IMMUNE CELLS IN
BOVINE MESENTERIC
ADIPOSE TISSUE

by

Jenna Roos Wilson

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Pre-Veterinary Medicine and Animal Biosciences with Distinction

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ABSTRACT

Modern nutritional programs in the dairy industry are designed to increase the size of the adipose mass during late lactation. The objective is to increase nutrient reserves that can be mobilized later to meet the overwhelming energy demands of heavy milk production in the next lactation. In addition, the energy density of rations is increased to elevate energy intake at the time of heavy lactation. In human and murine obesity models, these two practices are linked to metabolic disorders. Dietary elements trigger intestinal mucosal inflammation, erode intestinal permeability barrier functions, and enable endotoxins to translocate from the gut to the systemic circulation. Adipose depots overburdened with nutrients trigger immune cells to infiltrate the fat. Translocated endotoxins target these cell infiltrates and establish a pro-inflammatory milieu in the adipose depot. The inflammation generates Type 2 diabetes, lipolysis, dyslipidemia, and steatohepatitis.

Increasing morbidity due to metabolic disorders in heavily lactating cows is testimonial to the intractable nature of this problem in the dairy industry. Interactions between diet-induced shifts in the gut microbiome and the nutrient-laden adipose depot present an interesting approach to better understand the pathogenesis, and therefore to control this disorder in dairy cows.

Our objective was to determine the presence of helper T cell and dendritic cell populations in bovine mesenteric adipose tissue. Endogenous populations of T cells, T lymphocytes, and dendritic cells do exist in bovine mesenteric adipose tissue.
The results of this experiment confirm the presence of dendritic cells in the bovine mesenteric adipose tissue. The stromal cell fraction of the mesenteric adipose tissue (MAT) was made up of cells that expressed MHC II+ (10.62 ± 0.86%), CD209+ (2.05 ± 0.10%), CD209+/CD11b+ (1.45 ± 0.23%), and CD3+ (3.74 ± 2.16%). The results further suggest that immune function has an impact on metabolic homeostasis in the bovine.

The importance of this research is to determine the effects of the current nutritional standards of the dairy industry, which purposefully increase adiposity in dairy cows. Our data indicates that components of the innate and adaptive immune response are endogenous to the bovine mesenteric adipose depot. Their presence could contribute to insulin resistance, dyslipidemia, and chronic inflammatory disease coincident with nutrient overburdened adipocytes.
Chapter 1
INTRODUCTION

1.1 Metabolic Disease in Dairy Cows

Proper nutrition during the transition period of a cow (three weeks before and three weeks after calving) and body condition at calving can affect the negative energy balance during early lactation. Negative energy balance is a normal occurrence, however an excessive negative energy balance can result in poor health and performance. Cows with moderately lower BCS are more likely to transition into lactation adequately than cows with a higher BCS (Overton and Waldron, 2004). Cows that do not adequately transition into lactation can develop ketosis, an elevated level of ketone bodies in the blood that results when glycogen stores are depleted and hepatic β-oxidation of fatty acids elevates circulating acetate, acetoacetate, and β-hydroxybutyrate in the blood.

In a recent survey of 1,955 cows from four herds in Wisconsin and New York, the incidence of hyperketonemia between 3 and 16 days in milk was 45.7%, and by the first 3 to 5 days in milk was 23.0% (McArt, et. al., 2013). In the same survey, the incidence of subclinical metabolic disturbances (moderate to slight elevations in circulating blood ketones) was 43.2% of 1,717 cows with peak prevalence of the subclinical disturbance occurring in 23.9% of the cows only 5 days post-calving (McArt et. al., 2012). Even the subclinical metabolic disturbances are associated with increased costs of other veterinary surgical/medical problems, diminished reproductive efficiencies and milk yields, as well as increased risk of involuntary culling from the
herd (McArt et. al., 2012). These types of surveys confirm that metabolic disorders in modern dairy cows are a widespread problem impacting cows immediately after the onset of lactation. Costs of this intractable metabolic disorder cannot simply be assigned to the metabolic problems. Rather, ancillary problems such as increased involuntary culling from the herd, decreased milk yields, increased veterinary surgical/medical costs, decreased reproductive efficiency, and increased mortality compound costs of metabolic disturbances. The clinical metabolic disorder has been proposed to cost $200 per animal, while the clinically inapparent metabolic disorder costs approximately $78 per animal (Geishauser et. al., 2001). Therefore, dry period nutritional management can affect post-partum health, increasing non-esterified fatty acid (NEFA) levels and increasing the likelihood of developing ketosis, both of which are detrimental to immune function.

