted for at least 4 d after the initial exposure to LPS.

Body temperature was greater in CA than in CH and UH heifers during C1. However, pro-inflammatory cytokine profiles either did not differ or were greater in CH (TNF-α) or UH (IL-6) than CA heifers. In addition, these pro-inflammatory cytokine responses differed between the Holstein genotypes despite their similar BT response. Thus, although these cytokines responded as expected when LPS was administered, their relative response differed among the 3 genotypes. The reasons for these differences in individual pro-inflammatory cytokine responses within genotype and for the differences

between the Holstein genotypes are not clear but the results do indicate the potential for differential innate immune signaling among genotypes.

Interleukin-8, IL-6 and TNF-α, are pro-inflammatory cytokines that are released by host immune cells when exposed to inflammatory stimuli (Arango Duqe and Descoteaux, 2014). Benjamin et al. (2016) identified greater IL-8 responses in contemporary Holsteins than in contemporary Angus when fibroblasts from 19 month old heifers were challenged with LPS or IL-1β. Additional fibroblast incubations (Kerr and Crooker, unpublished data) indicated the IL-8 response of 19 month old contemporary and unselected Holstein fibroblasts to IL-1β did not differ and that both were greater than the IL-8 response of fibroblast from Black Angus. The IL-8 response of unselected Holstein fibroblast to LPS was intermediate to the contemporary Holstein and Angus fibroblasts. An RNA-Seq analysis of the contemporary Holstein and Angus fibroblasts revealed that expression of TLR4, the pathogen recognition receptor for LPS, was also greater in contemporary Holstein than in Angus heifers before and after LPS stimulation (Benjamin et al., 2016). These fibroblast results and our whole animal TNF-α results indicate contemporary Holstein heifers might be able to detect and induce a more vigorous inflammatory cascade in response to Gram-negative bacteria than contemporary Angus heifers. Thus, the relative ‘hyper’ response of contemporary Holstein heifers might indicate a benefit over the ‘hypo’ response of contemporary Angus heifers because the pro-inflammatory TNF-α profile was more robust but returned to pre-challenge means about the same time in all 3 genotypes. This hyper-hypo consideration does not explain the different TNF-α profiles and similar IL-6 profiles in our CH and CA heifers or the similar IL-8 response

by the CH and UH fibroblasts but does indicate components of the immune response in Holsteins have been altered by the last 50 years of selective breeding.

Glucocorticoids enhance the immune system response by up-regulation of cytokine receptors to increase cytokine binding and subsequent induction of acute phase protein production by the liver (Wiegers and Reul, 1998). Cortisol concentrations increased within 1 h in all genotypes in response to LPS and during C1 remained elevated through 8 h as previously reported for heifers, cows and steers (Steiger et al., 1999; Waldron et al., 2003; Carroll et al., 2009, 2011). Interestingly, peak cortisol response occurred 2 h later in CA and the response was not maintained through 4 h as it was for UH and CH heifers. Carroll et al. (2011) suggested that a reduced cortisol response to LPS might identify animals that can mount a more robust innate immune response, characterized by greater concentrations of the pro-inflammatory cytokines IL-1β and TNF-α, that would allow these animals to return to homeostasis earlier. However, concentrations of TNF-α or IL-6 were lower in CA compared to CH or UH heifers. In addition, other than a numerical reduction in IL-6 concentration at 8 h, there was no indication that CA heifers had an earlier return to homeostasis for any of the parameters measured in this study. Our results indicate a delayed cortisol response was not associated with a more robust immune response to LPS.

Plasma SAA response profiles in our heifers are similar to those reported by Kahl and Elsasser (2006) and Carroll et al. (2009) in steers. Plasma concentrations of SAA were greatest at 24 h after LPS administration in C1 and in C2 and were greater in CA than in UH or CH heifers. Although both TNF-α and IL-6 stimulate APP production (Hagihara et

al., 2004), genotype differences in plasma concentrations of SAA (CA > UH or CH at 24 h) were not associated with genotype differences in plasma TNF-α (CH > CA) or IL-6 (UH > CA) concentration profiles. The greater BT and SAA concentrations and reduced pro-inflammatory cytokine profiles in CA heifers might indicate BT and SAA responses in CA heifers are more sensitive to TNF-α and IL-6 than either Holstein genotype. However, additional investigation is needed to clarify the relationships and factors responsible for the cytokine and APP differences among these genotypes.

Glucose is the preferred substrate for immune cell metabolism (Pithon-Curi et al., 2004; Calder et al., 2007) and the initial stress response is characterized by increased plasma cortisol concentrations, a rapid increase in glucose to fuel the innate immune response, a concomitant increase in insulin, and a subsequent anorexic phase reflected by increased lipolysis (Steiger et al., 1999). These typical metabolic changes after LPS administration (Steiger et al., 1999; Burdick Sanchez et al., 2013) occurred in all 3 genotypes evaluated in this study. The initial hyperglycemia is due to increased glycogenolysis and gluconeogenesis to meet immediate immune cell needs and this increase in glucose availability is supported by insulin resistance in peripheral tissues (Lang et al., 1990; Waldron et al., 2003). Although glucose availability is increased, the caloric needs of the immune system are substantial. Kvidera et al. (2017) estimated that the activated immune system of lactating dairy cows and growing steers and pigs uses between 0.7 and 1.1 g of glucose/kg of $BW^{0.75}$/h Thus, a phase of hypoglycemia is frequently observed. The greater increase in insulin concentrations in CH than in CA heifers was likely due to the greater glucose response to LPS. In contrast, the small

difference in glucose response (only differed by 20.4 mg/mL at 1 h) between CH and UH heifers does not appear to be sufficient to explain their different insulin responses. Magnitude of the insulin resistance induced in peripheral tissue by LPS administration is associated with magnitude of the TNF-α response (Youd et al., 2000; Hivert et al., 2010). Although the greater TNF-α and insulin responses in CH heifers might indicate a selection-induced alteration in insulin-resistance when they are challenged with an endotoxin like LPS, there are no data available to support this.

It is estimated that a 1 ºC increase in BT increases energy usage by 10 to 15% in animals (Kluger, 1989). Greater BT in CA than CH and UH heifers during C1 could reflect a more robust immune response in this genotype that would lead to greater glucose utilization and this might explain the reduced glucose response in CA heifers. However, the slower cortisol response and reduced concentrations of TNF-α and IL-6 compared to CH and UH heifers, are not consistent with a more robust response in CA heifers. In addition, there are no data to indicate that Angus and Holstein heifers differ in their glycogenolytic and/or gluconeogenic capacities.

Plasma NEFA concentrations were greatest 8 h after LPS administration and were greatest in CA heifers and in C2 and these responses were fairly consistent among heifers within a genotype. Others have also detected increased NEFA concentrations 6 to 8 h after LPS administration (Kushibiki et al., 2009; Lippolis et al., 2017) when glucose supply is diminished and intake is depressed. The reason(s) for the greater increase in NEFA in CA heifers and the greater increase during C2 are not known.

In contrast to previous reports of similar plasma IGF-I concentrations in UH and CH heifers (Baumgard et al., 2002; Weber et al., 2005), IGF-I concentrations tended to be greater in CH than in UH heifers. In an earlier preliminary evaluation of the immune system of UH and CH heifers (Chapter 2), IGF-I concentrations tended to be greater in UH than in CH heifers. These discrepancies are likely due to the small number of animals used in these LPS challenge studies. Growth hormone and IGF-I can augment PMNL bactericidal capacity through increased phagocytosis and intracellular ROS production (Inoue et al., 1998). Cellular ROS can be assessed indirectly via plasma XO concentration and XO is involved in the generation of NOx which impacts the immune response to infection (Godber et al., 2000; Kahl and Elsasser, 2004). In our study, ROS production, as assessed by XO concentrations, increased even though IGF-I concentrations were reduced. Others have reported that LPS reduced IGF-I concentrations (Spurlock, 1997) and increased XO concentrations (Kahl et al., 1997; Kahl and Elsasser, 2004) but there appear to be no cattle studies that measured both IGF-I and ROS production. Plasma NOx also increased in all 3 genotypes after LPS administration and this increase is consistent with results reported by Kahl and Elsasser (2004). Plasma NOx content can be used to estimate NO production (Kahl et al., 1997) and NOx itself has antimicrobial properties (Jones et al., 2010). Plasma NOx tended to be greater in the Holstein heifers at least during C1 and this agrees with the premise that Holsteins might respond more vigorously against Gram-negative bacteria than Angus heifers (Benjamin et al., 2016).

Depending on the interval between challenges, administration of a repeated LPS challenge allows evaluation of acute responses by the immune system from activation through recovery and an assessment of endotoxin tolerance (Kahl et al., 2011). Endotoxin tolerance is considered to be a protective mechanism against septic shock due to excessive inflammation when consecutive exposures to endotoxins like LPS occur (Cavaillon et al., 2003; López-Collazo and Del Fresno, 2013). We focused our efforts on evaluation of the acute phase of challenges separated by 4 d which was sufficient to demonstrate the refractory state of endotoxin tolerance (Cavaillon et al., 2003; Kahl et al., 2011; López-Collazo and Del Fresno, 2013).

Tolerance to LPS was evident in this study as stress and inflammatory responses were less during C2 than during C1. The reduced magnitude and duration of these responses agree with previous reports (Kahl and Elsasser, 2006; Kahl et al., 2011). Although there were differences among genotypes during C1, the similar cytokine responses among genotypes during C2 might indicate the endotoxin tolerance mechanisms were not challenged sufficiently to elicit differences or that the tolerance mechanisms did differ among genotypes such that the genotype differences observed during C1 were ameliorated during C2.

6. CONCLUSION

Heifer genotype affected metabolic, endocrine and immunological responses to LPS. The more rapid increase in cortisol concentrations in Holsteins and the greater concentrations of TNF-α in CH and IL-6 in UH are consistent with Holstein heifers having a more robust innate immune response than Angus heifers. Differences between

the UH and CH heifers indicates selection has altered aspects on the innate immune system in Holsteins. Additional studies, including those with a reduced intra-challenge interval are needed to elucidate the impact of selection on the innate immune response and endotoxin tolerance in cattle.

Table 1. Effect of genotype (G), challenge (C), time (T) and their interactions on response[1] of heifers[2] to repeated administration of lipopolysaccharide (LPS)[3]

	C1			C2				*P*-values[4]					
Item	UH	CH	CA	UH	CH	CA	SEM	G	C	GC	GT	CT	GTC
BT[5], °C	38.7[b]	38.5[b]	39.0[a]	39.0[a]	39.0[a]	39.1[a]	0.1	0.119	< 0.001	0.044	< 0.001	0.002	0.008
Glucose[6], mg/dL	80.8	85.8	73.0	73.7	79.4	73.9	3.2	0.021	0.116	0.397	0.002	< 0.001	0.011
Insulin[7], µIU/mL	73.9[xy]	114.9[x]	48.8[y]	47.6[y]	47.7[y]	57.1[y]	17.8	0.347	0.041	0.084	0.326	0.022	0.012
IGF-I[8], ng/mL	180.6	191.9	153.3	225.9	231.7	170.7	15.9	0.088	< 0.001	0.239	0.523	< 0.001	0.216
NEFA[6], mEq/L	104.6	74.6	104.7	163.2	124.1	183.7	16.2	0.125	< 0.001	0.376	0.001	< 0.001	0.799
Cortisol[6], µg/dL	6.1	6.4	5.2	3.6	3.8	3.3	0.4	0.253	< 0.001	0.441	< 0.001	< 0.001	0.776
TNF-α[9], ng/mL	2.4[b]	3.9[a]	2.5[b]	1.0[c]	0.9[c]	1.1[c]	0.3	0.153	< 0.001	0.024	0.107	< 0.001	0.025
IL-6[7], ng/mL	1.9[a]	1.4[ab]	1.2[bc]	0.9[bc]	0.7[c]	1.0[bc]	0.2	0.515	< 0.001	0.002	0.011	< 0.001	0.569
SAA[8], µg/mL	73.9	68.7	101.7	67.5	69.5	104.9	11.6	0.015	0.934	0.912	0.049	< 0.001	0.769
XO[10], mU/mL	3.5[x]	2.3[y]	2.4[y]	2.1[yz]	1.5[z]	1.7[z]	0.3	0.145	< 0.001	0.097	0.682	< 0.001	0.137
NOx[8], µM	5.5	4.9	3.2	4.7	3.6	3.8	0.7	0.100	0.370	0.366	0.574	0.092	0.275

[a,b,c] Genotype by challenge means within row with different superscript differ ($P \leq 0.05$).
[x,y,z] Genotype by challenge means within row with different superscript differ ($P < 0.10$).
[1]Response presented in least square means ± SEM.
[2]Heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins and contemporary Red-Black Angus (CA) cows.
[3]Identical LPS challenges (0.5 µg LPS/kg BW) were administered via jugular catheter on day 1 (C1) and 5 (C2).
[4]Effect of time was $P < .0001$ for all except BT ($P = 0.002$), insulin ($P = 0.022$) and NOx ($P = 0.092$).
[5]BT = rectal body temperature. Measured at -1, -0.5, 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h relative to challenge.
[6]Measured at -1, -0.5, 0, 1, 2, 3, 4, 6, 8 and 24 h relative to challenge.
[7]Measured at -1, 0, 1, 2, 3, 4, 6, and 8 h relative to challenge.
[8]Measured at 0, 4, 8 and 24 h relative to challenge.
[9]Measured at 0, 1, 2, 3, 4 and 6 h relative to challenge.
[10]Measured at 0, 8 and 24 h relative to challenge.

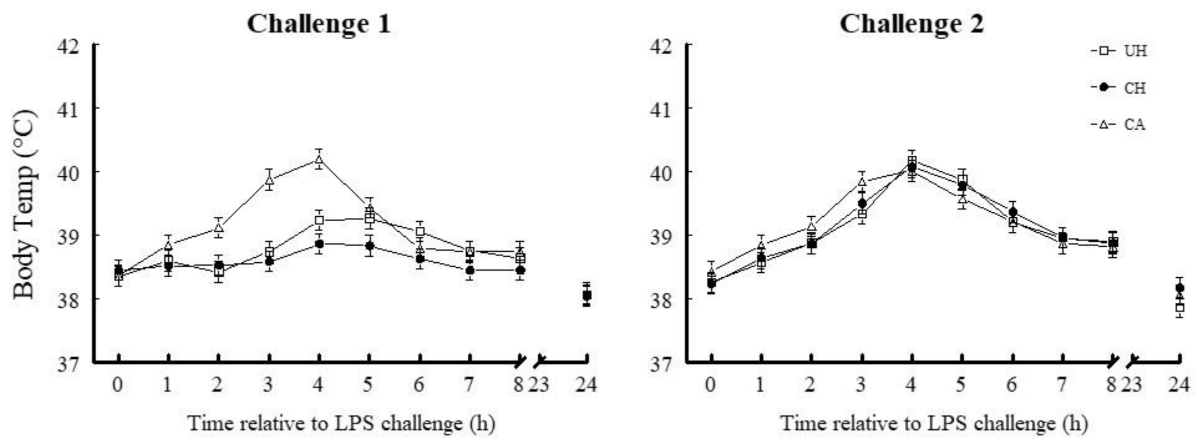

Figure 1. Rectal body temperature (BT) of heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins and contemporary Red-Black Angus (CA) cows after identical LPS challenges (iv administration of 0.5 μg LPS/kg BW) on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least square means ± SEM. There was a genotype by challenge by time interaction ($P = 0.008$) as BT of CA heifers was greater ($P < 0.013$) than that of UH or CH heifers at 2, 3 and 4 h during C1 but did not differ among genotypes ($P = 0.599$) during C2.

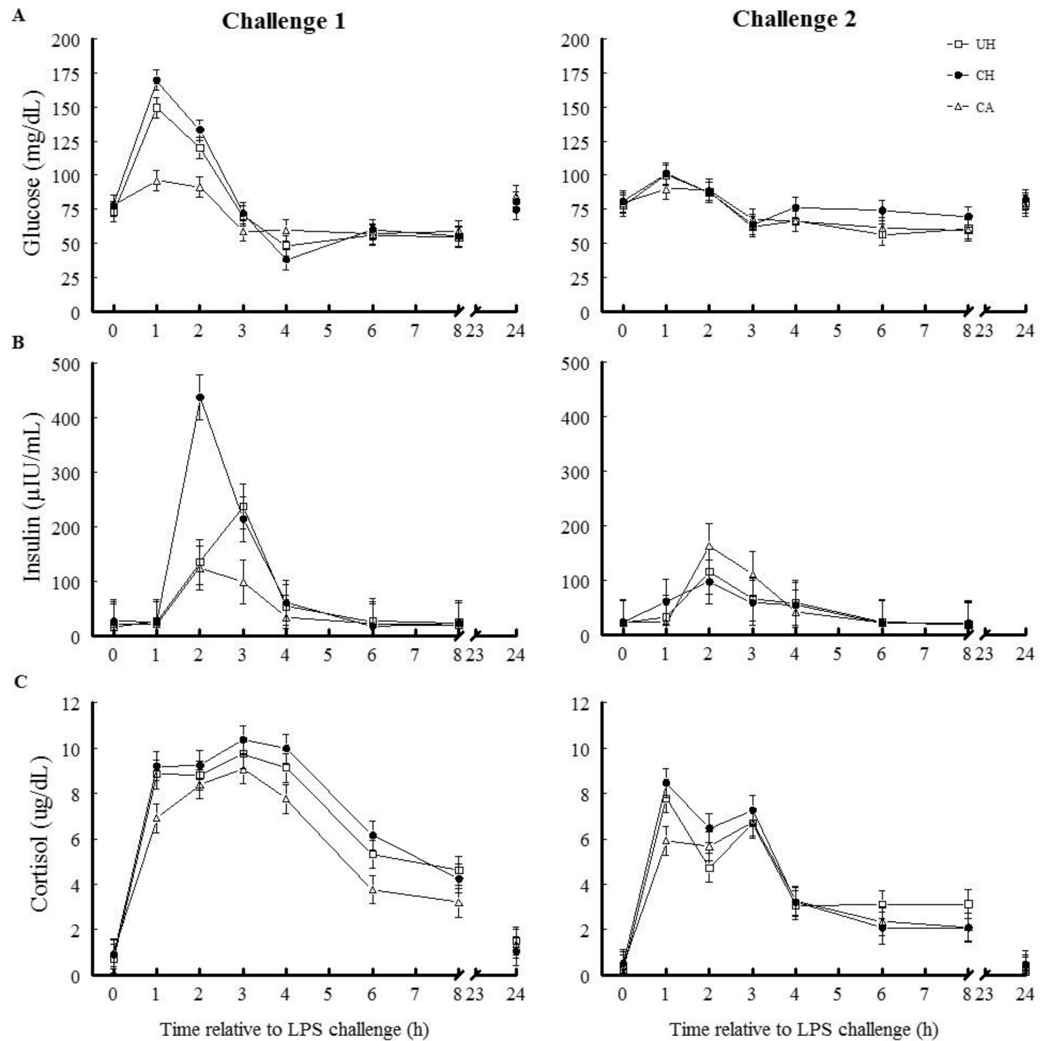

Figure 2. Plasma glucose (A), insulin (B) and cortisol (C) concentrations in heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins and Red-Black Angus (CA) cows after identical LPS challenges (iv administration of 0.5 µg LPS/kg BW) on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least square means ± SEM. There was a genotype by challenge by time interaction ($P < 0.012$) for glucose and insulin. Glucose concentrations increased less ($P = 0.046$) in CA than UH or CH during C1, but did not differ ($P > 0.216$) among genotypes during C2. Insulin concentration increased more (P = 0.014) in CH than UH and CA during C1 but did not differ ($P > 0.711$) among genotypes during C2. Cortisol concentration was greater in C1 than in C2 ($P < .0001$). There was also a genotype by time interaction ($P < 0.001$) for cortisol as cortisol concentrations peaked 1 h post-LPS in UH and CH heifers, while concentrations in CA heifers peaked at 3 h.

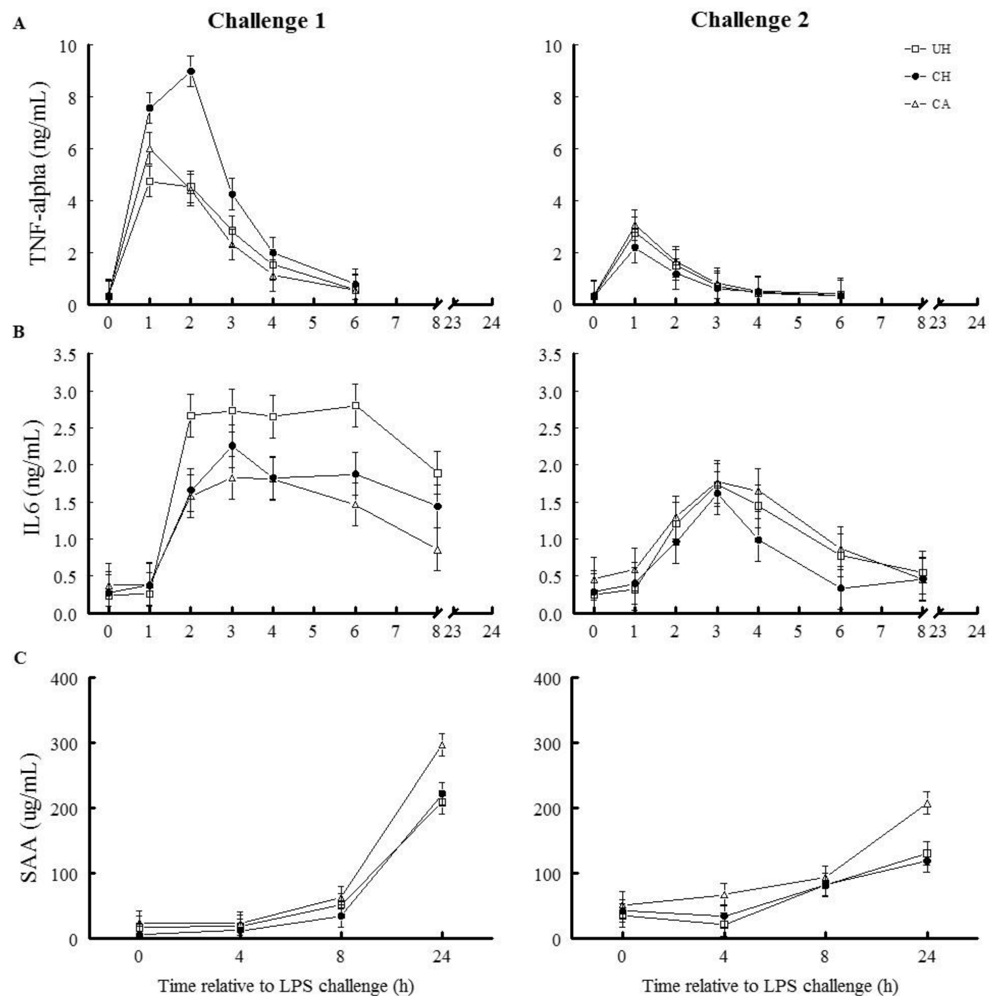

Figure 3. Plasma TNF-α (A), IL-6 (B) and SAA (C) concentrations in heifers (n = 4/genotype) from unselected (UH) and contemporary (CH) Holsteins and Red-Black Angus (CA) cows after identical LPS challenges (iv administration of 0.5 µg LPS/kg BW) on day 1 (Challenge 1) and 5 (Challenge 2). Data represent least square means ± SEM. There was a genotype by time and challenge interaction ($P = 0.025$) for TNF-α as concentrations were greater and peaked later in CH than UH and CA heifers during C1. Concentrations of TNF-α did not differ among genotypes during C2 ($P = 0.506$). There was a genotype by time ($P = 0.011$) and a genotype by challenge interaction ($P < 0.002$) for IL-6 as IL-6 concentrations were greater ($P = 0.051$) in UH than in CA heifers during C1 but did not differ among genotypes in C2 ($P > 0.387$). There was a genotype by time interaction ($P = 0.049$) for SAA as SAA concentrations were greater in CA than in UH or CH heifers at 24 h. There was also an interaction of time and challenge ($P < .0001$) for SAA as SAA concentrations tended to be greater ($P = 0.066$) at 0 h, were greater ($P = 0.013$) at 8 h, and were reduced (P < .0001) at 24 h during C2 relative to the corresponding times in C1.

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CHAPTER 4: Effect of milk yield genotype on response to repeated lipopolysaccharide (LPS) administration to lactating Holstein cows

1. SYNOPSIS

This study was designed to assess the impact of milk yield genotype on the innate immune system of multiparous cows and its interactions with endocrine and metabolic components. Cows (n = 12/genotype) from unselected (stable milk yield since 1964, UH) and contemporary (CH) Holsteins that differed by more than 4,500 kg milk/305 d, were fed the same diet ad lib and housed together for more than 4 months before being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an *iv* administration of saline or 0.25 μg LPS (Escherichia coli 055:B5) per kg BW. Cows were synchronized to be at day 8 of their estrous cycle for the first challenge (C1) at 70-84 days in milk. A second identical challenge (C2) and sampling was conducted 4 d later. Plasma samples were analyzed for concentrations of glucose, insulin, IGF-I, NEFAs, cortisol, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), serum amyloid A (SAA) and haptoglobin (Hp). Fresh blood samples were used for white blood cell count (WBC), analysis of polymorphonuclear leukocyte (PMNL) L-selectin and CD11b/CD18 expression, and phagocytic and oxidative burst capacities. In addition, isolated fibroblast from dermal biopsies collected 3-4 d after C2 were challenged with either LPS or IL-1β for 24 h, and IL-6 and IL-8 protein production was measured. Rectal BT and concentrations of all plasma components, WBC, adhesion molecules expression and PMNL activity were altered by time. During C1, plasma concentrations of glucose, insulin, TNF-α and IL-6 after LPS were greater in UH than CH cows. Expression of CD11b/CD18, phagocytic and oxidative burst capacities were also greater in UH cows.

BT, SAA and Hp concentrations, WBC and L-selectin expression did not differ between the genotypes after LPS. During C2, glucose, insulin and IL-6 concentrations were greater in UH than CH cows. The induction of tolerance to LPS was evident in UH cows that had a reduced pro-inflammatory response during C2 compared to C1, however this was not the case for CH cows where responses did not differ between challenges. IGF-I concentrations were greater in UH than CH cows; however, there were no interactions of genotype with either time, treatment or challenge. IL-8 protein production by isolated dermal fibroblast was greater in CH cows compared to UH after IL-1β treatment. Results indicate the last 50+ years of selection has reduced the acute innate immune (pro-inflammatory) and reduced development of tolerance (anti-inflammatory) responses of Holsteins to LPS and supports the premise that the contemporary cow is more susceptible to pathogenic challenges than her ancestors.

Keywords: Holstein genotype, milk yield, lipopolysaccharide, innate immunity

2. INTRODUCTION

Since the mid-1960s, selective breeding of dairy cows has focused primarily on increasing milk yield and production traits. This has undoubtedly benefitted producers and consumers in the United States and worldwide. It has increased production efficiency and profitability while also greatly reducing the impact of the dairy industry on the environment (Capper et al. 2009). However, selection for health components has been insufficient and there is great concern that genetic selection practices have made the contemporary cow more susceptible to disease and metabolic disorders (Pritchard et al., 2013; Egger-Danner et al., 2015).

As milk yield per cow increases, endocrine and metabolic adaptations occur to enable the cow to meet the metabolic demands of increased milk synthesis (Crooker et al. 2001) and these adaptations can have a detrimental impact on the immune system (Pritchard et al., 2013; Egger-Danner et al., 2015). Greater need to treat contemporary Holsteins for mastitis has been demonstrated when comparing to unselected Holstein (Jones et al., 1994). This unselected vs. contemporary Holstein animal model has also demonstrated that selection practices have altered circulating concentrations of endocrine and metabolic components (Weber et al., 2007) and that aspects of the immune system have been altered (Chapter 2 & 3).

Continued genetic selection for increased milk yield has reduced the ability of contemporary Holsteins to effectively combat infections (Oltenacu & Broom, 2010). Studies on the relationship between milk performance and health in dairy cattle showed a genetic correlation between milk yield and incidence of ketosis (0.26–0.65), ovarian cyst (0.23–0.42), mastitis (0.15–0.68) and lameness (0.24–0.48), indicating that continued selection for greater production increased incidence rates for these disorders and diseases (Ingvartsen et al., 2003).

