The objective was to unravel the peripartum immune and metabolic changes associated with metritis in Holstein cows. Holstein cows (n = 128) had blood collected at −14, 0, 3, and 7 d relative to parturition (DRP). Flow cytometry was used to evaluate blood leukocyte counts, proportions, and activation. Total cells, live cells, single cells, monocytes (CD172α+/CD14+), polymorphonuclears (CD172α+/CD14-/SSC high), B-cells (CD21+/MHCII+), CD4+ T-cells (CD4+), CD8+ T-cells (CD8+), and γδ T-cells (γδTCR+) were evaluated. Both CD62L and CD11b were used as markers of cell activation. Major histocompatibility complex class II was used as a marker of antigen presentation in monocytes. A Milliplex Bovine Cytokine/Chemokine 08-plex kit was used to evaluate plasma concentrations of IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and tumor necrosis factor-α. The body weight (BW) change prepartum was calculated as the difference between calving BW and prepartum BW divided by the number of days between measurements. Plasma fatty acids (FA) were measured at −14 and 0 DRP using untargeted gas chromatography with time-of-flight mass spectrometry. Data were analyzed by ANOVA for repeated measures. Cows that developed metritis (n = 57) had greater prepartum BW, prepartum BW loss, and greater FA concentrations at calving. Plasma FA at calving was positively correlated with IL-1β. Cows that developed metritis had persistent systemic inflammation, which was demonstrated by greater B-cell activation, greater pro-inflammatory cytokine concentrations, and greater cell damage pre- and postpartum. Postpartum, we observed greater polymorphonuclear cell activation and extravasation but lesser monocytes and CD4+ T-cells activation and extravasation, which suggests postpartum immune tolerance. Greater prepartum adiposity in cows that developed metritis may lead to systemic inflammation pre- and postpartum and immune tolerance postpartum, which may lead to failure to prevent bacterial infection, and development of puerperal metritis.

Key words: inflammation, uterine health, lipolysis, cytokines, transition period

INTRODUCTION

Metritis is a highly prevalent disease in Holstein cows shortly after calving (Sheldon et al., 2006), causing severe economic losses to the dairy industry (Pérez-Báez et al., 2021). Metritis is characterized by a dysbiosis of the uterine microbiome in which pathogenic bacteria overtake the uterine commensals (Jeon et al., 2015, 2016; Galvão et al., 2019). Whether or not the pathogenic bacteria thrive and cause metritis is most likely determined by how efficiently the immune system respond to invading pathogens. Upon pathogen recognition, endometrial cells and resident macrophages produce pro-inflammatory cytokines, such as IL-6 and IL-8, leading to a massive influx of leukocytes into the uterine lumen, particularly neutrophils and monocytes (Singh et al., 2008; Galvão et al., 2012; Sheldon et al., 2014), which are responsible for bacterial clearance (Auffray et al., 2009).

The recruitment of neutrophils and monocytes from the peripheral blood to the site of inflammation is mediated by cell adhesion molecules expressed on the surface of leukocytes and endothelial cells from local blood vessels such as CD62L and CD11b (Ley, 2003; Nourshargh and Alon, 2014). Furthermore, endometrial antigen presenting cells, such as monocytes, dendritic...
cells, and B-cells, take up antigens, travel to regional lymph nodes, and present processed antigens in the context of major histocompatibility complex molecules to prime naive T-helper cells (Swain et al., 1991). Primed T-helper cells secrete pro-inflammatory cytokines, such as IL-4, to activate T- and B-cells and induce proliferation in regional lymph nodes (Swain et al., 1991). Activated T- and B-cells shed CD62L, driving the traffic of activated lymphocytes away from lymphoid organs and toward inflamed tissues (Ivetic et al., 2019). This inflammatory response, when working optimally, leads to pathogen clearance, and resolution of inflammation (Chen et al., 2017).

Nonetheless, the tug of war between the immune system and the pathogens occurs when cows are transitioning into lactation, a period characterized by marked physiological changes that affect immune competence (Hammon et al., 2006). Several studies reported a reduction in feed intake, greater loss of BW, and greater accumulation of circulating fatty acid and ketone bodies during the transition period in cows that develop metritis (Hammon et al., 2006; Chebel et al., 2018). These signs have been associated with impaired leukocyte function such as lesser cytokine production by monocytes (Galvão et al., 2012) and impaired neutrophil phagocytosis and killing capacity (Hammon et al., 2006; Grinberg et al., 2008; Ster et al., 2012). Furthermore, over-conditioned cows have greater BW loss during the prepartum period than well- and under-conditioned cows (Chebel et al., 2018). In nonruminants during obesity, necrotic adipocytes release damage-associated molecular patterns (DAMP), which reach the peripheral blood and activate immune cells (Guillerey et al., 2012). Activated immune cells infiltrate the adipose tissue and surround the necrotic adipocytes in so called “crown like structures” inciting an inflammatory response, which leads to local and systemic persistent low-grade inflammation (Winer et al., 2014). Crown like structures have been reported in adipose tissue from cows with displaced abomasum undergoing negative energy balance (Contreras et al., 2015), therefore, it is possible that a similar inflammatory state occurs in cows which may predispose cows to develop metritis.