Dry matter intake (DMI) decreases over 30% during the last three weeks of gestation, limiting the availability of energy sources, such as glucose, amino acids, and fatty acids, which are required in high demand post-partum (McArt, 2013). This dynamic period of transition for dairy cows is a time during which most metabolic diseases could occur, with those cows struggling to adapt at the highest risk. Incidence of metabolic disease can lead to reduced milk production, removal from the herd, and economic losses. Therefore, it is important to understand the role immunology plays in regulating metabolic disease in dairy cows.

1.2 Adipocytes

Adipocytes are the primary cell type making up adipose (fat) tissue. There are three primary functions of adipocytes: store lipids, respond to insulin, and secrete hormones that affect other tissues. Lipid storage in adipose tissue represents excess
energy consumption in comparison to energy expenditure. Expanding adipose tissue can occur through hyperplasia (an increase in cell number) or by hypertrophy (an increase in cell size). The latter is considered to be a metabolically unhealthy means of expansion, whereas hyperplasia underpins how some individuals with nutrient overburdened adipose depots can remain healthy. It is now known that adipocytes play an important role in energy homeostasis and are involved in the pathogenesis of various metabolic diseases (Stephens, 2012).

Adipokines (cytokines produced in the adipose tissue) can signal satiety in the hypothalamus (leptin), generate a pro-inflammatory response in surrounding tissue and activate macrophages (TNF-α), regulate glucose uptake and fatty acid metabolism (adiponectin), and various other functions. Anti-inflammatory signals favor insulin sensitivity, lipogenesis, and preadipocyte expansion. Pro-inflammatory signals generated by adipocytes promote insulin resistance, lipolysis, and suppression of preadipocyte development. Adipokines play a major role in maintenance of adipose homeostasis.

Mammals have adipose depots located throughout the body; fat pads in distal limbs and digits, subcutaneous fat depots, and visceral fat depots (Rosen, 2006). The latter are located within the body cavity, such as in the intestinal mesentery and surrounding various organs.

1.3 Stromal Cell Fraction

The stromal cell fraction (SCF) of adipose tissue is comprised of preadipocytes, fibroblasts, stem cells, lymphocytes, macrophages, and dendritic cells. Healthy murine SCF is composed of approximately 10% macrophages—primarily the M2 anti-inflammatory phenotype. However, in states of adipose inflammation, the
murine SCF is composed of about 50% macrophages—primarily the M1 pro-inflammatory phenotype (Weisberg et. al., 2003). Excessively hypertrophic adipocytes initiate secretion of chemotactic factor (monocyte chemotactic protein 1, MCP1) that establishes a chemokine gradient. This gradient draws monocytes circulating in the vasculature into the adipose depot of obese fat stores. Once in the adipose tissue, the monocytes are triggered by MCP1 to develop into M1 pro-inflammatory macrophages. Accordingly, M1:M2 ratios swing toward the M1 phenotype, converting the adipose milieu from an anti-inflammatory steady state toward a new pro-inflammatory state.

1.4 Body Condition Score

Body Condition Score (BCS) is a useful tool for dairy herd management. It objectively evaluates the “fatness” of cows on a five-point scale. It can be used in increments of 0.25 (i.e. a cow can be a 2, 2.25, 2.5, etc.). A breakdown of the scoring system can be found in Table 1. Cows should be at a BCS of 3.5 to 4.0 at calving in order to have enough fat to support the energy demands of heavy milk production during early lactation. BCS is highly correlated with adipocyte size in the mesenteric adipose depot of dairy cows (Clark, 2014).