Despite focused research emphasis on the periparturient period over the past several decades, the incidence of problems during this period has not decreased (Drackley, 1999; Oliver et al., 2005; LeBlanc, 2010) as 30% to 50% of dairy cows are affected by some form of metabolic disorder or disease around the time of calving (Leblanc, 2010). Producers are continually challenged to modify management practices to meet the ever increasing needs of the dairy cow (Bjerre-Harpøth et al., 2012), and this

trend is expected to continue as maximum productive capacity of the dairy cow has not been reached (Bauman, 2000; Baumgard et al., 2017).

During early lactation, homeorhetic adaptations are needed to support lactation and these adaptations alter circulating concentrations of metabolites and hormones. Among the many changes is an increased proportion of saturated long chain fatty acids in circulation. Saturated fatty acids can activate inflammatory pathways by binding to Toll-like receptor 4 (TLR4, the primary lipopolysaccharide -LPS- receptor) rendering the cows more susceptible to metabolic disorders and diseases (Wong et al., 2009). In addition, the greater energy and nutrient content of the postpartum dairy cow diet alters rumen fermentation towards increased acid production which increases the presence of Gram-negative bacteria and their release of LPS and other endotoxins into the intestinal lumen where they can be absorbed (Zebeli and Metzler-Zebeli, 2012).

Binding of TLR4 by ligands such as saturated fatty acids or LPS can activate the downstream TLR4-signaling pathway initiating an acute immune response (Steiger et al., 1999; Netea et al., 2004; Zebeli & Metzler-Zebeli, 2012). A wide range of inflammatory mediators and cytokines are synthesized by host immune cells after ligand recognition (Arango Duque & Descoteaux, 2014). Changes in circulating concentrations of immune, metabolic and endocrine components during the immune activation induces multiple physiological changes including fever, increased vascular permeability and hepatic production of acute phase proteins (APP; Carroll et al., 2009; Mihara et al., 2012). However, a tolerance to LPS can be developed when the host is subjected to repeated or prolonged exposure to the ligand. This is a natural mechanism that regulates the magnitude and duration of the ligand-induced inflammatory response to avoid septic

shock that can be caused by excessive inflammation (Cavaillon et al., 2003; López-Collazo & Del Fresno, 2013).

Health problems in dairy cows not only are a significant cause of poor welfare but they can also limit production (Denholm et al., 2017). An improved understanding of how selection for milk yield has altered the immune system and its interactions with endocrine and metabolic components will contribute to efforts to improve cow health and enhance food safety and security. The primary objective of this study was to examine the impact of LPS administration on acute and tolerance aspects of the innate immune response in the Holstein cow. A novel aspect of this study is that we compared contemporary Holsteins (CH) with unique Holsteins that have not been subjected to selection since 1964 (unselected Holsteins, UH; Weber et al., 2007). Identical LPS challenges were administered on day 1 and 5 of study to assess impact of genotype on the acute innate immune response and on the development of a refractory (endotoxin tolerance) state. This animal model provides the opportunity to assess the impact of 50+ years of selection on the immune response of Holsteins.

3. MATERIAL AND METHODS

3.1. Animals, Experimental Design, and Treatments

Cows were from two genetic lines of Holsteins. Development of the static, unselected (UH) and a contemporary (CH) Holstein was initiated in 1964 by Dr. Charles Young as a component of a multistate, north central regional project (NC-2; Young, 1977; Weberet al., 2007). Genetic merit for milk yield of the UH cows has remained stable, while that of CH cows has continued to increase and is similar to that of U.S.

Holsteins. Current milk yield of UH cows is about 55% of CH cows (6,700 vs. 12,000 kg/305 d lactation).

Twenty-four multiparous cows (n = 12/genotype) were fed the same diet ad lib and housed together for more than 4 months before being assigned to one of 6 blocks (2/genotype) by DIM and randomly assigned within genotype to receive an iv administration of saline or 0.25 µg LPS (*Escherichia coli* 055:B5, Sigma-Aldrich L2880) per kg BW on d 1 (challenge 1, C1) and d 5 (C2) of study. Treatment assignments were not switched between challenges so C1 and C2 were identical. Estrous cycles were synchronized following the 5-day CIDR Synch protocol (Bridges et al., 2008) so that cows were at day 8 of their estrous cycle on d 1. Cows were between 70 and 84 DIM on d 1. Each cow was fitted with an indwelling jugular catheter at least 24 h before d 1. Blood samples (10 mL) were collected at -1, -0.5, 0, 1, 2, 3, 4, 6, 8, 12, and 24 h relative to treatment administration. Body temperatures (BT) were determined at these times and at 5 and 7 h. Blood was mixed with heparin (20 µL of 10.000 IU/mL), placed on ice and centrifuged (1500 x g, 15 min at 4 °C) within 30 min. Plasma was aliquoted, and stored at -20 °C until assayed. Liver biopsies (Carriquiry et al., 2009) and blood for flow cytometry (10 mL vacutainers containing 158 USP heparin, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and hemogram (3 mL vacutainers containing EDTA, Kendall Monoject, Mansfield, MA) assays were obtained at 0, 4 and 24 h relative to treatment administration. Dermal biopsies for fibroblast collection (Green et al., 2011) were obtained 3 to 4 d after C2. Cows were milked at 12-h intervals and daily yields were determined from recorded milk weights. Cows were observed daily throughout the study

and health abnormalities treated when warranted. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

3.2. Sample Analyses

Plasma glucose (Autokit Glucose, Wako Life Sciences, Mountain View, CA) and NEFA (NEFA-HR (2), Wako Life Sciences, Mountain View, CA) concentrations were determined by colorimetric assays with volumes modified for use in a 96-well plate. Samples were analyzed in duplicate. Intra and interassay coefficients of variation were 3.9 and 4.9% and 3.6 and 10.1% for glucose and NEFA, respectively. Plasma cortisol, insulin and progesterone concentrations were measured by RIA (ImmunoChem Cortisol, MP Biomedicals, Orangeburg, NY; ImmunoChem Insulin, MP Biomedicals, Solon OH; Coat-A-Count Progesterone, Siemens Healthcare Diagnostic Inc., Los Angeles, CA). The assays were validated for recovery and linearity of dilution with bovine plasma. Samples were analyzed in duplicate. The minimal detectable concentration of cortisol was 0.17 µg/dL. Intra and interassay coefficients of variation were 9.6 and 7.1%, respectively. The minimal detectable concentration of insulin was 4.60 µIU/mL. Intra and interassay coefficients of variation were 5.3 and 7.4%, respectively. The minimal detectable concentration of progesterone was 0.02 ng/mL and the intra-assay coefficient of variation was 9.7%. Plasma IGF-I concentrations were quantified by a validated double-antibody RIA (Weber et al., 2007). Samples were analyzed in triplicate. The minimal detectable concentration of IGF-I was 0.20 ng/mL. Intra and interassay coefficients of variation were 9.9 and 3.4%, respectively.

Plasma tumor necrosis factor-alpha (TNFα) concentrations were measured by a specific double antibody RIA (Kenison et al., 1990). The minimum detectable TNFα was

4.00 pg/assay tube. Samples were assayed in duplicate, and the intra and interassay coefficients of variation were < 15%. Plasma interleukin-6 (IL-6) concentrations were determined by ELISA (Kandasamy & Kerr, 2012). The minimum detectable concentration was 0.1 ng/mL. The intra assay coefficient of variation was < 10%. In addition, IL-6 and IL-8 production by fibroblast cultures isolated from dermal biopsies was assessed (Green et al., 2011) after 24 h exposure with either 100 ng/mL of LPS (isolated from *E. coli* O111:B4, Sigma-Aldrich, St. Louis, MO) or 10 ng/mL of IL-1β (AbD Serotec, Raleigh, NC).

Plasma serum amyloid A (SAA) and haptoglobin (Hp) were determined by ELISA (Phase™ Range, Tri-Delta Diagnostics, Inc., Morris Plains, NJ; HAPT-11, Life Diagnostics, Inc., West Chester, PA). Samples were analyzed in duplicate according to the manufacturer's instructions. Both ELISA kits were validated for recovery and linearity of dilution with bovine plasma. The minimum detectable concentration of SAA was 1.50 µg/mL and the interassay coefficient of variation was 12.3%. The minimum detectable concentration of haproglobin was ~ 3.70 ng/mL the intra and interassay coefficients of variation were 4.1 and 1.9%, respectively.

Liver biopsies were aliquoted and either immediately frozen in liquid nitrogen and stored at −80 °C or placed in RNA*later* (100 mg of tissue/mL; Ambion, Austin, TX) and stored for 7 to 21 d at −20 °C. After removing RNA*later*, tissue was stored at −80 °C until nansoString analysis (Chapter 5). Hemogram analyses were determined with a Vet Scan HM2 (Abaxis, Union City, CA).

3.3. Peripheral polymorphonuclear Analyses by Flow Cytometry

Ex-vivo innate immune responses were determined by measuring peripheral polymorphonuclear leukocytes (PMNL) surface expression of adhesion molecules L-selectin and CD11b/CD18, and PMNL phagocytic and oxidative burst capacity. Direct immunofluorescence staining was used to determine expression of adhesion molecules L-selectin (also known as CD62L) and CD11b/CD18 (β_2 integrin family member) by peripheral PMNL with and without phorbol 12-myristate 13-acetate (PMA, sc-3576, Santa Cruz Biotechnology, Santa Cruz, CA) activation *in-vitro*. Polymorphonuclear leukocytes were obtained from whole blood lysed with ammonium-chloride-potassium (ACK) lysing solution (prepared with 8.29 g NH_4Cl, 1 g $KHCO_3$, and 37.2 mg Na_2EDTA in 1 L of sterile water, filtered and its pH adjusted to 7.2 - 7.4). Hemocytometer counting chamber was used to adjust the lysed samples to have 1-2 million cells/0.1 mL.

Aliquots (0.1 mL) of PMNL were left unstimulated or were stimulated by incubating with 40 ng/mL of PMA for 30 min at 37°C. After incubation all tubes were filled with FACS buffer (2 mM Azide, 2% Goat Serum in 1XPBS), centrifuged (5 min at 8°C and 524 x g) and decanted to remove PMA. The residual buffer (~0.01 mL) and PMNL pellet were mixed with 0.02 mL of anti-CD62L R-PE-conjugate (#261-050, Ancell Corporation, Bayport, MN) and 0.02 mL of monoclonal mouse anti-bovine CD11b:FITC (#MCA1425F, Bio-Rad AbD Serotec, Raleigh, NC) and incubated for 20 min at 4°C in the dark to label L-selectin and CD11b/CD18 molecules. These PMNL mixtures were washed with FACS buffer as previously described to remove unbounded Abs and fixed with 0.1 mL of 1% paraformaldehyde and kept covered and on ice until flow cytometry analyses. Unstained PMNL and PMNL stained with monoclonal mouse

IgG2a:R-PE (#281-050, Ancell Corporation, Bayport, MN) and monoclonal mouse IgG2b:FITC (#MCA69IF, Bio-Rad AbD Serotec, Raleigh, NC) were used as negative controls. Phagocytic and oxidative burst capacity of peripheral PMNL were determined as previously described by Hulbert et al. (2011). All samples were analyzed by flow cytometry within 1 h of being labeled. Flow cytometry was conducted on a BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ) and data analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA). Forward versus side scatter gating was used to identify PMNL based on their size and granularity. For each sample, ten thousand events of labeled PMNL were acquired.

Expression of L-selectin and CD11b/CD18 molecules, and phagocytosis and oxidative burst capacities are reported as geometric mean fluorescence intensity (GMFI). Oxidative burst intensity is an indirect indicator of the amount of reactive oxygen species produced via oxidation of DHR123. L-selectin and CD11b/CD18 expression after *in-vitro* re-stimulation with PMA are reported as the percentage difference in expression between the PMA activated and the unstimulated sample.

3.4. Statistical Analyses

Dependent variables were checked for the assumption of normality using the UNIVARIATE procedure of SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC). Hemogram cell counts and flow cytometric data for phagocytic and oxidative burst capacity were square root transformed to meet normality assumptions. Daily milk yields were summarized within genotype and treatment by day of lactation ± 7 d relative to the challenges.

Plasma profile data were analyzed as a randomized block design by repeated measures using PROC MIXED by SAS with time as the repeated effect. The spatial power law for unequally spaced data was specified as the covariance structure. The model included genotype, time, treatment, challenge and their interactions as fixed effects, and block as a random effect. Overall response for each cow was calculated as area under the time by concentration curve (AUC) after the concentrations were corrected by subtracting the cow's pre-challenge mean from each post-administration value. The initial positive and subsequent negative AUC responses of glucose are reported, however not all cows had a glucose response to LPS so the data sets for these responses were incomplete. Response AUC data were analyzed as described above with genotype, treatment, challenge and their interactions as fixed effects and cow as a random effect. Effects of genotype, treatment, challenge and their interactions from the repeated measures and AUC analyses were comparable for all components, therefore; only the repeated measures results and glucose AUCs are reported. Results are reported as least squares means. Means were considered to differ when $P \leq 0.05$ and trends identified when $0.05 < P \leq 0.10$.

4. RESULTS

Transient characteristic signs of mild systemic immune response such as labored breathing, increased salivation, nasal discharge, coughing, mild diarrhea and lethargy were observed within 30 min of each LPS administration but were resolved within 6 to 7 h later. On challenge days, plasma progesterone concentration exceeded 1.0 ng/mL in all cows except 2 CH cows that had < 1 ng/mL on both challenge days but had greater progesterone concentration on day 5 than on day 1. Although these 2 cows were below

the typical threshold used to identify anestrous cows, cows subjected to the same synchronization procedure (Chebel et al., 2006) with progesterone < 1.0 ng/mL that did not displayed signs of estrus were considered anestrous cows.

Rectal BT peaked 4 h after LPS administration ($P < 0.001$) and increased 0.74 and 0.48 ± 0.08 ºC in C1 and C2, respectively (Fig. 1). There was a genotype by time interaction ($P = 0.009$) due to lower pre-challenge BT means ($P < 0.001$) in UH than CH, and a trend for UH to have lower BT (P < 0.058) at 5, 7 and 24 h after LPS than CH. There was also a genotype by treatment and challenge interaction (P = 0.035) for BT due to greater BT in CH saline cows at 7 and 12 h during C2.

There was a genotype effect ($P < 0.001$) for milk yield as milk production was greater in CH than UH cows (35.6 vs. 21.8 ± 2.2 kg/d, respectively; Fig. 2). There was also a day by treatment interaction ($P < 0.001$) for milk yield as yield decreased after LPS administration in both genotypes and the decrease was more pronounced during C1 than C2 (day 1 vs. day 5: $P = 0.006$). There were no interactions of genotype with day or treatment ($P > 0.329$) for milk yield.

Plasma glucose concentrations after LPS administration varied among cows. Administration of LPS increased glucose concentrations in 6 UH cows and 3 CH cows during C1 but only in 2 UH and 2 CH cows during C2. Although data from all cows were used for the repeated measure analysis, only data from the cows that had an initial hyperglycemic response were used to determine the AUC response.

Infusion of LPS caused an initial hyperglycemia followed by hypoglycemia that was similar for C1 and C2, however, during C1 glucose peaked at 1 h while in C2 it peaked at 2 h (Fig. 3). A genotype by time and treatment interaction ($P = 0.021$) for

glucose was observed as mean concentrations after LPS peaked higher and remained above pre-challenge mean longer (2 h vs. 1 h) in UH than CH. There was also a genotype by time, treatment and challenge trend ($P = 0.078$) as glucose peak concentrations were greater in C1 than C2 for UH (102.0 vs. 95.2 ± 4.0 mg/dL; $P < 0.001$) but lower for CH (71.8 vs. 81.4 ± 4.0 mg/dL; $P = 0.079$). However, since not all CH cows had a hyperglycemic response after LPS during C1, means of LPS and saline CH treated cows did not differ during the first 4 h of C1 ($P > 0.288$). The inflection time (2.7 and 2.2 ± 0.3 h, in C1 and C2 respectively) was not affected by genotype ($P = 0.334$), challenge ($P = 0.328$) or their interaction ($P = 0.737$). There was a genotype effect ($P = 0.003$) for the initial positive glucose response as AUC was greater in UH than CH (75.9 and 9.1 ± 11.9 mg•h/dL, respectively) but there was no impact of challenge ($P = 0.361$) or their interaction ($P = 0.228$) on this phase of the glucose response. There was no impact ($P > 0.200$) of genotype, challenge or their interaction on the negative phase of the glucose response.

There was a genotype by time and treatment interaction ($P = 0.002$) for plasma insulin as concentrations increased in UH but not CH cows ($P < 0.01$) after LPS administration. In UH cows, insulin concentrations peaked at 3 h (C1) and 2 h (C2) (Fig. 3B) and returned to pre-challenge means by 6 h after LPS administration. There was a genotype effect ($P = 0.007$) for IGF-I as concentrations were greater in UH than CH (147.4 vs. 112.5 ± 9.7 ng/mL, respectively). There was a time by treatment effect ($P < .001$) for IGF-I as concentrations at 24 h were greater in LPS than in saline but there was no effect for genotype or challenge on plasma IGF-I concentrations. Plasma NEFA concentrations fluctuated above and below the pre-challenge concentrations during C1

and C2. Although there were some differences, there were no clear patterns due to genotype, treatment, time, or challenge.

There was a genotype by time and treatment interaction ($P = 0.037$) for cortisol as concentrations increased similarly in UH and CH cows treated with LPS but remained elevated longer in UH cows (Fig. 4A). There was a time by challenge interaction ($P = 0.027$) for cortisol as concentrations from 2 to 6 h after LPS administration were greater during C1 than C2.

There was a genotype by treatment and challenge interaction ($P = 0.024$) and a trend for a genotype by time, treatment and challenge interaction ($P = 0.062$) for TNF-α as concentrations increased more in UH than in CH (0.82 vs. 0.51 ± 0.07 ng/mL, respectively; Fig. 4B) during C1 but did not differ ($P = 0.227$) among genotypes during C2. In addition, TNF-α concentration after LPS was greater during C1 than C2 ($P < 0.001$) for UH cows while response by CH cows did not differ between challenges ($P = 0.797$). Concentrations of TNF-α peaked 1 h post-LPS in both genotypes during both challenges. By 6 h in C1 and 4 h in C2, TNF-α concentrations did not differ from the pre-LPS administration means ($P > 0.454$).

There was a genotype by time and challenge interaction ($P = 0.011$) for IL-6 as concentrations were greater in UH than in CH cows during both challenges (Fig. 4C). Concentrations were greater during C1 than C2 in UH cows while the overall response by CH cows did not differ between challenges. During C1, IL-6 concentrations in UH cows started to increase at 2 h, peaked at 4 h (17.9 ± 1.7 ng/mL) and were still above the pre-LPS mean 8 h after LPS administration. In CH cows, IL-6 concentrations increased between 2 and 4 h and peaked at 4 h (4.0 ± 1.7 ng/mL), by 6 h concentrations were back

to the pre-LPS mean. During C2, UH cows responded to LPS; IL-6 concentrations peaked at 2 h and at 4 h did not differ from the pre-LPS mean concentrations. Interleukin-6 protein production by dermal fibroblast cultures 24 h after LPS or IL-1β exposure did not differ between the genotypes (Fig. 5A). Interleukin-8 production by dermal fibroblast cultures after 24 h of IL-1β treatment was lower in UH than CH ($P < 0.010$) but there was no effect of genotype on fibroblast IL-8 response to LPS (Fig. 5B).

Serum SAA concentrations did not differ between genotypes ($P = 0.506$) but there was a time by treatment and challenge interaction ($P < 0.001$) as SAA concentrations increased after LPS in both challenges but concentrations during C1 were less ($P < 0.007$) than those during C2. Serum Hp concentrations did not differ between genotypes ($P = 0.137$) but there was a time by treatment and challenge interaction ($P = 0.040$) for Hp, as concentrations at 24 h were greater in LPS than in saline treated cows during C1 but not C2. Response of Hp to LPS did not differ between the genotypes.

There was a time by treatment interaction ($P < 0.001$) for white blood cell count (WBC) as WBC decreased 4h (7.71 vs. $2.60 \pm 0.02 \times 10^9$/L) after LPS. By 24 h WBC was greater than the pre-LPS mean ($9.88 \pm 0.02 \times 10^9$/L; P = 0.003). There was a trend for a genotype by treatment and challenge interaction ($P = 0.059$) for WBC as counts were greater during C1 than C2 in CH but not UH cows ($P = 0.055$) after LPS administration. Counts did not differ between challenges in UH cows ($P = 0.944$). There was a genotype effect ($P < 0.006$) for hemoglobin (HGB) and hematocrit percentage (HCT%), there were also genotype by time and challenge interaction ($P = 0.006$) for HGB and a trend ($P = 0.056$) for HCT%, as both were greater in UH than CH cows (11.29 vs. 9.54 ± 0.35 g/dL; and 29.72 vs. 25.81 ± 0.84%, respectively). However, values

in both genotypes were within the accepted range for both parameters (8 – 15 g/dL for HGB and 24 – 46% for HCT). There was no effect of genotype or any genotype interaction on any of the other hemogram parameters ($P > 0.110$).

Expression of L-selectin by PMNL decreased ($P < 0.001$) 4 h after LPS administration in both genotypes and by 24 h expression did not differ from 0 h values ($P = 0.379$; Fig. 6A). There was a genotype by time and treatment interaction ($P = 0.035$) for L-selectin as expression at 0 h was greater in CH LPS than CH saline cows ($P = 0.035$). There was no effect of genotype or any genotype interaction for expression of L-selectin when PMNLs were re-stimulated *in-vitro* with PMA (Fig. 7A; $P > 0.243$). Expression of CD11b/CD18 by PMNL increased ($P < 0.001$) 4 h after LPS administration in both genotypes and by 24 h expression did not differ from 0 h values ($P = 0.315$; Fig. 6B). There was a genotype by time and treatment interaction ($P = 0.009$) for CD11b/CD18 as expression at 4 and 24 h after LPS was greater in UH than in CH cows. Expression of CD11b/CD18 in PMA stimulated PMNL was greater in UH than in CH cows (Fig. 7B; $P = 0.003$). There was a time by treatment interaction, because expression of CD11b/CD18 after PMA stimulation was greater in saline than in LPS treated cows at 4 and 24 h. There was a genotype by treatment trend ($P = 0.057$) for CD11b/CD18 after PMA activation, as expression was greater in UH saline treated cows than the rest of the groups ($P < 0.001$). There was a genotype by challenge trend ($P = 0.088$) as expression in UH was lower in C1 than in C2 ($P = 0.009$) while in CH expression did not differ between challenges ($P = 0.191$).

Polymorphonuclear leukocytes phagocytic capacity tended to be greater in UH than CH cows ($P = 0.058$) and lower during C1 than C2 ($P = 0.086$; Fig. 8A). However,

phagocytic capacity was not altered by LPS administration in either genotype (P = 0.568). The PMNL oxidative burst (OxB) capacity was greater in UH than CH cows (P = 0.046) and lower during C1 than C2 (P = 0.012; Fig. 8B).

5. DISCUSSION

Keeping cows healthy minimizes the need for therapeutic interventions which can reduce the use of antibiotics, reduce the cost of producing milk, and contribute to a continued supply of safe, wholesome milk for consumers. However, homeorhetic adaptations needed to support high milk yield production can have a detrimental effect on the immune system, and there is considerable concern that the contemporary cow is more susceptible to disease and metabolic disorders than her ancestors (Pritchard et al. 2013; Egger-Danner et al., 2015). In dairy cattle, selection for increased milk production had a negative effect on reproduction (Pryce et al., 2004) and it has been positively correlated with increased occurrence of metabolic disorders and disease (Jones et al., 1994; Pryce et al, 1997; Pritchard et al., 2013; Hagiya et al., 2014). In this study, unique Holstein cows that have not been selected for milk yield since 1964, and contemporary Holstein cows were used to examine the impact of milk yield genotype on innate immune response to a repeated challenge with LPS and its interactions with endocrine and metabolic components.

Lipopolysaccharide is a potent inducer of inflammation. After its recognition, host immune cells are activated to synthesize and secrete pro-inflammatory cytokines, such as TNF-α and IL-6, that are key regulators of the innate immune response. Cytokines are directly responsible for the clinical signs that accompany infections (Kelley et al., 2003) as they modulate the febrile response and the production and secretion of APP such as

SAA and Hp by the liver (Netea et al., 2004; Arango Duque & Descoteaux, 2014). In this study, differences in innate immune responses support the premise that contemporary cows are more susceptible to pathogenic challenges than her ancestors.

Differences in milk production between the genotypes reflect the effects of 50+ years of genetic selection with a substantial focus on increased milk yield per cow (Miglior et al., 2005; Oltenacu & Broom, 2010; Baumgard et al., 2017). As expected, the endotoxemia response decreased milk yield by both genotypes (Waldron et al., 2003; Ning et al., 2018; Zhao et al., 2018). Increased circulating concentrations of pro-inflammatory cytokines can decrease milk production (Shuster et al., 1995; Yuan et al., 2013; Bradford et al., 2015) and when the systemic inflammatory response is activated, more nutrients are used to support pro-inflammatory events which decreases the flow of nutrients to the mammary gland for milk synthesis.

Endotoxin challenges induce fever through prostaglandin-mediated actions of pro-inflammatory cytokines, primarily IL-6 (Harden et al., 2006), on the hypothalamus (Kozak et al., 1998). Interestingly, IL-6 concentrations after LPS were greater in UH than CH cows although BT was either not affected or tended to be lower in UH cows. In addition, concentrations of IL-6 in UH cows were significantly reduced during C2 relative to those during C1 but BT was not. Greater concentrations of 2 pro-inflammatory cytokines, IL-6 and TNF-α appeared to have no effect on the systemic thermic response in UH-LPS cows. Although the reasons behind this phenomenon are not clear, results indicate the potential for innate immune signaling differences between the genotypes.

Despite differences in their cytokine profiles, increases in BT after LPS administration did not differ between genotypes and were similar to what we observed in

heifers (Chapter 2 & 3), and what others observed with heifers and cows when LPS was administered by *iv* infusion or as a single *iv* bolus (Steiger et al., 1999; Waldron et al., 2003; Carroll et al., 2009; Jing et al., 2014; Ning et al., 2018). The LPS dose (0.25 µg/kg of LPS) used in our study was lower than what we previously administered to heifers and what others had administered to cattle. Our initial efforts (not reported) with 0.50 µg LPS/kg produced a rather severe response that included early signs of a septic shock. This was especially evident in a CH cow that produced essentially no milk during the 12 h following LPS administration. We did not want the dose to overwhelm the system, so we reduced the dose in order to better evaluate the impact of genotype on the innate immune responses.

Differences among studies in BT response after administration of similar or lower doses of LPS could be due to the *E. coli* serotype used. For example, *E. coli* O111:B4 serotype has been reported to generate a more potent hyperthermic response than other *E. coli* serotypes (Akarsu & Mamuk, 2007). However, it is not clear why similar BT changes occurred with a lower dose of a less potent serotype (055:B5) or why BT increased to the same extent in both challenges and therefore more research on this subject is needed.

Previous studies (Heriazon et al., 2011; Hine et al., 2012; Thompson-Crispi et al., 2013) reported that cows possessing a robust immune response, cell- and antibody-mediated, have a decreased occurrence of metabolic disorders and disease. Conversely, cows with an exacerbated TNF-α response to endotoxins, classified as “hyper-responsive,” are more likely to develop more severe clinical cases of infection and have longer recovery times (Elsasser et al., 2005). A characteristic of these hyper-responsive

animals is that their ability to develop tolerance to the endotoxin is reduced (Elsasser et al., 2005). Concentrations of TNF-α were greater in UH than CH cows during C1. However during C2, the response by UH cows decreased while response of CH cows did not differ from their response during C1. This indicates UH cows developed tolerance to the repeated LPS administration, and it also indicates that this might not have occurred in CH cows. Greater pro-inflammatory cytokine concentrations by UH cows after LPS does not represent a "hyper-response" as tolerance to LPS was developed in this genotype. Response in UH cows appeared rather as a prompt, regulated and short-lasting inflammatory reaction which could be advantageous since it can lead to a faster resolution of infections (Burvenich et al., 2003).

