BRANCHED CHAIN AMINO ACIDS OR BRANCHED CHAIN KETO ACIDS ALTER NEUTROPHIL IMMUNOMETABOLIC GENE EXPRESSION IN EARLY LACTATION DAIRY COWS

By

Isabelle R. Bernstein

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Animal Science – Master of Science

2023

ABSTRACT

Dairy cattle undergo both metabolic and physiological changes in the early lactation period that result in inflammation and increase their risk of disease. The effects of branched chain amino acids (BCA) and branched chain keto-acids (BCK) on neutrophil phagocytic and oxidative burst function as well as antioxidant defense, toll-like receptor pathways, and adhesion and migration gene expression were determined in one experiment. In this experiment, 36 multiparous dairy cows, enrolled using a randomized block design, were abomasally infused for the first 21 days post-partum with 1 of 3 treatments: 1) CON (saline); 2) BCA, consisting of 67g valine, 50g leucine, and 34g isoleucine; and 3) BCK, consisting of 77g keto-valine, 57g keto-leucine, and 39g keto-isoleucine. No changes in the proportion of neutrophils undergoing phagocytosis or oxidative burst were found between treatments or between days 10 and 20 of lactation. BCA treatment increased neutrophil expression of *GPX1* and decreased expression of *MPO* compared to CON cows. Abomasal infusion of BCK increased neutrophil expression of *IRAK1* and *IL10RA* compared to CON infusion. No changes in neutrophil adhesion and migration gene expression were observed between treatments. I conclude that supplementation of branched chain amino acids alters neutrophil antioxidant defense and lysosomal protein gene expression while branched chain keto-acids alter neutrophil cytokine and toll-like receptor pathway gene expression in early lactation multiparous dairy cows.

ACKNOWLEDGMENTS

I have so many people that helped me through my graduate degree at Michigan State and continued to encourage and inspire me. I'd like to begin with Dr. Zhou, who initially took me on as an undergraduate research assistant. If not for his willingness to accept someone who had very minimal large animal experience, I would not have gained the interest I had in dairy nutrition nor would I have learned so much in this field. I would also like to thank my committee members, Dr. Contreras, Dr. Bradford, and Dr. VandeHaar for both their patience and support while I've navigated my way through my program, as well as their willingness to offer a helping hand.

I'd also like to acknowledge how grateful I am for Cynthia Collings and all her support both in the lab and at the farm. If not for her, I would not have been able to learn or complete any of the lab work needed for my project. Not only has her knowledge both guided and inspired me, but she has been an amazing emotional support during more troublesome times. Dave Main also used lots of his time to assist at the farm and gave us his insight on how to improve our biopsy and catheterization techniques, which I am extremely grateful for. Thank you also to Jayasimha Daddam, who assisted me with my writing and finding sources when I was stuck. He helped keep me on track when I needed an extra push and I appreciate everything he has done since joining us this Spring.

We also had a plethora of undergraduate students that assisted both at the farm and in the laboratory. I am so amazed at how eager and helpful they were and extremely proud of the amount they learned about dairy cows and animal handling. Without them, this project would not have been possible. I would also like to add a special thank you to fellow graduate student and friend, Sarah Naughton, who immediately after starting her PhD, came and assisted with this project. She added so much enthusiasm and a positive energy that carried us through the toughest

days at the farm. Sarah saved us so much time and energy that we were beginning to run out of, and I am proud to have received her assistance and friendship.

I would like to end by saying how eternally thankful I am for my (now graduated) lab member and mentor Kristen Gallagher. She taught me so much about dairy cows and management, practical farm skills, laboratory skills, and data management beginning when I was still an undergraduate student and into my graduate studies. Kristen inspired me to go to graduate school and I will always be grateful for her encouragement. She made every day of the project so much easier, and her confidence and knowledge helped keep me on track when I was doubtful.

Overall, I am so thankful to everyone who has been in my life during the entirety of my graduate studies – my parents, fellow graduate students, other Animal Science faculty, and friends from all around. You all have made this experience such a wonderful and memorable time. Although my family and non-animal science friends may not always understand what I'm doing or what I'm talking about, they have continued to be there for me and encourage me.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC: allophycocyanin

BCA: branched chain amino acid

BCAT: branched chain amino acid transferase

BCK: branched chain keto acid

BCKDH: branched chain α keto acid dehydrogenase

BHBA: β-hydroxybutyrate

CXCR2: C-X-C Motif Chemokine Receptor 2

CXCR4: C-X-C Motif Chemokine Receptor 4

DMI: dry matter intake

FITC: fluorescein isothiocyanate

FSC: forward scatter

G-CSF: granulocyte colony-stimulating factor

GPX1: glutathione peroxidase 1

ICAM: intercellular adhesion molecule

IKK: inhibitor of kappa b kinase

IL-10: interleukin 10

IL-1β: interleukin 1β

IL-4: interleukin 4

IL-6: interleukin 6

IκB: inhibitor of kappa b

LFA1: lymphocyte function-associated antigen 1

LPS: lipopolysaccharide

MAC1: macrophage-1 antigen

MFI: mean fluorescent intensity

MPO: myeloperoxidase

NEFA: non-esterified fatty acid

NET: neutrophil extracellular traps

NFκB: nuclear factor κB

NOD: nucleotide oligomerization domain

NSAID: non-steroidal anti-inflammatory drug

PAMP: pathogen associated molecular pattern

PBS: phosphate-buffered saline

PE: phycoerythrin

PI: propidium iodine

PMNL: polymorphonuclear leukocyte

PRR: pattern recognition receptors

ROS: reactive oxygen species

SOD: superoxide dismutase

SSC: side scatter

TLR: toll-like receptor

TrxR: thioredoxin reductase

CHAPTER 1:

REVIEW OF THE LITERATURE

LITERATURE REVIEW

Early lactation cows and disease

Dairy cattle experience many metabolic and physical changes during the time immediately before and after parturition. This period, between 3 weeks before and 3 weeks after parturition, is defined as the transition period (Drackley, 1999). During the 3 weeks after parturition, cows are at the highest risk for infection and metabolic diseases, except the first few weeks of their own life (Bradford et al., 2015). In fact, most infections and metabolic diseases occur during this period, with approximately 50% of cows suffering from at least one subclinical disorder post-calving (Bradford et al., 2015; Drackley, 1999). The incidence of disease and illness is costly to producers, as it results in decreased milk production often during the entire lactation period and subsequent lactations (Drackley, 1999). Producers also often have to cull affected cows, as the 2 weeks after parturition accounts for an estimated 50% of morbidity on dairy farms, which is not economically preferable (Bradford et al., 2015). Unfortunately for the dairy industry, most disorders that commonly occur during the early lactation period are just as pervasive as they were 20 years ago (Goff, 2006).

Oxidative stress in the early lactation cow

Dairy cattle are put under substantial amounts of metabolic and physiological stress during their adaptation from pregnancy to lactation. Nutrients must meet the increase in demand for lactation and maintenance. This elevated demand of metabolites results in an increase in oxidation of energy-producing substrates and the body begins to accumulate reactive oxygen species (**ROS**) (Sordillo & Aitken, 2009). When there is insufficient antioxidant capacity to neutralize ROS, cows experience oxidative stress (Sordillo & Aitken, 2009). Some ROS include superoxide and hydrogen peroxide. ROS are free radicals that are formed as an end product of

the electron transport chain and can result in tissue damage by attacking DNA, lipids, proteins, etc. (Sordillo & Aitken, 2009). The oxidative stress that early lactation dairy cattle experience increases their susceptibility to disease, especially mastitis. ROS are important in facilitating pathogen clearance, but they may also promote the breakdown of the blood–milk barrier and induce mammary epithelial tissue damage (Schukken et al., 2011). Bradford et al. (2015) concluded that oxidative stress likely interferes with resolution of inflammation, resulting in the associated susceptibility of disease. Dairy cows also undergo extensive tissue remodeling during the transition period and must use body reserves as an energy source. This lipid mobilization is associated with increased plasma non-esterified fatty acid (**NEFA**) concentrations. Contreras et al. (2012) showed that increasing concentrations of physiological NEFA increased ROS in bovine endothelial cells. Additionally, lipid peroxides are formed when intracellular lipids come into contact with ROS – lipid peroxidation deteriorates cell membrane integrity (Bradford et al., 2015). The sudden transition to lactation as well as higher body condition are closely associated with increased lipid peroxidation (Bernabucci et al., 2005). Despite this, ROS are still important as a host defense mechanism against pathogens produced by neutrophils and macrophages (Sordillo & Aitken, 2009).

Inflammation during the early lactation period

The mechanism causing the high incidence of disease during early lactation likely involves inflammation. Parturition is a stressful event and the days following involve systemic inflammation as a response (Bradford et al., 2015). It has been argued whether inflammation is beneficial or detrimental to the cow during early lactation, and the answer to this is not simple. Proinflammatory cytokines not only induce immune cell activation, but also symptoms of sickness such as fever, fatigue, and loss of appetite. This communication via cytokines is

completed by either the endocrine system through the blood or the brain through the vagus nerve (Dantzer & Kelley, 2007). It is theorized that the inflammatory status of post-partum cows may help reduce incidence of disease, as well as supporting reproductive function and homeorhesis. Dairy cows have a high energy demand during early lactation and as a result must mobilize lipids from adipose tissue, which also is linked to higher incidence of disease (Drackley, 1999). Another role of inflammation during this period is to facilitate parturition, which is distinguished by a large influx of macrophages and neutrophils to the uterus. This leads to dilation of the cervix, contraction, bursting of the fetal membrane, and expelling the placenta; all of which are essential for parturition to occur (Challis et al., 2009; van Engelen et al., 2009).

Inflammation and milk production

It is important to note that an excessive amount of inflammation can impede lactation performance, concluded in both observational and controlled studies (Ballou, 2012; Bradford et al., 2015). Mireles et al. (2005) and Tang et al. (2010) suggested that inflammation repartitions nutrient usage and decreases energy available for production. The mechanisms behind decreased milk yield in relation to inflammation are still poorly defined. It is also hypothesized that systemic inflammation during the early lactation period is necessary for allocating glucose to the mammary gland as well as other nutrients, such as amino acids and other glucogenic compounds, that are in high demand (Bradford et al., 2015; Drackley, 1999; Sadri et al., 2017). The link between inflammation, nutrient partitioning, and disease are not very well defined, but it was found that challenging cows with inflammatory medium can disrupt metabolic homeostasis by repressing dry matter intake (**DMI**) (Gott et al., 2015). Overall, reducing inflammation, rather than eliminating it, is theorized to be the best solution to the health problems incurred while preserving high production during early lactation (Bradford et al., 2015).

Lipopolysaccharides and inflammation

Lipopolysaccharides (**LPS**), a component of the outer membrane of gram-negative bacteria, are the main pathogen component that initiates an inflammatory response during infection (Hogan & Smith, 2003). This triggers an inflammatory response and evokes leukocyte migration to the target (Schukken et al., 2011). LPS are also released in the rumen from breakdown of bacteria, which brings on inflammation (and subsequently decreased feed intake and milk production). This problem can be exacerbated by a sudden shift in diet, such as a dry cow would shift into a high-grain lactation diet during the transition period (Gott et al., 2015). A commonly studied phenomenon, known as endotoxin tolerance, results from repeated subacute doses of LPS and has been found to decrease generation of proinflammatory mediators in tolerant animals (Zebeli et al., 2013).

Cytokines in the inflammatory response

To begin the inflammatory cascade, the body will release inflammatory mediators such as cytokines, chemokines, adhesion molecules, complement proteins, and eicosanoids (Newton & Dixit, 2012). Pro-inflammatory cytokines, including tumor necrosis factor α (**TNFα**), interleukin 1β (**IL-1β**), and interleukin 6 (**IL-6**), are produced by different immune cells, but mostly produced by macrophages and mast cells. These proinflammatory cytokines recruit leukocytes and activate endothelial cells while additionally activating the acute phase response (Bannerman et al., 2009). Ohtsuka et al. (2001) observed increased serum TNFα activity in cows with moderate to severe fatty liver. Resolving signals (involved in reducing inflammation) include interleukin 10 (**IL-10**), interleukin 4 (**IL-4**) and omega-3 fatty acid derivatives (resolvins and protectins) (Banchereau et al., 2012; Fiorentino et al., 1991; Spite et al., 2014). Scavenging of chemokines and cytokines is another mechanism that functions to reduce inflammatory processes

and stop neutrophil migration (Liew & Kubes, 2019). Repeated subacute administration of cytokines has impaired production and markers of health in cows (Bradford et al., 2015), suggesting that chronic subacute inflammation is systemically detrimental. Kushibiki et al. (2003) demonstrated that subacute doses of TNFα decreased feed intake, elevated plasma NEFA concentrations, and increased markers of oxidative stress in mid-lactation cows.

Pattern recognition receptors

Pattern recognition receptors (**PRR**s) also play a role in the induction of cytokine production and other inflammatory mediators by activation with pathogen association molecular patterns (**PAMP**s). Different PRRs include toll-like receptors (**TLR**s), C-lectin receptors, and nucleotide-binding oligomerization domain (**NOD**)-like receptors, each activated by different molecules (Takeuchi & Akira, 2010). One of the most notable TLRs is TLR4, which is commonly known as the LPS receptor. Interestingly, TLR4 is also activated by saturated fatty acids, which are at a high concentration in early lactation (Mamedova et al., 2013). Although neutrophils are recruited by TLR4 signaling, activation of TLR on immune cells causes them to systemically release multiple chemokines that can distract neutrophils from locating the bacteria in an infectious site (Andonegui et al., 2009; Liew & Kubes, 2019).

Chemokines in the inflammatory response

Chemokine signaling along the endothelium guides neutrophils to exit the vasculature and toward an end target (usually being the site of infection). This involves a second chemokine gradient, although the means by which different chemokines signal through the same receptor is not established (Liew & Kubes, 2019; Massena et al., 2010; Zimmerman et al., 1997). Chemokines such as CXCL8, CXCL1, CXCL2, and CXCL5 all signal via CXC chemokine receptor 2 (**CXCR2**) on neutrophils, which results in a conformational change of the integrins

(Abram & Lowell, 2009; Pruenster et al., 2009; Sadik et al., 2011; Sanz & Kubes, 2012; Williams et al., 2011). The integrins lymphocyte function-associated antigen 1 (**LFA1**) and macrophage-1 antigen (**MAC1**) bind to intercellular adhesion molecule 1 and 2 (**ICAM1**, **ICAM2**) on endothelial cells after conformational change (Phillipson & Kubes, 2011).

