Immune cell populations residing in mesenteric adipose depots and mesenteric lymph nodes of lean dairy cows

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ABSTRACT

Inconsistent evidence of inflammatory immune cell infiltrates in adipose tissues with extensive triglyceride mobilization raises the possibility that regulatory or anti-inflammatory immune cell populations reside within the mesenteric adipose tissue (MAT) and mesenteric lymph nodes (MLN). These resident immune cell populations may be involved in attenuating the inflammatory response. We explored the immune cell population of MAT and MLN collected from lean, lactating Holstein cows without apparent disease in an abattoir ($n = 42$). Lean cows had a body condition score of 2.6 ± 0.1 (mean $\pm$ SD) with a greater frequency of adipocyte area occurring in small rather than large adipocytes. Cells were labeled with monoclonal antibodies specific to bovine leukocyte antigens for enumeration by flow cytometry. Within both lymph node and adipose tissues, relatively large subpopulations of cells expressed the $\beta 2$ integrins CD11b and CD11c, class II major histocompatibility antigens (MHCII), and the SIIRP-1 α receptor (CD172a) typical of dendritic cells and macrophages. Macrophage/dendritic cell heterogeneity was marked by $\beta 2$ integrin expression alone or in conjunction with CD172a or MHCII across subpopulations from both tissues; CD209, the DC-SIGN c-type lectin receptor of dendritic cells, was not detected by fluorescence-activated cell sorting in either tissue. Lymphocytes comprised $74.1 \pm 3.7\%$ and $13.7 \pm 3.7\%$ of the MLN and MAT cell populations, re-

spectively, and CD3⁺CD4⁺ lymphocytes accounted for $49.8 \pm 9.9\%$ of the MLN and $6.13 \pm 1.23\%$ of the MAT cells. Fox P3⁺ regulatory lymphocytes comprised $15.3 \pm 1.1\%$ and $6.73 \pm 0.52\%$ of the MLN and MAT cells, whereas $\gamma\delta$ ⁺ lymphocytes accounted for $6.65 \pm 0.74\%$ and $3.91 \pm 0.43\%$ of the MLN and MAT cells, respectively. Subpopulations of CD3⁺CD8⁺ cytotoxic T cells and CD3⁺CD11c⁺ innate lymphocytes were present in MLN but not MAT. These results show that subpopulations of resident tissue macrophages, dendritic cells, T helper lymphocytes, regulatory T lymphocytes (Tregs), and $\gamma\delta$ lymphocytes reside in mesenteric lymph nodes and adipose tissues. Balance in the innate and adaptive immune functions embedded in these tissues could support metabolic health.

Key words: mesenteric adipose, mesenteric lymph node, lymphocyte, macrophage, dendritic cell

INTRODUCTION

Compared with normally conditioned cows, overconditioned dairy cows are more prone to severe negative energy balance during the postpartum period (Dann et al., 2006; Janovick et al., 2011). Failure of homeorhetic regulation of metabolic activity during the postpartum period leads to dysregulated metabolic states and increased risk of metritis, ketonemia, displaced abomasum, retained placenta, and susceptibility to infection (Ospina et al., 2010). A signature event associated with insulin resistance in adipose depots of obese monogastrics is the induction of inflammation (Weisberg et al., 2003; Xu et al., 2003; Deiluiis et al., 2011) following reductions in resident regulatory or anti-inflammatory immune cells (Osborn and Olefsky, 2012; Mathis, 2013; Panduro et al., 2016) that normally counterbalance tissue inflammation (Mukesh et al., 2010; Vatier et al., 2012; Depreester et al., 2018). In contrast to monogastrics, results in bovines indicate that substantial numbers (Contreras et al., 2015) or trace amounts (Aker

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et al., 2012) of inflammatory cell infiltrates appear in postpartum adipose depots of overconditioned dairy cows. The conflicting findings suggest that inflammatory disease and insulin resistance in the adipose depot can be unpredictably divorced from metabolic disorders in overconditioned cows. This raises compelling questions about the degree of balance or imbalance in the immune homeostasis of resident adipose immune elements achieved across experimental designs.

Although much is known about the balance between regulatory and proinflammatory tissue-fixed immune elements and bystander adipose tissue injury (insulin resistance) in health or obesity, much less is known about the relationship between steady-state adipose-fixed immune elements and mesenteric lymph node (MLN)-fixed immune elements or those trafficking to the MLN from the blood, adipose, and intestine tissues. Migratory dendritic cells and macrophages transport adipose tissue-specific (Weitman et al., 2013) or commensal microbial-specific antigens (Macpherson and Uhr, 2004; Mazmanian et al., 2005) to the draining MLN. In healthy, steady-state conditions, these cells promote regulatory or anti-inflammatory T lymphocyte (Treg) responses due to their highly tolerogenic nature. These regulatory effector lymphocytes disseminate from the MLN back to the adipose tissue and intestine, where their regulatory activities rein in inflammation in the adipose depot and intestine mucosa (Lathrop et al., 2011; Cebula et al., 2013). The Treg in the adipose tissue prevent insulin resistance, whereas Treg in the MLN maintain oral tolerance to symbiotic intestinal commensals (Hadis et al., 2011) and sustain the integrity of intestinal permeability barriers (Strauch et al., 2005; Atarashi et al., 2011; Round et al., 2011). Populations of proinflammatory macrophages, dendritic cells, and T lymphocytes increase in MLN of adipose depots with hypertrophied adipocytes. Coincidentally, the size of the Treg population decreases in these MLN (Magnuson et al., 2017). The pivotal function of MLN is to sustain immune homeostasis in the intestinal mucosa and secondarily to orchestrate steady-state homeostasis in the mesenteric adipose. These nodes would be expected to harbor dominant amounts of immune regulatory cell phenotypes in lean, healthy, steady-state dairy cows.