Dermal fibroblast have been used and accepted as an *in-vitro* model to investigate the innate immune response, as fibroblast responses to LPS appear to be consistent with the individual animal response to an *in-vivo* LPS challenge (Green et al., 2011). Although plasma IL-6 concentrations differed between UH and CH cows, IL-6 concentrations in media from dermal fibroblast cultures exposed to LPS or IL-1β for 24 h did not differ between the genotypes. In contrast, IL-8 concentrations in culture media were lower in UH than CH after IL-1β treatment. This response was the opposite of what we had previously observed in non-pregnant heifers, where fibroblasts from UH heifers tended ($P = 0.08$) to have greater concentrations of IL-8 after IL-1β treatment (Chapter 2). This difference in IL-8 response by fibroblasts could be due at least in part to differences in age of the animals from which they were collected (Green et al., 2011). Although a positive correlation has been reported for LPS and IL-1β response by dermal fibroblasts (Green et al., 2011) and the signal cascades from LPS and IL-1β recognition end in the

upregulation of NFκB responsive genes, the receptors (TLR4 for LPS and IL-1R for IL-1β) and their signaling pathways differ (Verstrepen et al., 2008). Differences in the relative presence of the components in the IL-1R pathway of the UH and CH cows could contribute to IL-8 concentration differences between the genotypes after IL-1β stimulation.

Interleukin-1β increases cellular antimicrobial activity and stimulates the production of other cytokines such as IL-6, IL-8 and TNF-α (Zanotti et al., 2002). Interleukin-8 is an important cytokine of the innate immune response that acts as a chemo-attractant of neutrophils (Arango Duque & Descoteaux, 2014). Kandasamy & Kerr (2012) classified "low-" and "high-responding" cows based on fibroblast media concentrations of IL-6 and IL-8 after 24 h of LPS or IL-1β exposure . Media concentrations of IL-8 after IL-1β treatment in our UH and CH cows would fit their classification as "low" and "high" responders, respectively. The authors suggested that the low-responding fibroblasts are associated with cows that have a more effective acute response. These results add to the premise that genetic selection altered the innate immune response of the contemporary Holstein cows, and suggest that these alterations might have been detrimental.

Circulating concentrations of acute phase proteins, SAA and Hp, also increased after LPS in both genotypes. Concentrations of both proteins were greatest at 24 h after LPS in both genotypes and challenges but Hp concentrations were also high in the saline cows during C2. Considerable variation in baselines, patterns and changes after endotoxin exist for APP in the literature (Jacobsen et al., 2004; Kahl & Elsasser, 2006; Carroll et al., 2009; Kvidera et al., 2016) and individual variation on Hp concentrations

on both saline and LPS treated cows (Jacobsen et al., 2005) had an impact on the final results.

Similar to previous reports (Jacobsen et al., 2004; Kahl & Elsasser, 2006; Carroll et al., 2009; Kvidera et al., 2016), serum SAA and Hp concentrations in our cows increased during C1 and C2. However, the lack of a decrease in APP concentrations during C2 relative to C1 disagrees with previous reports (Kahl & Elsasser, 2006). In addition, despite differences in serum pro-inflammatory cytokine concentrations between UH and CH cows, SAA and Hp concentrations did not differ between the genotypes. Additional investigation is needed to clarify the relationships and factors responsible for the cytokine and APP differences among these genotypes.

Repeated exposure of the host to an endotoxin results in the development of a tolerant state that regulates the magnitude and duration of the toxin-induced inflammatory response (Lopez-Collazo et al., 2013). Assessing responses to repeated endotoxin exposures during a 2 week interval (Suojala et al., 2008) allows evaluation of the innate immune system from activation through recovery and establishment of an endotoxin tolerant state (Kahl et al., 2011). Reduced concentrations of inflammatory markers in UH cows during C2 indicates tolerance and agrees with previous LPS-cattle studies where a repeated challenge approach was used (Kahl & Elsasser, 2006; Kahl et al., 2009, 2011). However, the similar inflammatory marker response during C1 and C2 by CH cows indicates the CH cows did not establish tolerance or that the time interval for this to occur differed from that of the UH cows. These results agree with our previous heifer study in that the mechanisms for establishing tolerance appear to have been altered by 50+ years of selection

Total WBC is a common and easy tool used to measure an animals relative immune status (Jacobsen et al., 2005; Kvidera et al., 2016; Horst et al., 2018). Leukopenia probably occurs due to the extravasation of leukocytes into tissues (Kvidera et al., 2016; Horst et al., 2018) and was evident 4 h after LPS. By 24 h, WBC exceeded pre-LPS means in both genotypes. Subsequent counts that exceeded baseline by 12 to 18 h after LPS administration was previously observed (Horst et al., 2018), and this response appears to be related to an increment in neutrophil counts. Increased number of neutrophils in circulation results from either increased bone marrow release, release from the pulmonary marginated pool to the circulating pool, or decreased endothelial adherence that impairs extravasation (Jagels & Hugli, 1994; Horst et al., 2018). Expression of the adhesion molecules, L-selectin and CD11b/CD18, at 24 h after LPS administration did not differ from pre-challenge means in either genotype so decreased endothelial adherence, however does not seem to be the reason in our study.

Expression of L-selectin decreased and of CD11b/CD18 increased after LPS administration in both genotypes during both challenges. Expression of CD11b/CD18 however, was greater in UH than CH cows after LPS. Cell adhesion molecules and integrins control the process of rolling, adhesion, and transendothelial migration of leukocytes into infected sites (Iwasaki and Medzhitov, 2004; Laudanna et al., 2002). Activated PMNL shed L-selectin as a prerequisite for β_2 integrin-mediated tight adhesion (Kishimoto et al., 1989), therefore decreased expression of this molecule after LPS was expected and agrees with previous observations (Diez-Fraile et al., 2003). The CD11b/CD18 (also known as Mac-1 and CR3) molecule is one of the most important β_2 integrins involved in PMNL recruitment into inflamed tissues. It is translocated from

cytoplasmic granules to the cell surface after cellular activation and this increased expression aids PMNL binding to bind endothelial cells through interaction with ICAM-1, its specific ligand (Rambeaud and Pighetti, 2005). Greater expression of CD11b/CD18 by PMNLs from UH cows at 4 and 24 h after LPS could indicate superior function compared to PMNLs from CH cows and this was supported by the greater phagocytic and oxidative burst capacities of UH cows. Deficiency of macrophage CD11b/CD18 expression has been correlated with a marked inhibition of NFkB nuclear translocation in response to LPS (Perera et al., 2001). Therefore expression of CD11b/CD18 is not only essential for PMNL migration but also for optimal signaling after LPS recognition, and greater CD11b/CD18 expression by UH cows after LPS probably marks an advantage that was lost through 50+ years of selection.

After *in-vitro* re-stimulation of PMNL with PMA, expression of CD11b/CD18 was greater in UH cows and this was mainly due to UH-saline cows that had greater expression of CD11b/CD18 than the rest of the groups after PMA. Despite not being administered with LPS, saline cows were subject to stressors like being moved and handled during challenge days, and even though cortisol concentrations did not fluctuate in saline cows, minimal changes (maybe out of the scope of sensitivity of the cortisol assay used) might have “primed” the PMNL in these cows. Primed cells do not change the expression of their surface molecules which explains why expression in saline treated cows did not differ before PMA stimulation at any sampling time. However, upon being re-stimulated with PMA the PMNLs become fully activated (Hallett & Lloyds, 1995; El-Benna et al., 2016). Priming could thus explain why expression of CD11b/CD18 after PMA was greater at 4 and 24 h compared to 0 h in the saline cows. The lack of a PMA

effect on CD11b/CD18 expression in LPS treated UH and CH cows indicates their cells were already stimulated by the LPS administration and the re-stimulation had no additional effect on expression. Greater CD11b/CD18 expression in PMNLs from UH-LPS cows and from PMA activated PMNLs from UH-saline cows supports the premise that UH cows have better PMNL function than CH cows when encountering a stimulus.

Activation of the acute inflammatory response includes as described a rise in BT, recruitment of immune cells like neutrophils and macrophages and also activation of the hypothalamic-pituitary-adrenal (HPA) axis with the release of cortisol (Turnbull & Rivier, 1999). Cortisol concentrations in response to LPS in this study increased in both genotypes as previously reported for heifers, cows and steers challenged with LPS (Steiger et al., 1999; Waldron et al., 2003; Carroll et al., 2009; Carroll et al., 2011). However, cortisol peaked sooner and returned to pre-challenge means later in UH than CH cows. Elevated concentrations of cortisol ensure mobilization and supply of nutrients to support the immune system during inflammatory situations such as the systemic immune response induced by LPS in the present study (Munksgaard et al., 2006). Therefore, an earlier peak of cortisol could indicate the endocrine system in UH cows was more adept than that of CH cows to support activation of the innate immune system.

A delayed return of cortisol concentrations to pre-challenge means by UH cows compared to CH (8 vs. 6 h) could be detrimental for UH cows because it has been suggested that a reduced cortisol response may allow animals to return to homeostasis earlier (Carroll et al., 2011). However, there was no indication that UH cows had a delayed return to homeostasis as all parameters measured, with the exception of IL-6, returned to pre-challenge means at the same time as CH cows.

The initial acute immune response is usually characterized by a rapid increase in glucose, a concomitant increase in insulin, and a subsequent anorexic phase with increased lipolysis followed by decreased gluconeogenesis (Steiger et al., 1999; Waldron et al., 2003; Burdick Sanchez et al., 2013). Although these metabolic changes typically occur in response to endotoxemia, only 50% of the CH cows had a hyperglycemic response during C1 and their response was considerably less than that of the UH cows. In addition, there were no changes in insulin concentrations in these CH cows after LPS administrations.

Increased glycogenolysis and gluconeogenesis are responsible for the initial hyperglycemia needed to meet immediate energy needs of the immune cell. Glucose is the preferred substrate for immune cell metabolism, up to 1.1 g of glucose/kg of $BW^{0.75}$/h is used by the activated immune system of lactating dairy cows (Pithon-Curi et al., 2004; Calder et al., 2007; Kvidera et al., 2017). Low glucose concentrations after LPS in CH cows is probably due to altered hepatic gluconeogenic capacity (Garcia et al., 2015). Previous work by Garcia et al. (2015) showed that hepatic gluconeogenesis is impaired during inflammation in dairy cows regardless of stage of lactation (early vs. mid lactation). However, glucose availability increased as expected in UH cows after LPS. Even though milk production decreased in UH and CH cows after LPS administration, milk yield remained greater in the CH cows. This greater production required more glucose which might explain why glucose did not spike in the CH cows. This greater competition for available energy could also help explain the less robust immune response in the CH cows. However, other studies with lactating contemporary cows have

demonstrated increases in blood glucose after LPS administration (Waldron et al., 2003; Kvidera et al., 2017) so the actual reason remains unclear.

Reduced glucose concentrations in blood can clearly have a detrimental effect when fighting against pathogens as readily available energy is needed for proper function of the activated immune system. Further research on the immunometabolic responses of the liver during inflammation in these genotypes is warranted. However, there was a trend for hepatic expression of ($P = 0.095$) pyruvate carboxylase, a key enzyme of gluconeogenesis, to be upregulated more in UH than in CH after LPS administration. Our plasma and gene expression (Chapter 5) data could indicate UH cows had greater gluconeogenic capacity than CH cows after LPS. Although immune cells can utilize NEFA for energy (Contreras & Sordillo, 2011), serum concentrations of NEFA were also lower in CH than in UH cows.

The insulin response mimicked the glucose responses as insulin increased after LPS in the UH but not in the CH cows. Consistent with our previous (Weber et al., 2007) serum IGF-I concentrations were greater in UH than in CH cows. Although IGF-I administration can alter aspects of the immune response (Inoue et al., 1998), response of IGF-I to LPS administration has been variable (Spurlock, 1997; Waldron et al., 2003) with no consistent pattern or biological explanation. The greater TNF-α, IL-6, glucose and insulin responses in UH cows indicate selection altered metabolic, endocrine and immune adaptations when these animals are challenged with LPS. Data appear to indicate selection has produced CH cows that have adapted to prioritize and maintain milk production at the expense of supporting the immune system.

6. CONCLUSION

Results from this study indicate selection has altered the ability of the cow to respond to immunological challenges. Differences in plasma concentrations of pro-inflammatory cytokines, glucose, insulin and cortisol, and PMNL adhesion molecules expression and activity after LPS between the genotypes support a more robust innate immune response in UH than in CH cows. In addition, results indicate a greater development of tolerance to immunotoxin stimulation in UH than CH cows. Results indicate selection has altered aspects on the innate immune system in Holsteins and provides insight about systemic immune (pro-inflammatory cytokines) and metabolic (glucose metabolism) parameters that were possibly altered by genetic selection. Additional research with these genotypes designed to examine the pathways identified in this work as influencing immune response in the contemporary cow could help elucidate avenues for adjusting genetic selection programs to increase health and well-being of the contemporary Holstein cow.

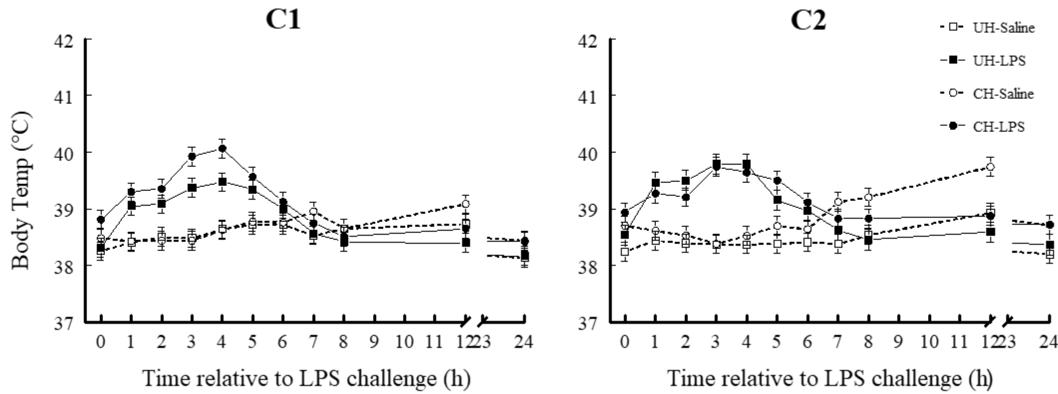

Figure 1. Rectal body temperature (BT) from unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 µg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). Data represent least square means ± SEM. There was a genotype by time interaction ($P = 0.009$) for BT as pre-challenge means were lower in UH than CH ($P < 0.001$), and a trend for UH to have lower BT ($P < 0.058$) at 5, 7 and 24 h after LPS than CH.

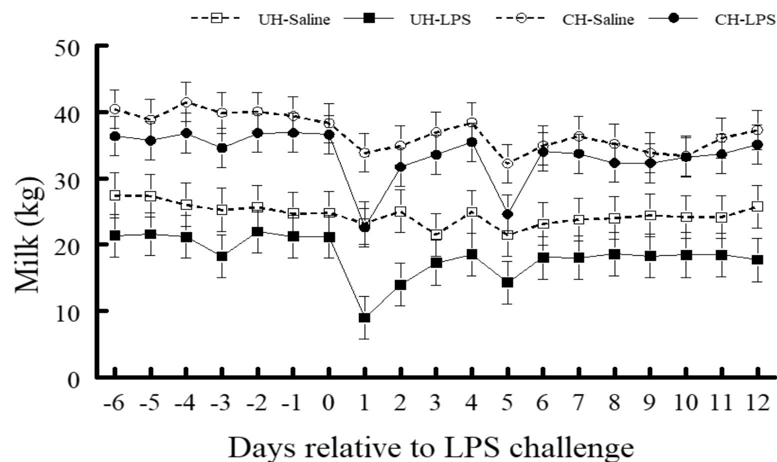

Figure 2. Milk yield in unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 µg LPS/kg BW on day 1 and 5. Data represent least square means ± SEM. Milk production by UH cows was lower than CH ($P < 0.001$). There a day by treatment interaction ($P < 0.001$) for milk yield as yield decreased after LPS administration in both genotypes and the decrease was more pronounced during C1 than C2 (day 1 vs. day 5: $P = 0.006$).

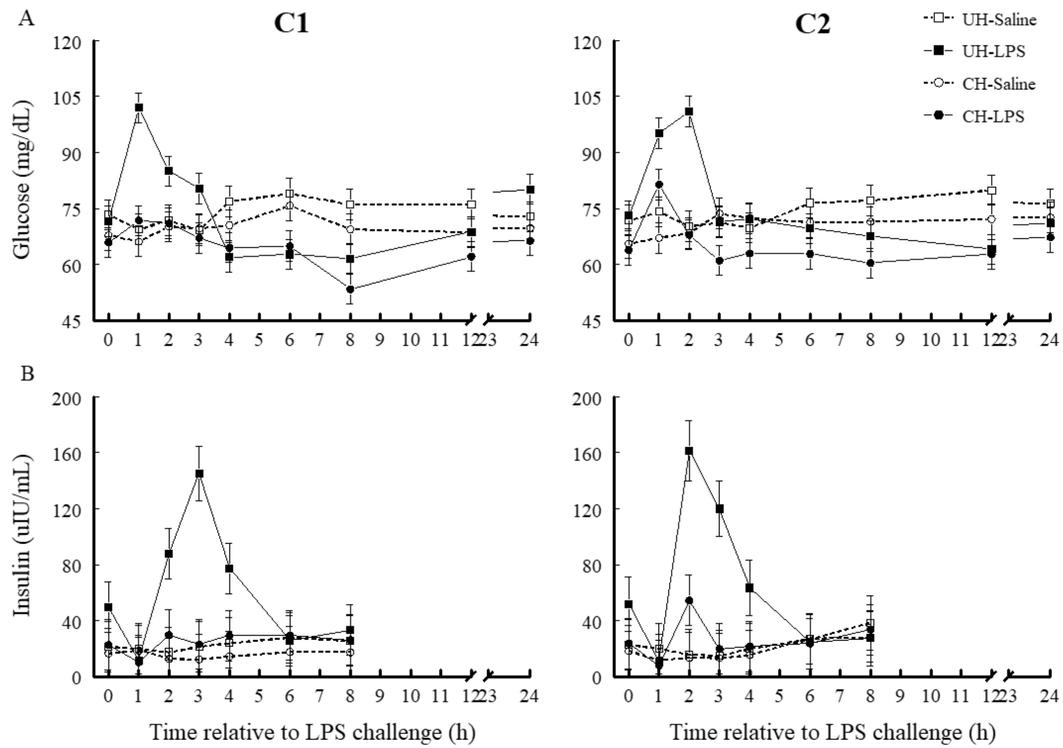

Figure 3. Plasma glucose (A) and insulin (B) concentrations in unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 µg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). Data represent least square means ± SEM. There was a genotype by time and treatment interaction ($P < 0.022$) for glucose and insulin. Glucose concentrations after LPS peaked higher and remained above pre-challenge mean longer in UH cows. There was also a trend ($P = 0.078$) for a genotype by time, treatment and challenge interaction for glucose as peak concentrations were greater in C1 than C2 for UH but lower for CH. Insulin concentrations were altered by LPS only in UH cows ($P < 0.01$) in both challenges. In UH LPS cows insulin concentrations peaked at 3 h during C1 and at 2 h during C2; by 6 h in both challenges concentrations were back to pre-challenge means.

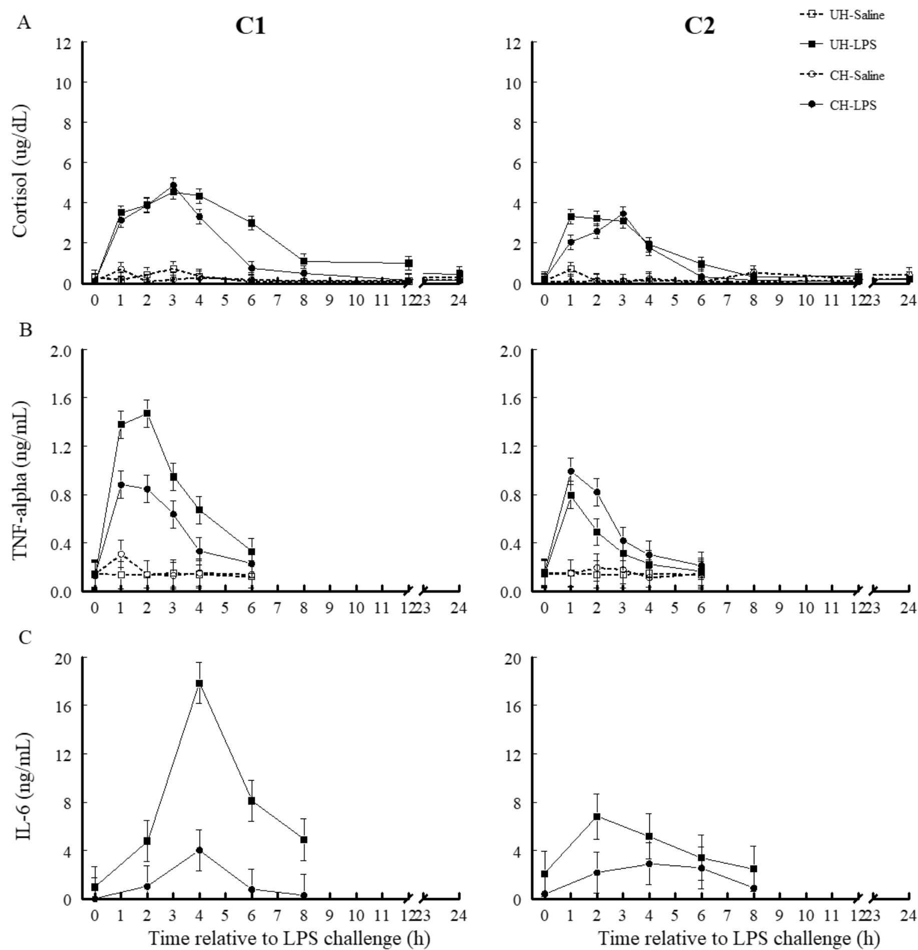

Figure 4. Plasma cortisol (A), TNF-α (B) and IL-6 (C) concentrations in unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 μg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). Data represent least square means ± SEM. There was a genotype by time and treatment interaction (P = 0.037) for cortisol as concentrations in UH increased 1 h post-LPS and remained high through 4 h while in CH, concentration did not peak until 3 h and had begun to decrease by 4 h. In addition, cortisol concentrations returned to pre-challenge means later in UH than CH (8 h vs. 6 h post-challenge, respectively). There was a genotype by treatment and challenge interaction ($P < 0.025$) for TNF-α and IL-6 concentrations. Both pro-inflammatory cytokines increased more in UH than in CH cows during C1. During C2, TNF-α did not differ ($P = 0.227$) between the genotypes and IL-6 only increased in UH cows after LPS. Concentrations of both cytokines were greater in UH during C1 than C2 while the overall response by CH did not differ between challenges.

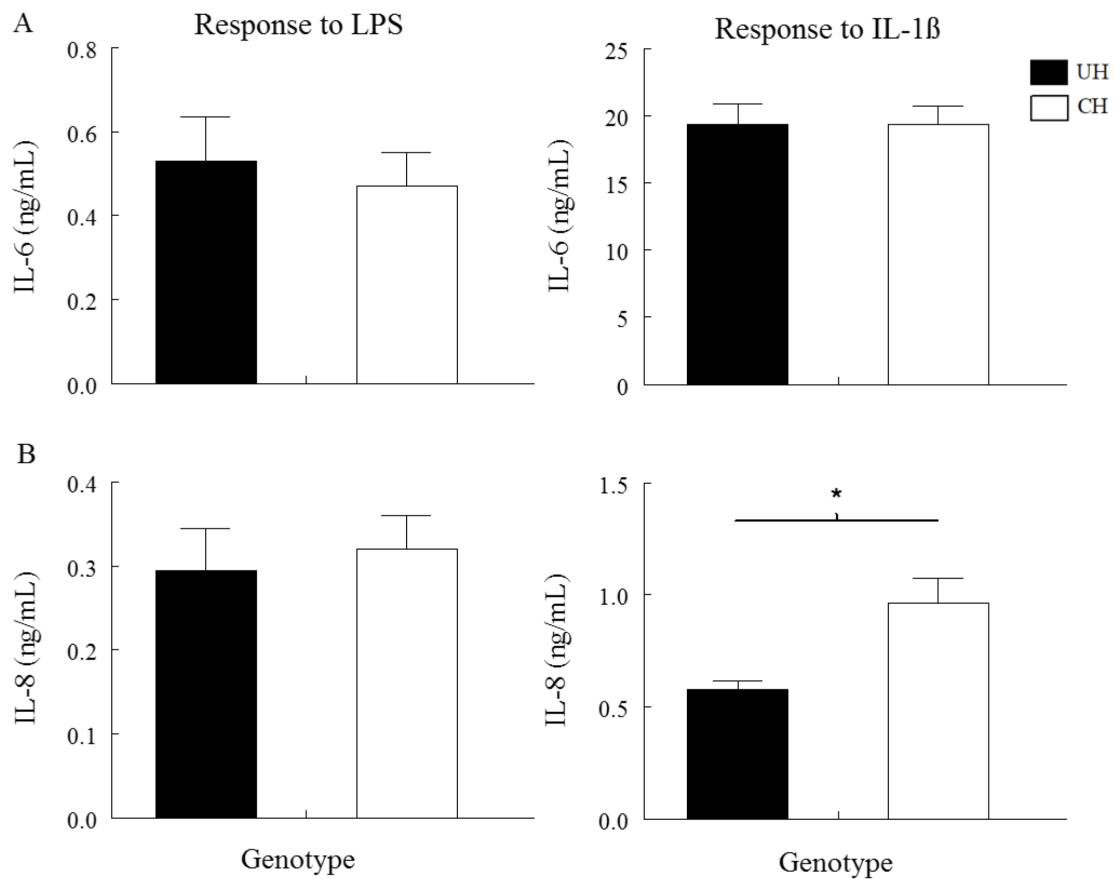

Figure 5. IL-6 (A) and IL-8 (B) protein production by cultured fibroblast in response to LPS (100 ng/mL) or IL-1β (1 ng/mL) treatment for 24 h. Fibroblast were obtained from unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive two consecutive, with 4 d apart, *iv* administrations of saline or 0.25 µg LPS/kg BW. Data represent least square means ± SEM. Fibroblast IL-6 protein production after both LPS and IL-1β exposure did not differ between the genotypes. IL-8 production was lower in UH than CH ($P < 0.010$) after IL-1β treatment but no significant differences were found for IL-8 between the genotypes after LPS treatment.