Figure 1. The left panel depicts an underconditioned cow, the middle panel depicts a lean body condition, and the right panel depicts an obese cow.
<table>
<thead>
<tr>
<th>Body Condition Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Severely under-conditioned; very thin, emaciated. Tailhead bones easily discernible and deep cavities around tailhead, prominent backbone, lumbar transverse processes clearly visible, hooks/thurl/pins very prominent and sharp with V-angle between hooks and pins, thin legs, poor muscle condition</td>
</tr>
<tr>
<td>2</td>
<td>Under-conditioned; thin. Tailhead prominent; somewhat hollow but has modest covering of flesh, limited skin cover, prominent backbone, limited skin cover on lumbar transverse processes (visible ¾ of distance), angular hooks/pins with prominent thurl and V-angle between hooks and pins</td>
</tr>
<tr>
<td>3</td>
<td>Good body condition. More flesh covering backbone, no deep depressions or fat deposits around tailhead, smooth tips of lumbar transverse processes, hooks and pins rounded, smooth. Soft V-angle between hooks, thurl and pins.</td>
</tr>
<tr>
<td>4</td>
<td>Over-conditioned; overweight. Backbone barely visible, lumbar transverse processes very smooth, tips barely visible. Hooks and pins very smooth/rounded but visible, flat, U-angle between hooks and pins. Rump and thurl flat, sacral and tailhead ligaments not visible.</td>
</tr>
<tr>
<td>5</td>
<td>Severely over-conditioned; obese. Backbone not visible, lumbar transverse processes flat, bones not visible, hooks/pins not visible/U-angle between hooks and pins, flat over rump and tailhead. All boney prominences from behind rounded and covered in fat, tailhead buried in fat, fat deposits seen on rump and legs.</td>
</tr>
</tbody>
</table>

Table 1. Body condition score (BCS) scale from 1-5 adapted from the Penn State Body Condition Scoring System.
Figure 2. Mesenteric adipocyte area is highly correlated to body condition score in lactating Holstein dairy cows.

1.5 Dendritic Cells

Dendritic cells (DCs) are antigen presenting immune cells that are named for their branched projections called dendrites. DCs survey the microenvironment. Even though there are many different phenotypic subclasses of DCs, tissue-fixed DCs are functionally viewed as immature/resting and mature/activated DCs. Immature/resting DCs are located across all tissues and in the normal “steady-state” of healthy tissue homeostasis, and induce and sustain anti-inflammatory, tolerogenic immune response across tissue compartments. In the unhealthy tissue environment, DCs bind, ingest, and digest foreign materials and pathogens. Bacterial, viral, fungal, and even tumorigenic pathogen-associated molecular motifs bind DC receptors that generate a number of intracellular events culminating in maturation/activation of the immature DC. Maintenance of the tolerogenic, anti-inflammatory state is attributed to quiescent/immature DCs, while induction of immunogenicity, inflammation, and pro-
inflammatory immune response is ascribed to activate/mature DCs (Pletincky et. al., 2011). They control and coordinate immune functions via antigen uptake and processing them into peptides, phospholipids, and glycolipid fragments. During this process, DCs differentiate into “mature” DCs by upregulating immunostimulatory structures. These include antigen major histocompatibility (MHC class I and II) molecules and T lymphocyte co-stimulatory molecules.

Antigen-loaded DCs migrate regionally to lymph nodes, where they present MHC restricted antigen to drive clonal expansion of T lymphocytes. Therefore, DCs act as messengers of the immune response. In the adaptive immune response, DC migration, interaction, and gate-keeping functions are signaled by the functions of DC-derived cytokines. Exposure to antigen causes a DC to produce T lymphocytes to shift towards either the Th1, Th2, or regulatory T lymphocyte (Treg) effector cell functions. To do this, DCs act through toll receptors and C-lectin type receptors. A balance between these two receptors types, and the cytokines they respond to, regulates the transition of the immune response to either a Th1 pro-inflammatory state or a Treg anti-inflammatory state.

1.6 Literature Review

1.6.1 Immune Cells in Adipose Depots

A research group (Atker et al., 2012) looked into the possibility of inflammatory and immune cells infiltrating the adipose tissue of bovine species. The results indicated that the nutrient-laden adipose depots from over-conditioned cows were not infiltrated with inflammatory or immune cells. However, this study used immunohistochemical methodologies with cell markers primarily developed for flow
cytometry. Furthermore, there were too many variables in genetics, diet, and age among the cattle. Based on the data, the authors concluded that inflammatory responses in adipose tissue were not relevant in the pathogenesis of metabolic disorders in the bovine model. The work of Atker et al. was inconsistent with human and murine models.