The presence of cluster of differentiation (CD) molecules on the cell surface allows identification of immune cell phenotypes. In bovines, macrophages and dendritic cells are identified by the expression of CD11b, CD11c, CD172a, CD209, and major histocompatibility complex class II (MHCII) molecules (Miyazawa et al., 2006; González-Cano et al., 2014). Macrophages and dendritic cells present antigen to naïve T cells to drive T-cell differentiation into regulatory, anti-inflammatory, or proinflammatory T-effector subsets identified by the

expression of CD3, CD4, CD8, FoxP3, and $\gamma\delta$ (Murphy and Reiner, 2002).

We hypothesized that subpopulations of regulatory/anti-inflammatory myeloid (macrophage/dendritic) and lymphoid (T lymphocyte) cells reside in lean bovine mesenteric adipose tissue (MAT) and MLN. Thus, the objective of this work was to more completely characterize tissue-resident myeloid and lymphoid cell subpopulations in these tissues of lean dairy cows.

MATERIALS AND METHODS

Animals and Tissue Samples

Lactating, mature Holstein cows lacking gross evidence of diseases ($n = 42$) were randomly selected from an abattoir population at the time of entry onto the slaughterhouse floor. An experienced bovine veterinarian (R. Dyer) evaluated every cow for clinical evidence of lameness and external, gross lesions. Cows lacking external evidence of disease were assigned a BCS (Ferguson et al., 1994) before slaughter. Animals with BCS 2.0 to 3.5 were chosen for tissue analysis.

Tissue Harvest

After killing animals by captive bolt, a single veterinarian (R. Dyer) evaluated all viscera to exclude any animals with evidence of septic intra-abdominal, thoracic, intrauterine, intramammary, or gastrointestinal disorders. Then, MLN and MAT were collected within 15 min of death after removal of the viscera from the carcass. Adipose tissue was excised from the great mesentery 15 to 20 cm from the mesenteric border of the jejunum and midway down the full length of the jejunum. The tissue was trimmed into pieces of approximately 30 g and placed in 150 mL of sterile PBS with 10 mM HEPES, pH 7.35 (38°C), for cell phenotyping. Hardening of the fat during transport (45 min) to the laboratory was prevented by placing MAT in an insulated box containing 38°C water. Mesenteric lymph nodes were removed from the mid-jejunum mesentery located within 7 to 10 cm of the adipose sample. Adipose tissue was trimmed off the MLN capsule and the node was placed in 150 mL of sterile PBS with 10 mM HEPES, pH 7.35 (4°C), for cell phenotyping. The MLN samples were placed on ice and transported to the laboratory within 45 min.

Isolation of MAT Stromal Cell Fraction and MLN Cells

Isolation of MAT Stromal Cell Fraction. All MAT samples were maintained at 38°C throughout tissue preparation. Six 1-g samples of MAT (6 g total of

MAT from a cow) were finely minced with a sterile scalpel in a sterile glass Petri dish. Any vascular structures were dissected from the adipose tissue and discarded. Minced MAT was transferred to 50 mL of PBS with 10 mM HEPES (hereafter, PBS-HEPES; 38°C, 1.0 g of adipose per 50 mL of PBS) and washed once (10 min, $800 \times g$, 25°C) in 50 mL of PBS-HEPES to remove blood contamination. Washed, minced MAT (1 g) was digested in flasks containing 2 mL of PBS-HEPES and 4 mg/mL collagenase type II (Sigma Chemical, St. Louis, MO; 38°C). The flasks were placed in a 38°C incubator and shaken (150 rpm/min) for 60 min. Supernatants were transferred to glass wool columns and the flasks washed 3 to 4 times with 5 mL of PBS-HEPES, 38°C. Eluents from the column were collected, and the cells were pelleted ($1,000 \times g$, 25°C, 20 min), pooled into one pellet, and washed (PBS-HEPES, 4°C). Pooled cells were suspended in divalent cation-free PBS with 10% acid citrate dextrose (ACD) and layered on top of a Percoll gradient (Sigma Chemical) consisting of 1.029 g of Percoll/mL over 1.10 g of Percoll/mL. Gradients were centrifuged (20 min, 25°C, $1,200 \times g$) and the cell-enriched layer collected at the Percoll gradient interface. Cells were washed in divalent cation-free PBS with 10% ACD. Aliquots of cells were counted and suspended in trypan blue for viability ($84.5 \pm 0.02\%$ live; mean $\pm$ SEM). Finally, the cell concentrations were adjusted to 1.0×10^7 viable cells/mL of divalent cation-free PBS with 10% ACD and 2% equine serum (ES; Sigma Chemical) at 4°C.

Isolation of MLN Cells. The MLN were placed in sterile Petri dishes containing PBS-HEPES (4°C) and minced with scraping using a sterile scalpel blade. The minced MLN were repeatedly suspended in PBS-HEPES (4°C) and the cell-enriched supernatants placed onto glass wool columns. Eluents were collected and cells washed once (PBS-HEPES at 4°C; $1,000 \times g$, 30 min, 25°C). Contaminating red blood cells were lysed (sterile distilled H₂O, 25 s), the MLN cells were washed 3 times (PBS-HEPES), and the pellets suspended in divalent cation-free PBS with 10% ACD and 2% ES (4°C; hereafter, PBS-ACD-ES) at 1.0×10^7 viable cells/mL.