In summary, cows experience immune dysregulation around parturition, and obesity and BW loss may affect the inflammatory state that predisposes cows to metritis. Therefore, our first hypothesis was that prepartum body weight (ppBW), body weight change (BWC) and circulating fatty acids would be greater in cows that develop metritis than in cows that do not. Our second hypothesis was that this greater lipolytic state would lead to persistent inflammation and promote the development of metritis. Therefore, the objective of this study was to unravel the immune and metabolic changes associated with metritis in dairy cows.

**MATERIALS AND METHODS**

This prospective observational study was conducted at the University of Florida Dairy Unit (Gainesville, FL) from September 2019 to March 2020. All procedures involving cows were approved by the Institutional Animal Care and Use Committee of the University of Florida, protocol number 201910623.

**Sample Size Calculation**

A sample size was calculated using JMP Pro (Version 15. SAS Institute Inc., Cary, NC) based on previous studies evaluating immune responses in dairy cows with and without metritis (Kasimanickam et al., 2013; Cui et al., 2019). The sample size was calculated based on tumor necrosis factor α (TNF-α), IL-6, IL-1β, and PMN, lymphocytes, and monocytes proportions. Differences in monocyte proportions required the largest sample size. Based on an anticipated difference of 2% in the proportion of monocytes between groups with a standard deviation of 2% (Cui et al., 2019), a sample size of 17 cows per group was determined to be sufficient for achieving 80% power at an α level of 0.05. With an expected incidence of metritis of at least 15% (Galvão et al., 2019), and an attrition rate of 10%, we would need to enroll 125 cows to have at least 17 cows with metritis in the study. A total of 128 Holstein cows (71 primigravid and 57 multigravid) were enrolled at 260 d of gestation and followed until 13 ± 1 d relative to parturition (DRP).

**Housing and Feeding**

All cows were housed in freestall barns with sand-bedded stalls pre- and postpartum. Prepartum, multigravid cows were fed a TMR formulated to meet or exceed the nutrient requirements for dry Holstein cows weighing 680 kg (NRC, 2001) twice daily. Postpartum, multiparous cows were fed a TMR formulated to meet or exceed the nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% FCM (NRC, 2001) twice daily. Nulliparous cows were housed in a freestall barn with individual feeding gates (Calan Broadbent Feeding System, American Calan Inc., Northwood, NH) since 241 d of gestation and were fed a TMR once daily. After parturition, primiparous cows were moved to a postpartum pen also equipped with individual feeding gates and each cow...
was assigned to an individual gate until 100 DRP. Nul-liparous animals were part of an experiment where they were assigned to a control, which was offered 100 g/cow per day ground corn as a top-dress to the total mixed diet, and to a treatment, which consisted of top-dress addition of calcidiol (Rovimix Hy-D 1.25%. 12.5 mg/g calcidiol; DSM Nutritional Products, Parsippany, NJ) at a concentration of 10 mg/kg ground corn from 248 d of gestation until 100 DRP. Postpartum, all cows were milked 2 times a day at 0600 and 1800 h. The rolling herd average milk yield was approximately 11,000 kg.

Case Definition and Diagnosis

Metritis was diagnosed by examination of the uterine discharge with a Metricheck device (Metricheck, Simcro, New Zealand) at 3 ± 1, 7 ± 1, 10 ± 1, and 13 ± 1 DRP using a 5-point scale as previously described (Jeon et al., 2016; 1 = not fetid normal lochia, viscous, clear, red, or brown; 2 = cloudy, pink, red, or brown mucoid discharge with flecks of pus; 3 = not fetid, pink red or brown mucopurulent discharge with <50% pus; 4 = not fetid, pink, red or brown purulent discharge with ≥50% pus; 5 = fetid red-brownish, watery discharge). Cows with a discharge score ≤4 were classified as healthy and cows with a score of 5 were classified as having metritis. Incidences of mastitis, digestive problems, respiratory disease, and antimicrobial treatments in the first 35 DRP were also recorded for individual cows, and cows with any of these diseases or submitted to antimicrobial treatment before metritis diagnosis were excluded from the study. A total of 9 cows were excluded. Four cows were excluded because they were treated with antimicrobials before metritis diagnosis. Three cows were excluded because of death. One cow was excluded because of uterine torsion and one cow was excluded because of peritonitis.

Blood Sample Collection

All cows had blood collected at −14, 0, 3, and 7 DRP. Blood was sampled from the jugular vein using a 20-gauge × 2.54-cm needle and 10-mL evacuated tube containing lithium heparin (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) at a concentration of 10 mg/kg ground corn from 248 d of gestation until 100 DRP. Postpartum, all cows were milked 2 times a day at 0600 and 1800 h. The rolling herd average milk yield was approximately 11,000 kg.