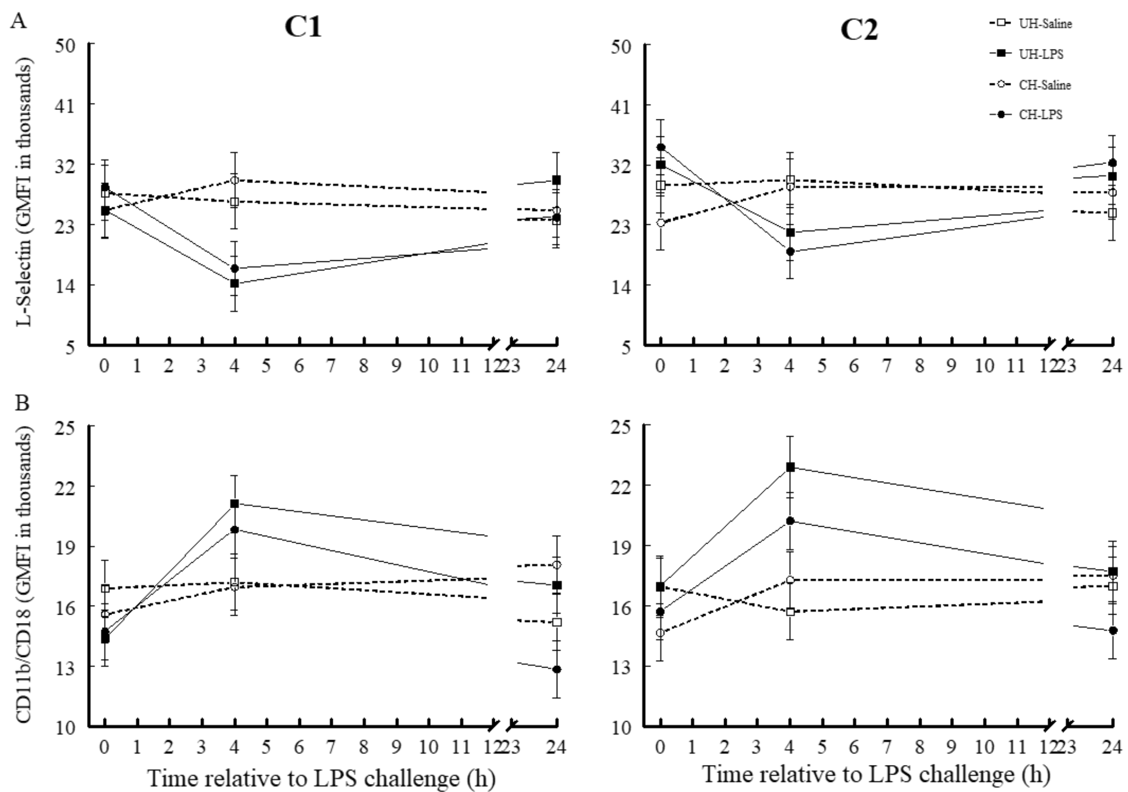

Figure 6. Expression of L-selectin (A) and CD11b/CD18 (B) on PMNL of unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 µg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). Data represent least square means ± SEM. Expression of L-selectin on PMNL decreased and of CD11b/CD18 increased ($P < 0.001$) in response to LPS at 4 h in both genotypes; by 24 h expression of both molecules did not differ from 0 h ($P = 0.379$). There was a genotype by time and treatment interaction ($P = 0.009$) for CD11b/CD18 as expression after LPS was greater in UH than CH at 4 and 24 h.

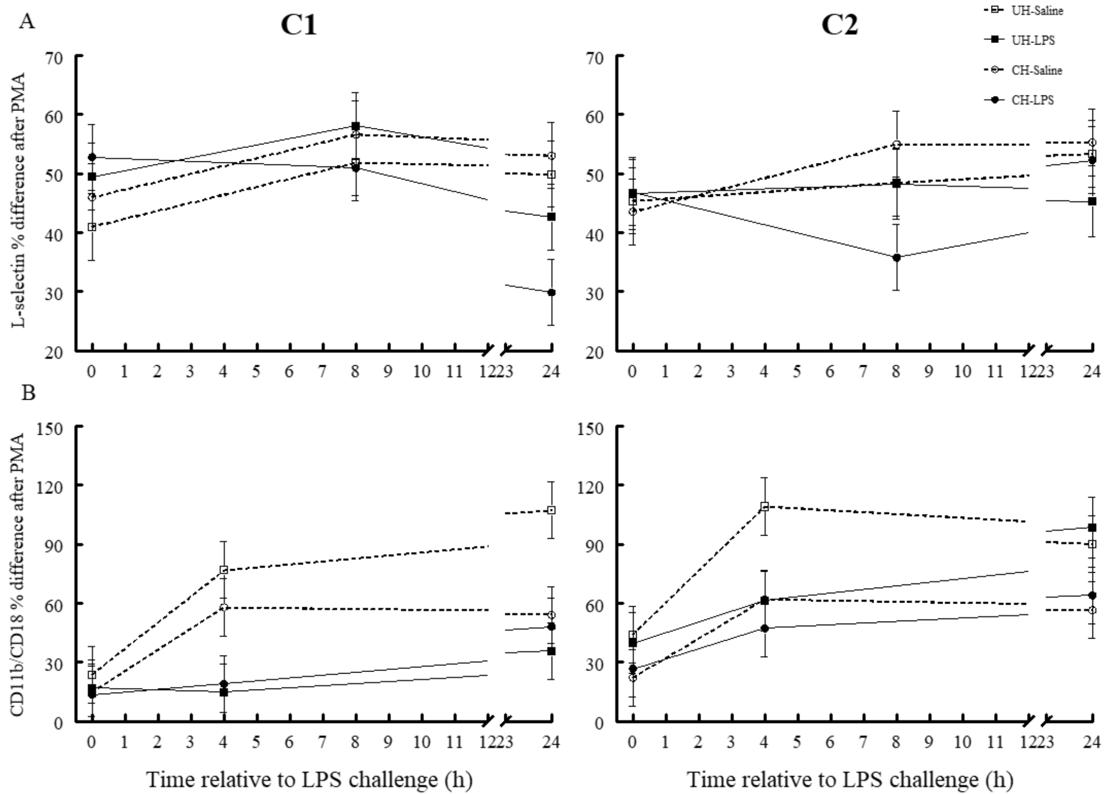

Figure 7. Expression difference of L-selectin (A) and CD11b/CD18 (B) after re-stimulation with PMA on PMNL of unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 μg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). There was no effect of genotype or any genotype interaction for expression of L-selectin when PMNLs were re-stimulated *in-vitro* with PMA, however, expression of CD11b/CD18 was greater in UH than in CH cows ($P = 0.003$). There were genotype by treatment ($P = 0.057$) and genotype by challenge ($P = 0.088$) trends for CD11b/CD18 after PMA activation, as expression was greater in UH saline treated cows than the rest of the groups and expression in UH was lower in C1 than in C2 ($P = 0.009$) while in CH expression did not differ between challenges.

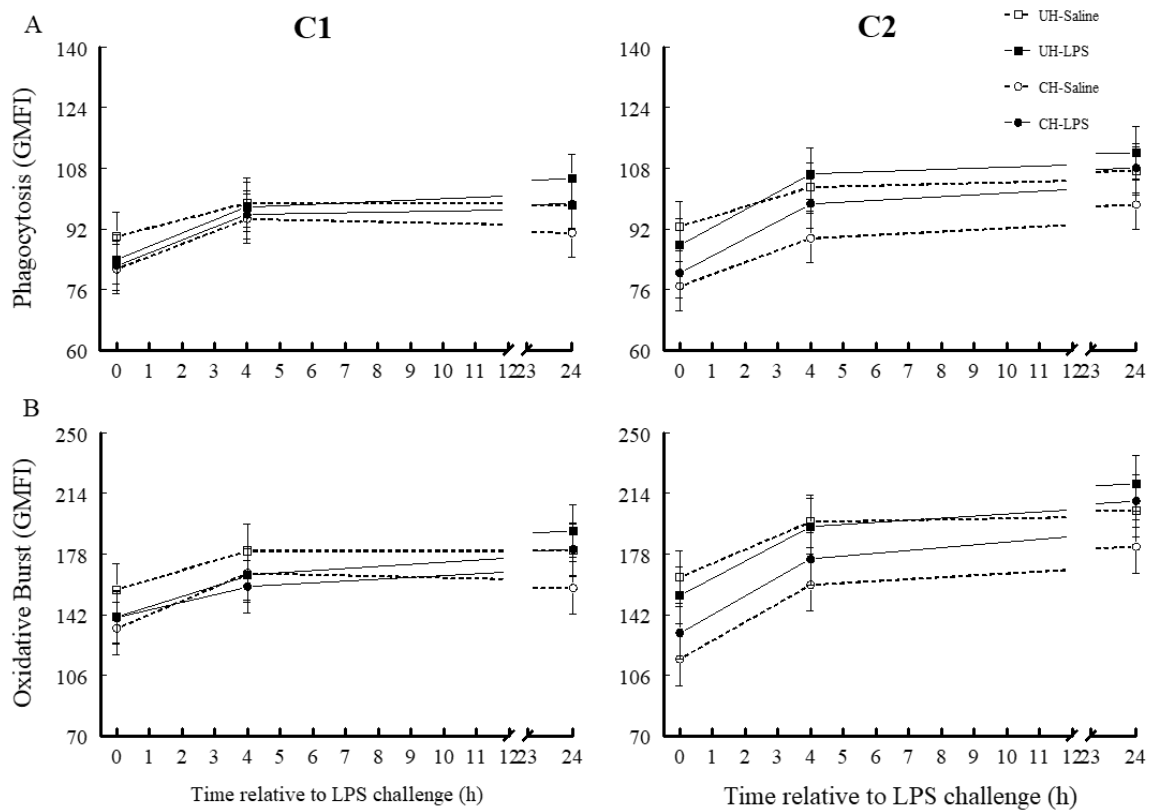

Figure 8. Phagocytic (A) and oxidative burst capacities (B) of PMNL of unselected (UH) and contemporary (CH) Holstein multiparous cows (n = 12/genotype) after being blocked (2/genotype) by DIM and randomly assigned within genotype to receive an identical *iv* administration of saline or 0.25 µg LPS/kg BW on day 1 (challenge 1; C1) and 5 (C2). Data represent least square means ± SEM. PMNL phagocytic capacity tended to be greater ($P = 0.058$) and oxidative burst was greater ($P = 0.046$) in UH than CH cows. Both were lower in C1 than C2 ($P < 0.087$). Phagocytic and oxidative burst capacities were not altered by LPS administration in either genotype ($P > 0.567$).

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CHAPTER 5: Milk Yield Genotype Affects Hepatic Gene Expression when Challenged with Lipopolysaccharide (LPS)

1. SYNOPSIS

Objectives were to determine effects of milk yield genotype on hepatic expression of genes related to innate immune response, somatotropic axis and glucose and lipid metabolism during a repeated LPS challenge. Multiparous cows (n = 10/genotype) from unselected (stable milk yield since 1964, UH) and contemporary (CH) Holsteins that differed by more than 4,500 kg milk/305-d were housed together and fed the same diet *ad lib* for more than 4 months before being blocked (2/genotype) by DIM and randomly assigned within genotype to receive *iv* saline or 0.25 μg/kg BW of LPS (Escherichia coli 055:B5). Cows were synchronized to be at day 8 of their estrous cycle for the first challenge (C1) at 70-84 DIM. Liver biopsies were collected at 0, 4 and 24 h relative to treatment. Acute innate immune responses were assessed in C1. A second identical challenge (C2) and sampling was conducted 4 d later to assess the impact of a repeated challenge. Expression of 67 genes associated with immunity and metabolism were determined by digital multiplexed analysis (nanoString nCounter). During the acute phase (C1), UH cows had a more robust expression of genes related with immune cell activation, cytokine and chemoattractant production than CH, however, expression of genes related to the somatotropic axis, glucose and lipid metabolism in response to LPS were similar for the low and high milk yield genotypes. Responses during C2 were diminished in both genotypes which indicate compensatory mechanisms invoked by C1 were still affecting the response to LPS. Results indicate milk yield genotype impacts the

response to LPS and contributes to a less robust immune response in the contemporary cow.

Keywords: gene expression; immunity; metabolism; bovine genotype; lipopolysaccharide

2. INTRODUCTION

Selective breeding practices have truly transformed the phenotypic and genotypic character of Holstein cows (Sonstegard et al., 2009). Over the past decades, the dairy industry has focused on maximizing milk yield (Sundrum, 2015) tremendously increasing milk and component yields per U.S. Holstein. Annual milk yield per cow increased over 4-fold in the last 75 years (Baumgard et al., 2017). These increases have undoubtedly benefitted producers and consumers in the United States and worldwide. It has improved production efficiency and profitability while also greatly reducing the impact of dairy production on the environment (Capper et al. 2009). However, these also has dramatically altered metabolic fluxes in cows to support the increased demand for nutrients to synthesize milk (Bauman, 2000) and there is great concern that genetic selection practices have made the contemporary cow more susceptible to disease and metabolic disorders, especially during the transition from pregnancy to lactation (Pritchard et al. 2013; Egger-Danner et al. 2015).

Unfavorable genetic correlations have been estimated between milk yield and diseases such as mastitis, milk fever, ketosis and retained placenta (Jones et al., 1994; Pryce et al., 1997; Schnitzenlehner et al., 1998; Lucy, 2001; Heringstad et al., 2005). As milk yield per cow increases, endocrine and metabolic adaptations occur to enable the cow to meet the metabolic demands of increased milk synthesis (Crooker et al. 2001), and

these adaptations can disrupt the immune system, compromising cow health and well-being (Pritchard et al., 2013; Egger-Danner et al., 2015). In high yielding cows, the immune status can be even further weakened due to augmented metabolic demands that support increased production (Crooker et al., 2001; Pritchard et al., 2013).

The immune dysfunction around the transition period provides opportunistic bacteria a greater opportunity to establish infections, including mastitis, metritis, and pneumonia (Burvenich et al., 1994) disrupting production, animal welfare and profits. Previous research with different milk yield genotypes (UMN unselected vs. contemporary Holstein cows; Jones et al., 1994; Weber et al., 2007) has shown that selection practices have altered circulating concentrations of endocrine and metabolic components (Weber et al., 2007), and that contemporary Holsteins have a greater need for mastitis treatments (Jones et al., 1994).

During bacterial infections, toll-like receptors (TLRs) on host cell membranes recognize conserved bacterial motifs (pathogen associated molecular patterns, PAMP) and activate signaling pathways that initiate the innate immune response (Kopp & Medzhitov, 2003). Detection of lipopolysaccharide (LPS) occurs via Toll-like receptor 4 (TLR4) and stimulation of the TLR4 pathway elicits an immune response that activates inflammatory pathways that aim to resolve the infection. However, excessive inflammation with chronic pro-inflammatory cytokines production can cause metabolic changes, including anorexia, lipomobilization, reduced insulin sensitivity and impaired hepatic function (Kushibiki et al., 2003; Bertoni & Trevisi, 2004; Bertoni et al., 2008). These changes predisposes the cow to metabolic disorders, including the metabolic syndrome (Dallmeier et al., 2012), nonalcoholic fatty liver disease and steatosis (Liu et

al., 2010). Estimates indicate 40 to 60% of high-producing contemporary dairy cows develop moderate to severe fatty liver during early lactation (Jorritsma et al., 2001; Starke et al., 2011).

The liver needs to adapt to the nutritional changes and increased metabolic demands of early lactation to redirect nutrients toward milk synthesis and minimize health disorders (Drackley, Overton, & Douglas, 2001). Regulation of hepatic transcription is an important mechanism of inflammation and metabolic control. Therefore, the primary objective of this study was to determine the impact of milk yield genotype on hepatic expression of genes related to the somatotropic axis, glucose and lipid metabolism and innate immune response during a double LPS challenge. Unique Holstein cows that have not been subjected to selection since 1964 (unselected Holsteins, UH; Weber et al., 2007) and contemporary Holsteins (CH) cows were challenged with identical LPS treatments on day 1 and 5 of study to assess the impact of genotype on hepatic gene expression. This animal model and a provocative double LPS challenge provide the opportunity to assess the impact of 50 years of selection on the hepatic transcriptional adaptations that support an acute innate immune response as well as the development of a refractory (endotoxin tolerance) state.

3. MATERIAL AND METHODS

3.1. Animals, Experimental Design, and Treatments

Cows were from two genetic lines of Holsteins. Development of the static, unselected (UH) and a contemporary (CH) Holstein was initiated in 1964 by Dr. Charles Young as a component of a multistate, north central regional project (NC-2; Young, 1977; Weber et al., 2007). Genetic merit for milk yield of the UH cows has remained

stable, while that of CH cows has continued to increase and is similar to that of U.S. Holsteins. Current milk yield of UH cows is about 55% of CH cows (6,700 vs. 12,000 kg/305 d lactation).

Twenty multiparous cows (n = 10/genotype) were fed the same diet ad lib and housed together for more than 4 months before being assigned to one of 5 blocks (2/genotype) by DIM and randomly assigned within genotype to receive an *iv* administration of saline or 0.25 μg LPS (*Escherichia coli* 055:B5, Sigma-Aldrich L2880) per kg BW on d 1 (challenge 1, C1) and d 5 (C2) of study. Saline treatments were incorporated to have a real assessment of the impact of LPS in hepatic gene expression without the confounding effect of repeated liver biopsies. Treatment assignments were not switched between challenges so C1 and C2 were identical. Cows were between 70 and 84 DIM on d 1. Estrous cycles were synchronized following the 5-day CIDR Synch protocol (Bridges et al., 2008) so that cows were at day 8 of their estrous cycle on d 1. Cows were observed daily throughout the study and health abnormalities treated when warranted. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

3.2. Samples, mRNA Extraction and Quality Assessment

Liver biopsies (Carriquiry et al., 2009) were obtained at 0, 4 and 24 h relative to treatment administration. Samples were aliquoted and either immediately frozen in liquid nitrogen and stored at −80 °C or placed in RNA*later* (100 mg of tissue/mL; Ambion, Austin, TX) and stored for 7 to 21 d at −20 °C before RNA*later* was removed and tissue stored at −80 °C until mRNA isolation.

Hepatic mRNA was extracted from 50 mg of tissue using RNeasy kits (Qiagen, Valencia, CA). Quantity and purity of the extracted mRNA were estimated using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies Inc., Wilmington, DE) at 260/280 and 260/230 nm absorbance waves. An aliquot of each RNA sample was diluted in RNase- DNase-free water (Qiagen, Valencia, CA) to achieve a consistent sample concentration of 100 ng/μL and stored at −80 °C. Purity and integrity of all mRNA isolates were assessed with an Agilent Nano chip and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) prior to gene expression analysis. All samples had an RNA integrity number (RIN) of > 7.

3.3. Gene Expression Analysis and Data Normalization

Expression of 75 genes (Suppl. 1) was assessed with NanoString nCounter® System (NanoString Technologies, Seattle, WA; Kulkarni 2011) and the nSolver™ 2.0 Analysis Software (NanoString Technologies, Seattle, WA). NormFinder software (Andersen et al., 2004) was used to evaluate 8 candidate reference genes (Suppl. 1) for stable expression throughout the range of sample mRNA counts for genes of interest. A combination of GAK, PPIA, SNAPC2 and *UXT* (Table 1) was identified as the most appropriate to normalize the gene expression data.

3.4. Statistical Analyses

Statistical analyses were conducted using SAS version 9.3 (SAS/STAT, SAS Inst. Inc., Cary, NC). All expression data were square root transformed and analyzed as a randomized block design by repeated measures using the MIXED procedure with time as the repeated effect. Models included genotype, time, and treatment with or without challenge as main effects. All interactions were included as fixed effects. The spatial

power law for unequally spaced data was specified as the covariance structure (except for CCL20 C1-only analysis where compound symmetry was specified as the covariance structure). Challenge 1 represents the acute responses to LPS and C2 represents results during a refractory period when the immune response was expected to be reduced. Although the least squares means are not affected, the effect of challenge and its interactions with genotype, time, and treatment were not always significant even though the responses in C1 and C2 represent distinctly different physiological conditions. This lack of interaction negated, in a strict statistical interpretation, the opportunity to assess some comparisons of biological importance during C1. Therefore, C1 data were analyzed separately to avoid the biologically inappropriate impact of the expected reduced response during C2 on interpretation of the C1 data. Using the combined C1 and C2 data was biologically appropriate to assess the attenuated responses during C2 because response during this refractory period is affected by the response during C1 (Ziegler-Heitbrock, 1995). However, discussion of the combined analysis is restricted to the C2 data. Results are reported as least squares means of the square root transformed total digital counts. Significance for the acute phase data is reported from the model that did not include challenge (C1 data only) and significance for the refractory phase data is reported from the model with challenge (C1 and C2 data). Means for C1 data obviously were not affected by the model and all comparable means were considered to differ when $P \leq 0.05$. Trends were identified when $0.05 < P \leq 0.10$.

4. RESULTS

Gene expression differed or tended to differ between genotypes for 12 immune-related genes during C1 and for 15 (9 immune-related and 6 metabolic-related) genes

when both challenges were analyzed together ($P < 0.10$; Table 2). There were genotype by time, genotype by treatment and genotype by time and treatment interactions or trends for 17, 7, and 11 genes respectively during C1 ($P < 0.10$; Suppl. 3). When both challenges were analyzed together, these numbers were 3, 7 and 1, respectively ($P < 0.10$; Suppl. 4). There were time by treatment interactions for 52 genes during C1 and 42 genes when C2 was included in the statistical model. When both challenges were analyzed together, there were 17 T*Trt*C, 5 G*T*C, 2 G*Trt*C and 2 G*T*Trt*C interactions ($P < 0.05$).

4.1. Immune-related genes - Acute phase (C1-only)

During C1 expression of 21 of the 49 immune related genes were affected by genotype interactions with time, treatment or both (Table 3; $P < 0.10$). These genotype interactions were due to the fact that at 4 h, all of these genes (Table 3), with the exception of CSF3, IL4, IL17C, IL17RC, SAA3 and SOCS3, had greater expression in UH relative to CH after LPS administration.

Expression of CSF3, IL4 and IL17C was very low but there was a genotype by time interaction (Table 3; $P < 0.03$) for each because their expression was greater in UH relative to CH at 4 h and greater in CH relative to UH at 24 h (Suppl. 2 & 3). Expression of IL17RC was greater ($P < 0.05$) in UH-LPS cows relative to CH-LPS cows before LPS administration (Suppl. 2 & 3). After LPS administration, expression of IL17RC decreased in UH cows and increased in CH cows at 24 h ($P < 0.05$; Suppl. 2). Expression of IL17RC increased ($P < 0.05$) in CH saline cows at 4 h but was unchanged ($P > 0.10$) in UH saline cows. Expression of SAA3 increased in both genotypes 4 h after LPS administration, and although expression from 4 to 24 h was unchanged in UH cows, it

continued to increase through 24 h in CH cows (Suppl. 2 & 3). Expression of SOCS3 increased at 4 h after LPS ($P = 0.01$) and returned to pre-challenge expression at 24 h in UH cows but was not altered in CH LPS cows (Suppl. 2 & 3). Expression of SOCS3 was greater ($P < 0.05$) at 4 and 24 h than at 0 h in CH saline cows and there was a trend ($P < 0.10$) for greater expression at 4 h than at 24 h in UH saline cows (Suppl. 2 & 3).

There was a genotype by time and/or treatment effect (Table 3) on genes associated with the TLR4 activation pathway (TLR4, CD14, MYD88 and NFKB1) and for genes associated with pro and anti-inflammatory cytokine production (IL1RN, IL6, TGFB1 and TNF). These genes are important in LPS recognition and in immune system activation and regulation and had greater expression in UH relative to CH 4 h after LPS (Fig. 1, 2 and 3).

4.2. Immune-related genes - Refractory phase (C1 and C2)

Analysis of the refractory phase results indicated expression of 8 of the 49 immune-related genes was greater during C1 than C2 (Suppl. 2 & 4; $P < 0.10$) and only CASP1 was expressed more in C2 than in C1 (P = 0.01). The only genotype by challenge effect ($P < 0.01$) occurred for CCL20 which was expressed more during C1 than C2 in UH cows (Fig. 2; Suppl. 2 & 4; $P < 0.01$) but did not differ between challenges in CH cows ($P = 0.60$). There was also a genotype by treatment and challenge interaction ($P < 0.01$) for CCL20 as expression after LPS was greater in UH than CH cows during C1 and lower in UH than CH cows during C2.

Expression of 6 immune related genes was affected by genotype interactions with time or treatment (Table 4; $P < 0.10$) and only S100A12 was affected by a genotype by time and treatment interaction (Fig. 4; $P = 0.04$). Expression of S100A12 increased 4 h

after LPS administration in both genotypes and challenges, however at 24 h expression decreased in UH while in CH expression remained elevated. There were genotype by time, treatment and challenge interactions ($P < 0.03$) for CASP1 and SOCS3 expression and a trend for these interactions for FGF21, IL1B and IL17RC (Suppl. 2 & 4; $P < 0.10$). Expression of CASP1 increased at 4 h in both genotypes during C1 but only increased in CH cows ($P = 0.04$) during C2. Expression of SOCS3 after LPS increased at 4 h in UH cows only during C1 ($P = 0.01$) and in CH cows only during C2 ($P = 0.01$). Expression of SOCS3 did not change in saline cows during C2 ($P > 0.10$).

Expression of FGF21 did not differ between genotypes and was not altered ($P > 0.11$) by LPS during C1, however expression of FGF21 at 24 h tended to be lower ($P = 0.08$) in CH LPS compared to CH saline cows. During C2, expression of FGF21 at 0 h was greater in UH LPS cows ($P < 0.05$) but there were no other differences between the genotypes during this challenge (Suppl. 2 & 4). Expression of IL1B during C1 increased in both genotypes 4 h after LPS and returned to pre-challenge means by 24 h (Fig. 2; Suppl. 2 & 4; $P < 0.01$). During C2, expression of IL1B was greater in UH relative to CH cows at 0 h ($P < 0.01$) and after LPS administration, expression of IL1B decreased in UH cows (Suppl. 2, 3 & 4; $P > 0.10$) and increased in CH cows at 4h ($P = 0.01$). Expression of IL17RC during C2 tended to decrease at 4h in UH but not CH cows after LPS administration ($P = 0.06$).

4.3. Metabolic-related genes - Acute phase (C1-only)

During C1 expression of 2 of the 18 metabolic related genes, ACOX1 and IGF1R, were affected by genotype interactions with time and treatment (Suppl. 2 & 3; $P < 0.04$) while there was only a trend ($P = 0.09$) for INSR-b. Expression of ACOX1 tended ($P =$

0.06) to be greater in UH relative to CH cows 4 h after LPS administration. However, counts at 4 h did not differ from those at 0 h for either genotype ($P > 0.22$). Expression of IGF1R decreased in CH ($P < 0.01$) and tended ($P = 0.08$) to be lower in UH than in CH cows 4 h after LPS administration. However, counts at 4 h did not differ between the genotypes ($P = 0.34$). Expression of IGF1R increased at 4 h ($P = 0.01$) and tended to be greater at 24 than at 0 h ($P = 0.09$) in CH than in UH saline cows. Expression of INSR-b decreased 4 h after LPS administration ($P < 0.01$) in both genotypes and by 24 h had returned to pre-challenge expression in CH cows but remained low in UH cows ($P = 0.02$). Expression of INSR-b increased in CH saline cows at 4 h ($P = 0.01$).

Expression of IGFBP3 had a genotype by time trend ($P = 0.09$) as it increased 4 h after LPS administration in both genotypes ($P < 0.03$) but returned to pre-challenge means at 24 h in UH cows but not in CH cows. Expression of XDH had a genotype by treatment trend ($P = 0.07$) as expression after LPS administration was greater in UH relative to CH cows (Suppl. 2 & 3).

4.4. Metabolic-related genes – Refractory phase (C1 and C2)

With the combined model, expression of 5 of the 18 metabolic-related genes was greater during C1 than C2 (Suppl. 2 & 4; $P < 0.10$) and IGFBP5 was expressed more in C2 than in C1 ($P = 0.03$). None of the metabolic-related genes presented a genotype by challenge effect.

There was a genotype by treatment interaction for XDH ($P = 0.03$) and a trend for PC1 ($P = 0.095$) as expression of both genes increased after LPS in UH cows only. None of the 18 metabolic-related genes showed a significant interaction of G*T, G*T*Trt or G*T*Trt*C (Suppl. 2 & 4; $P > 0.10$).

5. DISCUSSION

Selection practices have truly transformed the phenotypic and genotypic character of the Holstein cow (Sonstegard et al., 2009) and have tremendously increased milk and component yields per U.S. Holstein since the 1960s. These increases have dramatically altered metabolic fluxes in the cow to support the increased demand for nutrients to synthesize milk (Bauman, 2000). Greater partitioning of available nutrients and energy towards milk production can decrease the amount available for other physiological functions (Bauman and Currie, 1980; Esposito et al., 2014; Baumgard et al., 2017). There is concern that contemporary cows are more prone to disease and metabolic disorders than their ancestors (Pritchard et al. 2013; Egger-Danner et al., 2015). Multiparous unselected and contemporary Holsteins that differed by more than 4,500 kg milk/305-d (Weber et al., 2007) were used to examine the effects of selection for milk yield on hepatic expression of genes when the cows were treated with LPS. The LPS was administered to generate an acute innate immune response and again 4 d later when responses were reduced due to the development of endotoxin tolerance.

A systemic inflammatory response was achieved by LPS administrations, as evidenced by changes in gene expression as well as the increased rectal temperature and pro-inflammatory cytokine concentrations in blood from these cows (Chapter 4). The upregulation of genes of the TLR4 signaling pathway and of genes that encode pro- and anti-inflammatory cytokine production (Table 3; Figure 1-3) indicate the acute immune response was activated during C1 and C2 in both genotypes. Interestingly, the magnitude of upregulation of these genes was greater in UH than in CH cows during C1 but these genotype differences were not observed during C2, the refractory phase.