Immune Cell Staining of CD Markers

Murine anti-bovine leukocyte antigen-specific mAb (Washington State Monoclonal Antibody, Washington State University, Pullman, WA) were used across all procedures (Table 1). Stock solutions of primary antibodies were prepared by dilution (15 μ g/mL) in PBS-ACD-ES. Stock solutions of each mAb were serially diluted in PBS-ACD-ES with the final dilution in complement-inactivated normal bovine serum. Preliminary work indicated that 50% complement-inactivated

bovine serum blocked the Fc receptor and nonspecific binding of all isotype and protein-matched irrelevant primary control antibodies. Secondary antibody reagents were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated caprine anti-murine IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Secondary reagents were diluted 1:100 in PBS-ACD-ES with 10 mM HEPES, and were used at a working dilution of 1:200 in the final reaction. A final working amount of 5% complement-inactivated normal goat serum (Jackson ImmunoResearch) blocked nonspecific binding of conjugated secondary antibodies. All primary and secondary antibody reactants (100 μ L) were added to wells of a 96-well, V-bottom microtiter plate with 1.0×10^6 viable cells (100 μ L) to achieve a final reaction volume of 200 μ L/well. Control wells included cells in wash buffer only, cells in primary murine mAb of no known reactivity (Life Technologies, Carlsbad, CA) matched by isotype and amount of protein to the primary leukocyte-specific mAb (Table 1), and a final working dilution (1:200) of secondary FITC- or PE-conjugated goat anti-murine isotype-specific antibody. Both primary and secondary antibody reactions were performed at 4°C for 30 min, followed by 3 washes in PBS-ACD-ES, with vortexing (10 s) between each wash. All cell preparations were fixed in PBS with 2% phosphate-buffered formaldehyde (4°C, 16 h) and passed into tubes through a 35- μ m cell strainer cap.

For intracellular Fox P3 detection, cells stained for cell surface markers and fixed in 2% formaldehyde (16 h, 4°C) were permeabilized by incubation in fixation/permeabilization buffer (eBiosciences, San Diego, CA; 60 min, 4°C) and then washed in PBS-ACD-ES. Permeabilized cells were stained with murine monoclonal anti-bovine FoxP3 following procedures previously outlined for primary and secondary reactions.

Flow Cytometric Analysis. Cell analysis was performed on a BD fluorescence-activated cell sorter (FACS) Calibur System using the BD Quest Pro Software (BD Biosciences, San Juan, CA). Forward and side scatter thresholds were set to optimize scatter of the MAT stromal cell fraction (SCF) and MLN cell preparations and to eliminate unnecessary debris. Gating was established for the populations of interest within the MAT SCF and MLN cells (data not shown). Fluorescent detectors were adjusted using unstained control preparations and the isotype- and protein-matched control preparations for each set of cells. Compensation settings were adjusted for single FITC- or PE-stained cells to minimize spectral overlap and place the stain of interest in the appropriate position on the green (FL1) or red (FL2) axis. Data acquisition was performed until 10,000 gated events were counted.

Table 1. Monoclonal antibodies, specificities, and dilution of primary antibody stock

Specificity ¹	Monoclonal antibody catalog number ²	Isotype	Final working dilution
CD3	MM1A	IgG ₁	1:4
CD172a	DH59B	IgG ₁	1:4
CD11b	MM10A	IgG _{2b}	1:32
CD8 α	CACT130A	IgG ₃	1:4
CD4	IL11A	IgG _{2a}	1:32
$\gamma\delta$ TCR1-N24 δ	G-GB21A	IgG _{2b}	1:32
FoxP3	Fox5A	IgG ₁	1:4
MHCII	TH14B	IgG _{2a}	1:32
CD209	209MD26A	IgG _{2a}	1:8
CD11c	BAQ153A	IgM	1:4
CD11c	BAQ151A	IgG ₁	1:4
Isotype control			
None	MG100	IgG ₁	1:4
None	MGM00	IgM	1:4
None	PA5-33223	IgG _{2b}	1:32
None	PA5-33236	IgG _{2a}	1:32
None	MA1-10433	IgG ₃	1:4

¹CD = cluster of differentiation; MHC = major histocompatibility complex.

²Murine anti-bovine CD3, CD172a, CD11b, CD8 α , CD4, $\gamma\delta$ TCR1-N24 δ , FoxP3, MHCII, CD209, and Cd11c from Monoclonal Antibody Center, Washington State University (Pullman, WA). Isotype controls Mg100, MGM00, Pa5-33223, Pa5-33236, and MA1-10433 from Life Technologies (Carlsbad, CA).

Adipocyte Size Histomorphometry. Lactating, mature Holstein cows lacking gross evidence of diseases ($n = 53$) were randomly selected from an abattoir population at the time of slaughter. Some animals ($\text{BCS} \leq 3.5$, $n = 42$) included those used for MAT SCF and MLN cell isolation but others ($\text{BCS} > 3.0$) were separate from the set used for cell isolation. Samples of MAT (1 cm^2) were removed from the same site as used for MAT SCF isolation and placed in 3% phosphate-buffered formaldehyde to determine adipose area. Fixed MAT was trimmed, embedded in paraffin, sectioned at $5 \mu\text{m}$, placed on glass slides, and stained with hematoxylin and eosin. Adipocyte surface area was determined on 10 randomly selected adipocytes along the diagonal in each of 10 randomly selected $40\times$ fields (100 total adipocytes) for each MAT specimen using a Nikon DS-F2 camera and NIS Elements D image analysis software (Nikon Instruments Inc., Melville, NY). Accordingly, adipocyte area was determined as the mean of 100 randomly selected adipocytes for each MAT. Frequency distribution of adipocyte area was determined within successive areas of $1,000 \mu\text{m}^2$ and expressed as percent total counts ($n = 5,300$ and $4,200$ for all cows across all BCS and cows used for MAT SCF phenotype, respectively).