Nuclear factor kappa b

Nuclear factor kappa b (**NFκB**) is a family of transcription factor that play a large role in inflammation and signaling. NFκB is typically activated by the phosphorylation of inhibitor of kappa b (**IκB**) by IκB kinase (**IKK**) complex and in degraded. NFκB is then translocated into the nucleus in order to trigger various inflammatory transcriptional responses (Hoffmann & Baltimore, 2006). NFκB has been found to induce either negative feedback genes or positive feedback genes depending on the quantity of pathogen-associated molecular pattern molecules. Sung et al. (2014) examined LPS-induced NFκB activity in macrophages and observed that subcritical doses of LPS induce predominantly negative feedback genes, preventing sufficient response from macrophages, whereas macrophages responded well in response to high doses of LPS in which amplification of NFκB becomes a positive feedback loop and overcomes the negative feedback signals. Generally, however, NFκB is a proinflammatory transcription factor and its downregulation accompanies synthesis of anti-inflammatory cytokines IL-10 and IL-4 (Iribarren et al., 2003; Landen et al., 2016; Schottelius et al., 1999; Wynn & Vannella, 2016). Although inflammatory response can occur regardless of NFκB activity, lack of NFκB in mice models resulted in impaired cell proliferation (Cao et al., 2001).

Antioxidant defense

As host immune cells produce ROS to fight pathogens, the body must also have a mechanism to protect itself from damage. Selenium is well known as an important antioxidant

and is often incorporated into enzymes, known as selenoenzymes (Sordillo & Aitken, 2009). Glutathione peroxidase (**GPX1**) is one of such selenoenzymes and is frequently used as a tool to assess selenium status in dairy cattle as well as ROS production. It plays a large role in reducing hydrogen peroxide and lipid peroxides to water and alcohols by oxidizing glutathione (Sordillo & Aitken, 2009). Sordillo et al. (2007) found that GPX1 activity increases at calving and into the first 3 weeks after calving. This correlates with other studies that found that lipid peroxides and other biomarkers of oxidative stress increased from calving and through 3 weeks after (Bernabucci et al., 2005) indicating that GPX1 is needed to reduce concentrations of ROS. Thioredoxin reductase (**TrxR**) is another selenoenzyme that functions similarly to GPX1 (Sordillo et al., 2007). Another important group of important antioxidant defense enzymes are superoxide dismutases (**SODs**) which reduce superoxide anions into molecular oxygen and hydrogen peroxide (Younus, 2018).

Polymorphonuclear leukocytes

Neutrophils, known as one type of white blood cell, are a myeloid leukocyte that are one of the primary responders during inflammation and defend against pathogens such as bacteria, fungi, and protozoa (Liew & Kubes, 2019). They are also known as polymorphonuclear leukocytes (**PMNL**) due to their segmented nucleus consisting of 3 to 5 lobes and granules with digestive enzymes (Curi et al., 2020). Neutrophil cytoplasmic granules play an important role in killing pathogens. These granules consist of at least four types, including primary, secondary, and tertiary granules and secretory granules/vesicles (Lacy, 2006; Sheshachalam et al., 2014). Primary or azurophil granules contain the microbicidal enzyme myeloperoxidase (**MPO**) and neutral proteases cathepsin G, elastase, proteinase 3, and defensins. These primary granules release proteins and peptides that are used for pathogen killing and digestion; these are the first

to be produced during the development of the neutrophil (Sheshachalam et al., 2014). Secondary and tertiary granules contain overlapping contents, including lactoferrin and matrix metalloproteinase-9, which aid in breaking down the extracellular matrix and activating IL-1β (Lacy, 2006; Sheshachalam et al., 2014). Finally, secretory granules contain the negative acute phase protein albumin and some preformed cytokines (Sheshachalam et al., 2014). Granules are released in hierarchical order beginning with secretory granules, tertiary granules, secondary granules, and finally primary granules (Sengelov et al., 1993). Neutrophils are extremely malleable cells, and have also been found to change gene expression in response to varying intercellular signals (Newburger et al., 2000). Neutrophils display functions such as phagocytosis, degranulation, and neutrophil extracellular trap (**NET**) formation (Liew & Kubes, 2019) but also must first be recruited to the site of infection before performing these functions.

Neutrophil life cycle

Neutrophils have a very short life cycle, with maturation taking approximately 14 days and a half-life of 6-8 hours in humans and 11 hours in mice (Basu et al., 2002; Bonilla et al., 2020; Galli et al., 2011; Pillay et al., 2010). Granulopoiesis, the production of neutrophils, eosinophils, and basophils, originates in the bone marrow and during infection generation can increase to 10 times normal production rates (Liew & Kubes, 2019). The main regulator of neutrophil production is the growth factor granulocyte colony-stimulating factor (**G-CSF**) (Lieschke et al., 1994) which directs cells to myeloid lineage and influences development, function, and release of mature neutrophils from the bone marrow (Lord et al., 1989; Richards et al., 2003). Other transcription factors (such as STAT3) and proteins (such as S100A8) also regulate neutrophil development to varying degrees (Dahl et al., 2003; Nerlov & Graf, 1998; Rosmarin et al., 2005; Sasmono et al., 2007). Cytokines such as IL-6 and IL-4 have also been

shown to stimulate granulopoiesis in vivo (Metcalf et al., 1986; Metcalf et al., 1987; Pojda & Tsuboi, 1990). Mature neutrophils are able to develop outside of the bone marrow in response to molecules such as the acute phase protein serum amyloid A (De Santo et al., 2010) or in the spleen during infection (Deniset et al., 2017).

Neutrophil death and turnover are not well understood. Currently it is believed that neutrophils will undergo apoptosis after resolution of inflammation and macrophages and/or monocytes will phagocytose cell remains, occurring mainly in the liver, bone marrow, and spleen (Hong et al., 2012; Schmidtke & Diamond, 2000). In mice, approximately one-third of neutrophils are removed in the bone marrow (Martin et al., 2003). This results in reduced IL-23 production, which has been found to have a positive relationship with G-CSF synthesis (Hong et al., 2012; Schmidtke & Diamond, 2000) effectively reducing neutrophil production. Despite this belief, there is little evidence of phagocytosis of immune cells as removal of monocytes and macrophages has been found to have no effect clearance rate of neutrophils from sites of inflammation (Wang et al., 2017). More recent evidence has suggested that neutrophils may also leave the site of inflammation and reenter the vascular system (referred to as reverse transmigration), at least during times of sterile injury (Liew & Kubes, 2019). This may preserve a significant proportion of neutrophils and help the host maintain tissue homeostasis. Additionally, the receptor C-X-C chemokine receptor type 4 (**CXCR4**) has been found to increase expression on neutrophils before apoptosis to help guide neutrophils back to the bone marrow for destruction, however Eash et al. (2009) found that neutrophils that lack CXCR4 did not have a different half-life from wild-type neutrophils. This indicates that CXCR4 expression may not be the only factor affecting neutrophil clearance, although Martin et al. (2003) found that removing CXCR4 signaling inhibited bone marrow removal. In general, many ideas exist

about neutrophil clearance and there is a large body of evidence that suggests that it is not a simple process.

Neutrophil recruitment cascade

The neutrophil recruitment cascade begins with alterations on the surface of endothelial cells as a result of inflammatory mediators, such as cytokines, which are released by leukocytes that encounter pathogens (Ley et al., 2007; Phillipson & Kubes, 2011; Sadik et al., 2011). Neutrophils will then undergo behavior including rolling/crawling, tethering, and adhesion along the endothelium (Yipp et al., 2017). During neutrophil rolling, adhesive bonds must rapidly form and break as neutrophils roll on endothelial cells with a higher shear stress (Ramachandran et al., 2004; Sundd et al., 2012; Sundd et al., 2011). Selectins, a family of cell adhesion molecules, are the main mediators of rolling (Sadik et al., 2011). PRRs upregulate selectins such as P and E-selectin (Petri et al., 2008; Phillipson & Kubes, 2011). The act of rolling is important for neutrophils to increase encounters with chemokines immobilized on endothelial cells (Massena et al., 2010). Crawling and adhesion involve use of integrins such as LFA1 and MAC1, which bind to ligands ICAM 1 and 2 along the endothelium. The bond at the leading edge of the neutrophil is not particularly strong until it shifts to the rear of the cell while rolling, which creates a long tether consisting of integrins that "sling" to the front of the cell, pulling it forward and creating the "crawling" motion (Schmidtke & Diamond, 2000; Sundd et al., 2012; Sundd et al., 2010). Yipp et al. (2017) found that intravenous LPS challenge increased expression of MAC1 and induced neutrophil crawling activity. The interaction of the neutrophils with chemokines as mentioned above increases integrin affinity and induces adhesion (Sadik et al., 2011). Adhesion to the endothelium is crucial in order to allow neutrophils to transmigrate into tissue, also known as diapedesis (Liew & Kubes, 2019). Passage of neutrophils through the

endothelium occurs either via a paracellular route (between endothelial cells) or a transcellular route (through an endothelial cell) (Petri et al., 2008; Voisin & Nourshargh, 2013). It has been reported that neutrophils preferentially migrate through the paracellular route (Ley et al., 2007). In studies involving acute injury, inhibiting neutrophil recruitment resulted in hindered tissue repair, indicating that neutrophil recruitment is necessary for normal healing (McDonald & Kubes, 2016).

Phagocytosis

Once recruited to the site of inflammation, neutrophils will often phagocytose pathogens. Phagocytosis is the process in which neutrophils and macrophages engulf and destroy pathogens and cell debris. Particles that are to be phagocytosed may be opsonized (in which they are covered in immunoglobulins or complement proteins) or non-opsonized, where via differing pathways the neutrophils will recognize the particle and extend themselves before surrounding and entrapping it (Lee et al., 2003). The neutrophil is not antimicrobial until both primary and secondary granules fuse with the phagosome and release antimicrobial contents (Segal, 2005). Calcium, specifically located in the cytosol, triggers the fusion of granules to the phagosome and is required for granule secretion (Lee et al., 2003).

NETosis

Neutrophils are also able to release their DNA and use it in a "netlike" arrangement, known as neutrophil extracellular traps (**NETs**), which allows them to increase the possibility of catching pathogens (Brinkmann et al., 2004; Kolaczkowska et al., 2015; McDonald et al., 2012). This is known as NETosis. DNA is negatively charged, which allows for other NET components to bind via positive charge (including histones, proteases and other peptides)(Liew & Kubes, 2019). Previously it was believed that neutrophils will lyse and subsequently die following

NETosis (Liew & Kubes, 2019), but newer studies found that NET formation can occur via transport by vesicles or degranulation (Pilsczek et al., 2010). Platelets activated by TLR4 bound to neutrophils have also been found to prevent lysis of neutrophils following NETosis. Yipp et al. (2012) found that NETosis in neutrophils was also dependent on TLR2 opsonization. Although NETs aid in pathogen removal, they can damage other surrounding cells (Branzk et al., 2014; McDonald et al., 2012). In humans, increased plasma NETs were correlated with increased tissue injury and mortality rates (Lefrancais et al., 2018). Neutrophils that are overactivated may encourage tissue damage and harm surrounding tissue, further pushing the debate of whether inflammation is harmful or helpful.

Oxidative burst

A key source of ROS during the early lactation period is phagocytic leukocytes, such as neutrophils and monocytes, which particularly gravitate towards uterine tissue post-parturition (Sordillo et al., 2009). A majority of ROS is produced by NADPH oxidase, which pumps into phagocytic vacuoles that participate in killing pathogens (Segal, 2005). The granules in the cytoplasm of neutrophils, known as primary lysosomes, fuse with the phagocytic vacuoles and the ROS and hydrolytic enzymes contained within act as anti-bacterial agents (Curi et al., 2020). MPO is one of the main enzymes stored in granules and catalyzes the oxidation of chloride by hydrogen peroxide to form hypochlorous acid, a potent oxidizer (Parker & Winterbourn, 2012). This release of granule contents is known as oxidative burst. Transmigrated neutrophils that degranulate and release toxic mediators and cause tissue injury can further drive neutrophil recruitment (Lacy, 2006; Sheshachalam et al., 2014)

Neutrophil metabolism

Neutrophils, like other immune cells, rely on glucose to meet their metabolic needs. The glucose used by neutrophils comes from the circulating vasculature - during times of inflammation this can significantly divert nutrients that would normally be used by the host for production and normal metabolic needs (Odegaard & Chawla, 2012). This reallocation of nutrients to the immune cells is driven by insulin resistance which is directly caused by inflammation. These nutrients not only provide energy but also are precursors for generating new immune cells, effector molecules (such as antibodies, cytokines, and acute phase proteins), and antioxidant defense molecules (such as glutathione) (Calder, 2006). Neutrophils have few functional mitochondria, so little citric acid cycle activity and rates of oxidative phosphorylation occur (Curi et al., 2020). Additionally, neutrophils will rely on fatty acid metabolism in times of limited glucose availability. The amino acid glutamine is extremely important for normal neutrophil proliferation and inflammatory response (Curi et al., 2020). Not only is one of the main products of glutamine metabolism the antioxidant glutathione, but glutamine was found to regulate ROS production, delay apoptosis, and bactericidal activity (Curi et al., 2020). The citric acid cycle intermediate α-ketoglutarate has also been found to increase generation of ROS as well as MPO activity in neutrophils (Curi et al., 2020), suggesting that catabolic states are associated with increased capacity for inflammation (Calder, 2006).

Treatments of inflammation

Some research has been done on reducing inflammation in dairy cattle, but none has been substantially effective. Studies using nonsteroidal anti-inflammatory drugs (**NSAIDs**) during early lactation have found mixed results on production and incidence of disease, including Meier et al. (2014) and Priest et al. (2013) did not find any treatment effects on milk production, body

condition, incidence of disease, or reproductive performance while Carpenter et al. (2016) found a significant increase in daily milk yield and a decrease in somatic cell count during the first few months of lactation. There is, however, the concern of drug residues, where milk withdrawal times can be 3 to 5 days after the last administered dose. This can cause financial issues as well as general disorder in management (Bradford et al., 2015). There is a need to use other treatment methods such as supplementing compounds, biomolecules, etc., which can reduce inflammation. Additional work by Zebeli et al. (2013) found that administering LPS around calving improved metabolic state of cows and reduced plasma concentrations of NEFA and β-hydroxybutyrate (**BHBA**) while it also increased plasma glucose, suggesting an immune refractory state was induced by LPS treatment and exposure increased LPS tolerance.

BCA metabolism

The metabolism of branched chain amino acids (**BCAs**) leucine, isoleucine, and valine have been extensively studied. Although it is known that BCAs are oxidized by immune cells, evidence points to their use being toward protein synthesis rather than as an energy substrate (Calder, 2006) as the immune system is extremely dependent on protein synthesis for effector molecules. In human immune cells, most BCAs are found in lymphocytes, followed by eosinophils and neutrophils, with leucine having the highest uptake in B cells and valine having the lowest (Burns, 1975). Leucine acts as a signaling molecule during the regulation of overall amino acid and protein metabolism, as well as a stimulus of insulin secretion from β-cells in the pancreas (Sadri et al., 2017).