In a conflicting report, Contreras et. al. (2016) determined omental adipose depots from cows with metabolic disorders, secondary to gastric displacement, were infiltrated with a greater number of macrophages expressing higher amounts of the pro-inflammatory genes (CCL2, TNF-α, and IL-6) than adipose tissues from healthy cows. The authors speculated that the M1 type pro-inflammatory macrophage infiltrates in the adipose depot that occurred in association with metabolic disorders could contribute to the pro-inflammatory status of early lactation cows. Currently, it is unclear if the M1 macrophage infiltrates in the visceral adipose depots were unrelated to or an effect of the gastric displacement seen in the treatment groups. The authors proposed the loss of dry mater and energy intake that always occurs with gastric displacement in dairy cows triggered the catabolic state and metabolic disease in these cows.

The alternative explanation more in line with the murine and human models of metabolic disease is that the inflammatory cells infiltrated the adipose depots and augmented the onset of the metabolic disturbances prior to gastric displacement. Regardless, the data at least showed that bovine mesenteric adipose depots accommodate inflammatory infiltrates in association with metabolic disorders.

Based on the human and murine models of metabolic diseases associated with adiposity, we propose an endogenous population of immune cells exists in the
mesenteric adipose depots from lean dairy cows. Furthermore, we propose that, like lean murine and human individuals, these cells have matured into “gate keepers” that can direct effector lymphocyte development during an adaptive immune response. We hypothesize that the mature phenotype of endogenous DCs from lean adipose tissues retains the capability to dampen the immune and inflammatory responses in the fat of normal dairy cows. The maturation state of DCs in fat depots can potentially generate an anti-inflammatory barrier against metabolic disorders in cows. Metabolic disease, and problems such as insulin resistance, dyslipidemia, and chronic inflammatory disease, stem from overburdened adipocytes that polarize endogenous DC and macrophage phenotypes toward the pro-inflammatory phenotype.

In lean organisms, resident immune cells play a “housekeeping” role in adipose tissue. Cells affected by IL-4 and IL-13 differentiate toward M2 macrophages that are associated with an anti-inflammatory state. Additionally, regulatory Th2 macrophages are activated by toll-like receptors (TLRs), and produce the anti-inflammatory cytokines IL-10 and TGF-β.

As adipocytes become overburdened with lipids and follow the hypertrophic pathway, they will attract immune cells, such as macrophages and DCs, into the tissue. The maturation of these infiltrating cells directs the synthesis and secretion of pro-inflammatory cytokines and mediators that activate the immune response, and trigger a sterile, sustained, and unresolving inflammation in the adipose tissue. This can lead to insulin resistance, lipolysis, and various metabolic diseases.

Tumor necrosis factor-α (TNF-α) is a cytokine produced primarily by M1 macrophages, DCs, and Th1 lymphocytes (to a lesser extent) that are present in adipose tissue. TNF-α is elevated in an obese state. TNF-α can promote insulin-
resistance. Additionally, immune cells in the tissue can be further affected by γ-interferon, and shift toward M1 pro-inflammatory macrophages.

1.6.2 Dendritic Cells in Adipose Tissue

C-type lectin receptors constitute a broad number of cell surface receptors expressed across cells of myeloid origin—macrophages, monocyte-derived DCs, and DCs (Sancho and Sousa, 2012).

C-type lectin receptors, such as CD209 (DC-SIGN), modulate the pro-inflammatory immune response toward an anti-inflammatory, tolerogenic response by driving DC maturation toward the tolerogenic DC type. The secretory activity of DCs is pivotally important in initiating and orienting this immune response. The major cytokines that up-regulate C-type lectin receptors of DCs are IL-10, IL-4, and TGF-β. TNF-α, γ-interferon, and IL-12 down-regulate C-type lectin receptors and cause a shift towards a pro-inflammatory state.

Anthony et. al. (2013) conducted a study in which they developed knockout mice for the IL-4 cytokine. Wild-type mice were able to mount an anti-inflammatory immune response while the knockout mice could not, implying IL-4 is necessary for mounting an anti-inflammatory immune response.

A study by Chieppa et. al. (2003) demonstrated that cross-linking of a mannose receptor with a PAM-1 antibody cause DCs to mature. These DCs then produced more IL-10 and less IL-12, indicating a shift toward an anti-inflammatory state. Natural ligands of the mannose receptors of DCs are also capable of increasing production of IL-10 and suppressing IL-12.

DCs can also change the chemokine receptors that they express in order to also shift to a pro- or anti-inflammatory state. Upon recognition of antigen, DCs will
migrate to lymphoid tissue by chemotaxis. Chemokines CCR7 and CCL19 are associated with T cells in the lymph node, thus attracting the DCs to the T cells to promote an anti-inflammatory immune response (Chieppa et. al., 2003).