Statistical Analysis

For FACS, the level of fluorescence above background was evaluated by one-tailed Student's t -test. Fluorescent differences across MLN and MAT SCF were evalu-

ated by one-tailed Student's t -test. The correlation between BCS and adipocyte area was determined using the Pearson product-moment correlation coefficient t -test for correlations. Differences between means were declared at $P \leq 0.05$.

RESULTS

Adipocyte area correlated with BCS ($r = 0.49$, $P \leq 0.05$, $n = 53$). Body condition score and the area of MAT adipocytes used for FACS analysis were 2.6 ± 0.1 and $4,971.07 \pm 395 \mu\text{m}^2$, respectively ($n = 42$). Smaller adipocytes occurred with greater frequency than larger adipocytes in the MAT across both populations of cows. Numerically higher frequencies of smaller adipocytes occurred in the adipose tissues of cows used for isolation of MAT SCF (Figure 1). These outcomes are consistent with a moderately lean body condition and trends for smaller adipocytes of visceral adipose depots in later lactation (Akter et al., 2011; Kenez et al., 2015).

Myeloid Cells Are Indigenous Members of the Core Bovine MAT SCF and MLN Cell Populations

The cell surface markers CD11b, CD11c, CD172a, CD209, and MHCII are primarily but not exclusively expressed on cells from the macrophage and dendritic cell (myeloid) series. The $\beta 2$ integrin CD11b was expressed in (mean $\pm$ SEM) $24.9 \pm 2.4\%$ of the MLN and $13.9 \pm 4.3\%$ of the MAT SCF cell population (Figure

Figure 1. Frequency distribution of mesenteric adipose depot adipocyte area expressed as percent of total cell count for cows across all BCS ($n = 53$) and cows used for mesenteric adipose tissue stromal cell fraction phenotype ($n = 42$).

2, Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15156>). Expression of CD11b was 1.79-fold greater in MLN than in MAT SCF ($P \leq 0.05$).

Expression of CD172a, the signal regulatory phosphatase (SIRP) binding receptor, occurred in $6.37 \pm 0.90\%$ of the MLN and $16.3 \pm 5.0\%$ of the MAT SCF populations (Figure 2, Supplemental Figure S1). Interestingly, CD172a expression was 2.5-fold greater ($P \leq 0.05$) in the MAT SCF ($16.3 \pm 5.0\%$) than in the MLN. Because bovine myeloid or dendritic cells express CD11b and CD172a (Miyazawa et al., 2006; González-Cano et al., 2014), the coordinated expression of these 2 proteins in MLN and MAT SCF was of interest. Co-expression of CD11b and CD172a occurred in only $5.00 \pm 0.59\%$ of the MLN and $7.11 \pm 2.07\%$ of the MAT SCF cell populations (Figure 2) in these cows. Within the CD172a⁺ populations, the fraction of cells ($76.2 \pm 4.1\%$) that expressed CD11b⁺ was greater in the MLN ($42.9 \pm 9.3\%$) than in the MAT SCF ($P \leq 0.05$, Figure 2). Moreover, $6.74 \pm 1.00\%$ of the MLN cell population was CD172a⁺, CD11b⁺, with a much larger percentage ($17.27 \pm 3.07\%$) of the MAT SCF population expressing CD172a in the absence of the CD11b integrin (Figure

2; $P \leq 0.05$). Thus, CD172a expression was greatest in the MAT SCF, occurred evenly within the CD11b⁺ cell populations in both the MAT SCF and MLN, and was higher in the CD11b⁺ cell populations in MAT SCF. We also assessed co-expression of CD11b with CD209, the DC-SIGN C-type lectin receptor expressed on myeloid-derived, conventional dendritic cells, and some macrophages (Figure 3, Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15156>). Subpopulations of cells from both the MLN and MAT SCF expressed CD11b as described above. Expression of CD209 and therefore CD11b CD209 co-expression was not detected in the MLN or MAT SCF cell populations (Figure 3, Supplemental Figure S1).

We evaluated CD11c and MHCII expression on myeloid subpopulations in the MLN and MAT SCF. A subpopulation of MLN cells ($25.7 \pm 0.6\%$) and MAT SCF cells ($9.83 \pm 0.57\%$) expressed CD11c (Supplemental Figure S2; <https://doi.org/10.3168/jds.2018-15156>), and MHCII was a dominant cell surface protein ($20.0 \pm 1.53\%$; Supplemental Figure S3, <https://doi.org/10.3168/jds.2018-15156>) in the MLN. Compared with MLN cells, a smaller subpopulation (10.8

Figure 2. Representative fluorescence-activated cell sorting plots of cluster of differentiation (CD)11b and CD172a co-expression in mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for CD11b, for CD11b and CD172a, or for CD172 in MLN and MAT ($n = 5$; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

$\pm 0.8\%$) of the MAT SCF ($P \leq 0.05$) expressed MHCII (Supplemental Figure S3; $P \leq 0.05$). In contrast, MHCII⁺ CD11c⁺ co-expression occurred in a relatively smaller subpopulation ($10.0 \pm 0.6\%$) of the MLN and an even smaller population ($1.16 \pm 0.37\%$) of the MAT SCF (Figure 4; $P \leq 0.05$). Altogether, cell populations spanning the CD11c⁺ MHCII⁺, CD11c⁺ MHCII⁻, and CD11c⁻ MHCII⁺ phenotypes existed in MLN and MAT. The relative size of the different CD11c and MHCII subpopulations in MAT SCF was CD11c⁺ MHCII⁻ =