Upon pathogen recognition, pathogen recognition receptors (PRRs) such as TLR4 signal the presence of the PAMP to the host and trigger pro-inflammatory and antimicrobial responses by activating intracellular signaling pathways (Akira & Takeda, 2004). The induced transduction pathways result in the activation of genes that lead the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira et al., 2006), which together initiate the innate immune response (Mogensen, 2009). Greater expression in UH cows of critical genes involved in the recognition of Gram-negative bacteria such as TLR4 and CD14, and of genes involved in immune activation, such as MYD88, NFKB1, TNF and IL6, indicates this genotype elicited a more robust innate response than CH cows. Benjamin et al. (2016) identified high vs. low response phenotypes from fibroblast of young vs. old Holstein and Holstein vs. Angus after LPS stimulation. The higher response phenotype was characterized by greater expression of TLR4, IL8, CCL2, CCL5, CCL20 and TNF. Although IL8 and CCL2 were not included in our analysis, and CCL5 expression showed no differences between genotypes (Suppl. 2 & 3), greater expression of TLR4, CCL20, TNF, and other immune-related genes in UH during C1 (Table 3) points to UH cows as high responders and CH cows as low responders. Benjamin et al. (2016) concluded that high response animals are potentially more likely to develop more severe cases of infection as observed in their studies with cases of coliform mastitis. However, a prompt but regulated and short-lasting inflammatory reaction is considered advantageous for early resolution of infection (Burvenich et al., 2003). Having greater expression of genes involved in inflammation during the acute phase of the reaction with a return to pre-challenge expression means by 24 h might indicate the UH cows are more adept in

pathogen recognition and immune activation than the CH cows which could lead to a more rapid pathogen cleaning and resolution of infection.

A high response has also been associated with "hyper-responsive" animals (Elsasser et al., 2005). These animals produce more TNF-α, are more likely to develop severe cases of infection, have longer recovery times and fail to establish immune tolerance to repeated endotoxin (LPS) administration (Elsasser et al., 2005). Permanent or repetitive exposure of the host immune cells to even low doses of endotoxins leads to a state of hypo-responsiveness known as endotoxin tolerance (López-Collazo & Del Fresno, 2013). This state is characterized by lack of pro-inflammatory mediator production after re-stimulation with the endotoxin. Lower expression of up-regulated genes during C2 compared to C1 was observed in both of our genotypes. The UH cows had greater expression during the acute phase (C1) and this resulted in greater plasma concentrations of pro-inflammatory cytokine in UH than in CH cows (Chapter 4). However, during C2, the expression of these genes and blood concentrations of cytokines were less than during C1, indicating that immune tolerance was stablished in this genotype. Developing tolerance to the repeated LPS administration does not agree with the hyper-response defined by Elsasser et al. (2005). This indicates neither the UH or the CH cows in our study would be classified as hyper-responsive. Further research is needed to clarify whether a high or low response phenotype has an advantage during acute, repeated or prolonged exposure to endotoxins.

Several mechanisms are involved in the induction of endotoxin tolerance, and although a full understanding of this phenomenon remains elusive (López-Collazo & Del Fresno, 2013), part of the immune suppression observed during this period is due to the

anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β (Thomson & Knolle, 2010) that are coded by the IL10 and TGFB1 genes. Activation of TLR4 by LPS triggers the expression of these anti-inflammatory cytokines which function to suppress pro-inflammatory cytokine production (Thomson & Knolle, 2010) and thus protect the host from LPS-induced toxicity (Pengal et al., 2006). In our study, expression of IL10 increased more during C1 than C2 but there were no differences between the genotypes. Conversely, TGFB1 expression was not altered by the repeated challenge but there was a genotype by treatment interaction in both statistical analyses (C1-only and C1 and C2; Table 3 & 4; Fig. 3) as its expression after LPS was greater in UH than in CH cows at 4 h in both challenges.

Binding of ligands to TGF-β initiates signaling by binding to serine/threonine kinases receptors on the cell surface activating downstream phosphorylation of the Smad proteins which translocate into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes (Shi & Massagué, 2003). TGF-β signaling controls a diverse set of cellular processes, including cell proliferation, recognition, differentiation and apoptosis (Shi & Massagué, 2003). It also has a pivotal role in peripheral tolerance, assuring that self-reactive T cells do not cause autoimmunity (Li & Flavell, 2008). More recently TGFβ1 gene has been identified as a negative regulator of the TLR-induced inflammation (Yang et al., 2012) highlighting its potential as a target for the treatment of inflammatory diseases in humans (Yang et al., 2015). Greater expression of TGFB1 in UH cows after LPS could indicate differences in the anti-inflammatory response by these genotypes. Moreover, genotype differences in plasma concentrations of pro-inflammatory cytokines (Chapter 4) and TGFβ1 expression

might contribute to the differences between these genotypes in their development of tolerance to endotoxin.

Another interesting gene which expression differed between the genotypes and challenges was CCL20. Expression of CCL20 increased after LPS in both genotypes and challenges, however, compared to C1 expression during C2 was lower in UH while there were no differences between challenges for CH. In addition, expression of CCL20 was greater in CH than UH cows during C2. CCL20 gene codes for CCL20 chemokine. Chemokines are a family of small cytokines which have the properties of both chemotactic mediators and cytokines (Luster, 1998). CCL20 is the only chemokine known to interact with the CC chemokine receptor 6 (CCR6). The ligand-receptor pair CCL20-CCR6 is responsible for the chemoattraction of immature dendritic cells, T-cells and B-cells (Schutyser et al., 2003) to inflammation sites. However, hepatic expression of CCL20 and serum concentrations of CCL20 in humans and mice have been associated with disease severity, inflammation and hepatocellular injury as CCL20 mediates infiltration of immune cells into the injured liver exacerbating inflammation (Affò et al., 2014). Expression of CCL20 was greater during C2 in CH-LPS cows and although the significance of greater expression during consecutive challenges is not clear, it could indicate that during C2, CH cows had more hepatocyte injury with subsequent increased immune cells infiltration after LPS.

There were no physiological signs of hepatic injury in either genotype or challenge, however, another gene, CASP1, also had greater expression in CH cows after LPS in C2. CASP1 codes for caspase-1, a cysteine protease involved in cellular death by apoptosis and pyroptosis (Miao et al., 2011). Greater expression of both CCL20 and

CASP1 by CH cows during C2 could therefore indicate hepatocyte injury. Conversely, expression of ICAM1, gene that codes for the intracellular adhesion molecule ICAM-1, which is involved in leukocyte transmigration into tissues (Dustin et al., 1986) did not differ between the genotypes during C2. This indicates immune cell infiltration into the liver, and thus hepatic injury, might not have differed between the genotypes.

Caspase-1 is the central effector protein of the inflammasome complex and when activated by LPS it participates in the cleavage of the IL-1β precursor protein (Schumann et al., 1998). Greater expression of CASP1 by CH cows during C2 could therefore be associated with the greater IL1B gene expression by CH cows after LPS during this challenge. Interestingly, although IL1B expression patterns differed between the genotypes during C2, expression of IL1RN, which codes for the interleukin-1 receptor antagonist (IL-1Ra), did not differ between the genotypes during this challenge. IL-1Ra binds to IL-1 receptors but does not induce any intracellular signals (Lamacchia et al., 2010). However, by binding to the receptors it functions as an endogenous negative feedback down-regulator of the pro-inflammatory effects of IL-1 (Lamacchia et al., 2010). These differences in expression between genotypes indicate hepatic production of IL-1 and its antagonist differ between UH and CH cows. This, in addition to the greater expression of TGFB1 in UH cows, seems to indicate that the anti-inflammatory mechanisms between these genotypes are different.

In connection with anti-inflammatory mechanisms, expression of SOCS3 (suppressor of cytokine signaling-3) also differed between genotypes. After LPS administration, SOCS3 expression increased in UH cows during C1 and in CH cows during C2. Expression of SOCS3 also increased in saline cows during C1. SOCS3

regulates cytokine or hormone signaling by binding to the JAK kinase and cytokine receptors, which results in the inhibition of STAT3 activation (Carow & Rottenberg, 2014). There was a genotype by time and treatment trend (Table 3) for JAK2 expression due to greater expression in UH-LPS cows during C1 at 4 h that could be associated with the greater expression of SOCS3 in these cows. However, expression of STAT3, which increased after LPS, did not differ between the genotypes (Suppl. 2-4). Several cytokines, growth factors and hormones utilize the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway to transmit their information into the cell nucleus (O'Shea & Murray, 2008; Cai et al., 2015). Therefore, SOCS3 is crucial in restraining inflammation and regulating optimal immune responses to protect against infections (Carow & Rottenberg, 2014). The UH cows had greater expression of genes that code for pro-inflammatory cytokines and greater plasma concentrations of TNF-α and IL-6 during C1 (Chapter 4). However, greater expression of SCOS3 and a trend for JAK2 during this challenge probably controlled the responses of these cytokines regulating its production. The reason behind a greater expression of SOCS3 during C2 in CH cows and expression changes in saline cows are not clear.

The S100A12 gene was the only gene that had a genotype by time and treatment interaction when both challenges were included in the statistical analysis. An up-regulation of S100A12 expression after LPS administrations as observed in our genotypes agrees with previous studies in piglets (Xia et al., 2018) and with bovine mammary epithelial cells (Strandberg Lutzow et al., 2008). However, in our study expression of S100A12 at 24 h was reduced in UH cows while it remained high in CH cows. The S100 proteins are small, acidic, calcium-binding proteins that exert

antimicrobial effects and have been used as markers of inflammation (Donato et al., 2013). The S100A12 gene is considered a pro-inflammatory factor that is up-regulated at sites of inflammation (Donato et al., 2013) and it can have direct and indirect extracellular antimicrobial activity against Gram-negative and positive bacteria (Haley et al., 2015; Realegeno et al., 2016). However, overexpression of S100A12 has been associated with several vascular smooth muscle cell dysfunctions (Bowman et al., 2010) and it also contributes to the pathogenesis of acute and chronic inflammatory responses in humans (Yang et al., 2001). Although the consequences of greater expression of S100A12 at 24 h after LPS in CH cows remains elusive, differences in this gene could translate in differences in calcium-binding proteins and between their antimicrobial capacities.

The liver plays a central and critical role in homeostatic and homeorhetic regulation of inflammation and metabolism during acute immune responses (Heymann & Tacke, 2016; Robinson et al., 2016). Previous reports indicated that activation of the TLR4 signaling pathway by LPS and increased pro-inflammatory cytokine concentrations can decrease gluconeogenesis and promote triglyceride storage during an acute response (Jiang et al., 2008; Bradford et al., 2009; Wang et al., 2015). In our study, expression of genes related with glucose metabolism changed after LPS, however, expression of ACOX1, involved in fatty acid β-oxidation (Hashimoto et al., 1999), did not change after LPS in either genotype. Expression of acyl-CoA oxidase 1 (ACOX1) tended to be greater in UH than CH cows 4 h after LPS, however this gene is mainly regulated by PPARa (Varanasi et al., 1996; Hashimoto et al., 1999), which did not differed between the genotypes.

Pyruvate carboxylase (PC1) and phosphoenolpyruvate carboxykinase 1 (PCK1) genes code for gluconeogenic enzymes that catalyze the mitochondrial transformation of pyruvate into oxaloacetate and the cytosolic transformation of oxaloacetate into phosphoenolpyruvate, respectively. Expression of PC1 increased in UH-LPS cows during C1 and C2 and expression of PCK1 decreased in both genotypes and challenges (Suppl. 2-4). Reduced PCK1 expression also occurred when dairy cows received intramammary LPS and systemic TNF-α challenges (Jiang et al., 2008; Bradford et al., 2009). However, these treatments did not increase PC1 expression. Greater PC1 expression in UH cows seems to indicate that the gluconeogenic capacity in these genotypes differs. This was further supported by the greater LPS induced initial hyperglycemia in UH than in CH (Chapter 4).

Circulating concentrations of GH and IGF-I differ between the UH and CH genotypes during the periparturient period (Weber et al., 2007) but LPS administration did not alter circulating IGF-I (Chapter 4) or hepatic expression of IGF-I. During C1, LPS administration decreased IGF1R and increased IGFBP3 expression in both genotypes. However, the down regulation of IGF1R was more pronounced in CH cows and expression of IGFBP3 had not returned to pre-challenge expression by 24 h in the CH cows. The IGF1R primarily signals through the MAP Kinase and PI3 Kinase/Akt pathways that modulate multiple cellular functions including cell growth, proliferation, differentiation and migration via endocrine, paracrine and autocrine mechanisms (Annunziata et al., 2011). Binding of IGF-I to IGF-I binding protein 3 (IGFBP3) prolongs IGF-I half-life (Rajaram et al., 1997). IGFBP-3 is a well-documented inhibitor of cell growth and a promoter of apoptosis (Jogie-Brahim et al., 2009; Agostini-Dreyer et

al., 2015). Although the true meaning of the IGFBP3 expression differences between the genotypes is not clear, sustained up-regulation of IGFBP3 by CH cows at 4 and 24 h could be indicating cellular growth was inhibited and apoptosis promoted after LPS in this genotype.

Dairy selection programs recognize the critical need to enhance the cow's ability to resist and minimize the incidence of metabolic disorders and diseases (Cole & VanRaden, 2018) and efforts to identify genes associated with enhanced resistance include identifying animals with natural superior immunity (Mallard et al., 2011). Differences between UH and CH cows demonstrates genetic selection has modified immune, metabolic and endocrine signaling pathways and identifies genes that have potential to enhance future selection efforts. For example, key genes involved in pathogen recognition and inflammation such as TLR4, MYD88, NFKB1, IL6, TNF and TGFB1 are candidates that could be useful as biomarkers in selective breeding programs for Holsteins (Bishop & Woolliams, 2014; Benjamin et al., 2016). Future investigation of the whole genome with RNA-seq and the incorporation of other omics technologies in these genotypes will further help improving future genetic programs to increase dairy cow health and well-being.

6. CONCLUSION

The innate immune response to LPS differed between the UH and CH genotypes indicating signaling pathways have been altered by five decades of selective breeding. The greater expression of genes in the TLR4 signaling pathway and of genes involved in the production of pro and anti-inflammatory mediators (IL6, TNF, IL1RN, TGFB1) in UH cows during the acute innate immune response, indicate UH cows have a more robust

response than CH cows. However, this response seemed to be controlled, indicating that UH cows are not only more adept in immune activation but also have a greater ability to minimize negative effects of prolonged inflammation. The LPS induced expression of genes involved with the somatotropic axis and metabolism of glucose and lipids were similar for the UH and CH genotypes indicating selection had a greater impact on the innate immune response than on these primary regulators of metabolism. Potential candidate genes for additional evaluation were identified. More research on TGFB1 gene expression, Smad proteins and their signaling pathway in these genotypes will help elucidate anti-inflammatory and endotoxin tolerance differences between the genotypes. These results indicate the last 50 years of selection have increased milk yield but have decreased the innate immune capacity of the contemporary cow.

Table 1. Selected control genes for nanoString nSolver™ expression normalization

Gene Symbol	Gene Name	Accession (RefSeq ID)	Average sqrt count
SNAPC2	Small nuclear RNA activating complex, polypeptide 2	NM_001034516	11
GAK	Cyclin G associated kinase	NM_001046084.1	72
UXT	Ubiquitously-expressed transcript	NM_001037471	181
PPIA	Peptidylprolyl isomerase A	NM_178320.2	1575

Table 2. Effect of genotype (G) after saline (SAL) or lipopolysaccharide (LPS) treatment administration during challenge 1 (C1) and C1-C2 analysis on hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation

Gene	*P*-values	
	G in C1	G in C1-C2
Immunity		
CD14	0.098	0.040
CD40	<.001	0.160
ICAM-1	0.001	0.005
IL1B	0.097	0.157
IL1RN	0.022	0.339
IL6	<.001	0.043
IRAK1	0.284	0.078
IRF1	0.003	0.303
MYD88	0.137	0.084
NFKB1	0.079	0.202
NFKBIA	0.059	0.234
NLRP3	0.003	0.022
TGFB1	0.282	0.088
TLR4	0.039	0.413
TNF	0.071	0.065
TNFRSF1A	0.120	0.098
Meatabolism		
GHR-1A	0.139	0.025
IGF1	0.551	0.068
IGFBP2	0.148	0.006
INSR-b	0.209	0.041
INSR	0.197	0.023

Table 3. Genotype (G) interaction with time (T) and/or treatment (Trt; with saline -SAL- or lipopolysaccharide -LPS-) during challenge 1 (C1) on immune-related hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation

Gene[1]	***P*-values**		
	GT	**GTrt**	**GTTrt**
CCL20	0.037	0.094	0.055
CD14	0.081	0.716	0.234
CD40	0.447	0.010	0.457
CSF3	0.023	0.989	0.872
ICAM-1	0.051	0.002	0.094
IL1RN	0.045	0.063	0.140
IL1RN.2	0.046	0.082	0.054
IL4	0.023	0.989	0.872
IL6	0.001	<.001	0.056
IL17C	0.028	0.741	0.843
IL17RC	0.044	0.491	0.009
IRF1	0.219	0.054	0.327
JAK2	0.202	0.309	0.091
MYD88	0.050	0.189	0.309
NFKB1	0.048	0.334	0.160
NFKBIA	0.026	0.200	0.049
SAA3	0.089	0.824	0.343
SOCS3	0.707	0.548	0.003
TGFB1	0.231	0.012	0.374
TLR4	0.090	0.208	0.063
TNF	0.007	0.245	0.433

[1]All genes were sqrt transformed, and data represent P-values of LSM transformed values.

Table 4. Genotype (G) interaction with time (T) and/or treatment (Trt; with saline -SAL- or lipopolysaccharide -LPS-) during both challenges (C1-C2) on immune-related hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation

Gene[1]	*P*-values		
	GT	GTrt	GTTrt
FASLG	0.123	0.066	0.457
ICAM-1	0.312	0.003	0.633
IL1B	0.322	0.091	0.271
IL6	0.063	0.075	0.110
S100A12	0.003	0.577	0.045
TGFB1	0.593	0.004	0.335
TNF	0.078	0.272	0.892

[1]All genes were sqrt transformed, and data represent P-values of LSM transformed values.

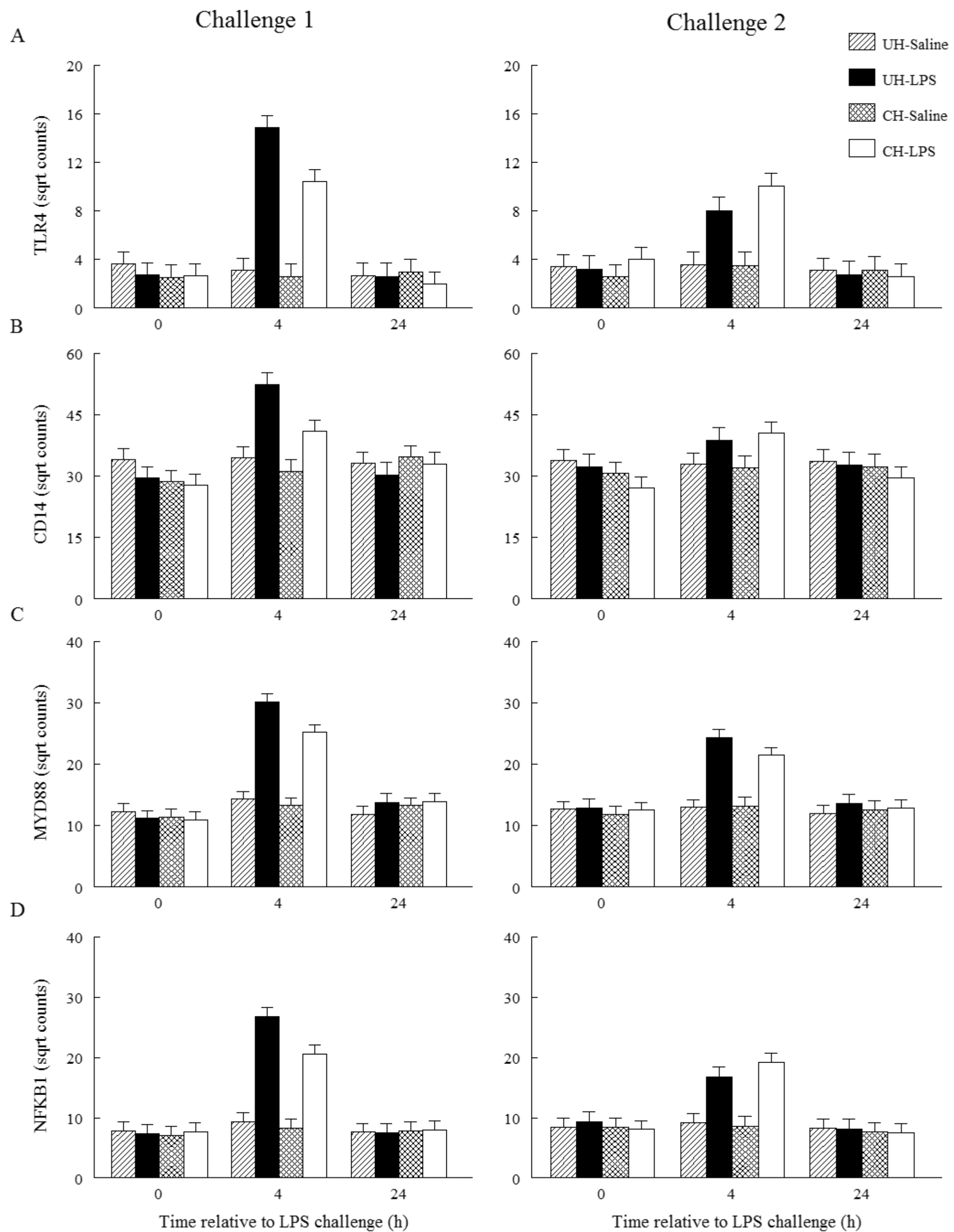

Figure 1. Liver toll-like receptor 4 (TLR4; A), cluster of differentiation 14 (CD14; B), myeloid differentiation primary response 88 (MyD88; C) and nuclear factor kappa light polypeptide enhancer in B-cells 1 (NFkB1; D) gene expression as square root transformed total digital counts in unselected (UH) and contemporary Holstein cows (CH; n=10/genotype) after two consecutive, with 4 d apart, challenges (0.25 μg LPS/kg BW, iv.). Data represent least square means of G*T*Trt*C ± SEM. During C1, there was a G*T*Trt trend (P = 0.06) TLR4 and G*T interactions or trends (P < 0.10; Table 3) for

CD14, MYD88 and NFKB1. When both challenges were included in the statistical model only CD14 presented a trend for the interaction of G*T*C ($P = 0.09$).

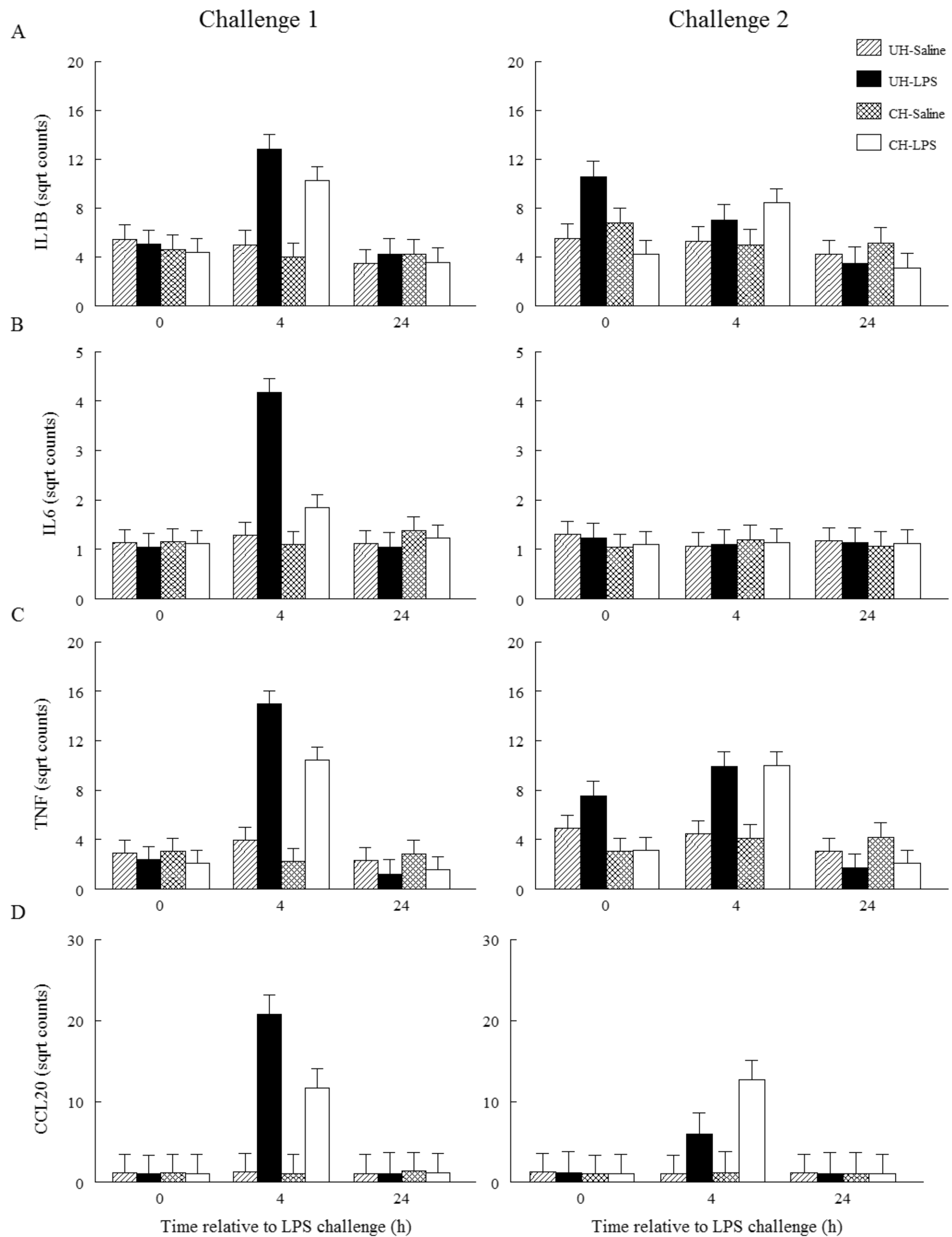

Figure 2. Liver interleukin-1β (IL1B; A), IL6 (B), tumor necrosis factor-alpha (TNF; C) and chemokine C-C motif ligand 20 (CCL20; D) gene expression as square root transformed total digital counts in unselected (UH) and contemporary Holstein cows (CH; n=10/genotype) after two consecutive, with 4 d apart, challenges (0.25 µg LPS/kg BW, iv.). Data represent least square means of G*T*Trt*C ± SEM. During C1, there were G*T interactions for IL6, TNF and CCL20 ($P < 0.05$) and IL1B presented a genotype trend ($P = 0.09$). When both challenges were included in the statistical model there were

G*T*C interactions ($P < 0.02$) for IL6 and TNF; G*Trt*C interactions ($P < 0.05$) for IL6 and CCL20; and a G*T*Trt*C trend ($P = 0.06$) for IL1B.