CD209 defines one class of C-type lectin receptors termed DC-SIGN (humans) or SIGNR1 or SIGNR3 (mice). This subclass of C-type lectins binds with molecular structures of microbes as well as damaged eukaryotic cellular components. CD209 binds mannose and fucose residues of bacterial surface proteins. DC-SIGN also mediates antigen endocytosis to form MHC I and II peptide complexes for antigen presentation. Aside from endocytotic functions, DC-SIGN augments TLR-mediated cytokine gene expression, particularly potentiating IL-10 expression (anti-inflammatory) and suppressing TLR-mediated IL-12 and IL-6 expression (pro-inflammatory). Therefore, DC-SIGN regulates both innate and adaptive immune responses in cooperation with TLR-dependent ligands. DC-SIGN also tends to polarize DC activity toward the IL-10 producing tolergenic DC.

It is important to note that the major function of C-type lectin receptors may have little to do with microbial pathogen recognition. Rather, these receptors endow macrophages and DCs with homeostatic function in maintaining normal tissue homeostasis. Most of these receptors bind self-ligands and function in myeloid cell migration, cell adhesion, and cell-to-cell interactions such as DC-T lymphocyte binding. C-type lectins bind self-glycan molecular motifs and directly clean up apoptotic and dead cells and oxidized lipids that are generated during tissue damage. Anti-inflammatory signaling by C-type lectin receptors such as CD209 could dampen self-directed immune response during tissue damage. Accordingly, CD209 expression could be considered a marker for M2 macrophages or tolergenic DC phenotypes.
1.7 Hypothesis and Objectives

We hypothesize that macrophages and DCs are endogenous cells of bovine MAT. MAT from lean dairy cows will be populated with DCs and macrophages polarized toward an anti-inflammatory phenotype that provide a barrier against metabolic disease induction triggered by transmural endotoxin translocation across the intestinal mucosa. The objectives of this project were to characterize the myeloid immune phenotypes in MAT from lean dairy cows.
Chapter 2

METHODS AND MATERIALS

2.1 Isolation of MAT Stromal Cell Fraction and MLN Cell Fraction

Samples of mesenteric adipose tissue (MAT) and mesenteric lymph node (MLN) were collected from abattoir from lactating, Holstein dairy cows. MAT samples were placed in Hank’s phosphate-buffered saline solution (HBSS), 10mM HEPES, and transported to the lab in a cooler (38°C). MLN samples were placed in HBSS, 10mM HEPES and transported to the lab (4°C).

MAT was minced into fine pieces at 38°C, and eight 1gram samples were washed (1X HBSS) and divided into 1gram samples. Minced MAT (1gram) was placed in HBSS with 10mg type 2 collagenase, and digested (60min, 38°C) with shaking (150rpm).

Digests were collected and layered on a glass wool column and washed 3x (1X HBSS). Column eluents were collected and layered onto a gradient of 20% and 60% Percoll. Gradients were centrifuged (800xg) and washed 1x (1X HBSS). Cells were counted, resuspended in HBSS (1.0x10^7 viable cells/ml) and distributed into 96 well plates at 1.0x10^5 cell per well for staining.

2.2 Cell Surface Staining for FACS Analysis

Cells were stained using murine monoclonal anti-bovine specific markers for macrophages, DCS, and lymphocytes. All murine monoclonal anti-bovine immune cell marker specific antibodies were obtained from Washington State Monoclonal
Antibody Center, Washington State University, Pullman, Washington. All fluorochrome conjugated caprine anti-murine Fc fragment specific antibodies were obtained from Jackson Immunochemicals, West Grove, Pennsylvania.

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>CD3</td>
<td>IgG</td>
</tr>
<tr>
<td>CD209</td>
<td>IgG2a</td>
</tr>
<tr>
<td>CD11b</td>
<td>IgG2b</td>
</tr>
</tbody>
</table>

Table 2. List of monoclonal antibodies.

### 2.3 Primary Monoclonal Antibodies

All primary antibodies and isotype matched primary control antibodies were diluted in HBSS, 2% equine serum (ES), and 10% acid-citrate-dextrose (ACD) to working stock solutions of 15µg/ml. Final working dilutions for each primary antibody (1:32) were established from serial dilutions of the primary antibody, MLN cell preparations, and a 1:200 final working dilution of the PE or FITC conjugated caprine anti-murine Fc secondary antibody.