CD11c⁻ MHCII⁺ > CD11c⁺ MHCII⁺. Within the MAT CD11c⁺ cell populations, the CD11c⁺ MHCII⁻ pool of cells ($5.92 \pm 1.64\%$) was 5-fold greater than the population of CD11c⁺ MHCII⁺ cells ($1.16 \pm 0.37\%$; Figure 4, $P \leq 0.05$). The MHCII⁺ CD11c⁻ subpopulation ($4.66 \pm 1.40\%$) was 4-fold larger than the MHCII⁺ CD11c⁺ subpopulation in the MAT SCF (Figure 4, $P \leq 0.05$). In the MLN, the relative size of the subpopulations was CD11c⁺ MHCII⁻ $\gg$ CD11c⁺ MHCII⁺ > CD11c⁻ MHCII⁺. Within the MLN CD11c⁺ cell populations, the

CD11c⁺ MHCII⁺ pool of cells ($10.0 \pm 1.2\%$) was half the size of the CD11c⁺ MHCII⁻ cell population ($20.2 \pm 2.4\%$; Figure 4, $P \leq 0.05$).

Lymphocytes Are Dominant Members of the Core Resident Bovine MAT SCF and MLN Cell Populations

As expected, most ($74.1 \pm 3.7\%$) of the MLN cell populations consisted of CD3⁺ lymphocytes. In contrast, only $13.7 \pm 3.7\%$ of the MAT SCF consisted of CD3⁺ lymphocytes (Supplemental Figure S4; <https://doi.org/10.3168/jds.2018-15156>). The size of the MLN

lymphocyte population was considerably greater than that of the MAT SCF ($P < 0.05$). To characterize the lymphocyte phenotypes further, we used FACS analysis of the SCF stained with anti-CD4, anti-CD8, and anti- $\gamma\delta$ antibodies. In the MLN, $48.02 \pm 3.0\%$ of the cells were CD4⁺; a much smaller portion ($7.78 \pm 0.49\%$) of the MAT SCF population was CD4⁺ (Supplemental Figure S5; <https://doi.org/10.3168/jds.2018-15156>; $P < 0.05$). A CD3⁺CD4⁺ double-positive cell population accounted for $49.8 \pm 9.9\%$ of the MLN cells and a smaller double-positive cell population was detected in the MAT SCF ($6.13 \pm 1.23\%$; Figure 5; $P < 0.05$). These

Figure 3. Representative fluorescence-activated cell sorting plots of cluster of differentiation (CD)11b and CD209 co-expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for CD11b, for CD11b and CD209, or for CD209 in the MLN and MAT SCF ($n = 5$; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

results show that approximately 65% (48.0% of 74.1%) of the $CD3^+$ T lymphocyte cell lineage in the MLN co-expressed $CD4^+$. Similarly, 45.1% (6.1% of 13.7%) of the $CD3^+$ T lymphocyte lineage in the MAT SCF was $CD3^+CD4^+$ double positive. The $CD8^+$ subpopulation within the MLN accounted for $17.49 \pm 1.99\%$ of the cell population. In contrast, only $2.52 \pm 1.22\%$ of the MAT SCF population was $CD8^+$ (Supplemental Figure S6; <https://doi.org/10.3168/jds.2018-15156>). The MLN cell populations consisted of $12.97 \pm 3.35\%$ $CD3^+CD8^+$ double-positive lymphocytes. Only $1.07 \pm 0.52\%$ of the MAT SCF cells were $CD3^+CD8^+$ double positive

(Supplemental Figure S6). The number of $CD8^+$ and $CD3^+CD8^+$ cells in the MAT SCF was no greater than background staining ($P > 0.05$). The ratio of CD4 to CD8 lymphocytes within the $CD3^+$ pool of cells was approximately 3.8 in MLN and >42.1 in MAT SCF.

Suspensions of MLN cells also contained $15.3 \pm 1.1\%$ FoxP3 $^+$ cells, whereas MAT SCF expressed FoxP3 in $6.73 \pm 0.52\%$ of cells (Figure 6). In addition, $CD4^+$ FoxP3 $^+$ double-positive cells comprised $4.64 \pm 0.58\%$ of the MLN cell populations and $0.03 \pm 0.01\%$ of the MAT SCF (Figure 6). The amount of double-positive staining cells in the MAT SCF was no

Figure 4. Representative fluorescence-activated cell sorting plots of cluster of differentiation (CD)11c and major histocompatibility complex class II (MHCII) co-expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for CD11c, for CD11c and MHCII, or for MHCII in the MLN and MAT SCF (n = 4; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

Figure 5. Representative fluorescence-activated cell sorting plots of cluster of differentiation (CD)4 and CD3 co-expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for CD3, for CD3 and CD4, or for CD4 in the MLN and MAT SCF ($n = 5$; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

greater than background ($P \geq 0.05$). Analysis by FACS revealed that $6.65 \pm 0.74\%$ of the MLN cell population consisted of $\gamma\delta^+$ lymphocytes, whereas $3.91 \pm 0.43\%$ of the lymphocyte pool in the MAT SCF were $\gamma\delta^+$ T lymphocytes (Figure 7). Because subpopulations of FoxP3⁺ and FoxP3⁻ $\gamma\delta^+$ regulatory lymphocytes have been described, we also assessed FoxP3 expression in $\gamma\delta$ cells from MAT SCF and MLN. The size of the $\gamma\delta^+$ FoxP3⁺ double-positive lymphocyte subpopulation was $0.84 \pm 0.33\%$ in MLN and $0.00 \pm 0.07\%$ in MAT

SCF (Figure 7). The amount of $\gamma\delta^+$ FoxP3⁺ expression in the MLN but not the MAT SCF was greater than background ($P \leq 0.05$). Thus, a small subpopulation of $\gamma\delta^+$ FoxP3⁺ lymphocytes existed in the MLN.