During the early lactation period of dairy cows, there is an increased demand for protein in the mammary gland to support lactation (NASEM, 2021). Circulating BCAs are used for cell and milk protein synthesis and constitute up to 50% of all essential amino acids in milk (Leal

Yepes et al., 2019). Higher concentrations of insulin have been found to stimulate synthesis of muscle protein and significantly reduce concentration of BCAs in the blood (NASEM, 2021), agreeing with findings by Zinicola and Bicalho (2019) that showed that cows classified as high insulin had higher milk protein content on days 0 and 10 of lactation. Additionally, BCAs make up approximately 33% of the essential amino acids in muscle proteins (J. H. Lee et al., 2017) suggesting that BCAs are vital to the body for normal maintenance.

The catabolism of BCAs begins with mitochondrial branched chain amino acid transaminase (**BCAT**(m)), encoded by the *BCAT2* gene, in which BCAs and α-ketoglutarate are converted into branched chain α-keto acids (**BCKs**) and glutamate (Lynch & Adams, 2014). The glutamate created during this step is highly used in neutrophils, presumably to convert into other amino acids (such as aspartate and alanine) as well as for nucleic acid synthesis (Mann et al., 2021). The transamination by BCAT(m) occurs in peripheral tissues, mainly the muscle, as the liver lacks this enzyme. Despite this, most BCK oxidation takes place in the liver (Lynch & Adams, 2014). The oxidation of BCKs is catalyzed by mitochondrial branched chain α-keto acid dehydrogenase complex (**BCKDC**) which is irreversible and rate-controlling. The activity of BCKDC is highly regulated and is inhibited via phosphorylation by branched chain α-keto acid dehydrogenase kinase and activated by mitochondrial protein phosphatase 1K (Lynch & Adams, 2014). Oxidative stress plays a large role in BCKDC activity as well, with long-chain fatty acids and other NEFAs inhibiting BCKDC by affecting the body's redox state or concentration of acetyl-CoA (necessary as a coenzyme) (Lynch & Adams, 2014).

BCA supplementation effects

Some studies have been done to observe BCA supplementation on immune function and metabolism. In general, BCA supplementation has been associated with positive effects on

regulation of body weight, muscle protein synthesis, and glucose metabolism in humans (Lynch & Adams, 2014). Regarding glucose metabolism, leucine has been shown to have an insulinotropic effect in both humans and mice (Sadri et al., 2017). This may be through its oxidation in the mitochondria as well as allosterically activating glutamate dehydrogenase in pancreatic β-cells (Sadri et al., 2017). Supplementation of leucine was found to affect some intermediary metabolic pathways by increased amino acid oxidation (Sadri et al., 2017). Due to this, it is thought that leucine may stimulate cellular uptake of BCAs and other amino acids, which would increase the ability of protein synthesis and amino acid oxidation.

Role of BCA and BCK in inflammation

The effects of BCAs on inflammation have been moderately studied with positive changes associated with antioxidant defense and inflammatory mediators (Cruzat et al., 2014). J. H. Lee et al. (2017) found that *in vitro* supplementation of BCAs decreased mRNA expression of IL-6 as well as cyclooxygenase-2, both inflammatory mediators, in mice macrophages. Additionally, they found that BCAs inhibited LPS-induced ROS production, with leucine having the largest effect. Similarly, mice fed a BCA-deficient diet for 3 weeks showed increased susceptibility to infection, decreased antibody production, and decreased plasma complement C3 (Petro & Bhattacharjee, 1981) suggesting the importance of BCAs in the involvement of pathogen defense. It is thought that the effect of BCAs on immune function relies on L-glutamine metabolism, which is heavily dependent on conversion of BCAs to BCKs through BCAT (Cruzat et al., 2014). Bassit et al. (2000) supplemented male triathletes with BCAs and found that BCA supplementation attenuated the typical reduction in serum glutamine observed after intense exercise compared to non-supplemented athletes, further supporting the belief that BCAs are highly connected to L-glutamine metabolism. Furthermore, they found that BCA

supplementation reversed the decrease in lymphocyte production of IL-1 and 2, TNFα, and IFNγ seen in non-supplemented athletes after exercise, all of which are involved in immune cell recruitment and activation.

Conclusions

Dairy cattle in early lactation are prone to many metabolic and infectious diseases due to the nutrient and energy requirements of lactation and inflammation associated with parturition (Bradford et al., 2015; Drackley, 1999). Although inflammation may be beneficial to facilitate parturition and reduce incidence of infectious diseases (Challis et al., 2009; van Engelen et al., 2009), it also has been found to impede on lactation performance when in excess (Ballou, 2012; Bradford et al., 2015). Supplementation of nutrients such as BCAs can potentially increase capacity for protein synthesis in inflammatory response such as immune cell generation, effector molecules, and antioxidant defense (Calder, 2006) so that a healthy degree of inflammation is still allowed while not completely eliminating its effects. Neutrophils are a primary topic of interest as they are heavily implicated in innate immunity and are capable of rapid changes in gene expression (Newburger et al., 2000) and microbicidal function as the dairy cow progresses through lactation (LeBlanc, 2020). The effect of supplementing either BCAs or BCKs on neutrophil function in dairy cattle is pertinent to further understanding their effects on inflammation.

CHAPTER 2:

ABOMASAL INFUSION OF BRANCHED-CHAIN AMINO ACIDS OR BRANCHED-CHAIN KETO-ACIDS ALTER NEUTROPHIL IMMUNOMETABOLIC GENE EXPRESSION IN EARLY LACTATION DAIRY COWS

ABSTRACT

Inflammation greatly impacts health and performance of dairy cows during early lactation. The objectives of this study were to determine the effect of branched-chain amino acids (BCA) and branched-chain ketoacids (BCK) on neutrophil phagocytic and oxidative burst function as well as mRNA expression of genes regulating neutrophil adhesion, chemotaxis and migration, Toll-like receptor pathway, and antioxidant metabolism in the first 3 weeks postpartum. Thirty-six multiparous Holstein cows were used in a randomized block design experiment. Cows were abomasally infused for 21 d after parturition with solutions of saline (CON, n = 12); BCA (n = 12) including 67 g valine, 50 g leucine, and 34 g isoleucine; and BCK (n = 12) including 77 g ketovaline, 57 g ketoleucine, and 39 g ketoisoleucine. All cows received the same diet. Neutrophils were isolated from blood collected on d 10 and d 20 of lactation. Treatment effects were determined using PROC GLIMMIX in SAS. No treatment differences ($P > 0.48$) were observed for phagocytosis or oxidative burst activity upon pathogen challenge. Cows that received BCA treatment had significantly higher expression of antioxidant enzyme *GPX1* compared to both CON and BCK ($P \leq 0.01$), suggesting enhanced reduction of pro-oxidant with CON. Expression of lysosomal protein *MPO* was lower in BCA cows compared to CON ($P = 0.03$), which likely was associated with reactive oxygen production in neutrophils. Expression of pro-inflammatory cytokine receptor kinase *IRAK1* was higher in BCK cows compared to CON or BCA cows ($P \leq 0.05$). Expression of anti-inflammatory cytokine receptor *IL10RA* was also increased in BCK cows compared to CON ($P = 0.03$). Overall, results suggest that neutrophil transcriptome respond differently to BCA and BCK supplementation during early lactation. Further work is required to understand underlying the mechanisms of the changes observed in this study.

INTRODUCTION

The early lactation period in dairy cattle is when cows are the most prone to infection and metabolic disease aside from the first few weeks of their life (Bradford et al., 2015). Most infections and disease that a dairy cow may suffer from occurs during this time, with a minimum of 50% suffering from at least one disorder (Bradford et al., 2015; Drackley, 1999). This notable susceptibility to disease is due to both high energy and nutrient demand needed for lactation (Drackley, 1999) as well as the inflammation needed to facilitate parturition (Challis et al., 2009; van Engelen et al., 2009). Although some inflammation is necessary to fight off infection, excess can cause fever, fatigue, and decreased dry matter intake (Dantzer & Kelley, 2007) which subsequently affects production. The suggested solution to this problem is to better equip the body with ways to handle inflammation rather than completely eliminate it (Bradford et al., 2015).

The increased oxidation of energy producing substrates during early lactation has been associated with increased reactive oxygen species (ROS) production (Sordillo & Aitken, 2009). This as well as release of ROS from immune cells during inflammation associated with the early lactation period may cause cellular and tissue damage if not reduced by antioxidants (Schukken et al., 2011), such as glutathione. The imbalance of ROS production and antioxidant defense is known as oxidative stress (Sordillo & Aitken, 2009) and can result in disease and fertility problems in dairy cattle (Pedernera et al., 2010). Cows in early lactation may experience negative energy balance due to the high nutrient and energy demand associated with milk production (Pedernera et al., 2010). Increased negative energy balance during the early lactation period has been highly correlated with increased oxidative stress (Pedernera et al., 2010). Ways

to increase antioxidant defense to better handle ROS generation is pertinent to decrease incidence of disease and improve overall health of early lactation dairy cattle.

Currently, methods administering nonsteroidal anti-inflammatory drugs (NSAIDs) during early lactation have been studied to minimize overall inflammation (Bradford et al., 2015). Although some research found a decrease in mRNA expression of inflammatory mediators in response to NSAID treatment (Caldeira et al., 2019), most did not see any treatment effects on milk production, reproductive performance, or incidence of disease (Meier et al., 2014; Priest et al., 2013) and have mostly been focused on mammary immune response. Drug residues in milk is also a concern for producers, which results in milk being dumped and is consequently not financially practical (Bradford et al., 2015). Therefore, strategies involving nutritional supplements rather than pharmaceuticals are of particular interest in managing inflammation in dairy cattle.

Neutrophils are the primary immune cell responders comprising host defense in innate immunity (non-specific immune response) (LeBlanc, 2020). The microbicidal function of neutrophils is known to decrease during the early lactation period (LeBlanc, 2020) which has been associated with mastitis, retained placenta, and displaced abomasum (Burton et al., 2005; Goff & Horst, 1997; Kimura et al., 2002). Increased plasma NEFA and BHBA concentrations in early lactation have been found to decrease neutrophil phagocytic and microbicidal capacity in vitro (Graugnard et al., 2012). They have been found to undergo rapid changes in gene expression (Newburger et al., 2000); it is hypothesized that gene expression profiles in neutrophils are impacted by negative energy balance in early lactation (Graugnard et al., 2012; Zhou et al., 2015). The importance of neutrophils in preventing disease in early lactation as well

as their functional plasticity make them a topic of interest in improving health and milk production in early lactation dairy cattle.

Research involving supplementation of branched chain amino acids (BCAs) on milk production and plasma metabolites in early lactation dairy cows (Leal Yepes et al., 2019) found that BCA treated cows had significantly fewer plasma samples classified as ketotic. Additional work supplementing isoleucine and leucine in mid-lactation dairy cows noted an increase in mammary blood flow and udder affinity for essential amino acids (Yoder et al., 2020), suggesting higher uptake in the mammary gland. Studies in humans found positive effects on body weight regulation, muscle protein synthesis, and glucose metabolism with BCA supplementation (Lynch & Adams, 2014). Branched chain keto-acids (BCKs) are BCA metabolites created by de-amination with mitochondrial branched chain amino acid transferase (BCAT(m)) which can be converted back to BCAs or further catabolized (Harper et al., 1984). Gallagher (2022) previously measured milk production parameters and markers of health in multiparous early lactation dairy cows abomasally infused with BCAs and BCKs. BCA treatment improved lactation performance, with increased yield of milk, milk fat, lactose, milk solids, energy corrected milk, fat corrected milk, increased raw efficiency (milk:DMI) and plasma BHBA concentrations, while it decreased plasma glucose concentrations compared to non-supplemented cows. Additionally, BCK treatment decreased liver triglyceride content compared to non-supplemented cows. These results suggest altered nutrient partitioning as well as mammary gland and liver metabolism in response to BCA and BCK supplementation. Based on the observed changes in lactation performance and liver triglyceride content in response to BCA and BCK supplementation reported by Gallagher (2022), we wanted to additionally evaluate the study's effects on neutrophil function.

Some research has been done on the effect of BCA supplementation on inflammation in neutrophils related to the immune system, showing positive effects on antioxidant defense and expression of inflammatory mediators in human and mouse models (Cruzat et al., 2014; J. H. Lee et al., 2017). Synthesis of effector molecules (such as antibodies, cytokines, and acute phase proteins) and generation of new immune cells during periods of inflammation requires nutrients (Calder, 2006), which suggests that the immunological effects of BCA supplementation are mostly a result of protein synthesis. In B cells, it has been found that leucine has the highest rate of uptake out of the BCAs, followed by isoleucine and then valine (Glassy & Furlong, 1981). They were found to be taken up the highest in the S phase followed by the G1 phase of cell development, coinciding with highest protein synthesis activity (Calder, 2006), which further supports that effects of BCA supplementation rely on their incorporation in proteins. Human neutrophils have been found to contain branched-chain alpha keto acid dehydrogenase (BCKDH) and can oxidize BCK to generate NADH (Burns, 1975). Since Schafer and Schauder (1988) showed that supplementation of keto-leucine increases activity of BCKDH in lymphocytes, it is possible that BCK supplementation can alter neutrophil function in early lactation.

We therefore hypothesized that abomasal infusion of BCA or BCK will improve neutrophil function and alter gene expression in early lactation dairy cows. Our objective was to determine if abomasal infusion of BCA or BCK for 21 d postpartum would affect phagocytosis, oxidative burst activity as well as expression of genes related to antioxidant defense, cytokine production and receptors, adhesion and migrationthe in multiparous Holstein cows.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol no. PROTO202000206). Experimental design used for this project has been previously described in Gallagher (2022). The experiment was conducted as a randomized complete block design with multiparous (lactation ≥ 2) Holstein dairy cows (n = 36) from the Michigan State University Dairy Cattle Teaching and Research Center (Lansing, MI) between November 2021 and March 2022. A total of 36 cows were blocked according to the expected calving date into 3 blocks. Treatments were balanced for parity and previous lactation 305 mature equivalent milk yield. All cows enrolled underwent a rumenotomy procedure prepartum (46 ± 2 d before expected calving date) unless previously cannulated (n = 6). Within each block, cows were randomly assigned to 1 of 3 treatments: control (**CON**, n = 12) receiving daily abomasal saline infusion without branched-chain amino acids or ketoacids; branched-chain amino acid (**BCA**, n = 12) receiving daily abomasal infusion of 67 g L-valine, 50 g L-leucine, and 34 g L-isoleucine (>99.9% purity, ACP Chemicals, Montreal, QB); and branched-chain ketoacid (**BCK**, n = 12) receiving daily abomasal infusion of 77 g 2-ketovaline calcium salt, 57 g 2-ketoleucine calcium salt, and 39 g 2-ketoisoleucine calcium salt (> 99% purity, Stru Chem, Wujiang City, China). Dosage of BCA and BCK was based on a preliminary block of 6 cows (data not presented) that successfully increased plasma BCA concentration by approximately 30%. The daily dose of BCAs and corresponding BCKs were matched on a 1:1 molar ratio across BCA and BCK treatments. Calcium chloride dihydrate (> 98% purity, Fischer Chemical) was added to CON and BCA infusate to match the Ca level in the BCK treatment. Treatments were delivered to cows daily via continuous abomasal infusion using a peristaltic pump (Spires

et al., 1975; Westreicher-Kristen & Susenbeth, 2017). Seven liters of aqueous solution were administered to each cow daily at an adjusted pH of 2.5 to mimic pH in abomasum. Calving day was considered d 0. Initiation of abomasal infusion for all animals occurred at 1200 h on d 1 after calving. Abomasal infusions were halted during milking for approximately 3 – 4 h/d. Abomasal infusion lines were flushed with 120 mL water after stopping and before continuing infusion to ensure treatment delivery to the abomasum.