Caprine anti-murine Fc specific antibodies were diluted in HBSS, 3% goat serum (GS), and 10% ACD solution. Final working dilutions of PE and FITC conjugated antibodies were titrated to 1:400.

Fc-blocking reagents included normal bovine serum (heat inactivated 56°C, 30min, and stored –20°C).

### 2.4 Cell Labeling for FACS Analysis

All cell preparations, reagents, and labeling procedures were maintained at 4°C. Primary anti-CD209 or anti-CD11b monoclonal antibody in HBSS, 2% ES, and
10% ACD was placed in appropriate wells of a 96 well plate to obtain the final working dilutions of 1:32. Heat inactivated normal bovine serum was placed into each well to achieve a final Fc-blocking concentration of 50% serum in each well. Cells (1.0x10^7/ml in HBSS, 2%ES, and 10%ES) were finally added to each well (1.0x10^5 cells/well). Control wells included cells, Fc-blocking serum (50%), and the isotype protein-matched irrelevant murine monoclonal antibody. The plates were vortexed (30sec), incubated (30 min, 4°C), and washed 3x (200µl HBSS, 10% ACD).

2.5 FACS Analysis

Stained cells populations were strained through 1um cell filters, pelleted, and re-suspended in 1ml HBSS, 3% buffered formaldehyde. Marker expression was performed on 10,000 gated events using a Becton Dickson flow cytometer.
Chapter 3

RESULTS

3.1 How to Read a FACS Histogram

3.1.1 Lymph Node Population Histogram

Using an unstained MLN population for control, a histogram of the cell fraction represents where the gated cell fraction is located (Figure 3). The high density of events in the gated area is considered to contain viable cells to be used in FACS analysis. Events scattered along the vertical axis are considered to be dead cells or cellular debris, and were not gated.

Figure 3. Representative histogram of LN cells circled within a gate to be used for FACS analysis.
3.1.2 Adipose Stromal Cell Fraction Population Histogram

Using an unstained MAT stromal cell fraction (SCF), a histogram of the cell fraction represents where the gated cell fraction is located (Figure 4). The size and shape of the area of gated events is different from the MLN histogram to exclude large amounts of presumably dead cells and cellular debris. The cells in the gated area are considered intact cell representatives of the SCF that could be employed in the FACS analysis. Events distributed in areas of low side and forward side scatter are considered to be dead cells or cellular debris, and were excluded from the analysis.

![Figure 4. Representative histogram of MAT cells circled within a gate to be used for FACS analysis](image)

3.1.3 Stained Cell Populations

When interpreting a FACS histogram, it is important to know what each quadrant represents. Double negative cells reside in the lower left (LL) quadrant, and double positive cells reside in the upper right (UR) quadrant. Single positive cells with
green fluorescence lie in the LR quadrant, and single positive cells with red fluorescence lie in the UL quadrant.

3.2 Cell Marker Results

3.2.1 MLN Immune Cell Profile

Cells expressing MHC II represent a mean 21.03 ± 2.16% of cells in the MLN (Aylward, 2015). CD209 was expressed at a mean 1.86 ± 0.20% of the MLN cells. CD209+/CD11b+ cells represent a mean 1.58 ± 0.30 of cells in the MLN. Additionally, there was a population of CD3+ cells in the MLN (60.76 ± 10.36%).

Figure 5. Representation of MHC II+ cells in the MLN. n=4.
Figure 6. Expression of CD209$^+$, CD11b$^+$, and CD209$^+$/CD11b$^+$ cells in the MLN. n=5.

Figure 7. Expression of CD3$^+$ cells in the MLN. n=4.
3.2.2 MAT Immune Cell Profile

Cells in the bovine MAT expressing MHC II represent a mean $10.62 \pm 0.86\%$ of the SCF (Aylward, 2015). Cells in bovine MAT expressing CD209 represent a mean $2.05 \pm 0.10\%$ of the SCF. Double stained CD209$^+/CD11b^+$ cells in the MAT represent a mean $1.45\pm0.23\%$ of the SCF. There was also a substantial population of CD3$^+$ cells in the MAT ($3.74 \pm 2.16\%$).