The CD11c specificities were not limited to the MHCII⁺ myeloid cell population in the MLN. This $\beta 2$ integrin was also co-expressed with CD3 on $10.61 \pm 0.62\%$ of the MLN cells but only $0.19 \pm 0.18\%$ of the MAT SCF (Figure 8). The level of double-positive cells in the MAT SCF was not above background ($P > 0.05$).

DISCUSSION

The present investigation established that resident immune elements in MAT tissues and MLN of lean, lactating dairy cows consist of subpopulations of myeloid and lymphoid anti-inflammatory phenotypes that counterbalance proinflammatory components of the immune response.

Expression of CD11b, CD11c, CD172a, and MHCII in subpopulations of the MAT depot and MLN denotes

the presence of resident tissue macrophages and dendritic cells (Miyazawa et al., 2006; González-Cano et al., 2014). The CD11c protein enables monocyte trafficking (Ley et al., 2007) into adipose tissues to provide sentinel, scavenger, and reparative functions sustaining tissue integrity. Populations of anti-inflammatory macrophages expressing CD206, CD301, and Arg1 in visceral bovine adipose depots (Contreras et al., 2015, 2016) are subpopulations of the CD11b⁺ CD11c⁺ cells described herein. We note that CD11c is expressed on

Figure 6. Representative fluorescence-activated cell sorting plot of FoxP3 expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for cluster of differentiation (CD)4, for FoxP3 and CD4, or for FoxP3 in the MLN (n = 8) and MAT SCF (n = 7; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

tissue-fixed CD11b⁺CD11c⁺F4/80⁺/CD64⁺ macrophages and peripheral blood monocytes (Hope et al., 2000; Tamoutounour et al., 2012; Cho et al., 2016) but is also constitutively expressed across all lineages of dendritic cells (Steinman et al., 1979; Nussenzweig et al., 1981; Metlay et al., 1990; Merad et al., 2013). This distinction is important because macrophages focus on tissue integrity and remodeling, whereas dendritic cells modulate immune responses in adipose depots and the gastrointestinal tract.

Phenotypic diversity inherent within myeloid populations of either tissue was exemplified in populations co-expressing MHCII with CD11c and CD11b with

CD172a. The CD11b⁺ and CD11b⁻ populations both expressed CD172a, whereas other CD11b⁺ subpopulations lacked CD172a expression. The size of the CD172a⁺ cell population in the adipose SCF of lean cows is consistent with those reported in nonlactating, healthy Holstein cows and lactating cows with displaced abomasum (Contreras et al., 2015, 2016). The CD11b^{high}, CD172a^{high} phenotypes occur in macrophages; CD11b⁻ CD172a^{high} and CD11b^{low} CD172a^{high} phenotypes appear in immature and mature dendritic cells (McKeever et al., 1991; Miyazawa et al., 2006; Seo et al., 2009a) and is consistent with earlier work showing that CD172a-expressing cells support clonal

Figure 7. Representative fluorescence-activated cell sorting plot of $\gamma\delta$ expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for $\gamma\delta$, for $\gamma\delta$ and FoxP3, or for FoxP3 in the MLN (n = 14) and MAT SCF (n = 14; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

Figure 8. Representative fluorescence-activated cell sorting plots of cluster of differentiation (CD)11c and CD3 co-expression in the mesenteric lymph node (MLN; A) and mesenteric adipose tissue (MAT) stromal cell fraction (SCF; B) and percent of cells positive for CD3, for CD3 and CD11c, or for CD11c in the MLN and MAT SCF ($n = 4$; C). Data are expressed as mean $\pm$ SEM. Means with different letters (a, b) within staining group differ ($P \leq 0.05$) and means with * differ from background staining ($P \leq 0.05$).

expansion of T helper (Th)1/Th2 effector (Brooke et al., 1998) and $\gamma\delta^+$ FoxP3 $^-$ regulatory lymphocytes (Guzman et al., 2014). Clonal expansion of Th2 and regulatory $\gamma\delta$ lymphocytes supports regulatory/anti-inflammatory functions in the adipose and oral tolerance in the intestine.

The CD172a $^-$ and CD172a $^{\text{high}}$ cells in the MLN stem from migrating lamina propria-derived dendritic cells that have sampled intestinal microbiome (Turnbull et al., 2005; Jang et al., 2006) and trafficked in bovine afferent lymph (Howard et al., 1997; Gliddon and Howard, 2002; Stephens et al., 2003) to the MLN (Liu et al., 1998; Cerovic et al., 2013).

Subpopulations of CD11c $^+$ and CD11c $^-$ cells co-expressed or failed to express MHCII, consistent with phenotypes of steady-state, tissue-fixed dendritic cells and macrophages. Pools of CD11c $^+$ MHCII $^+$ circulating monocytes, dendritic cells, bone marrow-derived stem cells, and migrating dendritic cells (Howard et al., 1997; González-Cano et al., 2014; Cho et al., 2016) sustain these populations of tissue-fixed CD11c $^+$ MHCII $^+$ CD172a $^+$ and CD11b $^+$ macrophages and dendritic cells. In the steady state, activated dendritic cells and macrophages trafficking into the MLN and MAT dampen CD11c and upregulate MHCII expression (Hope et al., 2000; Seo et al., 2009a) during maturation into antigen-

presenting cells. Subpopulations of MHCII^{high} CD11c^{high} found in the MLN likely arise from MHCII^{high} CD11b^{high} CD11c^{high} dendritic cells trafficking from the intestinal mucosa via afferent mesenteric lymphatics (Gliddon and Howard, 2002; Turnbull et al., 2005; Worbs et al., 2006). Dendritic cells and macrophages in the murine intestinal lamina propria continuously pass pseudopods between mucosal epithelium to sample the microbiome (Farache et al., 2013). The cells exit the mucosa and traffic, via the mesenteric lymphatics, to the MLN. This pattern of dendritic cell trafficking underpins anatomic and functional relationships between CD11c⁺ MHCII⁺ dendritic cells in afferent lymphatic vessels (Howard et al., 1997), the cells described herein in the MLN, and those in the intestine (Fries et al., 2011; González-Cano et al., 2014). Functional characterization of these phenotypes would begin to define how regulatory and proinflammatory phenotypes orchestrate immune homeostasis in adipose (Kotas and Medzhitov, 2015; Panduro et al., 2016) and intestine tissues (Brooke et al., 1998; Turnbull et al., 2005; Bekiaris et al., 2014).