Blood Collection and PMNL Isolation

Blood was sampled for subsequent analysis from the jugular vein on d 10 and d 20 relative to parturition at 9 am before feed was delivered. Approximately 120 mL of blood drawn for polymorphonuclear leukocyte (PMNL) isolation was collected into two 60 mL conical tubes with 1000 IU/mL of sodium heparin (cat. no. 411212500, Acros Organics, Geel, Belgium) and immediately inverted. An additional 10 mL evacuated tube containing sodium heparin (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ) was collected for phagocytosis and oxidative burst. Blood collected for PMNL isolation was kept on ice and immediately processed following the procedure described by Yuan et al. (2014). The final neutrophil pellet was resuspended in 5mL of 1x phosphate-buffered saline (**PBS**), containing 137mM sodium chloride and 2.7mM potassium chloride, and vortexed. Approximately 2 mL was transferred to 1.5 mL RNAse-free tube containing 500 µL of RNAlater (cat. no. AM7021, Thermo Fisher Scientific) and stored in -80 °C for later RNA extraction. The other portion was left suspended and 100 µL of CH138A primary anti-bovine granulocyte monoclonal antibody (cat. no. BOV2067, Washington State University, Pullman) was added to identify neutrophils and incubated with cells for 15 min on ice. Samples were then washed with 2 mL of 1x PBS, vortexed, centrifuged at 2100 rpm for 3 min at 4 °C, and supernatant was aspirated until approximately 100 µl was left in the tube.

Afterwards, 50 µL of the fluorophore phycoerythrin (**PE**) (cat. no. 1020-09S, SouthernBiotech, Birmingham, AL) was added and incubated with the cells for 15 min on ice. Cells were again washed as described above and resuspended in 1x PBS. A separate aliquot of 200 µL of the cell suspension in PBS was transferred to a 5 mL sterile culture test tube (cat. no. 14-956-3C, Thermo Fisher Scientific) for flow cytometric analysis of viability and PMNL cell concentration. Cells were gated by forward and side scatter (**FSC**, **SSC**) to identify singlets, then subsequently gated using the PE channel to identify CH138A positive cells – indicating neutrophils. After analysis, cells were spot checked for viability by adding 1 µL of Trypan blue (cat. no. T10282, Life Technologies Corp, Eugene, OR) to 10 µL of the cell suspension and read on the Countess® IIFL Hemacytometer (Life Technologies Corp). Each sample was read 2 times to verify viability.

Whole-Blood Phagocytosis and Oxidative Burst

The phagocytic capacity and oxidative burst activity of peripheral neutrophils and mononuclear phagocytes were determined upon challenge with enteropathogenic bacteria (Escherichia coli 0118:H8) as described by Zhou et al. (2018). Briefly, 200 µL of whole blood with 40 µL of 100 µM dihydrorhodamine 123 (DHR123) (Sigma-Aldrich, St. Louis, MO) and 40 µL of propidium iodine (**PI**)-labeled bacteria (10^9 cfu/mL) were incubated at 38.5 °C in a water bath for 10 min. After red blood cells were lysed with ice-cold Milli-Q water (Millipore, Billerica, MA), cells were resuspended in 10x PBS solution. Subsequently, neutrophils were stained with CH138A primary anti-bovine granulocyte monoclonal antibody (cat. no. BOV2067, Washington State University, Pullman) and Brilliant Violet 421™ anti-mouse IgM antibody (cat. no. 406518, BioLegend), and mononuclear phagocytes were marked with allophycocyanin (**APC**) labeled anti-CD14 antibody (cat. no. 301808; Biolegend). Last, the cells were resuspended in PBS solution for flow cytometry analyses (LSR II; Becton Dickinson).

Neutrophils and mononuclear phagocytes were distinguished by gating based on their side scatter properties in combination with the PE and APC, respectively. Compared with the negative controls, the neutrophils and mononuclear phagocytes from E. coli–stimulated samples with greater emissions of PI were considered positive for phagocytosis. Similarly, compared with negative controls, the E. coli–stimulated samples with greater emissions of rhodamine 123 in fluorescein isothiocyanate (**FITC**) channel were considered positive for oxidative burst. Gating of neutrophils versus mononuclear phagocytes, CH138A positive neutrophils, unchallenged and challenged phagocytic neutrophils, unchallenged and challenged phagocytic neutrophils undergoing oxidative burst, and a histogram of mean fluorescent intensity (**MFI**) of unchallenged and challenged neutrophils are pictured below in Fig 2.1.

RNA Isolation, cDNA Synthesis, and q-RT PCR

Total RNA was extracted from PMNL using the Quick-RNA Miniprep Kit (cat. no R1055, Zymo Research, Irvine, CA) according to manufacturer's instructions. Before extraction, RNAlater was discarded so only the PMNL pellet remained – 600 µL of RNA lysis buffer from the kit was added and samples were sonicated for 15 seconds at 70% power using the Fisherbrand™ Model 120 Sonic Dismembrator (Thermo Fisher Scientific). Concentration and quality of RNA was confirmed by optical density $(OD)_{260nm}/OD_{280nm}$ (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE).

Samples were diluted to 100 ng/µL with RNase free water in preparation for cDNA synthesis. Twelve samples (CON, n = 1; BCA, n = 7; BCK, n = 4; d10, n = 6; d20, n = 6) out of seventy-two collected were removed from subsequent analysis due to RNA concentrations being too low to synthesize cDNA. Synthesis of cDNA was completed using the High Capacity cDNA Reverse Transcription Kit (cat. no. 4368813, Applied Biosystems, Waltham, MA) according to

manufacturer's instructions. Samples of cDNA were synthesized to a concentration of 50 ng/µL. Quantitative PCR was performed using 4 µL of cDNA diluted to 1.25 ng/µL combined with a 6µL mix comprised of 4 µL 1x SYBR Green master mix (Applied Biosystems) and 1µL each of 10 µM forward and reverse primers in a 384-Well Reaction Plate (cat. no. 37942, Thermo Fisher Scientific). Each sample was run in triplicate and a 4-point relative standard curve was used. The reactions were performed using a QuantStudio™ 7 Flex (Applied Biosystems) with the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing plus extension). The relative quantification data were calculated using the standard curve with QuantStudio™ Real-time PCR software (Applied Biosystems). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 65 °C for 15 s, then 95 °C for 15 s. Primer design and sequences used have been reported previously (Zhou et al., 2018) and are reported in Table 2.1. The final quantities were normalized by using the geometric mean of internal control genes (ICG) *GAPDH*, *Beta-actin*, and *RPS9*.

Statistical Analysis

All data were analyzed using PROC GLIMMIX of SAS v9.4 (SAS Institute, Cary, NC) with the model: $Y = \mu + b_i + T_j + D_k + TD_{jk} + A_{l:ij} + e_{ijklm}$ where Y = the dependent, continuous variable, μ = overall mean, b_i = random effect of block, T_j = fixed effect of treatment, D_k = fixed effect of time, TD_{jk} = interaction of treatment and time, $A_{l:ij}$ = random effect of the *l* th cow nested within block × treatment and e_{ijklm} = the residual error. Fixed effect of time is the day or week relative to parturition in which samples were collected for each variable. Parity (second vs. third lactation and greater) and previous 305-d milk yield were kept in the model as covariate for all variables when significant ($P \leq 0.05$) Normality was tested using Shapiro-Wilk test. When

normality assumption was rejected, data was log transformed for statistical analysis and back transformed for interpretation. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means. Data are reported as least squares means with associated standard errors. Significant effects are declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Phagocytosis and Oxidative Burst in Neutrophils and Mononuclear phagocytes

No changes in either phagocytosis or oxidative burst of neutrophils (Table 2.2a.) and mononuclear phagocytes (Table 2.3a.) were noted across both BCA and BCK treatments. We observed an increase in mean fluorescent intensity (MFI) from day 10 to day 20 for both neutrophils (Table 2.2b.) and mononuclear phagocytes (Table 2.3b.). An interaction between treatment and time was noted for mononuclear phagocytes (Table 2.3b.). No interaction of treatment and time was observed for neutrophils.

Expression of PMNL Antioxidant defense genes

Although no changes were observed in phagocytic and oxidative burst capability of neutrophils in response to BCA or BCK treatment, some changes in gene expression regarding antioxidant generation and microbicidal activity were observed (Table 2.4.). Glutathione peroxidase 1 (*GPX1*) expression significantly increased ($P < 0.01$) in response to BCA treatment compared to both CON and BCK. Similarly, treatment tended to effect myeloperoxidase (*MPO*) expression ($P = 0.09$), with higher expression in BCA treatment compared to CON. No changes ($P > 0.10$) were observed in expression of ROS production genes superoxide dismutase 1 or 2 (*SOD1*, *SOD2*). Additionally, no changes ($P > 0.10$) were observed in antioxidant metabolism

genes cystathionine beta-synthase (*CBS*), cystathionine gamma-lyase (*CTH*), glutathione-disulfide reductase (*GSR*), or S-adenosylhomocysteine hydrolase (*SAHH*).

Expression of Adhesion and Migration genes in PMNL

No changes ($P > 0.10$) in expression in response to treatment was observed in adhesion and migration genes (Table 2.4.) including CXC motif chemokine receptor (*CXCR2*), integrin subunit beta 2 (*ITGB2*), S100 calcium binding protein A8 (*S100A8*), or selectin L (*SELL*). There was, however, a tendency ($P = 0.10$) for increased expression on day 20 compared to day 10 observed for *S100A8.*

Expression of TLR pathways in PMNL

Changes in expression of toll-like receptor and cytokine gene were summarized in Table 2.4. Interleukin 1 receptor associated kinase 1 (*IRAK1*) had a significant increase ($P = 0.04$) in expression in response to BCK compared to CON and BCA, as well as a tendency ($P = 0.10$) for expression to decrease on day 20 compared to day 10. In addition, treatment tended to effect interleukin 10 receptor (*IL10RA*) expression ($P = 0.07$), with higher expression observed in BCK treatment compared to CON. No changes ($P > 0.10$) were observed in interferon gamma (*IFNG*), interleukin 1 beta (*IL1B*), interleukin 10 (*IL10*), nuclear factor kappa b subunit 1 (*NFKB1*), or toll-like receptor 2 or 4 (*TLR2*, *TLR4*).

DISCUSSION

Effects on antioxidant defense gene expression

Oxidative stress, a common ailment of early lactation dairy cows, is defined as an imbalance between antioxidants and oxidants. Selenium functions as an important antioxidant in dairy cattle, specifically regarding mammary gland health and reducing incidence as well as severity of mastitis (Smith et al., 1984; Sordillo & Aitken, 2009). This is due to the selenium

dependent enzymes that contain a selenocysteine residue, which reduce ROS and other harmful byproducts of fatty acid oxidation (Aitken et al., 2009). One of the most prominent selenoenzymes in cattle is cytosolic glutathione peroxidase (GPX1) (Aitken et al., 2009; Smith et al., 1997), which is commonly used as a measure of antioxidant status in dairy cows (Aitken et al., 2009). GPX1 functions to reduce hydrogen peroxide to water and lipid hydroperoxides to alcohols in order to prevent cellular damage (Smith et al., 1997).

Glutathione peroxidase activity is known to increase after calving (Sordillo et al., 2007), likely to protect cells from oxidative damage. Aitken et al. (2009) found that the expression of *GPX1* mRNA in bovine mammary tissue was sensitive to changes in ROS accumulation and proposed that changes in *GPX1* mRNA abundance are indicative of redox status of the tissue at the time of sample collection. The increase in expression of *GPX1* in neutrophils following BCA treatment suggests that the BCA treated cows had increased capacity to neutralize ROS and protect from potential oxidative damage. Interestingly, we did not observe any changes in *SOD1* and *SOD2* which catalyze the reduction of superoxide, as well as *CBS*, *CTH*, *GSR*, and *SAHH* which regulate glutathione biosynthesis (Forman et al., 2009). This suggests that supplementing BCAs may not alter glutathione production in early lactation dairy cows.

Effects on microbicidal gene expression

Neutrophil function is known to be impaired during the transition period (Kehrli et al., 1989; Kimura et al., 1999), including generation of bactericidal agents, chemotaxis, and adhesion. Myeloperoxidase (MPO), the most abundant enzyme stored in granules of neutrophils and released during oxidative burst, cell lysis, and NETosis, is expressed less during the transition period (Cai et al., 1994; Kehrli et al., 1989). It catalyzes oxidation of chloride by hydrogen peroxide to form hypochlorous acid, which is the main bactericidal mechanism of

oxidative burst (Parker & Winterbourn, 2012). MPO binds to extracellular components such as proteoglycans, proteins, and DNA as a means to slightly localize damage to specific locations, although it is known to induce cellular dysfunction and affect gene expression of host cells as a result of hypochlorous acid formation (Hawkins & Davies, 2021). The generation of oxidants by MPO is beneficial in the immune response and its ability to destroy pathogens; however "inappropriate" activation (i.e., excessive levels, activation when there is no infection, etc.) of MPO can result in tissue damage caused by oxidant formation (Davies, 2011). The purine and pyrimidine bases of DNA and RNA, as well as the side chains of amino acids are particular targets of hypochlorous acid (Hawkins & Davies, 2021), suggesting that MPO release should be highly controlled. It is known that MPO release is impaired during disease (such as metritis, mastitis, retained placenta) as well as during the transition period, although the mechanisms underlying this impairment is not well understood (LeBlanc, 2020).

Although MPO activity is used as a measure of neutrophil killing function (LeBlanc, 2020), no differences were observed in % of challenged neutrophils undergoing phagocytosis and oxidative burst nor MFI of oxidative burst, suggesting that BCA supplementation may not have effectively changed bactericidal capacity of circulating neutrophils. It is important to note that gene expression was measured in unchallenged neutrophils. Higher expression of *MPO* in an unchallenged setting could lead to increased generation of oxidants, damaging cells and tissue in the cow. Therefore, the decrease in expression of *MPO* observed in response to BCA treatment may be beneficial to the cow in early lactation as higher expression of *MPO* on unchallenged neutrophils could be damaging to cells and tissue.