Figure 8. Expression of MHC II$^+$ cells in bovine MAT. Mean BCS = 2.81, n=4.
Figure 9. Expression of CD209⁺, CD11b⁺, and CD209⁺/CD11b⁺ cell populations in the MAT. Mean BCS = 2.7, n=5.

Figure 10. Expression of CD3⁺ cells in the MAT. Mean BCS = 3.25, n=4.
Chapter 4
DISCUSSION

These data conclusively demonstrate the phenotypic presence of elements contributing to the innate and adaptive immune response. The innate response has a balance between the M1 and M2 macrophages. Lean fat most likely has an M2:M1 macrophage ratio > 1.0, indicating that balance is closer to an anti-inflammatory response. Macrophages in the bovine SCF probably follow that of the mouse, towards the M2 anti-inflammatory type. However, this remains to be shown.

The ligands most likely driving the innate DC and macrophage responses in the MAT of dairy cows would be gut-derived bacteria and bacterial components (endotoxin, peptidoglycans, DNA, lipotechoic acids, etc.) that translocated across the gut wall. There is some evidence that this may follow shifts in ration composition. Changes in rations favoring highly fermentable starches and lower content of fiber produce changes in bacterial community composition across the upper and lower gastrointestinal tract (Li et. al., 2012). Minimally, these changes have been determined to increase expression of endotoxin across the entire tract. Gut-derived endotoxin has been proposed in other models to trigger metabolic inflammatory diseases and metabolic disorders associated with obesity (Cani et. al., 2007). It is not yet known which antigens are driving the adaptive response, as those antigens that cross the bovine gut barrier remain to be defined.

The data in this thesis provides circumstantial evidence that the major components of the adaptive immune response are present in the bovine adipose depot. Given the CD209 C-type lectin receptor is expressed in the MAT—and at a higher
level than the MLN—it seems plausible that the antigens driving the adaptive immune response are endocytosed by the CD209 receptor of DCs, processed in the phagolysosome compartment, and subsequently combine with the MHC II proteins to form a peptide-MHC II complex. Presentation of the MHC II-peptide complex on DC and macrophage cell surfaces drives the adaptive T cell response in the fat. Indeed, data presented herein shows both CD209 receptors and MHC II antigen restricting elements are expressed in the mesenteric adipose depots of dairy cows.

CD209 binding could activate IL-10 expression while dampening TNF-a, IL-6, and IL-12 expression by DCs. This tolerogenic DC then drives Treg, adaptive anti-inflammatory response because of the dominance of the IL-10 from the DC presenting antigen to T lymphocytes. Thus, DCs have the ability to sculpt the effector T lymphocyte response toward the Treg phenotype, and establish an anti-inflammatory response in the adipose depot. Then, this adaptive immune phenotype would establish an endogenous, anti-inflammatory immune barrier protecting the adipose tissue against inflammation and insulin resistance, and therefore impede the production of metabolic disease.

The presence of CD3\(^+\), CD11b\(^+\), and CD209\(^+\), cells in the lymph node and SCF of the mesenteric adipose tissue is consistent with the presence of T lymphocytes and dendritic cells in both of these tissues. Together, the presence of these markers indicated both the MLN and MAT depot are endowed with immune cell populations capable of an innate and adaptive immune response. Following activation through a series of pathogen recognition receptors (PRRs), DCs up-regulate Class MHC II molecules, T lymphocyte co-receptor molecules, increase cytokine synthesis and secretion, and switch chemokine receptors from CXCR1 to CXCR7. As a result,
maturation allows DCs to migrate from peripheral tissues to the nearest draining lymph node, and function as professional antigen presenting cells for T lymphocyte activation and expansion.

T lymphocyte-dendritic cell interaction via the T lymphocyte antigen receptor binding the MHC peptide complex on DCs augments DC maturation, further enhancing T lymphocyte activation, clonal expansion, and cytokine synthesis and secretion. It is clear that in addition to T lymphocyte activation, DCs also direct T lymphocytes toward a state of tolerance and non-responsiveness that counterbalances T lymphocyte activation and responsiveness. Directing T lymphocytes into tolerance is a function reserved for immature DCs, and is an indispensable mechanism of sustaining an unresponsive tolerant peripheral immune state in normal tissue homeostasis. In adipose tissue, the tolerogenic, anti-inflammatory state protects against Type 2 diabetes and metabolic inflammatory disorders.