The inability to detect CD209 in the MLN and MAT SCF is concordant with trace amounts of expression (0.74%) in bovine peripheral blood dendritic cells/monocytes (González-Cano et al., 2014; Park et al., 2015, 2016) but inconsistent with reports of expression in immature and mature dendritic cells from lymphoid tissues (Cheong et al., 2010; Park et al., 2016). A multifunctional integrin, CD209 also binds fucose or mannose residues (Feinberg et al., 2001; van Liempt et al., 2006), triggering signals that augment (mannose) IL-12 and IL-6 or suppress (fucose) proinflammatory cytokine secretion of dendritic cells (Gringhuis et al., 2009). The functional significance of the apparent absence of expression in mesenteric tissues is unclear.

The MLN and MAT SCF cell populations contained considerable numbers of CD3⁺ lymphocytes. Antigen specificity in the MLN ranges across gut pathogens, dietary antigens, and commensal microbial antigens. Antigen specificity in the MAT SCF remains an enigma. The CD3⁺CD4⁺ phenotypes in adipose tissue likely consist of a mix of Th1, Th2, Th17, and Treg effector lymphocytes dominated by regulatory and Th2 functions (Caspar-Bauguil et al., 2005; Winer et al., 2009). In lean adipose tissues, MHCII-mediated antigen presentation drives Th2 production of IL-4 and IL-13 (Winer et al., 2009) and Treg production of IL-10. Collectively, these cytokines sustain anti-inflammatory macrophage functions in bovine adipose depots (Contreras et al., 2015, 2016).

The percentage of bovine CD3⁺CD4⁺ and CD3⁺CD8⁺ cells in the MLN supports earlier reports (Sopp and Howard, 2001). The size of the CD3⁺CD4⁺ lymphocyte pool in the steady-state bovine MAT SCF supports

results from other reports (Caspar-Bauguil et al., 2005; Nishimura et al., 2009) but was greater than that reported by Feuerer et al. (2009) in mice. The absence of CD3⁺CD8⁺ cells in the MAT SCF is consistent with anti-inflammatory functions enabling physiologic rather than inflammatory control of metabolic homeostasis in lean MAT SCF (Caspar-Bauguil et al., 2005; Feuerer et al., 2009; Nishimura et al., 2009).

With one exception (Gerner et al., 2010), the size of the CD4⁺ FoxP3⁺ MLN subpopulation is consistent with pool sizes in peripheral blood (Hoek et al., 2009; Suzuki et al., 2013). The size of the FoxP3⁺ MLN subpopulation was 5-fold greater than estimates generated by *in situ* staining (Roussey et al., 2016). This discrepancy likely reflects differences in experimental methodology, genetics, diet, or housing environment. The relatively large size of the FoxP3⁺ regulatory T lymphocyte population in the MLN implies that a state of tolerance dominates mucosal immune homeostasis.

FoxP3⁺ subpopulations in the bovine MLN are likely generated by commensal bacterial antigens (Lathrop et al., 2011; Cebula et al., 2013) restricted off the CD11c⁺ MHCII⁺, CD11c⁻ MHCII⁺, and CD11b⁺ and CD11b⁻ cells (Coombes et al., 2007; Welty et al., 2013; Loschko et al., 2016) described herein. This subset of FoxP3 lymphocytes initiates and sustains the oral mucosal tolerance (Worbs et al., 2006; Hadis et al., 2011) that blocks inflammatory bowel disease (Mucida et al., 2005; Hadis et al., 2011). Existence of this FoxP3 subpopulation in the bovine MLN raises an important question about its role in sustaining enteric mucosal tolerance during diet-induced shifts in commensal bacterial populations in the intestine.

The size of the subpopulation of FoxP3⁺ cells in lean bovine MAT SCF is consistent with reports in murine visceral adipose tissues (Deiluiis et al., 2011; Cipolletta et al., 2015). The relative proportion of resident adipose lymphocytes existing as FoxP3⁺ lymphocytes remains to be formally established but data reported herein suggest that 40 to 50% (6.73% FoxP3⁺ cells, 13.72 ± 0.69% CD3⁺ cells) of the CD3⁺ lymphocytes are regulatory. We propose that the MHCII⁺ CD11c⁺ macrophage/dendritic cell populations found in MAT SCF and MLN are the antigen-restricting cells supporting Treg clonal expansion (Feuerer et al., 2009; Cipolletta et al., 2015; Kolodin et al., 2015). The relative size of the Treg population implies that regulatory functions dominate immune homeostasis in lean fat and provides the quintessential anti-inflammatory/regulatory environment that is the cornerstone of physiologic rather than inflammatory control of metabolic variables in the adipose depot (Panduro et al., 2016).