Effects on TLR gene expression

BCA are catabolized by branched-chain amino acid transaminase (BCAT(m)) located in the mitochondria, occurring in extra-hepatic tissue. The following step in BCA catabolism is the oxidation of BCAs to BCKs by mitochondrial branched-chain alpha ketoacid dehydrogenase complex (BCKDC) (Lynch & Adams, 2014). Calder (2006) states that, despite being oxidized by immune cells, in vitro results showed that BCAs are unlikely to be used as energy substrates for the immune system and that their essentiality is more related to protein synthesis. Instead, neutrophils will more often convert BCAs to glutamate via BCAT where it can either be used to make α-ketoglutarate and generate energy from the TCA cycle, generate glutamine, aspartate, alanine for protein synthesis, or provide nitrogen required for nucleotides and nucleic acids (Mann et al., 2021). Based on these findings, it was hypothesized that BCA and/or BCK cows would have higher expression of genes involved with cytokines and their receptors as there would be higher availability of nutrients and energy from their oxidation available to generate these effector molecules.

Unexpectedly, there were no significant differences seen in pro-inflammatory cytokine gene expression (*IFNG*, *IL1B*) or anti-inflammatory cytokine gene expression (*IL10*) between treatments. Additionally, there were no changes in expression of toll-like receptor genes *TLR2* and *TLR4* as well as the transcription regulator *NFKB,* which is activated by TLRs. Although other pro-inflammatory cytokine genes (*TNFA*) and multifunctional cytokine genes (*IL6*) were undetected in samples from this study, we observed a significant increase in expression of interleukin 1 receptor association kinase 1 (*IRAK1*) in BCK-treated cows compared to both CON and BCA, as well as an increase in expression for interleukin 10 receptor subunit alpha (*IL10RA*) in BCK-treated cows compared to CON.

Interleukin 1 receptor associated kinases (IRAK) function alongside TLRs and activates NFκB; thereby regulating expression of inflammatory genes in various immune cells (Jain et al., 2014). They are activated by interaction of the adaptor protein myeloid differentiation factor 88 (MyD88) and TLRs, and auto-phosphorylation of the proST domain (containing serine, proline, and threonine residues) which allows IRAK to dissociate from MyD88 and initiate signaling (Jain et al., 2014). As the results show that there was no change in expression of *TLR2* or *TLR4*, BCK supplementation may have affected MyD88 function upstream, or it is possible it induced greater phosphorylation of *IRAK1* resulting in the observed increased expression. Although there was no increase in expression of *IL1B* or *TLR2* and *TLR4* observed across treatments, increased expression of *IRAK1* in neutrophils may allow for increased activation of IL-1 and TLR signaling pathways. Currently there has been little research done on BCA and BCK treatment effect on cytokine receptor expression in cattle to support the mechanisms behind the increase in expression in our studies. Future work investigating the effect of supplementing BCAs and BCKs to early lactation dairy cows on TLR and interleukin pathways in pathogen challenged neutrophils is required to confirm these results.

IL10RA is a receptor for IL-10, which is an essential anti-inflammatory cytokine. It closely interacts with the transcription factor STAT3, where IL-10 binding to IL10RA causes phosphorylated STAT3 to translocate to the nucleus and activate expression of anti-inflammatory effector genes (Hutchins et al., 2013) that function to block activation of proinflammatory cytokine synthesis (Crepaldi et al., 2001). In humans, expression of *IL10RA* in neutrophils is low at basal levels compared to monocytes or lymphocytes, but stimulation via LPS or interleukin 4 upregulates expression (Shouval et al., 2014). Crepaldi et al. (2001) found that the capacity of neutrophils to respond to IL-10 is highly dependent on expression of *IL10RA*.

We did not observe a significant change in *IL10* expression across treatments, so the increase in *IL10RA* expression in BCK-treated cows compared to CON suggests that BCK treatment may allow for increased response of neutrophils to IL-10.

Phagocytosis and oxidative burst

Phagocytosis is a function of neutrophils that is essential for their microbicidal capabilities. It is a process in which neutrophils (as well as macrophages) engulf pathogens and cell debris via a vacuole, the phagosome (Lee et al., 2003). Although many other functions of neutrophils are impaired during the onset of lactation and disease, little evidence has been found that indicates lower phagocytic ability (LeBlanc, 2020). Instead, neutrophil killing via ROS formation is more commonly impaired (LeBlanc, 2020). Oxidative burst, also known as respiratory burst, is the method in which neutrophils generate superoxide anions by superoxide dismutase (SOD), ROS formed by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), and hypochlorous acid by MPO (Chen & Junger, 2012; Elbim & Lizard, 2009). LeBlanc (2020) concluded that oxidative burst is the most widely impaired function of neutrophils in dairy cows during the early lactation period relative to the demands of lactation.

We observed that BCA and BCK treatment caused no changes in quantity of neutrophils undergoing phagocytosis and oxidative burst. Similar results were seen in mononuclear phagocytes (data shown in Table 2.3a.). As expected, we observed a significant increase in mean fluorescent intensity (MFI) of oxidative burst from day 10 to day 20 in neutrophils ($P = 0.02$) and mononuclear phagocytes ($P = 0.01$) which aligns with other studies that also showed a significant increase in oxidative burst capacity following the first week after calving (Kimura et al., 2014; LeBlanc, 2020). The lack of change in proportion of cells undergoing phagocytosis was also observed by Baakhtari et al. (2022) in both neutrophils and monocytes in racehorses

supplemented with BCAs. We also observed a treatment by time interaction for mononuclear phagocytes undergoing oxidative burst ($P = 0.02$) (Table 2.3b.) This interaction seems to be driven by BCA treatment, as Figure 2.2. shows that the proportion of mononuclear phagocytes undergoing phagocytosis increased from day 10 to day 20 while there was not a clear difference for BCK treatment across time points. The decrease we observed across time points seen in the CON group is similar to the results found by Kimura et al. (2014) for their control group, where there was a peak in proportion of neutrophils undergoing oxidative burst around days 7-10 and a decline following that period. Although their study only followed neutrophil function and we only observed an interaction with monocytes, it is possible that monocytes follow a similar pattern to neutrophils in early lactation. Impairment of oxidative burst function in neutrophils is found to be associated with health disorders in early lactation (such as retained placenta and metritis) (LeBlanc, 2020), so the increase in proportion of mononuclear phagocytes undergoing oxidative burst in BCA cows and the attenuated decline in BCK cows may be beneficial to the cow's health.

CONCLUSIONS

Abomasal infusion of BCA in the first 3 wk of lactation increased expression of *GPX1* while decreasing expression of *MPO.* Infusion of BCK increased expression of both *IRAK1* and *IL10RA* compared to CON. BCA and BCK treatment did not affect phagocytosis or oxidative burst activity when challenged by pathogens. Data from a preliminary study effectively increased circulating concentrations of BCAs by 30%, and we observed significant increases in milk yield and components with BCA treatment and a significant decrease in liver triglyceride content with BCK treatment (Gallagher, 2022). Although only changes in gene expression were observed, these results suggest that BCA supplementation could potentially enhance capacity of

antioxidant response and improve cytoprotective mechanisms in circulating neutrophils in multiparous dairy cows during early lactation. BCK supplementation may have increased ability of circulating neutrophils in early lactation multiparous dairy cows to respond to signals in IL-1, TLR and IL-10 pathways. Additional work is required to determine the effect of BCA supplementation on serum ROS concentrations in early lactation dairy cows as well as the mechanisms behind which BCK supplementation upregulates specific cytokine receptors. Furthermore, gene expression of genes related to antioxidant defense, cytokine pathways, and cell recruitment in challenged neutrophils from early lactation cows supplemented with BCA or BCK would aid in investigating the immunometabolic effects of these treatments.

CHAPTER 3:

IMPLICATIONS AND CONCLUSIONS

IMPLICATIONS AND CONCLUSIONS

Presently, pharmaceutical strategies do not effectively decrease incidence of disease while decreasing effects of inflammation in early lactation dairy cattle (Bradford et al., 2015). Despite being a response to parturition in order to reduce incidence of disease, immune cell response during inflammation is dysregulated from high nutrient and energy demands of lactation (Drackley, 1999). This relationship is still poorly defined, and exploring it will help improve health of cows and reduce losses for producers. By studying the response of neutrophil phagocytosis and oxidative burst activity as well as expression of genes supporting antioxidant defense, cytokines and receptors, and adhesion and migration to BCAs and BCKs, we may better understand ways in which nutrition can improve the immune function of dairy cattle.

The goal of this work is to assess how BCAs or BCKs supplementation changes the neutrophil gene expression in early lactation dairy cattle. The study evaluated the effect of BCA or BCK supplementation via abomasal infusion on neutrophils isolated from fresh multiparous Holstein cows. Parameters such as phagocytosis, oxidative burst, and gene expression of immunomodulatory genes were measured to evaluate neutrophil function. BCA supplementation significantly increased *GPX1* and decreased *MPO* expression compared to CON but had no effect on phagocytosis or oxidative burst activity. BCK supplementatation significantly increased expressesion of *IRAK1* and *IL10RA* and similarly had no effect on phagocytosis or oxidative burst activity. Considering neutrophils are found to have decreased microbicidal capacity during the transition period, BCA and BCK supplementation may enhance functional capacity of neutrophils using different mechanisms.

This study concludes that further work should be done to determine if BCA or BCK supplementation would alter neutrophil gene expression upon pathogen challenge. Work

investigating ROS concentrations and enzymatic activity in neutrophils following BCA or BCK supplementation is warranted. Evaluation of BCA or BCK on neutrophil function will help improve the current problem with inflammation and susceptability to disease in early lactation dairy cows. With this study, we conclude that BCA supplementation affects expression of genes related to antioxidant defense while BCK supplementation affects expression of genes related to cytokine and toll-like receptors.

BIBLIOGRAPHY

Abram, C. L., & Lowell, C. A. (2009). The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol, 27*, 339-362. doi:10.1146/annurev.immunol.021908.132554

Aitken, S. L., Karcher, E. L., Rezamand, P., Gandy, J. C., VandeHaar, M. J., Capuco, A. V., & Sordillo, L. M. (2009). Evaluation of antioxidant and proinflammatory gene expression in bovine mammary tissue during the periparturient period. *J Dairy Sci, 92*(2), 589-598. doi:10.3168/jds.2008-1551

Andonegui, G., Zhou, H., Bullard, D., Kelly, M. M., Mullaly, S. C., McDonald, B., . . . Kubes, P. (2009). Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J Clin Invest, 119*(7), 1921-1930. doi:10.1172/jci36411

Baakhtari, M., Imaizumi, N., Kida, T., Yanagita, T., Ramah, A., Ahmadi, P., . . . Yasuda, M. (2022). Effects of branched-chain amino acids on immune status of young racing horses. *J Vet Med Sci, 84*(4), 558-565. doi:10.1292/jvms.21-0529

Ballou, M. A. (2012). Growth and Development Symposium: Inflammation: Role in the etiology and pathophysiology of clinical mastitis in dairy cows. *J Anim Sci, 90*(5), 1466-1478. doi:10.2527/jas.2011-4663

Banchereau, J., Pascual, V., & O'Garra, A. (2012). From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat Immunol, 13*(10), 925-931. doi:10.1038/ni.2406

Bannerman, D. D., Rinaldi, M., Vinyard, B. T., Laihia, J., & Leino, L. (2009). Effects of intramammary infusion of cis-urocanic acid on mastitis-associated inflammation and tissue injury in dairy cows. *Am J Vet Res, 70*(3), 373-382. doi:10.2460/ajvr.70.3.373

Bassit, R. A., Sawada, L. A., Bacurau, R. F., Navarro, F., & Costa Rosa, L. F. (2000). The effect of BCAA supplementation upon the immune response of triathletes. *Med Sci Sports Exerc, 32*(7), 1214-1219. doi:10.1097/00005768-200007000-00005

Basu, S., Hodgson, G., Katz, M., & Dunn, A. R. (2002). Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood, 100*(3), 854-861. doi:10.1182/blood.v100.3.854

Bernabucci, U., Ronchi, B., Lacetera, N., & Nardone, A. (2005). Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *J Dairy Sci, 88*(6), 2017-2026. doi:10.3168/jds.S0022-0302(05)72878-2

Bonilla, M. C., Fingerhut, L., Alfonso-Castro, A., Mergani, A., Schwennen, C., von Kockritz-Blickwede, M., & de Buhr, N. (2020). How Long Does a Neutrophil Live?-The Effect of 24 h Whole Blood Storage on Neutrophil Functions in Pigs. *Biomedicines, 8*(8). doi:10.3390/biomedicines8080278

Bradford, B. J., Yuan, K., Farney, J. K., Mamedova, L. K., & Carpenter, A. J. (2015). Invited review: Inflammation during the transition to lactation: New adventures with an old flame. *J Dairy Sci, 98*(10), 6631-6650. doi:10.3168/jds.2015-9683

Branzk, N., Lubojemska, A., Hardison, S. E., Wang, Q., Gutierrez, M. G., Brown, G. D., & Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol, 15*(11), 1017-1025. doi:10.1038/ni.2987

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., . . . Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science, 303*(5663), 1532-1535. doi:10.1126/science.1092385

Burns, C. P. (1975). Isoleucine metabolism by leukemic and normal human leukocytes in relation to cell maturity and type. *Blood, 45*(5), 643-651.

Burton, J. L., Madsen, S. A., Chang, L. C., Weber, P. S., Buckham, K. R., van Dorp, R., . . . Earley, B. (2005). Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. *Vet Immunol Immunopathol, 105*(3-4), 197-219. doi:10.1016/j.vetimm.2005.02.012

Cai, T. Q., Weston, P. G., Lund, L. A., Brodie, B., McKenna, D. J., & Wagner, W. C. (1994). Association between neutrophil functions and periparturient disorders in cows. *Am J Vet Res, 55*(7), 934-943.