The immature DCs dominate peripheral immune tolerance by induction and maintenance of regulatory (Foxp3⁺) T lymphocyte (Treg) cells. Tregs are induced by immature DCs through expression of anti-inflammatory cytokines (IL-10) and a class of co-receptors (CD80, CD86) that bias naïve T lymphocyte effector development toward an anti-inflammatory Treg, rather than a pro-inflammatory (Th1) function. These regulatory T lymphocytes maintain a peripheral, tolerant immune state in tissues such as the adipose depot, skin, and mucosal surfaces of the gastrointestinal and respiratory tracts (Pletincky et. al., 2011).

Under normal conditions of tissue homeostasis, the immature DC phenotype sustains T lymphocyte unresponsiveness by limiting Th1 pro-inflammatory immune activity. Exposure to pathogen molecular motifs (i.e. bacterial endotoxin absorbed
from the gut) activates DCs by triggering DC tol receptors. Upon tol receptor activation, DCs move from an immature to a mature state, and lose the ability to develop and sustain Treg anti-inflammatory functions. Mature DCs down-regulate tolerant-inducing T lymphocyte co-receptors (CD80, CD86) and replace them with CD40 co-receptors. These co-receptors activated Th1 pro-inflammatory T lymphocyte development. In addition, mature DCs down-regulate anti-inflammatory cytokine production (TGF-β) that was produced by immature DCs, while up-regulating pro-inflammatory cytokine production (TNF-α, IL-6, IL-12, and IL-18) during transformation into mature DCs. The presence of MHC II on CD11c+ and CD11b+ cells argues that macrophages and DCs in both tissues can generate MHC II peptide complexes that provide the antigen-riven interactions between these cells and the CD3+ Th1, Th2, and/or Treg lymphocytes. The data set implies therefore the adipose depot and mesenteric lymph node possess the cellular machinery with potential to drive and sustain pro-inflammatory or anti-inflammatory immune responses.

The presence of CD209 C-type lectin receptors has important functional implications (Sancho and Sousa, 2012). DCs exhibit a variety of PRRs that aid in the recognition of microbial and endogenous molecular motifs. Hundreds of these receptors exist across four functional classes: tol receptors, C-type lectin receptors, caspase recruiting domain helicases, and nucleotide-binding oligomerization domain proteins. The PRRs individually trigger distinct types of innate immune responses, but collectively, cross talk across all the receptors sculpts an adoptive immune response that has characteristics contributed from all the classes of PRRs. Tol receptors bind protein, lipoprotein, and dimers and polymers of microbial nucleotides and trigger pro-inflammatory cytokine secretions. High sugars and polysaccharides of microbial
origin trigger C-type lectins whose downstream effect is the modification of cytokine synthesis and secretion that are activated by tol receptor ligands.

As a result, relative amounts of pro-inflammatory Th1, Th2, and Th17 effector lymphocytes and anti-inflammatory Tregs conducting the adoptive immune response will vary depending upon changes in composition across microbial types (Probst et al., 2014). Mannose or fucose ligand binding to the C-type lectin receptor (CD209) does not trigger cytokine synthesis and secretion by DCs. Rather, signals through CD209 modify other signal cascades activated by tol receptor ligands (LPS) for cytokine gene expression. CD209 signals can augment TLR receptor-initiated cytokine gene expression thereby increasing DC output of IL-6, CXCL8, IL-12, and IL-10. Thus, the signature function of high mannose-ligand-CD209 interaction is not the induction of cytokines, but the potentiation of TLR-mediated cytokine responses, particularly IL-10.

Fucose-based ligand binding to CD209 markedly increases IL-10 output while blocking IL-12 and IL-6 output (Gringhous et al., 2009). Other CD209 ligands decrease IL-6, IL-12, and TNF-α secretion by blocking gene transcription (IL-12) and simultaneously augmenting the degradation of IL-6 and TNF-α. Thus, the outcome of a ligand-CD209 interaction is dependent upon ligand composition, but tends to diminish pro-inflammatory signals. This drives DC maturation while augmenting anti-inflammatory signals that sustain DC immaturity. The net effect favors production of anti-inflammatory signals that sustain DC immaturity and favors Treg development. The immunologic consequences of sustained DC immaturity is induction and persistence of an anti-inflammatory tolerogenic state in peripheral tissues. Targeting
CD209 could be a strategy to diminish adipose inflammation and reverse the onset of metabolic inflammatory disease in dairy cows.
REFERENCES