The size of the MLN $\gamma\delta^+$ lymphocyte population was larger than earlier estimates for bovines (Wilson et al.,

1996; Sopp and Howard, 2001). The size of the $\gamma\delta^+$ lymphocyte population in the MAT SCF was comparable to those of lean murine adipose (Caspar-Bauguil et al., 2005; Mehta et al., 2015). The function(s) of these tissue-fixed lymphocytes in bovine MAT and MLN remains completely undefined. Elasticity in effector cell functions render $\gamma\delta$ lymphocytes producers of pro- and anti-inflammatory cytokines (IFN γ , IL-6, IL-17, IL-4, IL-13, transforming growth factor- β , IL-10, and granulocyte-macrophage colony-stimulating factor; Sopp and Howard, 2001; Bonneville et al., 2010; Guzman et al., 2012). In lean murine fat, a relatively small proportion of $\gamma\delta$ T lymphocytes exist as an MCP-1 $^+$ (monocyte chemoattractant protein-1), IFN γ^+ , IL-6 $^+$, IL-17 $^+$, or TNF- α^+ (tumor necrosis factor- α) proinflammatory phenotype (Zuniga et al., 2010; Mehta et al., 2015; Kohlgruber et al., 2018). Subpopulations of adipose IL-17 $^+$ $\gamma\delta$ T cells clonally expand Treg numbers by orchestrating IL-33 production from adipose stromal cells (Kolodin et al., 2015; Kohlgruber et al., 2018). Expanded populations of Treg favor anti-inflammatory polarization (Contreras et al., 2015, 2016) of CD11c $^+$ CD11b $^+$ MHCII $^+$ CD172a $^+$ macrophages described herein for lean adipose tissues. Obesity polarizes resident $\gamma\delta$ T cell pools (Caspar-Bauguil et al., 2005; Mehta et al., 2015) into producers of proinflammatory TNF- α , IL-17, and MCP-1 (Zuniga et al., 2010; Mehta et al., 2015) that transform CD11b $^+$, CD11c $^+$, MHCII $^+$, CD172a $^+$ macrophages into proinflammatory phenotypes (Eberl and Moser, 2009; Eberl et al., 2009; Contreras et al., 2015).

The MLN contained a minor population of FoxP3 $^+$ $\gamma\delta^+$ regulatory lymphocytes that support gastrointestinal tolerance (Casetti et al., 2009). The absence of FoxP3 $^+$ $\gamma\delta$ T cells in the MAT SCF, however, does not rule against regulatory functions in MAT SCF $\gamma\delta$ subpopulations (Seo et al., 2007, 2009b; Hoek et al., 2009) because some bovine FoxP3 $^-$ $\gamma\delta$ lymphocytes mediate transforming growth factor- β and IL-10 regulatory functions (Hoek et al., 2009; Guzman et al., 2014). Functional linkage of this phenotype to the tissue-fixed $\gamma\delta$ lymphocytes described herein could establish regulatory paradigms of immune homeostasis enabling physiologic rather than inflammatory controller adjustments of metabolic set points and tolerance in the fat and gastrointestinal tract, respectively (Rhodes et al., 2001; Hoek et al., 2009; Guzman et al., 2014).

Steady-state bovine MLN but not MAT harbor relatively large numbers of CD3 $^+$ CD11c $^+$ lymphocytes. Expression of CD11c occurs on CD2 $^+$ lymphocytes (Hall et al., 1993; Hope et al., 2000), invariant chain natural killer cells (iNK lymphocytes; Luo et al., 2007), $\gamma\delta$ lymphocytes (Hall et al., 1993), activated CD8 $^+$ regulatory lymphocytes (Hope et al., 2000; Seo et al., 2007;

Vinay et al., 2009), and innate lymphoid cells (type 1, 2, and 3). Their function in bovines deserves investigation because, in other species, innate lymphocytes regulate homeostasis in mucosal immune responses and support mucus (Oeser et al., 2015) and antimicrobial peptide secretion (Sonnenberg et al., 2012) by mucosal epithelial cells.

In summary, immune cell phenotypes described in the MAT and MLN provide circumstantial evidence that immune elements of myeloid and lymphoid origin provide regulatory environments that enable physiologic, rather than inflammatory, controls to set variables of metabolic activity and mucosal tolerance (Lumeng et al., 2007; Straus and Glass, 2007; Van Dyken and Locksley, 2013). The consistent yield of myeloid and lymphoid elements from MAT digests led us to conclude that immune elements can reside in all MAT, in contrast to results reported by Akter et al. (2012). The state of the regulatory environment could explain conflicting conclusions about adipose infiltrates of myeloid cells drawn from the immunohistochemical studies performed by Akter et al. (2012) and the immunohistochemical and flow cytometry studies performed by Contreras et al. (2015, 2016). The amount of regulatory activity in MAT could explain results supporting (Contreras et al., 2015; Vailati-Riboni et al., 2016, 2017) or refuting (Ji et al., 2012, 2014; Mann et al., 2015) proinflammatory gene expression in lipid-filled adipose tissues. Regulatory immune phenotype(s) in the MLN could generate the anti-inflammatory environments supporting the mucosal permeability barrier integrity that prevents trans-mucosal translocation of commensal bacterial elements triggering adipose inflammation (Cani et al., 2007, 2008). Although the majority of immune signals from the intestinal mucosa influence cellular structure and function in the MLN, experimental evidence shows that the phenotypic structure of the MLN is also transformed by signals arising from the mesenteric adipose depot (Magnuson et al., 2017). Even within the limited realm of available reagents, results from this study set the foundation upon which the functional phenotypes of regulatory/anti-inflammatory elements in fat and the intestine can be explored. Reining in unrestrained proinflammatory immune activity in the intestinal mucosa associated with dietary shifts could support a more seamless metabolic transition through energy, ration, and production stressors of the postpartum period.

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