Caldeira, M. O., Bruckmaier, R. M., & Wellnitz, O. (2019). Meloxicam affects the inflammatory responses of bovine mammary epithelial cells. *J Dairy Sci, 102*(11), 10277-10290. doi:10.3168/jds.2019-16630

Calder, P. C. (2006). Branched-chain amino acids and immunity. *J Nutr, 136*(1 Suppl), 288S-293S. doi:10.1093/jn/136.1.288S

Cao, Y., Bonizzi, G., Seagroves, T. N., Greten, F. R., Johnson, R., Schmidt, E. V., & Karin, M. (2001). IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell, 107*(6), 763-775. doi:10.1016/s0092-8674(01)00599-2

Carpenter, A. J., Ylioja, C. M., Vargas, C. F., Mamedova, L. K., Mendonca, L. G., Coetzee, J. F., . . . Bradford, B. J. (2016). Hot topic: Early postpartum treatment of commercial dairy cows with nonsteroidal antiinflammatory drugs increases whole-lactation milk yield. *J Dairy Sci, 99*(1), 672-679. doi:10.3168/jds.2015-10048

Challis, J. R., Lockwood, C. J., Myatt, L., Norman, J. E., Strauss, J. F., 3rd, & Petraglia, F. (2009). Inflammation and pregnancy. *Reprod Sci, 16*(2), 206-215. doi:10.1177/1933719108329095

Chen, Y., & Junger, W. G. (2012). Measurement of oxidative burst in neutrophils. *Methods Mol Biol, 844*, 115-124. doi:10.1007/978-1-61779-527-5_8

Contreras, G. A., Raphael, W., Mattmiller, S. A., Gandy, J., & Sordillo, L. M. (2012). Nonesterified fatty acids modify inflammatory response and eicosanoid biosynthesis in bovine endothelial cells. *J Dairy Sci, 95*(9), 5011-5023. doi:10.3168/jds.2012-5382

Crepaldi, L., Gasperini, S., Lapinet, J. A., Calzetti, F., Pinardi, C., Liu, Y., . . . Cassatella, M. A. (2001). Up-regulation of IL-10R1 expression is required to render human neutrophils fully responsive to IL-10. *J Immunol, 167*(4), 2312-2322. doi:10.4049/jimmunol.167.4.2312

Cruzat, V. F., Krause, M., & Newsholme, P. (2014). Amino acid supplementation and impact on immune function in the context of exercise. *J Int Soc Sports Nutr, 11*(1), 61. doi:10.1186/s12970-014-0061-8

Curi, R., Levada-Pires, A. C., Silva, E. B. D., Poma, S. O., Zambonatto, R. F., Domenech, P., . . . Pithon-Curi, T. C. (2020). The Critical Role of Cell Metabolism for Essential Neutrophil Functions. *Cell Physiol Biochem, 54*(4), 629-647. doi:10.33594/000000245

Dahl, R., Walsh, J. C., Lancki, D., Laslo, P., Iyer, S. R., Singh, H., & Simon, M. C. (2003). Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol, 4*(10), 1029-1036. doi:10.1038/ni973

Dantzer, R., & Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain Behav Immun, 21*(2), 153-160. doi:10.1016/j.bbi.2006.09.006

Davies, M. J. (2011). Myeloperoxidase-derived oxidation: mechanisms of biological damage and its prevention. *J Clin Biochem Nutr, 48*(1), 8-19. doi:10.3164/jcbn.11-006FR

De Santo, C., Arscott, R., Booth, S., Karydis, I., Jones, M., Asher, R., . . . Cerundolo, V. (2010). Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils

differentiated with serum amyloid A. *Nat Immunol, 11*(11), 1039-1046. doi:10.1038/ni.1942

Deniset, J. F., Surewaard, B. G., Lee, W. Y., & Kubes, P. (2017). Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of S. pneumoniae. *J Exp Med, 214*(5), 1333-1350. doi:10.1084/jem.20161621

Drackley, J. K. (1999). ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? *J Dairy Sci, 82*(11), 2259-2273. doi:10.3168/jds.s0022-0302(99)75474-3

Eash, K. J., Means, J. M., White, D. W., & Link, D. C. (2009). CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood, 113*(19), 4711-4719. doi:10.1182/blood-2008-09-177287

Elbim, C., & Lizard, G. (2009). Flow cytometric investigation of neutrophil oxidative burst and apoptosis in physiological and pathological situations. *Cytometry A, 75*(6), 475-481. doi:10.1002/cyto.a.20726

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol, 147*(11), 3815-3822.

Forman, H. J., Zhang, H., & Rinna, A. (2009). Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med, 30*(1-2), 1-12. doi:10.1016/j.mam.2008.08.006

Gallagher, K. (2022). *Branched Chain Amino Acids or Branched Chain Keto Acids Improved Hepatic Metabolism and Production in Early Lactation Dairy Cows.* (M.S.), Michigan State University, Ann Arbor. Retrieved from http://ezproxy.msu.edu/login?url=https://www.proquest.com/dissertations-theses/branched-chain-amino-acids-keto-improved-hepatic/docview/2723521162/se-2?accountid=12598 (29390838)

Galli, S. J., Borregaard, N., & Wynn, T. A. (2011). Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol, 12*(11), 1035-1044. doi:10.1038/ni.2109

Glassy, M. C., & Furlong, C. E. (1981). Neutral amino acid transport during the cell cycle of cultured human lymphocytes. *J Cell Physiol, 107*(1), 69-74. doi:10.1002/jcp.1041070109

Goff, J. P. (2006). Major advances in our understanding of nutritional influences on bovine health. *J Dairy Sci, 89*(4), 1292-1301. doi:10.3168/jds.S0022-0302(06)72197-X

Goff, J. P., & Horst, R. L. (1997). Physiological changes at parturition and their relationship to metabolic disorders. *J Dairy Sci, 80*(7), 1260-1268. doi:10.3168/jds.S0022-0302(97)76055-7

Gott, P. N., Hogan, J. S., & Weiss, W. P. (2015). Effects of various starch feeding regimens on responses of dairy cows to intramammary lipopolysaccharide infusion. *J Dairy Sci, 98*(3), 1786-1796. doi:10.3168/jds.2014-8638

Graugnard, D. E., Bionaz, M., Trevisi, E., Moyes, K. M., Salak-Johnson, J. L., Wallace, R. L., . . . Loor, J. J. (2012). Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. *J Dairy Sci, 95*(4), 1749-1758. doi:10.3168/jds.2011-4579

Harper, A. E., Miller, R. H., & Block, K. P. (1984). Branched-chain amino acid metabolism. *Annu Rev Nutr, 4*, 409-454. doi:10.1146/annurev.nu.04.070184.002205

Hawkins, C. L., & Davies, M. J. (2021). Role of myeloperoxidase and oxidant formation in the extracellular environment in inflammation-induced tissue damage. *Free Radic Biol Med, 172*, 633-651. doi:10.1016/j.freeradbiomed.2021.07.007

Hoffmann, A., & Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. *Immunol Rev, 210*, 171-186. doi:10.1111/j.0105-2896.2006.00375.x

Hogan, J., & Smith, K. (2003). Coliform mastitis. *Vet Res, 34*(5), 507-519. doi:10.1051/vetres:2003022

Hong, C., Kidani, Y., N, A. G., Phung, T., Ito, A., Rong, X., . . . Bensinger, S. J. (2012). Coordinate regulation of neutrophil homeostasis by liver X receptors in mice. *J Clin Invest, 122*(1), 337-347. doi:10.1172/JCI58393

Hutchins, A. P., Diez, D., & Miranda-Saavedra, D. (2013). The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. *Brief Funct Genomics, 12*(6), 489-498. doi:10.1093/bfgp/elt028

Iribarren, P., Cui, Y. H., Le, Y., Ying, G., Zhang, X., Gong, W., & Wang, J. M. (2003). IL-4 down-regulates lipopolysaccharide-induced formyl peptide receptor 2 in murine microglial cells by inhibiting the activation of mitogen-activated protein kinases. *J Immunol, 171*(10), 5482-5488. doi:10.4049/jimmunol.171.10.5482

Jain, A., Kaczanowska, S., & Davila, E. (2014). IL-1 Receptor-Associated Kinase Signaling and Its Role in Inflammation, Cancer Progression, and Therapy Resistance. *Front Immunol, 5*, 553. doi:10.3389/fimmu.2014.00553

Kehrli, M. E., Jr., Nonnecke, B. J., & Roth, J. A. (1989). Alterations in bovine neutrophil function during the periparturient period. *Am J Vet Res, 50*(2), 207-214.

Kimura, K., Goff, J. P., Canning, P., Wang, C., & Roth, J. A. (2014). Effect of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil number and function in periparturient dairy cows. *J Dairy Sci, 97*(8), 4842-4851. doi:10.3168/jds.2013-7242

Kimura, K., Goff, J. P., & Kehrli, M. E., Jr. (1999). Effects of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. *J Dairy Sci, 82*(11), 2385-2392. doi:10.3168/jds.S0022-0302(99)75489-5

Kimura, K., Goff, J. P., Kehrli, M. E., Jr., & Reinhardt, T. A. (2002). Decreased neutrophil function as a cause of retained placenta in dairy cattle. *J Dairy Sci, 85*(3), 544-550. doi:10.3168/jds.S0022-0302(02)74107-6

Kolaczkowska, E., Jenne, C. N., Surewaard, B. G., Thanabalasuriar, A., Lee, W. Y., Sanz, M. J., . . . Kubes, P. (2015). Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat Commun, 6*, 6673. doi:10.1038/ncomms7673

Kushibiki, S., Hodate, K., Shingu, H., Obara, Y., Touno, E., Shinoda, M., & Yokomizo, Y. (2003). Metabolic and lactational responses during recombinant bovine tumor necrosis factor-alpha treatment in lactating cows. *J Dairy Sci, 86*(3), 819-827. doi:10.3168/jds.S0022-0302(03)73664-9

Lacy, P. (2006). Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol, 2*(3), 98-108. doi:10.1186/1710-1492-2-3-98

Landen, N. X., Li, D., & Stahle, M. (2016). Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci, 73*(20), 3861-3885. doi:10.1007/s00018-016-2268-0

Leal Yepes, F. A., Mann, S., Overton, T. R., Ryan, C. M., Bristol, L. S., Granados, G. E., . . . Wakshlag, J. J. (2019). Effect of rumen-protected branched-chain amino acid supplementation on production- and energy-related metabolites during the first 35 days in milk in Holstein dairy cows. *J Dairy Sci, 102*(6), 5657-5672. doi:10.3168/jds.2018-15508

LeBlanc, S. J. (2020). Review: Relationships between metabolism and neutrophil function in dairy cows in the peripartum period. *Animal, 14*(S1), s44-s54. doi:10.1017/S1751731119003227

Lee, Harrison, R. E., & Grinstein, S. (2003). Phagocytosis by neutrophils. *Microbes Infect, 5*(14), 1299-1306. doi:10.1016/j.micinf.2003.09.014

Lee, J. H., Park, E., Jin, H. J., Lee, Y., Choi, S. J., Lee, G. W., . . . Paik, H. D. (2017). Anti-inflammatory and anti-genotoxic activity of branched chain amino acids (BCAA) in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages. *Food Sci Biotechnol, 26*(5), 1371-1377. doi:10.1007/s10068-017-0165-4

Lefrancais, E., Mallavia, B., Zhuo, H., Calfee, C. S., & Looney, M. R. (2018). Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight, 3*(3). doi:10.1172/jci.insight.98178

Ley, K., Laudanna, C., Cybulsky, M. I., & Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol, 7*(9), 678-689. doi:10.1038/nri2156

Lieschke, G. J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., . . . Dunn, A. R. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood, 84*(6), 1737-1746.

Liew, P. X., & Kubes, P. (2019). The Neutrophil's Role During Health and Disease. *Physiol Rev, 99*(2), 1223-1248. doi:10.1152/physrev.00012.2018

Lord, B. I., Bronchud, M. H., Owens, S., Chang, J., Howell, A., Souza, L., & Dexter, T. M. (1989). The kinetics of human granulopoiesis following treatment with granulocyte colony-stimulating factor in vivo. *Proc Natl Acad Sci U S A, 86*(23), 9499-9503. doi:10.1073/pnas.86.23.9499

Lynch, C. J., & Adams, S. H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol, 10*(12), 723-736. doi:10.1038/nrendo.2014.171

Mamedova, L. K., Yuan, K., Laudick, A. N., Fleming, S. D., Mashek, D. G., & Bradford, B. J. (2013). Toll-like receptor 4 signaling is required for induction of gluconeogenic gene expression by palmitate in human hepatic carcinoma cells. *J Nutr Biochem, 24*(8), 1499-1507. doi:10.1016/j.jnutbio.2012.12.009

Mann, G., Mora, S., Madu, G., & Adegoke, O. A. J. (2021). Branched-chain Amino Acids: Catabolism in Skeletal Muscle and Implications for Muscle and Whole-body Metabolism. *Front Physiol, 12*, 702826. doi:10.3389/fphys.2021.702826

Martin, C., Burdon, P. C., Bridger, G., Gutierrez-Ramos, J. C., Williams, T. J., & Rankin, S. M. (2003). Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils

from the bone marrow and their return following senescence. *Immunity, 19*(4), 583-593. doi:10.1016/s1074-7613(03)00263-2

Massena, S., Christoffersson, G., Hjertstrom, E., Zcharia, E., Vlodavsky, I., Ausmees, N., . . . Phillipson, M. (2010). A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils. *Blood, 116*(11), 1924-1931. doi:10.1182/blood-2010-01-266072

McDonald, B., & Kubes, P. (2016). Innate Immune Cell Trafficking and Function During Sterile Inflammation of the Liver. *Gastroenterology, 151*(6), 1087-1095. doi:10.1053/j.gastro.2016.09.048

McDonald, B., Urrutia, R., Yipp, B. G., Jenne, C. N., & Kubes, P. (2012). Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe, 12*(3), 324-333. doi:10.1016/j.chom.2012.06.011

Meier, S., Priest, N. V., Burke, C. R., Kay, J. K., McDougall, S., Mitchell, M. D., . . . Roche, J. R. (2014). Treatment with a nonsteroidal antiinflammatory drug after calving did not improve milk production, health, or reproduction parameters in pasture-grazed dairy cows. *J Dairy Sci, 97*(5), 2932-2943. doi:10.3168/jds.2013-7838

Metcalf, D., Begley, C. G., Johnson, G. R., Nicola, N. A., Lopez, A. F., & Williamson, D. J. (1986). Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hematopoiesis in normal adult mice. *Blood, 68*(1), 46-57.

Metcalf, D., Begley, C. G., Williamson, D. J., Nice, E. C., De Lamarter, J., Mermod, J. J., . . . Schmidt, A. (1987). Hemopoietic responses in mice injected with purified recombinant murine GM-CSF. *Exp Hematol, 15*(1), 1-9.

Mireles, A. J., Kim, S. M., & Klasing, K. C. (2005). An acute inflammatory response alters bone homeostasis, body composition, and the humoral immune response of broiler chickens. *Poult Sci, 84*(4), 553-560. doi:10.1093/ps/84.4.553

National Academies of Sciences, Engineering, and Medicine. 2021. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition. Washington, DC: The National Academies Press. https://doi.org/10.17226/25806.