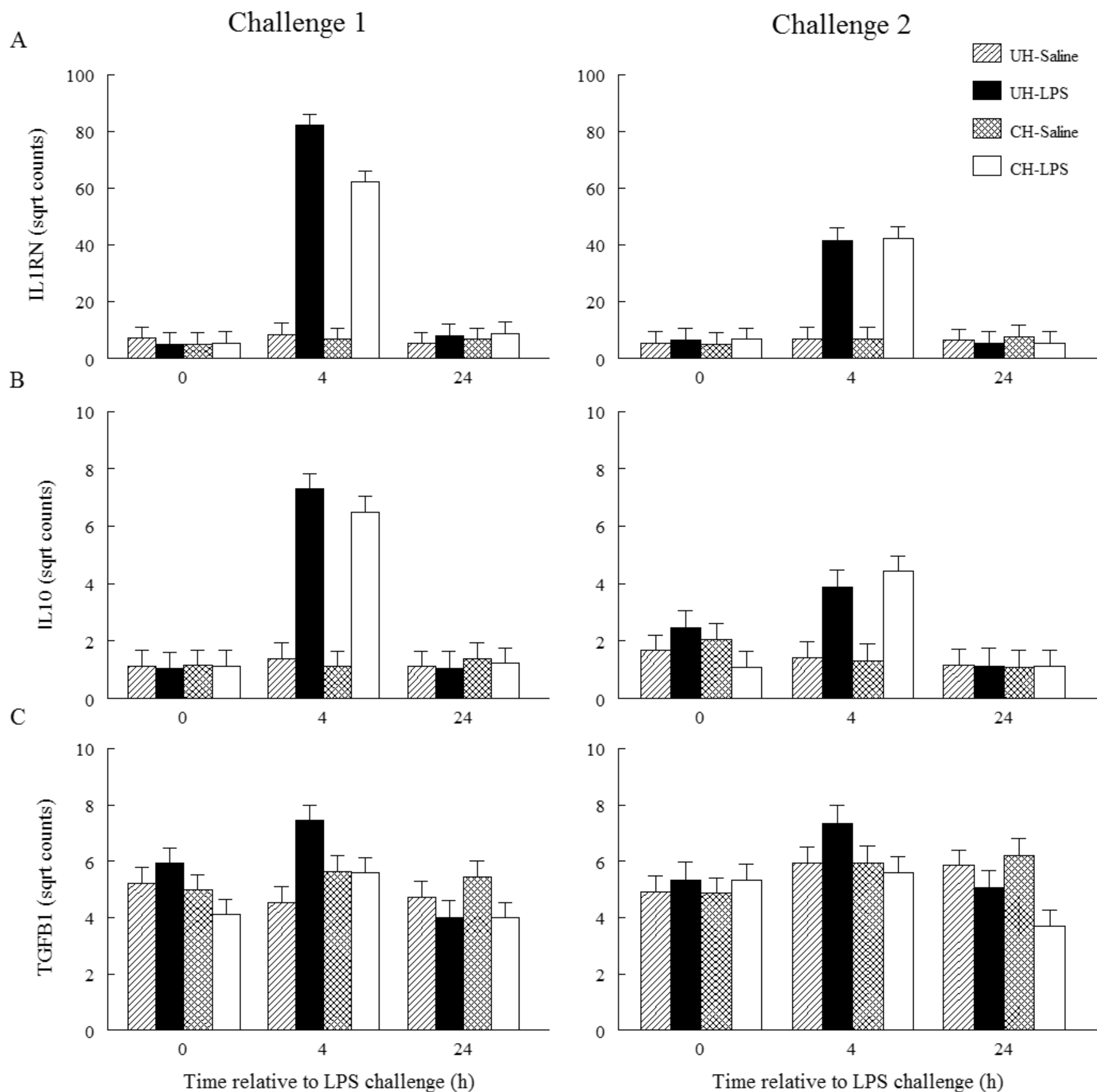

Figure 3. Liver IL-1 receptor agonist (IL1RN; A), interleukin-10 (IL10; B) and transforming growth factor-β (TGFB1; C) gene expression as square root transformed total digital counts in unselected (UH) and contemporary Holstein cows (CH; n=10/genotype) after two consecutive, with 4 d apart, challenges (0.25 μg LPS/kg BW, iv.). Data represent least square means of G*T*Trt*C ± SEM. During C1, there were G*Trt interactions for IL1RN and TGFB1 ($P < 0.07$) and G*T ($P = 0.05$) for IL1RN. When both challenges were included in the statistical model there was a G*Trt interaction ($P < 0.01$) for TGFB1; there were T*Trt*C interactions ($P < 0.02$) for IL1RN and IL10, however either IL1RN or IL10 presented interactions with genotype.

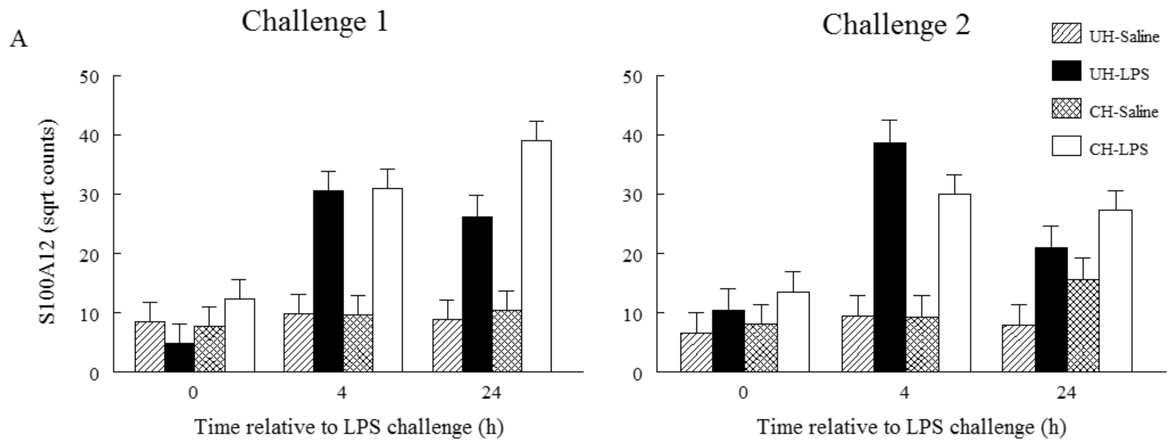

Figure 4. Liver S100 calcium binding protein A12 (S100A12) gene expression as square root transformed total digital counts in unselected (UH) and contemporary Holstein cows (CH; n=10/genotype) after two consecutive, with 4 d apart, challenges (0.25 μg LPS/kg BW, iv.). Data represent least square means of G*T*Trt*C ± SEM. When both challenges were included in the statistical model there were G*T*Trt interactions (*P* = 0.05) for S100A12 as expression increased 4 h after LPS administration in both genotypes and challenges, however at 24 h expression decreased in UH while in CH expression remained elevated.

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CHAPTER 6: ADAM17 protein expression and function in bovine polymorphonuclear leukocytes

1. SYNOPSIS

The tumor necrosis factor-alpha converting enzyme (TACE), also known as ADAM17 (a disintegrin and metalloproteinase-17), is a membrane-spanning metalloproteinase with a central role in cell regulation due to its powerful cleavage capacity and its ubiquitous presence in tissues. Although ADAM17 has been intensively studied in humans and mice, little information about ADAM17 exists for bovine cells and there appear to be no reports of functional antibodies for bovine ADAM17. This report provides information regarding functionality of several anti-ADAM17 antibodies and inhibitors and characterizes for the first time the presence and activity of ADAM17 protein on bovine polymorphonuclear leukocytes.

2. INTRODUCTION

The disintegrin and metalloproteinase (ADAM) enzymes are membrane-bound metalloproteases that belong to the adamalysin protein family (Black et al., 1997). The most important function of these proteolytic enzymes is to cleave ectodomains of numerous membrane-bound proteins (Black et al., 1997; Göoz, 2010; Mishra et al., 2017). There have been 40 ADAMs identified in the mammalian genome, however only 21 ADAMs have been described, and from those 21 only 13 have proteolytic activity (Edwards et al., 2008). Their cleavage functions are known to play important mediator roles in cell signaling events that regulate cellular fate. The TNF-α converting enzyme, TACE or ADAM17, was discovered and first described as an enzyme that releases membrane bound TNF-α precursor as a soluble form (Black et al., 1997; Moss et al.,

1997). Since its discovery, ADAM17 has been shown to be ubiquitously expressed in organs and cells and to cleave a diverse array of substrates including cytokines, growth factors and their receptors as well as adhesion molecules such as L-selectin (also known as CD62L; Li et al., 2006; Mishra et al., 2017). Indeed, ADAM17 is a crucial regulator involved with almost every cellular event from proliferation to migration (Göoz, 2010).

Although ADAM17 is one of the most studied ADAMs, information about its presence, activity and specific roles in bovine cells is lacking and the available information appears to be limited to gene expression measurements in granulosa and parietal cells (Portela et al., 2011; Mihi et al., 2013; Sayasith & Sirois, 2015). A major reason contributing to this lack of information is that there appear to be no reports of antibodies for bovine ADAM17, which impedes the ability to detect the protein in bovine cells.

Our primary objective was to identify antibodies that could detect ADAM17 in bovine cells, specifically in bovine polymorphonuclear leukocytes (PMNL). We evaluated anti-human antibodies for ADAM17 under the premise that there may be cross-reactivity considering the amino acid sequence homology between proteins from bovine and human is 85% (NCBI Blast). We successfully identified function-blocking antibodies for bovine ADAM17 and report for the first time, the presence of ADAM17 protein on bovine PMNL. We also assessed the relative ability of ADAM17 to cleave L-selectin from bovine PMNL.

3. MATERIALS AND METHODS

3.1. Antibodies and other reagents

Activity of ADAM10 was inhibited with GI254023X (#3995/1, R&D System, Minneapolis, MN), activity of ADAM17 was inhibited with BMS56394 (Bristol-Myers Squibb Company, Princeton, NJ; Ott et al., 2008; Wang et al., 2013), MEDI3622 (MedImmune, Gaithersburg, MD; Rios-Doria et al., 2015; Peng et al., 2016; Dosch et al., 2017), or D1(A12) (#MABT884, MilliporeSigma, Burlington, MA; Tape et al., 2011). MEDI3622 and D1(A12) are functional blocking monoclonal antibodies of ADAM17. Monoclonal mouse IgG2a:R-PE (#281-050, Ancell Corporation, Bayport, MN) and monoclonal mouse IgG2b:FITC (#MCA69IF, Bio-Rad AbD Serotec, Raleigh, NC) were used as PE- and FITC-isotype negative controls. Anti-CD62L conjugated to R-phycoerythrin (anti CD62L:R-PE, #261-050, Ancell Corporation, Bayport, MN) and anti-bovine CD11b conjugated to fluorescein isothiocyanate (anti CD11b:FITC, #MCA1425F, Bio-Rad AbD Serotec, Raleigh, NC) were used to detect the presence of L-selectin and CD11b/CD18 on PMNLs.

In addition to MEDI3622 and D1(A12) anti-ADAM17 Abs other ten TACE/ADAM17 ectodomain antibodies were evaluated for their capacity to stain bovine ADAM17. These included anti-human monoclonal human antibodies 111608 (#MAB9304), 111623 (#MAB9302), 111633 (#MAB9301) and 111636 (#MAB930) from R&D System (Minneapolis, MN); anti-human antibody M220 (Amgen, Thousand Oaks, CA; Doedens & Black, 2000); and anti-human antibodies that are not yet commercially available (605, 620, 622, 624 and 625 from R&D Systems, Minneapolis, MN). Anti-mouse IgG-PE (#715-116-150), a combination of anti-mouse IgG Biotin-SP (#115-066-062) and allophycocyanin (APC)-Streptavidin (#016-130-084), or APC-

F(ab')$_2$ donkey anti-human IgG (#709-136-149) from Jackson ImmunoResearch (West Grove, PA) were used as secondary antibodies. Anti-bovine CD18 primary Ab (BAQ30A; Jeyaseelan et al., 2000) was used as a positive control and human IgG1 λ (#I5029, MilliporeSigma, Burlington, MA) was used as a negative control for non-specific binding of the secondary antibodies to Fc receptors.

Phorbol 12-myristate 13-acetate (PMA, sc-3576) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and phosphate buffered saline (PBS, 17-517Q) from Lonza (Walkersville, MD). Ammonium-chloride-potassium (ACK) lysing solution was prepared with 8.29 g NH_4Cl, 1 g $KHCO_3$, and 37.2 mg Na_2EDTA in 1 L of sterile water, filtered and its pH adjusted to 7.2 - 7.4. FACS wash buffer consisted of 1% goat serum and 5 mM NaN_3 in 1X PBS.

3.2. Blood and leukocytes collection

All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. Blood from multiparous Holstein cows was collected in 10 mL heparinized evacuated tubes (Becton Dickinson Vacutainer System, Franklin Lakes, NJ), stored on ice and leukocytes isolation initiated within 30 min of collection. Whole blood (10 mL) was mixed with 40 mL of ACK buffer and incubated on ice for 10 min to lyse red blood cells. Lysed samples were centrifuged (5 min at 8°C and 524 x g) and the resulting leukocytes pellet was diluted with 2 mL of 1X PBS. A hemocytometer counting chamber was used to adjust the leukocytes suspension to 1-2 million cells/0.1 mL and 0.1 mL aliquots were transferred to a 5 mL Falcon tube and stored on ice until used within 10 min of isolation.

3.3. ADAM10 and ADAM17 inhibition by GI254023X or BMS56394 and L-selectin labeling procedure

Change in the prevalence of L-selectin on PMNL was used to determine the relative proteolytic activity of ADAM10 and ADAM17 in bovine PMNL. Proteolytic activity of ADAM10 and ADAM17 were inhibited by incubating the 1-2 million leukocytes aliquots with 5 µM of GI254023X or 10 µM of BMS56394, respectively for 30 min on ice. After inhibition of proteolytic activity, leukocytes aliquots were either left unstimulated or were stimulated by incubating with 40 ng/mL of PMA for 30 min at 37°C. All tubes were filled with FACS wash buffer, centrifuged (5 min at 8°C and 524 x g) and decanted to remove unbounded inhibitors and PMA. The leukocytes pellets were mixed with 0.02 mL of anti-CD62L R-PE-conjugate (0.5 mg/mL) and incubated for 20 min at 4°C in the dark to label L-selectin molecules. These leukocytes mixtures were washed with FACS buffer as previously described to remove unbounded Ab and re-suspended in 2 mL of 1X PBS for flow cytometry analysis. Unstained stimulated and unstimulated leukocytes were used as negative controls. Samples were analyzed by flow cytometry within 30 minutes of being labeled.

3.4. ADAM17 inhibition by MEDI3622 or D1(A12), and L-selectin and CD11b labeling procedure

As described above L-selectin was used to determine ADAM17 proteolytic activity and CD11b was used as a control of the selectivity of the blocking antibodies for ADAM17. Proteolytic activity of ADAM17 was inhibited by incubating the 1-2 million leukocytes aliquots with 5 µg/mL of MEDI3622 or D1(A12) for 1 hour on ice. After ADAM17 inhibition, leukocytes aliquots were PMA activated as previously described.

The residual buffer after activation and washes (~0.01 mL) and leukocytes pellets were mixed with 0.02 mL of either anti-CD62L R-PE-conjugate (0.5 mg/mL), anti-CD11b:FITC, IgG2a:R-PE isotype or IgG2b:FITC isotype. Mixes were incubated for 20 min at 4°C in the dark to label L-selectin or CD11b molecules. These PMNL mixtures were washed with FACS buffer as previously described to remove unbounded Ab and re-suspended in 2 mL of 1X PBS for flow cytometry analysis. Unstained stimulated and unstimulated leukocytes and PE- and FITC-isotypes were used as negative controls. Samples were analyzed by flow cytometry within 30 minutes of being labeled.

3.5. ADAM17 labeling procedure

Flow cytometry was performed to assess the ability of the anti-ADAM17 mAbs to recognize and bind bovine ADAM17. Each of the primary anti-ADAM17 mAbs except MEDI3622 and D1(A12) was mixed with an unstimulated and a PMA-stimulated aliquot of leukocytes and binding of primary antibodies was revealed with PE- or APC-conjugated secondary antibodies. The leukocytes aliquots were mixed with 5 µg/mL of an anti-ADAM17 mAb and incubated at 4°C for 30 min in the dark. The PMNL were washed as previously described to remove unbounded mAb and mixed with 0.1 mL of either PE-conjugated F(ab')2 donkey anti-mouse IgG or Biotin-SP F(ab')2 goat anti-mouse IgG plus APC-Streptavidin antibodies diluted 1:200 in FACS wash buffer and incubated at 4°C for 30 min in the dark. Leukocytes were washed with FACS wash buffer and the remaining aliquot after centrifugation and decanting was analyzed by flow cytometry. Unstained leukocytes were used as a negative control and anti-bovine CD18 Ab as a positive control (Jeyaseelan et al., 2000). Samples were analyzed by flow cytometry within 30 minutes of being labeled.

Anti-ADAM17 MEDI3622 and D1(A12) mAbs were evaluated in a similar manner except they were only labelled with secondary APC-F(ab')$_2$ donkey anti-human IgG Ab and only in unstimulated leukocytes. Aliquots of PMNL were mixed with 0.05 mL (1 mg/mL) of either MEDI3622, D1(A12) or human IgG and incubated at 4°C for 1 hour (MEDI3622) or 30 min (D1(A12) and IgG) in the dark. Leukocytes mixtures were washed with FACS wash buffer, and the residual mixed with 0.01 mL of 1X PBS. Bound MEDI3622, D1(A12) and IgG were revealed by incubating re-suspended aliquots with 0.03 mL of secondary APC-conjugated Ab at 4°C for 20 min in the dark. Unstained leukocytes and IgG plus secondary Ab were used as negative controls and leukocytes labeled with the secondary APC-conjugated Ab only were used to assess non-specific binding. Samples were analyzed by flow cytometry within 30 minutes of being labeled.

3.6. Flow Cytometry

Flow cytometry was performed on a BD FACSCanto II and a BD FACSCelesta cytometers (BD Biosciences, Franklin Lakes, NJ) and data analyzed using FlowJo 7.6.4 software (Tree Star Inc., San Carlos, CA). Forward versus side scatter gating was used to identify PMNL from the total leukocyte population based on their size and granularity (Figure 1; Bio-Rad, 2016). For each sample, ten thousand events of labeled PMNL were acquired.

4. RESULTS AND DISCUSSION

The proteolytic activity of ADAM17 is rapidly induced upon cellular activation by various stimuli, including cytokines, pathogen-associated molecular patterns, calcium influx changes or cell induction from chemicals like PMA (Walcheck et al., 2006; Horiuchi et al., 2007; Reiss & Saftig, 2009; Wang et al., 2011). In bovine, the presence of

ADAM17 mRNA has been detected on bovine gastric epithelial cells (Mihi et al., 2013) and granulosa cells (Portela et al., 2011; Sayasith & Sirois, 2015). This mRNA expression was up-regulated during gastric nematode infections (Mihi et al., 2013) and by luteinizing hormone, human chorionic gonadotropin or angiotensin-II treatment of granulosa cells (Portela et al., 2011; Sayasith & Sirois, 2015). However, to our knowledge, there are no reports that have identified the presence of ADAM17 protein in any bovine cells.

One of the many proteins that is cleaved from the surface of PMNLs by ADAM17 is L-selectin (Condon et al., 2001; Li et al., 2006; Mishra et al., 2017). L-selectin is an adhesion molecule known to promote the initial tethering and subsequent rolling of leukocytes along activated endothelial cells (Ivetic, 2013). L-selectin is rapidly shed from the PMNL outer membrane in response to a variety of stimuli, including cytokines, chemoattractants and PMA (Kishimoto et al., 1989; Springer, 1994; Fan & Derynck, 1999; Alexander et al., 2000), the same compounds that stimulate ADAM17 activity. L-selectin shedding is impaired in ADAM17-deficient cells and when ADAM17 activity in normal human and mouse cells is blocked by specific inhibitors such as BMS56394 (Walcheck et al., 2003; Wang et al., 2010, 2013). These results indicate a role for ADAM17 in the proteolytic cleavage of L-selectin and provides a way to measure relative ADAM17 activity.

Structural properties of the catalytic domain (Maskos et al., 1998) and the proteolytic properties (Vincent et al., 2001) of ADAM17 are similar to those of ADAM10. Although its amino acid sequence homology is only 30% (NCBI Blast; Gooz, 2010), ADAM10 is the closest ADAM relative of ADAM17. Gene expression studies

indicated ADAM10 cleaved TNF-α from bovine spleen cells (Lunn et al., 1997), but the PCR primers used broadly targeted mammalian disintegrin metalloproteases and were not specific for ADAM10 (Howard et al., 1996). Recent studies with human cell lines, indicate ADAM17, not ADAM10, is the main enzyme responsible for the cleavage of TNF-α and L-selectin (Black et al., 1997; Peschon et al., 1998; Bell et al., 2007). It is only when ADAM17 is completely inactive that ADAM10 can cleave some of the ADAM17 substrates such as TGF-α and TNF-α (Condon et al., 2001; Le Gall et al., 2009). Studies with mammalian cells that lacked one or more specific ADAMs have established that ADAM17 has a critical role in shedding TGF-α, TNF-α, L-selectin, heparin binding epidermal growth factor (EGF)-like growth factor (HB-EGF), amphiregulin and epiregulin while ADAM10 has a critical role in shedding EGF and betacellulin (Peschon et al., 1998; Sunnarborg et al., 2002; Sahin et al., 2004; Sanderson et al., 2005; Horiuchi et al., 2007). However, the specific enzymatic roles of ADAM10 and ADAM17 in bovine cells had not been elucidated until now.

Our results with bovine PMNLs show ADAM10 does not appear to be involved in L-selectin cleavage, as previously showed for humans and mice (Condon et al., 2001; Le Gall et al., 2009). L-selectin expression was reduced in PMA treated PMNL which indicates the proteases were stimulated and shed L-selectin from the PMNL surface (Figure 2). Expression of L-selectin was reduced to the same extent when ADAM10 activity was inhibited by GI254023X before PMNLs were exposed to PMA. This indicates ADAM10 had little to no role in L-selectin cleavage on bovine PMNLs. In contrast, when ADAM17 activity was inhibited with BMS56394 before PMNLs were activated with PMA, L-selectin expression was similar to that obtained with unstimulated

PMNL (Figure 2). The BMS56394 inhibitor is highly selective for ADAM17 in humans and mice (Ott et al., 2008; Y. Wang et al., 2013) so this lack of reduction in L-selectin expression provides indirect evidence that it inhibits ADAM17 in bovine and also provides indirect evidence of the presence of ADAM17 on bovine PMNL.

The information above suggests that ADAM17 plays a role in L-selectin shedding in bovine PMNLs. We next examined whether we could detect bovine ADAM17 using several anti-human ADAM17 mAbs. Mean fluorescence obtained when antibodies 111608, 111623, 11633, 11636, 605, 620, 622, 624, 625 and M220 were evaluated did not differ from the negative control (unstained PMNL; Figure 3). Indirect staining methods are more sensitive than direct methods (Chen et al., 2010). In this study both approaches were indirect, one involved an anti-mouse second stage and the other streptavidin. Both methods were used to prevent biases due to differential sensitivity, however the response was similar regardless of the staining procedure used (results not reported). Thus, despite the high sequence homology of human and bovine ADAM17, there was no bovine cross-reactivity by several anti-ADAM17 antibodies. The most likely reasons why none of these mAb recognized bovine ADAM17 appear to be that despite the high homology, species differences in the amino acid sequence within the binding site of these antibodies or in posttranslational modifications interfere with antibody recognition of the protein (Pedersen et al., 2002; Jefferis, 2016). It is also possible bovine ADAM17 is glycosylated differently and that this affected Ab binding to its epitopes (Chavaroche et al., 2014). This could also explain why the specific mouse and human Abs do not cross react to detect ADAM17 in the other species despite having a 90% homology.

The anti-ADAM17 monoclonal antibody MEDI3622 and D1(A12) Abs are functional blocking monoclonal antibodies of ADAM17 (Tape et al., 2011; Peng et al., 2016). MEDI3622 binds to a unique surface loop of the ADAM17 M-domain that is conserved between human, mouse and certain non-human primates (Rios-Doria et al., 2015; Peng et al., 2016; Dosch et al., 2017), and when comparing the protein sequence of human and bovine ADAM17 M-domain a 97% homology is obtained (Figure 4; Peng et al., 2016; NCBI Blast). D1(A12) is a "cross-domain" human antibody that has a new variable light chain that binds ADAM17 specific non-catalytic regions in addition to the catalytic domain making it a unique selective inhibitor of the complete ADAM17 ectodomain (Tape et al., 2011). Mean fluorescence obtained when MEDI3622 and D1(A12) were evaluated were greater than those obtained with unstained PMNL, the 2ndary APC-Ab when used alone and greater than the IgG isotype-matched control antibody (Figure 5). This indicates both MEDI3622 and D1(A12) cross-react with bovine ADAM17. The high specificity of MEDI3622 for a unique ADAM17 loop makes it a suitable antibody for at least 4 species and the unique variable light chain of D1(A12) makes this antibody also suitable to stain bovine ADAM17.

Both MEDI3622 and D1(A12) mAbs efficiently inhibit ADAM17 activity and block its cleavage of substrates such as EGFR-ligands and TGF-α in human cell lines (Richards et al., 2012; Rios-Doria et al., 2015; Peng et al., 2016; Dosch et al., 2017). In this study we show that both antibodies also inhibited bovine ADAM17 cleavage of L-selectin from PMNLs after PMA activation (Figure 6). When ADAM17 activity was inhibited by MEDI3622 or D1(A12) before PMNLs were activated with PMA, expression of L-selectin did not decrease and remained similar to expressions obtained in

unstimulated PMNL. In contrast to L-selectin expression, CD11b expression is rapidly upregulated upon neutrophil activation, as it transported from intracellular stores to the cell membrane. Neither MEDI3622 or D1(A12) blocked this process, demonstrating that these mAbs did not block PMNL activation in general.

The ectodomain of several inflammatory modulators including TNF-α and TGF-α, and of adhesion molecules such as L-selectin, ICAM-1 and VCAM-1 are shed from cells via ADAM17 activity (Tsakadze et al., 2006; Göoz, 2010). Therefore ADAM17 plays important roles in the coordination of pro- and anti-inflammatory activities and leukocyte transmigration during an immune response (Walcheck et al., 2006; Göoz, 2010). Having functional anti-ADAM17 Abs for bovine will contribute to efforts to improve our understanding of factors that affect regulation of inflammation in cattle.

This report identifies MEDI3622 and D1(A12) as ADAM17 antibodies that bind and selectively block bovine ADAM17 and characterizes for the first time the presence and activity of ADAM17 protein on bovine PMNL.

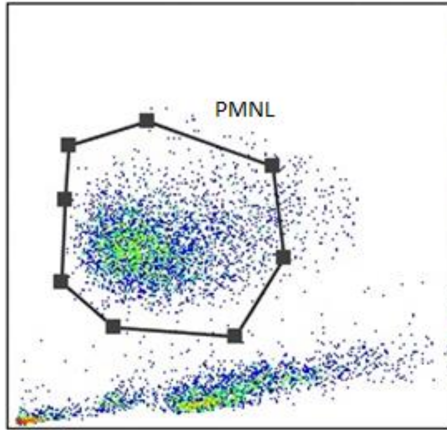

Figure 1. PMNL gating. For each sample ten thousand events of labeled PMNL were acquired and PMNL were gated based on cell size (forward scatter, y-axis) and cellular granularity (side scatter, x-axis).

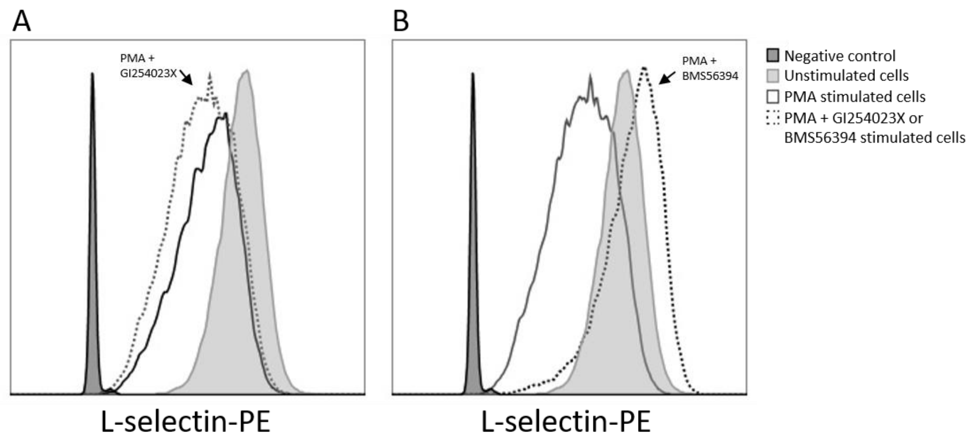

Figure 2. L-selectin expression in unstimulated and PMA-stimulated (40 ng/mL; 37°C for 30 min) bovine PMNL. Fluorescence intensity of L-selectin decreased (greater shedding of L-selectin) when PMNL were pretreated with GI254023X (A) but did not change when pretreated with BMS56394 (B).

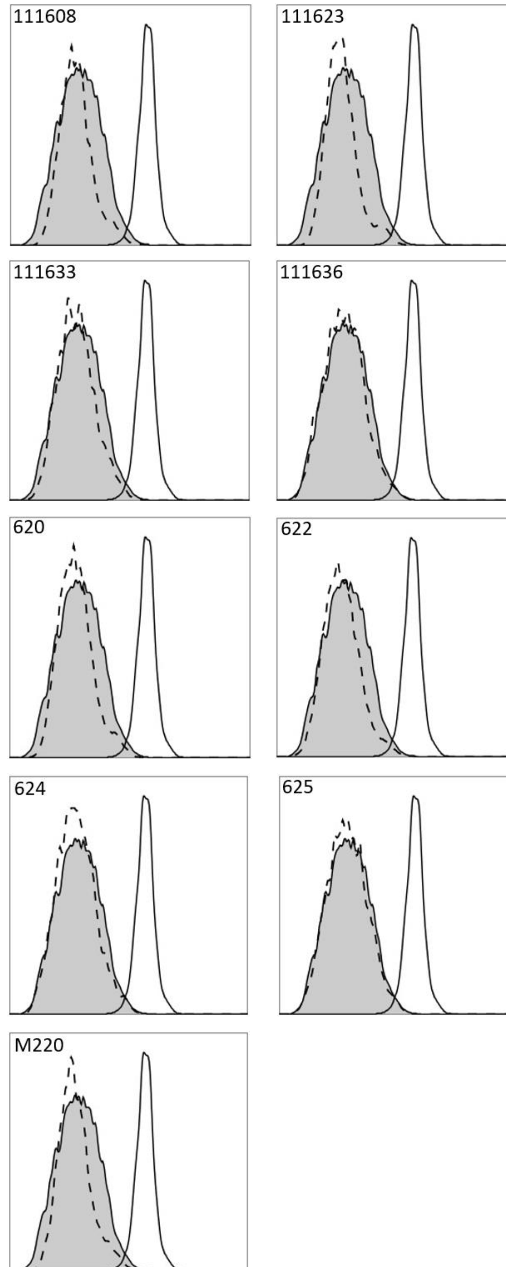

Figure 3. Ability of mAbs to recognize ADAM17. The 111608, 111623, 11633, 11636, 605, 620, 622, 624, 625 and M220 mAbs were incubated PMNL and labelled indirectly with secondary PE-conjugated Ab. Mean fluorescence (dashed lines) did not differ from the negative control (unstained PMNL; filled histogram). Anti-bovine CD18 (BAQ30A; Jeyaseelan et al., 2000) was used as the positive control (solid lines). Similar results were obtained with PMA activated PMNLs and IgG Biotin-SP plus APC-Streptavidin secondary staining.

Human M-domain RADP**D**P**M**KNTCKLLVVADHRFY**R**YMGRGEESTTTNYLIELIDRVDDIYRNTSWDNAGFKG 274
Bov. M-domain RADP**N**P**L**KNTCKLLVVADHRFY**K**YMGRGEESTTTNYLIELIDRVDDIYRNTSWDNAGFKG

Human M-domain YGIQIEQIRILKSPQ**E**VKPGE**K**HYNMAKSYPNEEKDAWDVKMLLEQFSFDIAEEASKVCL 334
Bov. M-domain YGIQIEQIRILKSPQ**V**VKPGE**R**HFNMAKSYPNEEKDAWDVKMLLEQFSFDIAEEASKVCL

Human M-domain AHLFTYQDFDMGTLGLAYVGSPRANSHGGVCPKAYYSP**V**GKKNIYLNSGLTSTKNYGKTI 394
Bov. M-domain AHLFTYQDFDMGTLGLAYVGSPRANSHGGVCPKAYYSP**I**GKKNIYLNSGLTSTKNYGKTI

Human M-domain LTKEADLVTTHELGHNFGAEHDPDGLAECAPNEDQGGKYVMYPIAVSGDHENNKMFSNCS 431
Bov. M-domain LTKEADLVTTHELGHNFGAEHDPDGLAECAPNEDQGGKYVMYPIAVSGDHENNKMFSNCS

Human M-domain KQSIYKTIESK**A**QECFQERS 474
Bov. M-domain KQSIYKTIESK**S**QECFQERS

Figure 4. Human and bovine ADAM17 M-domain protein homology. Their identical and different amino acids are shown as unbold and bold, respectively. ADAM17 M-domain sequences presented a 97% homology (NCBI Blast). Human M-domain sequene obtained from Peng et al., 2016.

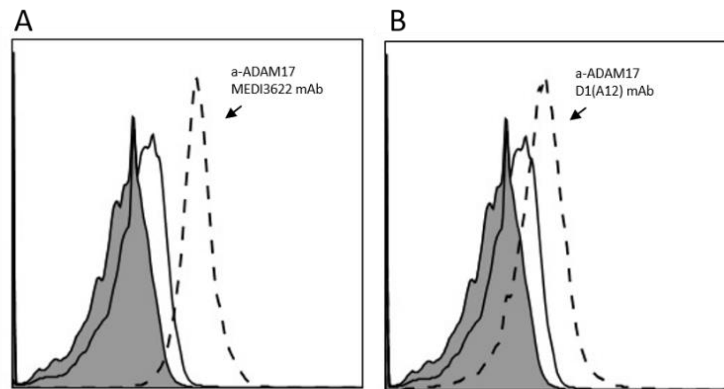

Figure 5. Expression of ADAM17 in PMNL bound to anti-ADAM17 MEDI3622 (A) or D1(A12) (B) mAbs and labelled indirectly with APC-F(ab')2 mAb. Mean fluorescence (dashed lines) of ADAM17 were greater than unstained PMNL (filled histograms) and the negative IgG controls (solid lines).

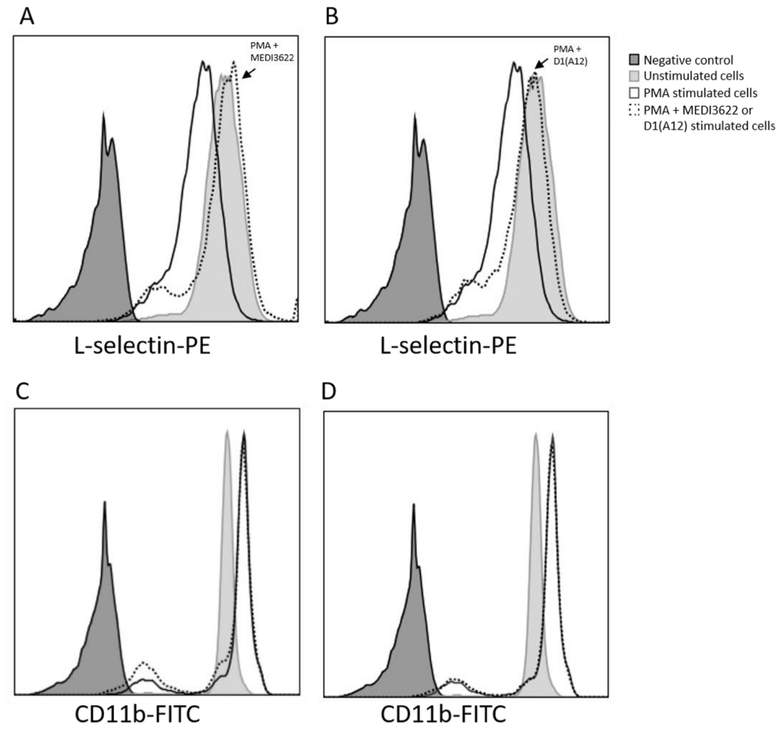

Figure 6. L-selectin and CD11b expression in unstimulated and PMA-stimulated (40 ng/mL; 37°C for 30 min) bovine PMNL. Fluorescence intensity of L-selectin did not change when PMNL were pretreated with MEDI3622 (A) or D1(A12) (B). Fluorescence intensity of CD11b increased (greater expression) when PMNL were pretreated with MEDI3622 (C) or D1(A12) (D).

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THESIS SUMMARY

Goals of the studies presented in this thesis were to increase understanding of how the innate immune system interacts with physiological alterations associated with increased milk yield. Studies were designed to assess biological alterations in innate immune responses and interactions of these responses with endocrine and metabolic components when growing heifers and multiparous lactating cows received iv administrations of lipopolysaccharide (LPS). A specific component of this evaluation was to assess the impact of selection of expression and function of ADAM17 in bovine PMNL.

A novel aspect of these studies was the comparison of unique Holsteins that have not been subjected to selection since 1964 (unselected Holsteins; UH) with contemporary Holsteins (CH). One of our animal models compared UH and CH heifers with heifers from contemporary Red-Black Angus cows (CA). Two of our experimental designs included replicated LPS challenges to assess impact of genotype on the acute innate immune response and on the development of a refractory (endotoxin tolerance) state. These studies assessed the impact of 50+ years of selection on the innate immune response of Holsteins.

The acute phase response (APR) of the innate immune system is stimulated by the release of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 from macrophages and monocytes at the site of injury or infection. Rapid induction of the APR serves to neutralize pathogens and reduce further pathogen invasion. This activation causes a number of physiological and metabolic changes and these alterations can be used to characterize the innate immune system. For

example, the more rapid increase in serum cortisol concentrations in Holsteins and the greater concentrations of TNF-α in CH and IL-6 in UH heifers following LPS administration indicate Holstein heifers might be able to detect Gram-negative bacteria more rapidly and induce a more vigorous and more controlled inflammatory cascade than Angus heifers. This may be beneficial for pathogen clearance because a fast, short-lasting and regulated inflammatory reaction is generally considered beneficial for early resolution of infection. Similar differences in IL-6 concentrations after LPS administration between the UH and CH heifers, regardless of pregnancy status, indicate the UH heifers may have more balanced pro- and anti-inflammatory functions during an acute response than CH heifers. Greater plasma concentrations of pro-inflammatory cytokines, glucose and cortisol, and PMNL adhesion molecules expression and activity after LPS in UH than CH cows indicate UH cows have a more robust innate immune response than CH cows. Although the magnitude of the response reductions during the second LPS challenge did not differ between UH and CH heifers, the greater reductions in UH than in CH cows indicate a greater development of tolerance to immunotoxin stimulation in the UH cows.

Hepatic expression of genes of the TLR4 signaling pathway and of genes involved in the production of pro and anti-inflammatory mediators (IL6, TNF, IL1RN, TGFB1) was greater in UH than in CH cows during the acute innate immune response. In addition, expression of these genes returned to pre-challenge means by 24 h in both genotypes. These results agree with the differences in plasma concentrations of pro-inflammatory cytokines and further support the premise that UH cows are not only more adept in immune activation but also have a greater response of anti-inflammatory

mediators to minimize negative effects of prolonged inflammation. During C2, results from the gene expression analysis also showed differences between the genotypes indicating that the development of tolerance to the repeated LPS administration is different between UH and CH cows. Expression of genes related to the somatotropic axis and metabolism of glucose and lipids did not differ between the UH and CH cows during the LPS challenge. Although the impact of selection on ADAM17 (a disintegrin and metalloproteinase-17) was not assessed, 2 functional blocking monoclonal anti-ADAM17 antibodies and 1 inhibitor were identified and characterized for cattle. Thus, for the first time, the presence and activity of ADAM17 protein in bovine PMNL has been confirmed.

Increased emphasis on functional and health traits of cows indicates the dairy industry recognizes the critical need to minimize the incidence of metabolic disorders and infectious diseases in dairy cows. Efforts to reverse the unintended negative impact of past selection practices through new selection strategies and marker assisted selection programs are important steps to continue to improve health and well-being of dairy cattle. Additional, more-focused, gene-assisted selection practices offer considerable potential to enhance these efforts. A better understanding of how the previous focus on selection for milk yield has altered the immune system and its interactions with endocrine and metabolic components will strengthen these gene-assisted selection programs to develop cows more suited to the rigors of increased milk yield. Results from these studies will contribute to efforts to provide a continued supply of safe, wholesome milk for consumers.

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Supplement 1. Genes and accession numbers according to National Center for Biotechnology Information (NCBI)

Gene symbol	Gene Name	Accession (RefSeq ID)
Immunity		
ADAM17	ADAM metallopeptidase domain 17	XM_002691486
AGP	Alpha-1-acid glycoprotein	AM403243.2
C3	Complement component 3	NM_001040469.2
CASP1	Caspase-1	AB285205.1
CCL5	Chemokine (C-C motif) ligand 5	NM_175827.2
CCL20	Chemokine (C-C motif) ligand 20	NM_174263.2
CD14	Clauster of differentiation 14 molecule	NM_175827.2
CD40	Clauster of differentiation 40 molecule	NM_174008.1
CD40LG	Clauster of differentiation 40 ligand	NM_174624.2
CEBPD	CCAAT/enhancer-binding protein delta	NM_001105611.2
CSF3	Granulocyte-colony stimulating factor 3	NM_174028.1
FASLG	Fas ligand	NM_001098859.2
FGF21	Fibroblast growth factor 21	XM_010815205.1
FGFR2c	Fibroblast growth factor 21 receptor isoform c	AJ413268.1
FOXO3	Forkhead box O3	NM_001206083.1
ICAM-1	Intercellular adhesion molecule 1	NM_174348.2
IL1B	Iinterleukin 1, beta	NM_174093.1
IL1RN	Interleukin 1 receptor antagonist	NM_174357
IL1RN.2	Interleukin 1 receptor antagonist	NM_174357.2
IL4	Interleukin 4	NM_173921.2
IL6	Interleukin 6	NM_173923.2
IL6R	Interleukin 6 receptor	NM_001110785.1
IL10	Interleukin 10	NM_174088.1
IL17C	Interleukin 17C	XM_010814721.1
IL17RC	Interleukin 17 receptor C	NM_001075178.1
IRAK1	Interleukin-1 receptor-associated kinase 1	NM_001040555.1
IRF1	Interferon regulatory factor 1	NM_001191261.2
JAK2	Janus kinase 2	XM_865133.3
LBP	Lipopolysaccharide binding protein	NM_001038674.2
LITAF	Lipopolysaccharide-induced TNF-alpha factor	NM_001046252.2
LYN	Tyrosine-protein kinase Lyn	AB562971.1
MYD88	Myeloid differentiation primary response 88	NM_001014382.2
NFKB1	Nuclear factor of kappa light polypeptide enhancer in B-cells 1	NM_001076409
NFKBIA	NF-kappaB inhibitor alpha	NM_001045868
NLRP3	NLR family, pyrin domain containing 3	NM_001102219.1
S100A12	S100 calcium-binding protein A12	NM_174651.3
SAA3	Serum amyloid A protein	NM_181016.3

SOCS3	Suppressor of cytokine singling 3	XM_002697964.3
STAT1	Signal Transducers and Activators of Transcription 1	NM_001077900.1
STAT3	Signal Transducers and Activators of Transcription 3	NM_001012671.2
STAT5a	Signal Transducers and Activators of Transcription 5 alpha	NM_001012673.1
STAT5b	Signal Transducers and Activators of Transcription 5 beta	NM_174617
TGFB1	Transforming growth factor, beta 1	NM_001166068
TLR2	Toll-like receptor 2	NM_174197.2
TLR4	Toll-like receptor 4	NM_174198.6
TNF	Tumor necrosis factor –alpha-	NM_173966.2
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	NM_174674.2
VEGF-A	Vascular endothelial growth factor A	NM_174216.1
XBP1	X-box binding protein 1	NM_001034727
Metabolism		
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	NM_001035289
GHR-1A	Growth hormone receptor 1-α	AY748827
GHRtot	Total growth hormone receptor	NM_176608.1
IGF1	insulin-like growth factor 1	NM_001077828.1
IGF1R	Insulin like growth factor 1 receptor	NM_001244612.1
IGF-ALS	Insulin-like growth factor binding protein, acid labile subunit	NM_001075963.1
IGFBP2	Insulin-like growth factor binding protein 2	NM_174555.1
IGFBP3	Insulin-like growth factor binding protein 3	NM_174556.1
IGFBP5	Insulin-like growth factor binding protein 5	NM_001105327.2
INSR-b	Insulin receptor B	XM_002688832
INSR	Insulin receptor	XM_002688832
KLB	beta-Klotho	NM_001205326.1
PC1	Pyruvate carboxylase	NM_177946
PCK1	Phosphoenolpyruvate carboxykinase 1	NM_174737.2
PPARa	Peroxisome proliferator-activated receptor alpha	NM_001034036
PPARd	Peroxisome proliferator-activated receptor delta	NM_001083636
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	NM_177945.3
XDH	Xanthine dehydrogenase	NM_173972.2
Control genes		
GAK	Cyclin G associated kinase	NM_001046084.1
PPIA	Peptidylprolyl isomerase A	NM_178320.2
RPS9	Ribosomal Protein S9	NM_001101152.2
SPRYD4	Protein sprouty homolog 4	NM_001076173.1
HNF4a	Hepatocyte nuclear factor 4, alpha	NM_001015557.1
HPRT	Hypoxanthine phosphoribosyltransferase	NM_001034035.1
UXT	Ubiquitously-expressed transcript	NM_001037471
SNAPC2	Small nuclear RNA activating complex, polypeptide 2	NM_001034516

Supplement 2. Least square means of the square root tranformed counts of hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation.

Gene	Genotype	Trt[1]	C1[2]			C2[2]			SEM
			0	4	24	0	4	24	
Immunity									
ADAM17	UH	SAL	8.7	8.9	9.1	9.5	8.9	9.1	0.7
		LPS	8.5	13.5	8.4	9.7	11.8	9.9	
	CH	SAL	7.7	8.8	9.8	9.0	8.8	8.6	
		LPS	8.2	11.6	9.4	9.5	12.2	9.6	
AGP	UH	SAL	202.9	213.0	242.6	222.2	197.7	229.3	39.3
		LPS	190.7	256.1	208.8	265.1	228.8	256.2	
	CH	SAL	199.1	225.5	276.4	208.4	203.2	232.5	
		LPS	172.3	196.8	215.7	176.4	224.0	208.4	
C3	UH	SAL	222.7	226.8	214.8	220.1	201.8	223.5	18.4
		LPS	219.6	257.9	168.9	228.3	211.7	202.2	
	CH	SAL	204.4	220.5	231.2	209.5	203.7	228.1	
		LPS	205.9	225.0	197.4	204.4	209.8	195.4	
CASP1	UH	SAL	3.3	2.9	2.9	3.5	4.5	4.2	0.7
		LPS	2.7	5.7	1.2	5.0	4.5	3.7	
	CH	SAL	2.6	3.6	3.4	3.9	3.5	3.5	
		LPS	2.9	5.2	1.7	3.9	5.6	3.0	
CCL5	UH	SAL	13.9	13.5	12.9	14.9	13.5	14.4	2.6
		LPS	12.2	43.6	18.9	15.2	36.9	20.7	
	CH	SAL	13.0	14.3	13.9	13.2	14.1	14.5	
		LPS	12.7	34.2	19.3	14.5	33.9	19.9	
CCL20	UH	SAL	1.1	1.3	1.1	1.3	1.1	1.2	2.4
		LPS	1.1	20.8	1.0	1.2	6.0	1.1	
	CH	SAL	1.1	1.1	1.4	1.0	1.2	1.1	
		LPS	1.1	11.7	1.2	1.1	12.7	1.1	
CD14	UH	SAL	33.9	34.4	33.1	33.8	32.8	33.6	2.8
		LPS	29.5	52.4	30.3	32.3	38.8	32.8	
	CH	SAL	28.7	31.2	34.6	30.6	31.9	32.3	
		LPS	27.7	40.8	33.0	27.0	40.4	29.5	
CD40	UH	SAL	3.9	3.9	4.0	5.0	6.3	3.9	1.9
		LPS	2.9	29.9	4.0	6.4	21.8	3.9	
	CH	SAL	2.8	3.3	3.5	4.4	4.9	3.4	
		LPS	2.3	22.8	2.7	3.9	24.1	3.5	
CD40LG	UH	SAL	3.3	3.6	3.5	3.4	2.5	3.9	0.7
		LPS	2.6	5.4	2.7	4.1	4.7	3.1	
	CH	SAL	2.5	3.1	4.3	2.4	2.4	3.8	

		LPS	2.5	4.5	1.6	2.7	4.4	2.5	
CEBPD	UH	SAL	17.0	18.4	14.8	14.7	18.0	14.4	2.1
		LPS	15.8	33.4	12.8	16.8	18.4	14.1	
	CH	SAL	15.7	21.5	16.7	17.0	19.2	16.2	
		LPS	16.4	29.2	13.7	17.2	20.2	14.2	
CSF3	UH	SAL	1.1	1.3	1.1	1.3	1.1	1.2	0.1
		LPS	1.1	1.3	1.0	1.2	1.1	1.1	
	CH	SAL	1.1	1.1	1.4	1.0	1.2	1.1	
		LPS	1.1	1.2	1.2	1.1	1.2	1.1	
FASLG	UH	SAL	1.2	1.3	1.3	1.6	1.3	1.3	0.2
		LPS	1.1	1.5	1.0	1.4	1.1	1.4	
	CH	SAL	1.1	1.3	1.4	1.0	1.3	1.1	
		LPS	1.3	2.3	1.2	1.5	1.6	1.1	
FGF21	UH	SAL	7.4	8.4	6.3	6.3	5.3	6.1	1.0
		LPS	6.6	6.5	4.4	9.4	5.3	5.1	
	CH	SAL	5.5	7.5	7.0	7.3	6.6	6.2	
		LPS	6.1	5.4	3.7	5.9	5.5	5.1	
FGFR2c	UH	SAL	7.3	7.1	7.4	8.2	6.8	7.8	0.6
		LPS	7.7	1.4	6.7	8.6	1.2	7.3	
	CH	SAL	7.4	7.6	7.4	8.2	7.4	7.1	
		LPS	6.9	1.6	8.0	7.5	2.4	7.5	
FOXO3	UH	SAL	9.5	8.1	7.7	8.2	7.4	8.1	0.7
		LPS	8.5	13.4	7.2	8.1	8.9	7.7	
	CH	SAL	8.0	8.4	8.4	8.1	7.5	8.1	
		LPS	8.1	13.2	8.5	8.9	9.5	7.7	
ICAM-1	UH	SAL	8.3	8.5	7.2	8.4	8.5	8.1	1.0
		LPS	7.4	22.2	7.3	10.8	15.0	7.2	
	CH	SAL	7.7	8.2	7.7	8.1	8.7	8.9	
		LPS	6.8	16.5	5.9	7.2	13.6	6.2	
IL1B	UH	SAL	5.5	5.0	3.4	5.5	5.3	4.2	1.2
		LPS	5.1	12.8	4.2	10.6	7.0	3.5	
	CH	SAL	4.6	4.0	4.2	6.8	5.0	5.1	
		LPS	4.4	10.2	3.6	4.2	8.4	3.1	
IL1RN	UH	SAL	7.1	8.4	5.3	5.4	7.0	6.4	4.0
		LPS	5.1	82.2	7.9	6.3	41.5	5.3	
	CH	SAL	5.1	6.8	6.9	5.1	6.8	7.5	
		LPS	5.4	62.3	8.8	6.7	42.4	5.5	
IL1RN.2	UH	SAL	12.4	13.3	10.8	10.5	12.4	11.9	3.7
		LPS	10.1	77.5	12.2	11.9	41.4	11.4	
	CH	SAL	11.2	13.7	13.4	10.2	12.8	13.0	
		LPS	10.2	59.5	13.0	11.9	40.8	11.0	

IL4	UH	SAL	1.1	1.3	1.1	1.3	1.1	1.2	0.1
		LPS	1.1	1.3	1.0	1.2	1.1	1.1	
	CH	SAL	1.1	1.1	1.4	1.0	1.2	1.1	
		LPS	1.1	1.2	1.2	1.1	1.2	1.1	
IL6	UH	SAL	1.1	1.3	1.1	1.3	1.1	1.2	0.3
		LPS	1.1	4.2	1.0	1.2	1.1	1.1	
	CH	SAL	1.1	1.1	1.4	1.0	1.2	1.1	
		LPS	1.1	1.8	1.2	1.1	1.1	1.1	
IL6R	UH	SAL	23.8	26.0	23.9	23.9	23.2	24.3	1.6
		LPS	23.4	26.1	20.9	23.8	19.6	23.1	
	CH	SAL	22.1	26.2	24.0	22.9	22.1	23.3	
		LPS	22.7	22.8	22.9	23.9	19.2	23.3	
IL10	UH	SAL	1.1	1.4	1.1	1.7	1.4	1.2	0.6
		LPS	1.1	7.3	1.0	2.5	3.9	1.1	
	CH	SAL	1.1	1.1	1.4	2.0	1.3	1.1	
		LPS	1.1	6.5	1.2	1.1	4.4	1.1	
IL17C	UH	SAL	1.1	1.3	1.1	1.3	1.1	1.2	0.1
		LPS	1.1	1.3	1.0	1.2	1.1	1.1	
	CH	SAL	1.1	1.1	1.4	1.0	1.2	1.1	
		LPS	1.0	1.2	1.2	1.1	1.2	1.1	
IL17RC	UH	SAL	17.2	17.3	17.7	17.7	17.1	17.4	1.0
		LPS	17.6	17.8	15.0	17.0	13.8	17.7	
	CH	SAL	16.1	18.4	17.1	17.6	17.8	17.7	
		LPS	14.9	14.5	18.1	16.2	14.0	16.8	
IRAK1	UH	SAL	7.6	7.4	7.4	6.2	7.1	8.0	0.5
		LPS	7.1	9.9	8.5	7.4	9.7	7.9	
	CH	SAL	6.7	7.2	7.4	6.7	6.6	8.0	
		LPS	6.3	8.9	9.3	6.9	8.2	7.1	
IRF1	UH	SAL	6.4	5.5	5.2	6.9	6.2	5.4	1.6
		LPS	5.2	19.8	2.6	8.3	10.0	3.9	
	CH	SAL	4.8	5.1	5.4	5.5	6.4	6.7	
		LPS	3.9	13.7	2.0	6.5	12.5	4.5	
JAK2	UH	SAL	6.3	5.6	5.6	6.9	6.0	6.1	0.9
		LPS	5.6	16.6	5.0	6.7	10.9	5.0	
	CH	SAL	5.9	6.4	6.0	6.2	6.7	6.2	
		LPS	5.8	13.5	6.2	6.6	13.3	5.8	
LBP	UH	SAL	52.0	61.2	45.1	40.0	44.9	56.0	12.0
		LPS	32.3	139.1	91.4	61.1	120.3	71.5	
	CH	SAL	40.4	49.5	62.7	38.3	46.0	69.9	
		LPS	33.6	116.4	70.6	40.3	114.3	58.8	
LITAF	UH	SAL	29.3	30.5	26.6	26.5	29.5	26.9	3.0