Nerlov, C., & Graf, T. (1998). PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev, 12*(15), 2403-2412. doi:10.1101/gad.12.15.2403

Newburger, P. E., Subrahmanyam, Y. V., & Weissman, S. M. (2000). Global analysis of neutrophil gene expression. *Curr Opin Hematol, 7*(1), 16-20. doi:10.1097/00062752-200001000-00004

Newton, K., & Dixit, V. M. (2012). Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol, 4*(3). doi:10.1101/cshperspect.a006049

Odegaard, J. I., & Chawla, A. (2012). Leukocyte set points in metabolic disease. *F1000 Biol Rep, 4*, 13. doi:10.3410/B4-13

Ohtsuka, H., Koiwa, M., Hatsugaya, A., Kudo, K., Hoshi, F., Itoh, N., . . . Kawamura, S. (2001). Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. *J Vet Med Sci, 63*(9), 1021-1025. doi:10.1292/jvms.63.1021

Parker, H., & Winterbourn, C. C. (2012). Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps. *Front Immunol, 3*, 424. doi:10.3389/fimmu.2012.00424

Pedernera, M., Celi, P., Garcia, S. C., Salvin, H. E., Barchia, I., & Fulkerson, W. J. (2010). Effect of diet, energy balance and milk production on oxidative stress in early-lactating dairy cows grazing pasture. *Vet J, 186*(3), 352-357. doi:10.1016/j.tvjl.2009.09.003

Petri, B., Phillipson, M., & Kubes, P. (2008). The physiology of leukocyte recruitment: an in vivo perspective. *J Immunol, 180*(10), 6439-6446. doi:10.4049/jimmunol.180.10.6439

Petro, T. M., & Bhattacharjee, J. K. (1981). Effect of dietary essential amino acid limitations upon the susceptibility to Salmonella typhimurium and the effect upon humoral and cellular immune responses in mice. *Infect Immun, 32*(1), 251-259. doi:10.1128/iai.32.1.251-259.1981

Phillipson, M., & Kubes, P. (2011). The neutrophil in vascular inflammation. *Nat Med, 17*(11), 1381-1390. doi:10.1038/nm.2514

Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., . . . Koenderman, L. (2010). In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood, 116*(4), 625-627. doi:10.1182/blood-2010-01-259028

Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., . . . Kubes, P. (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol, 185*(12), 7413-7425. doi:10.4049/jimmunol.1000675

Pojda, Z., & Tsuboi, A. (1990). In vivo effects of human recombinant interleukin 6 on hemopoietic stem and progenitor cells and circulating blood cells in normal mice. *Exp Hematol, 18*(9), 1034-1037.

Priest, N. V., McDougall, S., Burke, C. R., Roche, J. R., Mitchell, M., McLeod, K. L., . . . Meier, S. (2013). The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. *J Dairy Sci, 96*(7), 4323-4332. doi:10.3168/jds.2012-6266

Pruenster, M., Mudde, L., Bombosi, P., Dimitrova, S., Zsak, M., Middleton, J., . . . Rot, A. (2009). The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol, 10*(1), 101-108. doi:10.1038/ni.1675

Ramachandran, V., Williams, M., Yago, T., Schmidtke, D. W., & McEver, R. P. (2004). Dynamic alterations of membrane tethers stabilize leukocyte rolling on P-selectin. *Proc Natl Acad Sci U S A, 101*(37), 13519-13524. doi:10.1073/pnas.0403608101

Richards, M. K., Liu, F., Iwasaki, H., Akashi, K., & Link, D. C. (2003). Pivotal role of granulocyte colony-stimulating factor in the development of progenitors in the common myeloid pathway. *Blood, 102*(10), 3562-3568. doi:10.1182/blood-2003-02-0593

Rosmarin, A. G., Yang, Z., & Resendes, K. K. (2005). Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. *Exp Hematol, 33*(2), 131-143. doi:10.1016/j.exphem.2004.08.015

Sadik, C. D., Kim, N. D., & Luster, A. D. (2011). Neutrophils cascading their way to inflammation. *Trends Immunol, 32*(10), 452-460. doi:10.1016/j.it.2011.06.008

Sadri, H., von Soosten, D., Meyer, U., Kluess, J., Danicke, S., Saremi, B., & Sauerwein, H. (2017). Plasma amino acids and metabolic profiling of dairy cows in response to a bolus duodenal infusion of leucine. *PLoS One, 12*(4), e0176647. doi:10.1371/journal.pone.0176647

Sanz, M. J., & Kubes, P. (2012). Neutrophil-active chemokines in in vivo imaging of neutrophil trafficking. *Eur J Immunol, 42*(2), 278-283. doi:10.1002/eji.201142231

Sasmono, R. T., Ehrnsperger, A., Cronau, S. L., Ravasi, T., Kandane, R., Hickey, M. J., . . . Hume, D. A. (2007). Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. *J Leukoc Biol, 82*(1), 111-123. doi:10.1189/jlb.1206713

Schafer, G., & Schauder, P. (1988). Assessment of effects of amino acids and branched chain keto acids on leucine oxidation in human lymphocytes. *Scand J Clin Lab Invest, 48*(6), 531-536. doi:10.3109/00365518809085769

Schmidtke, D. W., & Diamond, S. L. (2000). Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. *J Cell Biol, 149*(3), 719-730. doi:10.1083/jcb.149.3.719

Schottelius, A. J., Mayo, M. W., Sartor, R. B., & Baldwin, A. S., Jr. (1999). Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem, 274*(45), 31868-31874. doi:10.1074/jbc.274.45.31868

Schukken, Y. H., Gunther, J., Fitzpatrick, J., Fontaine, M. C., Goetze, L., Holst, O., . . . members of the Pfizer mastitis research, c. (2011). Host-response patterns of intramammary infections in dairy cows. *Vet Immunol Immunopathol, 144*(3-4), 270-289. doi:10.1016/j.vetimm.2011.08.022

Segal, A. W. (2005). How neutrophils kill microbes. *Annu Rev Immunol, 23*, 197-223. doi:10.1146/annurev.immunol.23.021704.115653

Sengelov, H., Kjeldsen, L., & Borregaard, N. (1993). Control of exocytosis in early neutrophil activation. *J Immunol, 150*(4), 1535-1543.

Sheshachalam, A., Srivastava, N., Mitchell, T., Lacy, P., & Eitzen, G. (2014). Granule protein processing and regulated secretion in neutrophils. *Front Immunol, 5*, 448. doi:10.3389/fimmu.2014.00448

Shouval, D. S., Ouahed, J., Biswas, A., Goettel, J. A., Horwitz, B. H., Klein, C., . . . Snapper, S. B. (2014). Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol, 122*, 177-210. doi:10.1016/B978-0-12-800267-4.00005-5

Smith, K. L., Harrison, J. H., Hancock, D. D., Todhunter, D. A., & Conrad, H. R. (1984). Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. *J Dairy Sci, 67*(6), 1293-1300. doi:10.3168/jds.S0022-0302(84)81436-8

Smith, K. L., Hogan, J. S., & Weiss, W. P. (1997). Dietary vitamin E and selenium affect mastitis and milk quality. *J Anim Sci, 75*(6), 1659-1665. doi:10.2527/1997.7561659x

Sordillo, L. M., & Aitken, S. L. (2009). Impact of oxidative stress on the health and immune function of dairy cattle. *Vet Immunol Immunopathol, 128*(1-3), 104-109. doi:10.1016/j.vetimm.2008.10.305

Sordillo, L. M., Contreras, G. A., & Aitken, S. L. (2009). Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Anim Health Res Rev, 10*(1), 53-63. doi:10.1017/S1466252309990016

Sordillo, L. M., O'Boyle, N., Gandy, J. C., Corl, C. M., & Hamilton, E. (2007). Shifts in thioredoxin reductase activity and oxidant status in mononuclear cells obtained from transition dairy cattle. *J Dairy Sci, 90*(3), 1186-1192. doi:10.3168/jds.S0022-0302(07)71605-3

Spires, H. R., Clark, J. H., Derrig, R. G., & Davis, C. L. (1975). Milk production and nitrogen utilization in response to postruminal infusion of sodium caseinate in lactating cows. *J Nutr, 105*(9), 1111-1121. doi:10.1093/jn/105.9.1111

Spite, M., Claria, J., & Serhan, C. N. (2014). Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. *Cell Metab, 19*(1), 21-36. doi:10.1016/j.cmet.2013.10.006

Sundd, P., Gutierrez, E., Koltsova, E. K., Kuwano, Y., Fukuda, S., Pospieszalska, M. K., . . . Ley, K. (2012). 'Slings' enable neutrophil rolling at high shear. *Nature, 488*(7411), 399-403. doi:10.1038/nature11248

Sundd, P., Gutierrez, E., Pospieszalska, M. K., Zhang, H., Groisman, A., & Ley, K. (2010). Quantitative dynamic footprinting microscopy reveals mechanisms of neutrophil rolling. *Nat Methods, 7*(10), 821-824. doi:10.1038/nmeth.1508

Sundd, P., Pospieszalska, M. K., Cheung, L. S., Konstantopoulos, K., & Ley, K. (2011). Biomechanics of leukocyte rolling. *Biorheology, 48*(1), 1-35. doi:10.3233/BIR-2011-0579

Sung, M. H., Li, N., Lao, Q., Gottschalk, R. A., Hager, G. L., & Fraser, I. D. (2014). Switching of the relative dominance between feedback mechanisms in lipopolysaccharide-induced NF-kappaB signaling. *Sci Signal, 7*(308), ra6. doi:10.1126/scisignal.2004764

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell, 140*(6), 805-820. doi:10.1016/j.cell.2010.01.022

Tang, T., Zhang, J., Yin, J., Staszkiewicz, J., Gawronska-Kozak, B., Jung, D. Y., . . . Ye, J. (2010). Uncoupling of inflammation and insulin resistance by NF-kappaB in transgenic mice through elevated energy expenditure. *J Biol Chem, 285*(7), 4637-4644. doi:10.1074/jbc.M109.068007

van Engelen, E., de Groot, M. W., Breeveld-Dwarkasing, V. N., Everts, M. E., van der Weyden, G. C., Taverne, M. A., & Rutten, V. P. (2009). Cervical ripening and parturition in cows are driven by a cascade of pro-inflammatory cytokines. *Reprod Domest Anim, 44*(5), 834-841. doi:10.1111/j.1439-0531.2008.01096.x

Voisin, M. B., & Nourshargh, S. (2013). Neutrophil transmigration: emergence of an adhesive cascade within venular walls. *J Innate Immun, 5*(4), 336-347. doi:10.1159/000346659

Wang, J., Hossain, M., Thanabalasuriar, A., Gunzer, M., Meininger, C., & Kubes, P. (2017). Visualizing the function and fate of neutrophils in sterile injury and repair. *Science, 358*(6359), 111-116. doi:10.1126/science.aam9690

Westreicher-Kristen, E., & Susenbeth, A. (2017). Technical note: An improved tool to insert lines for abomasal infusion in rumen cannulated cattle. *J Dairy Sci, 100*(3), 1951-1954. doi:10.3168/jds.2016-12150

Williams, M. R., Azcutia, V., Newton, G., Alcaide, P., & Luscinskas, F. W. (2011). Emerging mechanisms of neutrophil recruitment across endothelium. *Trends Immunol, 32*(10), 461-469. doi:10.1016/j.it.2011.06.009

Wynn, T. A., & Vannella, K. M. (2016). Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity, 44*(3), 450-462. doi:10.1016/j.immuni.2016.02.015

Yipp, B. G., Kim, J. H., Lima, R., Zbytnuik, L. D., Petri, B., Swanlund, N., . . . Kubes, P. (2017). The Lung is a Host Defense Niche for Immediate Neutrophil-Mediated Vascular Protection. *Sci Immunol, 2*(10). doi:10.1126/sciimmunol.aam8929

Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., . . . Kubes, P. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med, 18*(9), 1386-1393. doi:10.1038/nm.2847

Yoder, P. S., Huang, X., Teixeira, I. A., Cant, J. P., & Hanigan, M. D. (2020). Effects of jugular infused methionine, lysine, and histidine as a group or leucine and isoleucine as a group on production and metabolism in lactating dairy cows. *J Dairy Sci, 103*(3), 2387-2404. doi:10.3168/jds.2019-17082

Younus, H. (2018). Therapeutic potentials of superoxide dismutase. *Int J Health Sci (Qassim), 12*(3), 88-93.

Yuan, K., Vargas-Rodriguez, C. F., Mamedova, L. K., Muckey, M. B., Vaughn, M. A., Burnett, D. D., . . . Bradford, B. J. (2014). Effects of supplemental chromium propionate and rumen-protected amino acids on nutrient metabolism, neutrophil activation, and adipocyte size in dairy cows during peak lactation. *J Dairy Sci, 97*(6), 3822-3831. doi:10.3168/jds.2013-7770

Zebeli, Q., Mansmann, D., Sivaraman, S., Dunn, S. M., & Ametaj, B. N. (2013). Oral challenge with increasing doses of LPS modulated the patterns of plasma metabolites and minerals

in periparturient dairy cows. *Innate Immun, 19*(3), 298-314. doi:10.1177/1753425912461287

Zhou, Z., Bu, D. P., Vailati Riboni, M., Khan, M. J., Graugnard, D. E., Luo, J., . . . Loor, J. J. (2015). Prepartal dietary energy level affects peripartal bovine blood neutrophil metabolic, antioxidant, and inflammatory gene expression. *J Dairy Sci, 98*(8), 5492-5505. doi:10.3168/jds.2014-8811

Zhou, Z., Ferdous, F., Montagner, P., Luchini, D. N., Correa, M. N., & Loor, J. J. (2018). Methionine and choline supply during the peripartal period alter polymorphonuclear leukocyte immune response and immunometabolic gene expression in Holstein cows. *J Dairy Sci, 101*(11), 10374-10382. doi:10.3168/jds.2018-14972

Zimmerman, G. A., McIntyre, T. M., & Prescott, S. M. (1997). Adhesion and signaling in vascular cell-cell interactions. *J Clin Invest, 100*(11 Suppl), S3-5.

Zinicola, M., & Bicalho, R. C. (2019). Association of peripartum plasma insulin concentration with milk production, colostrum insulin levels, and plasma metabolites of Holstein cows. *J Dairy Sci, 102*(2), 1473-1482. doi:10.3168/jds.2017-14029

APPENDIX

TABLES AND FIGURES

Table 2.1. List of primers used for RT-qPCR.