		LPS	24.9	72.2	28.2	30.6	56.6	28.2	
	CH	SAL	25.5	29.3	31.3	27.6	29.8	27.9	
		LPS	25.0	67.4	29.7	26.1	59.0	28.3	
LYN	UH	SAL	15.3	16.9	16.1	16.4	16.7	15.8	1.4
		LPS	13.9	30.5	17.2	16.6	23.9	17.0	
	CH	SAL	14.3	16.3	16.9	15.8	17.4	15.5	
		LPS	14.1	27.1	17.2	15.4	26.6	16.3	
MYD88	UH	SAL	12.2	14.2	11.8	12.6	12.9	11.9	1.3
		LPS	11.1	30.2	13.8	12.9	24.2	13.5	
	CH	SAL	11.4	13.2	13.2	11.8	13.1	12.5	
		LPS	10.9	25.1	13.8	12.5	21.4	12.9	
NFKB1	UH	SAL	7.9	9.4	7.6	8.4	9.2	8.2	1.5
		LPS	7.4	26.7	7.4	9.3	16.8	8.1	
	CH	SAL	7.1	8.3	7.9	8.4	8.6	7.6	
		LPS	7.7	20.6	8.0	8.1	19.2	7.5	
NFKBIA	UH	SAL	24.1	26.0	21.9	24.6	23.9	21.2	4.7
		LPS	19.7	80.4	19.9	30.6	39.9	19.1	
	CH	SAL	20.7	23.2	23.8	22.7	23.2	22.7	
		LPS	21.8	56.5	20.3	23.9	47.4	19.5	
NLRP3	UH	SAL	2.7	2.3	1.5	1.8	3.0	2.3	0.6
		LPS	1.9	5.6	1.4	3.9	3.1	2.4	
	CH	SAL	1.3	1.1	1.5	1.7	1.3	1.9	
		LPS	1.4	4.9	1.3	2.4	3.7	1.9	
S100A12	UH	SAL	8.6	9.9	8.9	6.7	9.6	8.0	3.4
		LPS	4.9	30.6	26.2	10.4	38.7	20.9	
	CH	SAL	7.8	9.7	10.5	8.1	9.3	15.6	
		LPS	12.3	30.9	39.0	13.6	30.0	27.3	
SAA3	UH	SAL	127.9	145.6	155.6	108.8	116.9	156.6	33.8
		LPS	84.6	248.6	267.0	184.5	230.7	271.8	
	CH	SAL	113.0	135.6	209.9	138.9	137.7	209.1	
		LPS	85.6	214.7	287.9	119.0	229.0	246.0	
SOCS3	UH	SAL	4.3	3.7	5.4	3.6	3.2	4.2	1.0
		LPS	3.4	4.9	3.5	5.3	5.2	4.0	
	CH	SAL	3.0	4.1	5.3	4.2	3.6	4.4	
		LPS	3.1	2.2	2.8	2.3	3.8	3.1	
STAT1	UH	SAL	18.2	18.1	16.2	20.0	21.0	19.9	2.2
		LPS	16.9	39.2	15.7	21.4	33.1	16.5	
	CH	SAL	17.1	19.0	18.7	17.9	19.3	20.6	
		LPS	16.6	34.5	17.3	20.4	32.1	18.1	
STAT3	UH	SAL	26.2	30.0	23.0	24.8	27.1	23.2	3.1
		LPS	21.7	66.7	26.4	28.6	53.9	26.3	

	CH	SAL	22.0	27.0	29.1	25.0	27.1	24.6	
		LPS	24.0	59.1	27.3	25.2	54.9	28.1	
STAT5a	UH	SAL	9.7	9.2	9.4	9.3	9.1	9.0	0.9
		LPS	8.5	21.1	8.8	10.1	16.0	8.8	
	CH	SAL	8.4	9.5	8.9	8.9	9.2	9.2	
		LPS	8.8	19.2	9.8	9.9	18.4	9.3	
STAT5b	UH	SAL	6.3	6.3	6.5	5.4	5.6	6.7	0.6
		LPS	5.8	3.7	5.5	7.1	3.5	6.2	
	CH	SAL	5.4	6.2	5.7	5.8	5.1	6.5	
		LPS	5.9	4.5	6.6	5.8	4.4	5.8	
TGFB1	UH	SAL	5.2	4.5	4.7	4.9	5.9	5.8	0.6
		LPS	5.9	7.4	4.0	5.3	7.3	5.1	
	CH	SAL	5.0	5.6	5.4	4.9	5.9	6.2	
		LPS	4.1	5.6	4.0	5.3	5.6	3.7	
TLR2	UH	SAL	6.5	7.7	6.3	7.2	7.3	6.6	1.6
		LPS	6.1	19.9	6.6	7.1	16.5	6.3	
	CH	SAL	6.2	6.6	7.4	7.0	7.5	7.1	
		LPS	5.5	18.1	5.3	6.6	18.7	6.5	
TLR4	UH	SAL	3.6	3.1	2.7	3.4	3.6	3.1	1.0
		LPS	2.7	14.8	2.6	3.2	8.0	2.7	
	CH	SAL	2.5	2.6	3.0	2.6	3.5	3.1	
		LPS	2.6	10.4	2.0	4.0	10.1	2.6	
TNF	UH	SAL	2.9	3.9	2.3	4.9	4.5	3.1	1.1
		LPS	2.4	15.0	1.2	7.5	9.9	1.7	
	CH	SAL	3.0	2.3	2.9	3.0	4.1	4.2	
		LPS	2.1	10.4	1.6	3.1	10.0	2.1	
TNFRSF1A	UH	SAL	17.4	19.4	16.9	17.2	18.2	16.8	1.9
		LPS	15.4	44.9	17.5	17.9	36.1	18.3	
	CH	SAL	14.9	17.2	17.4	16.3	17.0	16.4	
		LPS	15.1	38.5	18.5	16.5	36.0	16.8	
VEGF-A	UH	SAL	23.6	24.1	25.0	24.8	22.5	24.4	1.6
		LPS	23.0	28.5	20.2	25.4	22.5	22.5	
	CH	SAL	22.4	25.0	24.5	23.7	23.6	23.9	
		LPS	23.4	26.7	23.1	23.9	24.4	23.5	
XBP1	UH	SAL	36.1	43.8	32.8	30.2	32.1	35.2	5.7
		LPS	27.6	91.0	44.4	40.9	78.6	38.0	
	CH	SAL	28.1	31.6	40.7	28.5	29.8	35.4	
		LPS	27.3	81.7	38.4	27.4	80.8	33.9	
Metabolic									
ACOX1	UH	SAL	44.8	44.4	49.7	52.4	44.8	48.0	3.5
		LPS	46.5	50.8	39.3	48.6	35.1	49.7	

	CH	SAL	45.8	50.0	47.8	49.5	46.9	45.2	
		LPS	45.4	41.6	47.0	45.8	39.0	48.0	
GHR-1A	UH	SAL	21.3	21.2	19.8	20.8	18.7	20.3	1.9
		LPS	19.9	20.5	12.0	16.5	13.4	12.7	
	CH	SAL	17.7	17.1	17.1	18.6	16.5	13.8	
		LPS	19.5	17.3	13.2	15.1	13.1	12.8	
GHRtot	UH	SAL	36.1	38.9	35.8	36.6	32.9	34.7	2.4
		LPS	33.5	36.4	29.0	35.1	27.2	30.7	
	CH	SAL	31.2	34.7	35.6	33.5	32.8	30.8	
		LPS	34.0	32.1	31.1	31.4	29.6	31.3	
IGF1	UH	SAL	17.7	15.3	17.2	16.9	15.2	18.8	1.5
		LPS	18.5	21.0	16.1	16.0	16.0	17.6	
	CH	SAL	15.6	14.2	15.9	15.6	13.0	13.1	
		LPS	18.6	18.4	18.9	13.8	14.3	17.5	
IGF1R	UH	SAL	3.9	3.5	4.0	3.2	4.1	3.9	0.5
		LPS	3.6	2.6	2.8	3.7	1.4	4.2	
	CH	SAL	2.7	4.5	3.9	3.8	4.1	3.1	
		LPS	3.9	1.9	3.6	4.1	2.2	4.1	
IGF-ALS	UH	SAL	11.6	10.6	11.0	10.5	10.1	11.5	1.0
		LPS	11.2	8.8	6.7	10.4	7.6	9.1	
	CH	SAL	9.8	9.4	10.1	10.2	9.3	9.9	
		LPS	9.8	8.3	9.3	8.5	6.0	9.1	
IGFBP2	UH	SAL	41.1	42.6	39.3	38.2	38.4	38.2	4.7
		LPS	45.1	49.3	24.3	34.5	31.2	29.2	
	CH	SAL	45.9	51.3	45.4	42.6	44.0	48.5	
		LPS	46.3	49.3	37.3	44.8	37.3	39.3	
IGFBP3	UH	SAL	27.6	29.2	26.7	27.0	27.2	26.8	1.9
		LPS	26.6	33.0	32.3	31.4	28.5	34.2	
	CH	SAL	26.1	28.8	30.2	28.1	26.6	29.9	
		LPS	25.9	29.8	35.6	31.1	28.2	34.7	
IGFBP5	UH	SAL	1.1	1.3	1.0	1.3	1.3	1.2	0.4
		LPS	1.5	1.3	1.0	2.2	1.1	1.8	
	CH	SAL	1.1	1.1	1.4	1.2	1.7	1.7	
		LPS	1.6	1.2	1.2	2.5	1.5	1.8	
INSR-b	UH	SAL	10.0	9.6	10.1	9.5	9.8	9.8	0.7
		LPS	10.3	7.6	7.6	10.9	5.9	9.2	
	CH	SAL	9.3	11.6	10.3	10.5	10.9	10.4	
		LPS	10.0	7.4	10.1	10.6	6.5	10.3	
INSR	UH	SAL	15.5	16.4	15.3	15.7	14.8	15.9	1.2
		LPS	15.3	13.9	13.5	17.2	10.3	15.5	
	CH	SAL	15.0	18.4	17.0	16.0	17.2	16.7	

		LPS	15.7	13.2	16.8	17.1	13.1	16.2	
KLB	UH	SAL	19.5	17.8	21.1	21.0	18.0	20.1	1.8
		LPS	18.9	12.7	18.0	19.0	7.9	21.0	
	CH	SAL	18.1	19.3	21.1	19.6	17.8	19.8	
		LPS	20.1	9.2	22.0	18.4	8.5	20.9	
PC1	UH	SAL	18.6	19.1	17.5	14.3	15.6	17.2	2.4
		LPS	17.3	24.4	20.9	19.5	21.0	19.6	
	CH	SAL	18.5	22.0	21.4	17.0	18.1	22.7	
		LPS	16.3	23.1	20.3	16.9	19.8	17.9	
PCK1	UH	SAL	62.4	69.1	69.5	63.1	60.5	60.5	7.5
		LPS	73.0	23.2	34.3	64.5	16.4	55.7	
	CH	SAL	66.8	82.1	68.8	66.9	74.4	63.7	
		LPS	58.6	15.2	54.8	68.1	22.4	60.0	
PPARa	UH	SAL	27.0	27.7	29.3	29.8	24.0	28.1	1.8
		LPS	27.1	14.9	25.2	27.4	13.7	27.5	
	CH	SAL	24.8	26.5	26.9	26.5	23.8	26.7	
		LPS	26.2	14.4	27.4	28.1	16.0	27.8	
PPARd	UH	SAL	3.9	4.5	2.9	3.7	4.0	3.5	0.7
		LPS	3.2	6.8	4.1	3.2	6.3	4.1	
	CH	SAL	3.6	3.1	2.9	3.6	4.1	3.4	
		LPS	3.4	6.9	3.8	4.0	6.3	4.1	
PPARGC1A	UH	SAL	8.6	5.7	7.9	7.8	5.6	7.3	1.0
		LPS	7.3	19.7	7.0	7.0	12.9	8.5	
	CH	SAL	7.5	7.7	8.4	7.8	7.1	8.5	
		LPS	8.0	19.2	9.5	7.5	13.6	9.2	
XDH	UH	SAL	31.0	32.8	29.6	31.0	30.6	30.1	2.7
		LPS	30.4	45.6	31.1	34.3	37.3	34.1	
	CH	SAL	29.9	35.8	33.1	31.2	33.0	30.3	
		LPS	27.5	35.2	30.1	27.9	37.2	29.6	

[1]Treatment – cows were treated with iv saline (SAL) or LPS (0.25 µg LPS/kg BW) at time 0

[2]Challenge - identical saline or LPS challenges were conducted on day 1 and 5 (C1 and C2, respectively)

Supplement 3. Effect of genotype (G), time (T), treatment (Trt[1]) and its interaction during challenge 1 (C1) on hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation.

Gene	P-values						
	G	T	GT	Trt	GTrt	TTrt	GTTrt
Immunity							
ADAM17	0.472	<.001	0.133	0.006	0.763	0.000	0.388
AGP	0.872	0.003	0.427	0.501	0.523	0.152	0.271
C3	0.751	0.018	0.260	0.559	0.909	0.078	0.531
CASP1	0.735	<.001	0.701	0.777	0.905	0.000	0.255
CCL5	0.389	<.001	0.296	<.001	0.301	<.001	0.180
CCL20	0.101	<.001	0.037	<.001	0.094	<.001	0.055
CD14	0.098	<.001	0.081	0.097	0.716	<.001	0.234
CD40	0.000	<.001	0.447	<.001	0.010	<.001	0.457
CD40LG	0.405	0.000	0.812	0.757	0.592	0.001	0.468
CEBPD	0.901	<.001	0.765	0.029	0.388	<.001	0.130
CSF3	0.578	0.085	0.023	0.603	0.989	0.278	0.872
FASLG	0.180	0.039	0.607	0.374	0.200	0.058	0.544
FGF21	0.354	0.082	0.678	0.052	0.975	0.068	0.541
FGFR2c	0.572	<.001	0.385	<.001	0.951	<.001	0.432
FOXO3	0.920	<.001	0.186	0.002	0.650	<.001	0.708
ICAM-1	0.001	<.001	0.051	<.001	0.002	<.001	0.094
IL1B	0.097	<.001	0.343	<.001	0.326	<.001	0.760
IL1RN	0.022	<.001	0.045	<.001	0.063	<.001	0.140
IL1RN.2	0.156	<.001	0.046	<.001	0.082	<.001	0.054
IL4	0.578	0.085	0.023	0.603	0.989	0.278	0.872
IL6	<.001	0.000	0.001	<.001	<.001	<.001	0.056
IL6R	0.590	0.050	0.564	0.258	0.917	0.564	0.421
IL10	0.375	<.001	0.324	<.001	0.390	<.001	0.922
IL17C	0.590	0.037	0.028	0.538	0.741	0.287	0.843
IL17RC	0.263	0.606	0.044	0.073	0.491	0.510	0.009
IRAK1	0.284	<.001	0.232	0.006	0.960	<.001	0.468
IRF1	0.003	<.001	0.219	<.001	0.054	<.001	0.327
JAK2	0.690	<.001	0.202	<.001	0.309	<.001	0.091
LBP	0.413	<.001	0.223	0.007	0.534	<.001	0.163
LITAF	0.743	<.001	0.347	<.001	0.786	<.001	0.659
LYN	0.268	<.001	0.284	<.001	0.494	<.001	0.506
MYD88	0.137	<.001	0.050	<.001	0.189	<.001	0.309
NFKB1	0.079	<.001	0.048	<.001	0.334	<.001	0.160
NFKBIA	0.059	<.001	0.026	<.001	0.200	<.001	0.049
NLRP3	0.003	<.001	0.111	<.001	0.282	<.001	0.643

Gene							
S100A12	0.194	<.001	0.263	<.001	0.218	<.001	0.304
SAA3	0.916	<.001	0.089	0.109	0.824	<.001	0.343
SOCS3	0.269	0.288	0.707	0.179	0.548	0.201	0.003
STAT1	0.853	<.001	0.313	<.001	0.393	<.001	0.385
STAT3	0.598	<.001	0.122	<.001	0.757	<.001	0.246
STAT5a	0.458	<.001	0.707	<.001	0.799	<.001	0.274
STAT5b	0.925	0.058	0.473	0.074	0.127	0.003	0.853
TGFB1	0.282	0.012	0.231	0.806	0.012	0.012	0.374
TLR2	0.361	<.001	0.723	<.001	0.425	<.001	0.848
TLR4	0.039	<.001	0.090	<.001	0.208	<.001	0.063
TNF	0.071	<.001	0.007	<.001	0.245	<.001	0.433
TNFRSF1A	0.120	<.001	0.162	<.001	0.803	<.001	0.417
VEGF-A	0.901	0.022	0.690	0.971	0.675	0.040	0.409
XBP1	0.193	<.001	0.322	0.000	0.879	<.001	0.419
Metabolism							
ACOX1	0.865	0.812	0.621	0.383	0.588	0.445	0.037
GHR-1A	0.139	0.007	0.247	0.161	0.343	0.044	0.568
GHRtot	0.232	0.220	0.331	0.085	0.396	0.236	0.551
IGF1	0.551	0.740	0.428	0.034	0.481	0.023	0.203
IGF1R	0.966	0.255	0.393	0.026	0.670	0.004	0.036
IGF-ALS	0.464	0.016	0.252	0.055	0.294	0.194	0.537
IGFBP2	0.148	0.004	0.649	0.549	0.808	0.123	0.480
IGFBP3	0.858	0.001	0.100	0.028	0.696	0.050	0.638
IGFBP5	0.659	0.387	0.353	0.220	0.957	0.171	0.902
INSR-b	0.209	0.159	0.173	0.010	0.866	0.001	0.092
INSR	0.197	0.961	0.395	0.063	1.000	0.035	0.378
KLB	0.799	<.001	0.566	0.040	0.849	0.001	0.103
PC1	0.741	<.001	0.646	0.659	0.425	0.013	0.561
PCK1	0.653	0.001	0.402	<.001	0.577	<.001	0.199
PPARa	0.487	<.001	0.861	0.001	0.371	<.001	0.762
PPARd	0.495	0.007	0.868	0.005	0.502	0.049	0.732
PPARGC1A	0.373	<.001	0.582	<.001	0.770	<.001	0.311
XDH	0.409	0.000	0.474	0.482	0.074	0.045	0.154

[1]Treatment – cows were treated with iv saline (SAL) or LPS (0.25 µg LPS/kg BW) at time 0

Supplement 4. Effect of genotype (G), time (T), treatment (Trt[1]), challenge (C[2]) and its interaction on hepatic gene expression in unselected (UH) and contemporary (CH) multiparous Holstein cows at 10 weeks of lactation.

	P-values														
Gene	G	T	GT	Trt	GTrt	TTrt	GTTrt	C	GC	TC	GTC	TrtC	GTrtC	TTrtC	GTTrtC
Immunity															
ADAM17	0.278	0.001	0.478	<.001	0.891	<.001	0.804	0.149	0.872	0.269	0.192	0.360	0.509	0.310	0.715
AGP	0.539	0.084	0.356	0.850	0.382	0.149	0.991	0.854	0.678	0.054	0.072	0.516	0.936	0.866	0.113
C3	0.538	0.431	0.347	0.433	0.706	0.055	0.889	0.581	0.919	0.073	0.492	0.916	0.857	0.813	0.719
CASP1	0.803	<.001	0.835	0.406	0.928	0.001	0.776	0.013	0.513	0.104	0.705	0.626	0.813	0.104	0.029
CCL5	0.311	<.001	0.506	<.001	0.330	<.001	0.242	0.789	0.873	0.358	0.644	0.835	0.662	0.555	0.736
CCL20	0.547	<.001	0.953	<.001	0.599	<.001	0.971	0.001	0.000	0.368	0.220	0.002	0.000	0.351	0.280
CD14	0.040	<.001	0.359	0.110	0.712	<.001	0.787	0.334	0.717	0.204	0.087	0.402	0.877	0.348	0.267
CD40	0.160	<.001	0.874	<.001	0.597	<.001	0.962	0.575	0.417	0.336	0.359	0.440	0.388	0.149	0.206
CD40LG	0.109	0.002	0.588	0.581	0.468	<.001	0.633	0.947	0.809	0.065	0.378	0.296	0.868	0.862	0.652
CEBPD	0.423	<.001	0.931	0.088	0.377	0.001	0.716	0.021	0.533	0.001	0.817	0.140	0.741	0.005	0.333
CSF3	0.858	0.584	0.300	0.782	0.734	0.520	0.839	0.437	0.359	0.190	0.005	0.677	0.743	0.651	0.802
FASLG	0.445	0.123	0.123	0.251	0.066	0.185	0.457	0.799	0.201	0.119	0.835	0.846	0.924	0.184	0.736
FGF21	0.339	0.028	0.207	0.051	0.291	0.022	0.764	0.893	0.525	0.013	0.435	0.145	0.316	0.952	0.097
FGFR2c	0.635	<.001	0.166	<.001	0.864	<.001	0.198	0.288	0.702	0.283	0.435	0.780	0.937	0.785	0.861
FOXO3	0.582	0.000	0.608	0.000	0.415	<.001	0.819	0.001	0.697	0.004	0.245	0.096	0.935	0.039	0.878
ICAM-1	0.005	<.001	0.312	<.001	0.003	<.001	0.633	0.543	0.571	0.004	0.229	0.050	0.895	0.008	0.274
IL1B	0.157	<.001	0.322	0.003	0.091	<.001	0.271	0.785	0.787	0.016	0.227	0.146	0.476	0.045	0.060
IL1RN	0.339	<.001	0.276	<.001	0.420	<.001	0.419	0.001	0.250	0.000	0.285	0.002	0.373	0.000	0.343
IL1RN.2	0.405	<.001	0.328	<.001	0.249	<.001	0.341	0.002	0.401	0.000	0.364	0.007	0.363	0.000	0.404
IL4	0.858	0.584	0.300	0.782	0.734	0.520	0.839	0.437	0.359	0.190	0.005	0.677	0.743	0.651	0.802
IL6	0.043	0.005	0.063	0.005	0.075	0.003	0.110	0.001	0.154	0.003	0.015	0.005	0.043	0.003	0.209
IL6R	0.364	0.984	0.623	0.062	0.760	0.343	0.668	0.098	0.980	0.010	0.706	0.921	0.626	0.650	0.766

IL10	0.667	<.001	0.847	<.001	0.591	<.001	0.642	0.331	0.969	0.002	0.456	0.052	0.875	0.013	0.384
IL17C	0.825	0.383	0.267	0.747	0.895	0.505	0.984	0.558	0.364	0.095	0.006	0.642	0.579	0.635	0.681
IL17RC	0.352	0.228	0.229	0.000	0.289	0.093	0.139	0.813	0.482	0.136	0.170	0.335	0.949	0.484	0.066
IRAK1	0.078	<.001	0.367	0.000	0.347	0.000	0.695	0.177	0.850	0.766	0.277	0.538	0.309	0.027	0.803
IRF1	0.303	<.001	0.513	0.006	0.556	<.001	0.847	0.706	0.177	0.024	0.294	0.454	0.372	0.012	0.314
JAK2	0.466	<.001	0.564	<.001	0.844	<.001	0.563	0.557	0.196	0.174	0.220	0.051	0.100	0.198	0.267
LBP	0.342	<.001	0.695	<.001	0.252	<.001	0.432	0.671	0.774	0.054	0.253	0.986	0.835	0.042	0.320
LITAF	0.831	<.001	0.470	<.001	0.614	<.001	0.974	0.118	0.800	0.049	0.521	0.320	0.906	0.033	0.434
LYN	0.512	<.001	0.894	<.001	0.790	<.001	0.951	0.649	0.379	0.184	0.271	0.248	0.498	0.311	0.667
MYD88	0.084	<.001	0.175	<.001	0.109	<.001	0.482	0.110	0.754	0.071	0.646	0.239	0.846	0.245	0.982
NFKB1	0.202	<.001	0.713	<.001	0.752	<.001	0.926	0.281	0.288	0.096	0.197	0.065	0.341	0.159	0.318
NFKBIA	0.234	<.001	0.433	<.001	0.485	<.001	0.789	0.066	0.227	0.006	0.113	0.088	0.366	0.014	0.152
NLRP3	0.022	<.001	0.578	0.001	0.489	0.000	0.260	0.438	0.959	0.020	0.823	0.781	0.908	0.001	0.166
S100A12	0.157	<.001	0.003	<.001	0.577	<.001	0.045	0.970	0.597	0.313	0.585	0.809	0.198	0.064	0.884
SAA3	0.909	<.001	0.382	0.005	0.328	<.001	0.701	0.763	0.977	0.007	0.194	0.643	0.523	0.103	0.103
SOCS3	0.212	0.500	0.683	0.436	0.198	0.046	0.297	0.906	0.927	0.663	0.415	0.379	0.565	0.366	0.004
STAT1	0.688	<.001	0.320	<.001	0.814	<.001	0.639	0.270	0.844	0.073	0.911	0.386	0.476	0.061	0.682
STAT3	0.750	<.001	0.243	<.001	0.707	<.001	0.803	0.350	0.654	0.075	0.422	0.853	0.941	0.203	0.355
STAT5a	0.934	<.001	0.736	<.001	0.429	<.001	0.873	0.340	0.292	0.088	0.553	0.435	0.646	0.144	0.248
STAT5b	0.813	<.001	0.308	0.069	0.384	<.001	0.245	0.853	0.719	0.228	0.959	0.574	0.246	0.538	0.207
TGFB1	0.088	0.000	0.593	0.781	0.004	0.001	0.335	0.123	0.808	0.569	0.216	0.540	0.516	0.400	0.483
TLR2	0.852	<.001	0.950	<.001	0.789	<.001	0.848	0.801	0.420	0.624	0.643	0.816	0.548	0.754	0.901
TLR4	0.413	<.001	0.798	<.001	0.974	<.001	0.421	0.619	0.140	0.084	0.168	0.253	0.182	0.011	0.243
TNF	0.065	<.001	0.078	<.001	0.272	<.001	0.892	0.153	0.951	0.017	0.009	0.406	0.903	0.010	0.342
TNFRSF1A	0.098	<.001	0.474	<.001	0.834	<.001	0.803	0.305	0.637	0.057	0.360	0.462	0.898	0.196	0.492
VEGF-A	0.764	0.283	0.610	0.928	0.479	0.036	0.532	0.464	0.949	0.097	0.643	0.874	0.928	0.372	0.701
XBP1	0.135	<.001	0.625	<.001	0.635	<.001	0.544	0.297	0.776	0.177	0.263	0.727	0.789	0.426	0.363

Metabolism															
ACOX1	0.913	0.114	0.793	0.073	0.810	0.515	0.259	0.989	0.717	0.026	0.330	0.622	0.562	0.081	0.209
GHR-1A	0.025	0.002	0.896	0.002	0.125	0.234	0.593	0.032	0.991	0.204	0.399	0.234	0.916	0.289	0.701
GHRtot	0.120	0.414	0.594	0.010	0.240	0.385	0.903	0.072	0.792	0.064	0.204	0.987	0.916	0.398	0.572
IGF1	0.068	0.224	0.794	0.057	0.287	0.014	0.207	0.043	0.337	0.316	0.489	0.172	0.973	0.305	0.385
IGF1R	0.695	0.015	0.724	0.010	0.430	<.001	0.378	0.688	0.740	0.688	0.215	0.537	0.842	0.161	0.103
IGF-ALS	0.110	0.001	0.440	0.002	0.523	0.073	0.296	0.461	0.597	0.283	0.565	0.670	0.357	0.511	0.986
IGFBP2	0.006	0.081	0.563	0.098	0.975	0.067	0.571	0.076	0.644	0.016	0.863	0.485	0.675	0.348	0.654
IGFBP3	0.622	0.003	0.139	0.000	0.508	0.058	0.955	0.856	0.798	0.027	0.640	0.391	0.890	0.391	0.726
IGFBP5	0.296	0.321	0.891	0.075	0.952	0.085	0.755	0.027	0.472	0.688	0.750	0.381	0.984	0.459	0.911
INSR-b	0.041	0.000	0.219	0.000	0.815	<.001	0.160	0.919	0.917	0.121	0.668	0.902	0.622	0.326	0.331
INSR	0.023	0.056	0.292	0.006	0.973	0.000	0.690	0.965	0.924	0.061	0.289	0.678	0.975	0.812	0.521
KLB	0.983	<.001	0.733	0.001	0.739	<.001	0.378	0.576	0.685	0.410	0.642	0.646	0.964	0.460	0.360
PC1	0.553	<.001	0.607	0.321	0.095	0.036	0.826	0.201	0.931	0.068	0.951	0.767	0.667	0.176	0.584
PCK1	0.233	<.001	0.438	<.001	0.537	<.001	0.252	0.967	0.632	0.605	0.599	0.169	0.780	0.411	0.438
PPARa	0.476	<.001	0.634	<.001	0.116	<.001	0.906	0.815	0.708	0.155	0.810	0.357	0.853	0.379	0.715
PPARd	0.790	<.001	0.746	0.001	0.462	0.000	0.792	0.674	0.515	0.791	0.903	0.685	0.866	0.685	0.723
PPARGC1A	0.088	<.001	0.452	<.001	0.929	<.001	0.330	0.008	0.852	0.002	0.851	0.046	0.680	0.003	0.588
XDH	0.244	<.001	0.712	0.134	0.023	0.016	0.676	0.698	0.961	0.178	0.243	0.633	0.680	0.831	0.216

[1]Treatment – cows were treated with iv saline (SAL) or LPS (0.25 µg LPS/kg BW) at time 0

[2]Challenge - identical saline or LPS challenges were conducted on day 1 and 5 (C1 and C2, respectively)