Gene	F or R[1]	Sequence (5'-3')
CBS	F	GCCACCACCTCTGTCAAATTC
	R	GGACAGAAAGCAGAGTGGTAACTG
CTH	F	AAGTCCGCATGGAGAAGCATT
	R	GAAGGCAGCCCAGGATAAATAA
CXCR2	F	GGCACTGGGTCAAGTTCATATGT
	R	CGGAGTACGGTGGTTGATAGG
GPX1	F	CCCCTGCAACCAGTTTGG
	R	GAGCATAAAGTTGGGCTCGAA
GSR	F	CGCTGAGAACCCAGAGACTTG
	R	AAACGGAAAGTGGGAACAGTAAGTA
ICAM1	F	AGAATTAGCGCTGACCTCTGTTAAG
	R	CGGACACATCTCAGTGACTAAACAA
IFNG	F	CTTTTGGGTTTTTCTGGTTCTTAT
	R	GCTACATCTGGGCTACTTGCAT
IL1B	F	GAATCTATACCTGTCTTGTGTGAAAAA
	R	TCCTCTTGGGGTAGACTTTGG
IL10	F	GAAGGACCAACTGCACAGCTT
	R	AAAACTGGATCATTTCCGACAAG
IL10RA	F	GTATCGCAGCAATGGTTAC
	R	CCGTCAGAGTCACTTCAT
IRAK1	F	CCTCAGCGACTGGACATCCT
	R	GGACGTTGGAACTCTTGACATCT
ITGB2	F	GCACACAAACTGGCAGAAAG
	R	TGCAGACTTGGGGATGATCT
MPO	F	AAGTGGATACCTCGGTGGTG
	R	GGCCAAGCCACTGTGAAG
NFKB1	F	TTCAACCGGAGATGCCACTAC
	R	ACACACGTAACGGAAACGAAATC
S100A8	F	ACACCATGCTGACGGATCTG
	R	TCCCTATAGACGGCGTGGTAA

Table 2.1. (cont'd)

Gene	F or R[1]	Sequence (5'-3')
SAHH	F	TGTCAGGAGGGCAACATCTTT
	R	AGTGCCCAATGTTACACACAATG
SELL	F	CTCTGCTACACAGCTTCTTGTAAACC
	R	CCGTAGTACCCCAAATCACAGTT
SOD1	F	TGGGCCAAAAGATGAAGAGA
	R	ACGATGGCAACACCGTTT
SOD2	F	TGTGGGAGCATGCTTATTACCTT
	R	TGCAGTTACATTCTCCCAGTTGA
TLR2	F	CTGGCAAGTGGATTATCGACAA
	R	TACTTGCACCACTCGCTCTTCA
TLR4	F	TGCGTACAGGTTGTTCCTAACATT
	R	TAGTTAAAGCTCAGGTCCAGCATCT

[1]forward (F) or reverse transcription

Figure 2.1. Neutrophil and mononucelar subpopulations gated by FSC and SSC (A). Cells were stained with Brilliant Violent 421™ to identify CH138A positive neutrophils under Pacific Blue channel (B). Emission of PI under the PE channel identified unchallenged (C) and challenged (D) phagocytic CH138A positive neutrophils. Emission of rhodamine 123 under the FITC channel identified unchallenged (E) and challenged (F) phagocytic CH138A positive neutrophils undergoing oxidative burst. Histogram of mean fluorescent intensity (MFI) of unchallenged (G) and challenged (H) phagocytic CH138A positive neutrophils undergoing oxidative burst.

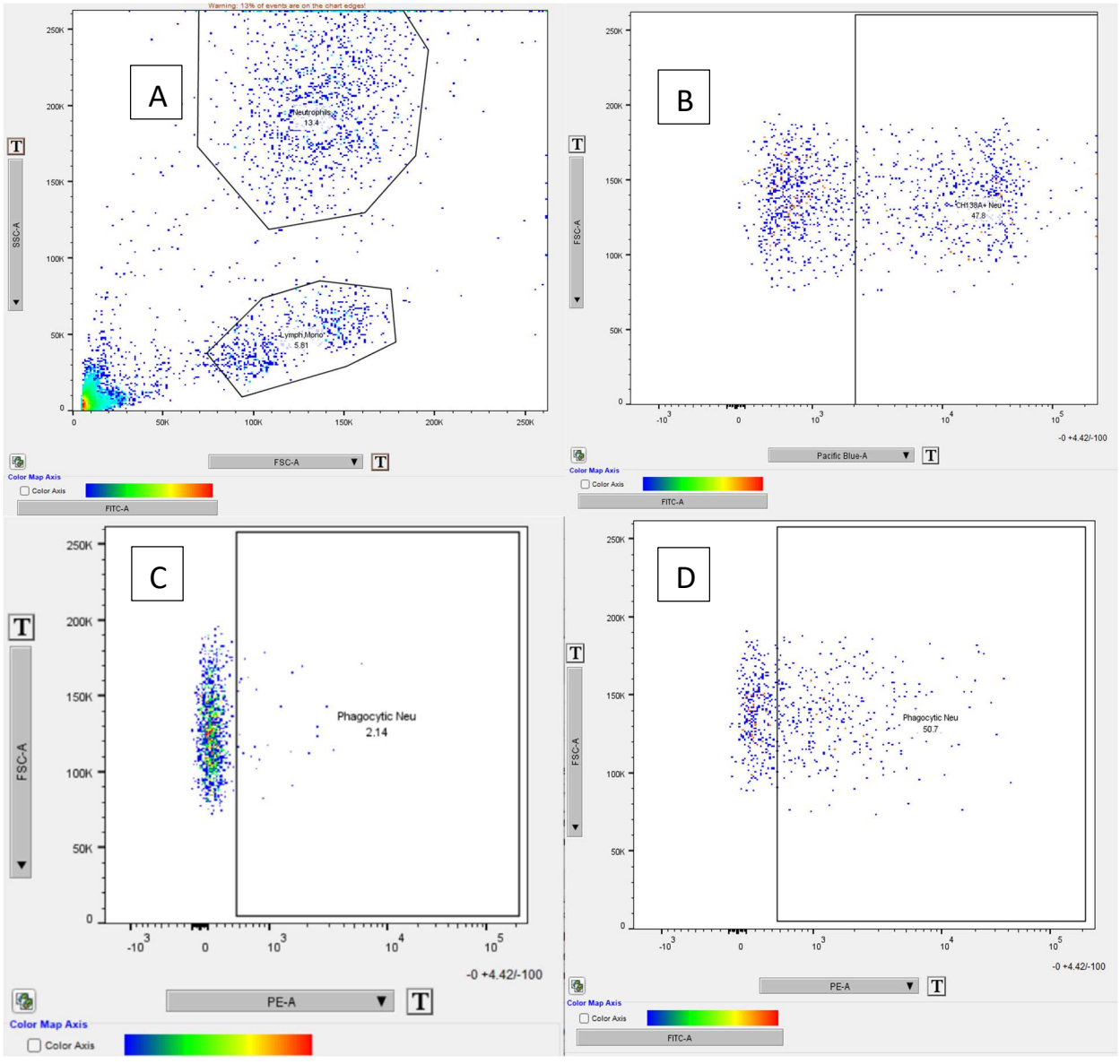

Figure 2.1. (cont'd)

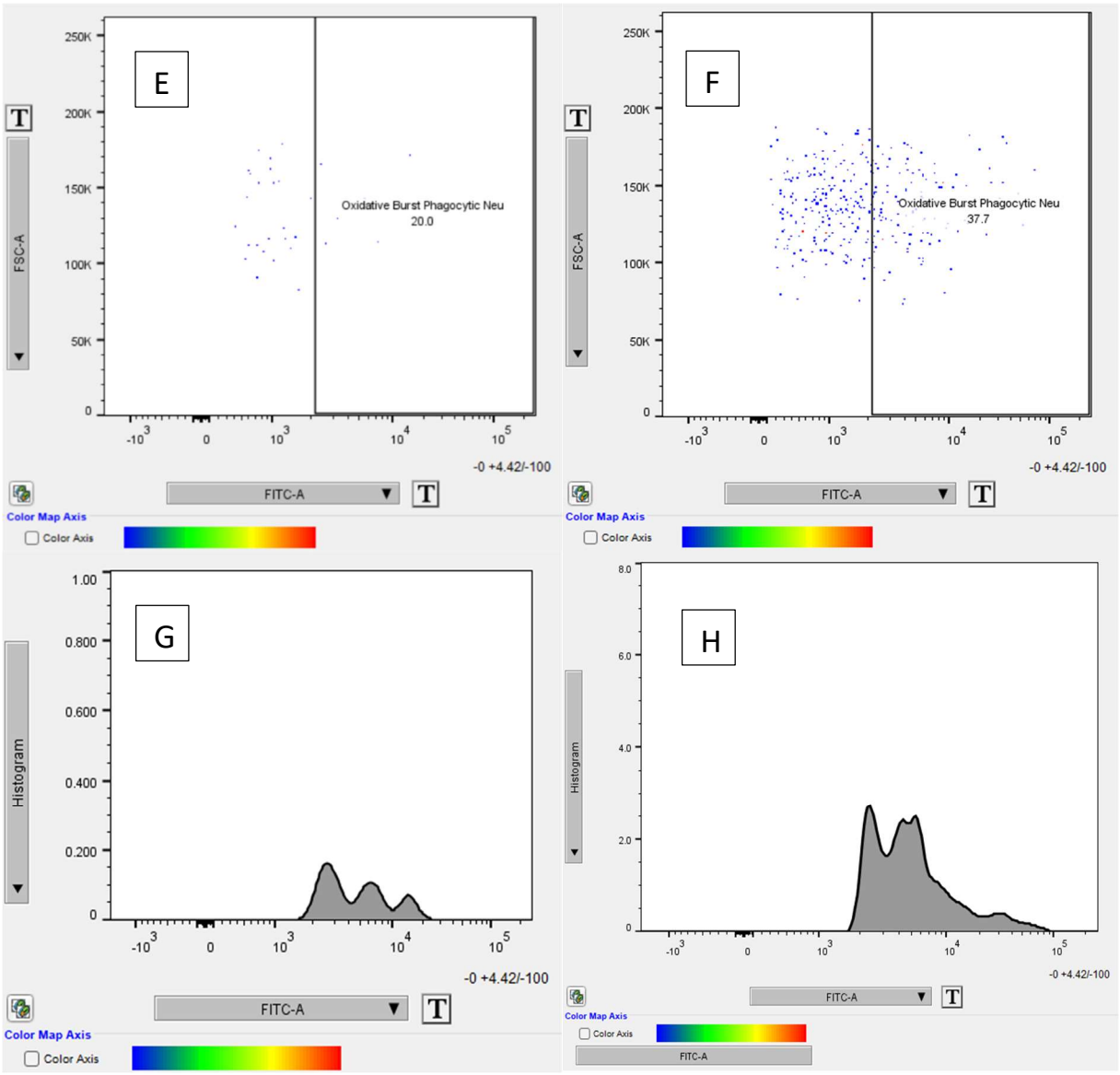

Table 2.2a. Effects (LSM and greatest SEM) of supplementing CON, BCA, or BCK[1] to early lactation multiparous Holstein cows on blood neutrophil phagocytosis, oxidative burst, and MFI[2] of oxidative burst.

	Treatment				*P*-value
Item (%)[3]	CON	BCA	BCK	SEM	Trt
Phagocytosis	63.8	60.1	58.1	4.69	0.48
Oxidative burst	60.5	63.7	63.8	4.50	0.72
MFI mean	4190	4050	3550	584	0.52

[1]CON = control (saline 0.9%); BCA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCK = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

[2]Mean fluorescent intensity

[3]Data reported as percentages of CH138A positive cells

Table 2.2b. Effects (LSM and greatest SEM) of supplementing CON, BCA, or BCK[1] to early lactation multiparous Holstein cows on blood neutrophil phagocytosis, oxidative burst, and MFI[2] of oxidative burst.

	Time			*P*-value	
Item (%)[3]	d10	d20	SEM	Time	Trt ×Time
Phagocytosis	59.7	61.6	2.42	0.52	0.68
Oxidative burst	60.9	64.4	2.53	0.33	0.76
MFI mean	3470[a]	4390[b]	309	0.02	0.78

[1]CON = control (saline 0.9%); BCA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCK = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)

[2]Mean fluorescent intensity

[3]Data reported as percentages of CH138A positive cells

[4]Tukey difference in means indicated with different superscript

Table 2.3a. Effects (LSM and greatest SEM) of supplementing CON, BCA, or BCK[1] to early lactation multiparous Holstein cows on blood mononuclear phagocyte phagocytosis, oxidative burst, and MFI[2] of oxidative burst.

	Treatment				*P*-value
Item (%)[3]	CON	BCA	BCK	SEM	Trt
Phagocytosis	43.6	50.7	49.8	5.69	0.41
Oxidative burst	42.4	47.1	46.4	5.71	0.68
MFI[2] mean	2300[a,b]	2600[b]	1970[a]	288	0.11

[1]CON = control (saline 0.9%); BCA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCK = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)
[2]mean fluorescent intensity
[3]Data reported as percentages of CD14 positive cells
[4]Tukey difference in means indicated with different superscript

Table 2.3b. Effects (LSM and greatest SEM) of supplementing CON, BCA, or BCK[1] to early lactation multiparous Holstein cows on blood mononuclear phagocyte phagocytosis, oxidative burst, and MFI[2] of oxidative burst.

	Time			*P*-value	
Item (%)[3]	d10	d20	SEM	Time	Trt ×Time
Phagocytosis	49.9	46.2	2.82	0.27	0.10
Oxidative burst	45.2	45.3	2.81	0.97	0.02
MFI[2] mean	2010[a]	2580[b]	159	0.01	0.72

[1]CON = control (saline 0.9%); BCA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCK = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)
[2]mean fluorescent intensity
[3]Data reported as percentages of CD14 positive cells
[4]Tukey difference in means indicated with different superscript

Table 2.4. Expression of genes in neutrophils from multiparous Holstein cows supplemented with BCA or BCK.

Genes (AU)[2]	Treatment[1]			SEM	*P*-value		
	CON	BCA	BCK		Trt	Time	Trt ×Time
Antioxidant response							
MPO	5.95[a]	3.53[b]	4.79[a,b]	1.11	0.09	0.46	0.79
SOD1	1.90	1.91	1.79	0.30	0.90	0.44	0.33
SOD2	1.04	0.92	1.12	0.16	0.48	0.83	0.37
CBS	1.87	1.83	2.01	0.20	0.66	0.11	0.40
CTH	2.18	1.78	1.84	0.36	0.47	0.39	0.32
GPX1	0.28[a]	1.88[b]	0.36[a]	0.18	<.01	0.74	0.72
GSR	2.43	2.39	2.56	0.32	0.86	0.95	0.46
SAHH	2.18	2.42	2.40	0.92	0.95	0.56	0.38
PMNL[3] adhesion							
ITGB2	0.47	0.43	0.64	0.14	0.31	0.99	0.82
CXCR2	1.01	0.95	1.17	0.12	0.14	0.44	0.55
ICAM1	1.93	1.85	2.16	0.22	0.38	0.50	0.78
S100A8	0.63	0.61	0.64	0.14	0.98	0.10	0.59
SELL	1.08	1.11	1.29	0.22	0.56	0.95	0.97
TLR[4] pathways							
IFNG	2.64	2.38	2.47	0.56	0.88	0.79	0.24
IL1B	0.29	0.38	0.29	0.12	0.75	0.83	0.42
NFKB1	3.93	5.20	4.05	1.25	0.54	0.59	0.85
IL10	2.74	3.30	4.37	0.89	0.16	0.42	0.14
IL10RA	0.55[a]	0.65[a,b]	0.95[b]	0.18	0.07	0.26	0.81
IRAK1	1.29[a]	1.43[a]	2.10[b]	0.33	0.04	0.10	0.12
TLR2	1.66	1.70	1.8	0.24	0.81	0.11	0.13
TLR4	1.47	1.63	1.47	0.28	0.80	0.56	0.26

[1]CON = control (saline 0.9%); BCA = branched chain amino acids (67 g valine, 50 g leucine, and 34 g isoleucine); BCK = branched chain keto-acids (77 g keto-valine, 57 g keto-leucine, and 39 g keto-isoleucine)
[2]arbitrary units
[3]polymorphonuclar leukocyte
[4]toll-like receptor
[5]Tukey difference in means indicated with different superscript

Figure 2.2. Interaction of treatment by time effect sliced by day in mononuclear phagocytes undergoing oxidative burst.