Blood Flow Cytometry Analysis

Blood immune cell populations were characterized using a Thermofisher Attune Nxt Flow Cytometer as previously described (Casaro et al., 2022). Briefly, once red blood cells were lysed, cells were incubated for 20 min at room temperature with a LIVE/DEAD Near-IR dead cell staining kit (Thermo Fisher Scientific, Waltham, MA) was used in both panels to stain dead cells.
Panel 1 contained Ab against CD4, CD8α, TCR-δ chain, and CD62L, and panel 2 contained Ab against CD21, major histocompatibility complex class 2 (MHCII), CD172α, CD14, CD11b, and CD62L (Table 1).

**Gating Strategy**

Gating strategy is depicted in Figure 1 and Supplemental Figure S1 (https://doi.org/10.17632/jynj857t3z.1; Casaro et al., 2023). On the first panel, once live and single cells were gated, myeloid cells were identified as CD172α+. Monocytes were identified as CD172α+/CD14−/SSChigh cells, and monocytes were identified as CD172α+/CD14+ cells. The proportion and MFI of CD11b+ or with greater MFI were activated. Monocytes with greater MHCII+ proportion or MFI were considered as being more capable to present antigenic peptides (Jakubzick et al., 2017). The B-cells with lesser CD62L+ proportion or MFI and greater CD11b+ proportion or MFI were considered as activated. (Kawai et al., 2005; Morrison et al., 2010). On the second panel, once live and single cells were gated, lymphocytes were identified using FSC and SSC parameters. Within the lymphocyte gated population, CD4+ T-cells were gated using CD4, CD8+ T-cells were gated using CD8α, and γδ T-cells were gated using γδTCR. The proportion of parent and MFI were recorded for CD4, CD8, and γδ T-cells. The proportion and MFI of CD62L were used as markers of cell activation on the previously gated populations. Lymphocytes lacking CD62L or with lesser MFI were considered as activated (Galkina et al., 2003).
Data were analyzed using FlowJo software (Version 10.0.7, Treestar, Palo Alto, CA). Once data tables were extracted from FlowJo, the average between the 2 panels live cells, singlets, and lymphocytes were calculated and used.

The flow cytometer settings were configured to collect a volume of 200 μL of sample or a maximum of 100,000 cells, whichever was reached first. The volume of fluid utilized to capture the 100,000 cells was recorded and whenever the total number of cells was less than 100,000, the total number of cells within the 200 μL sample was recorded.

Total cells/μL were calculated by dividing the number of cells recorded by the total volume acquired (in nL), and then, because samples were acquired from 100 μL of whole blood and resuspended in 500 μL of PBS, the result was multiplied by 5,000. The peripheral blood leukocyte counts were determined by multiplying the proportion of different population subsets by the total number of cells.

**Plasma Cytokine Analysis**

One frozen plasma aliquot was submitted to the University of Florida’s proteomics and mass spectrometry core (RRID:SCR_019151) of the Interdisciplinary Center for Biotechnology Research for multiplex analysis. A Milliplex Bovine Cytokine/Chemokine 08-plex kit (BCYT1–33 K-08; EMD Millipore Corporation, Billerica, MA) utilizing antibodies to bovine IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and TNF-α was used to screen one of the stored plasma aliquots. The assay was performed according to the manufacturer’s instructions by blinded technicians. Concentrations of markers were measured on the Luminex FlexMap 3D instrument (Luminex Corporation, Austin, TX). Quality control values ranged between 6.0 and 28.8, 12.6 and 70.2, 104.5 and 543.9, 618.0 and 3,334.6, 119.8 and 619.2, 129.1 and 531.8, 40.4 and 209.5, 426.7 and 2,594.6 for IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and TNF-α, respectively, which were within the ranges indicated by the manufacturer. Interassay coefficients of variation were 3%, 3%, 1%, 7%, 2%, 3%, 5%, and 4% for IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and TNF-α, respectively, which were within the ranges indicated by the manufacturer. Intra-assay coefficients of variation were 11.5%, 13.3%, 12.5%, 9.5%, 6.9%, 3.6%, 5.1%, and 14.0% for IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and TNF-α, respectively.

**Plasma Metabolome Analysis and Fatty Acids Calculation**

The second frozen plasma aliquot was submitted to the University of California’s West Coast Metabolomics Center in Davis, CA for metabolome analysis. Samples were analyzed by blinded technicians using untargeted gas chromatography with time-of-flight mass spectrometry (GC-TOF-MS) in a single batch as previously described (Fiehn et al., 2008; Fiehn, 2016). Fatty acids were calculated by the addition of all fatty acids present in plasma, which consisted of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), arachidonic acid (C20:5), and docosahexaenoic acid (C22:6).

**BW Data Collection**

Primigravid cows were weighed twice at 240 d of gestation, and the mean value was used as ppBW. The BW of multiparous cows were collected from the on-farm computer software (Afifarm management program, Afimilk Ltd., Kibbutz Afikim, Israel). Three consecutive weights were collected at −2, −1, and 0 d relative to dry-off from the lactation preceding enrollment. The 3 measurements were used to generate a mean ppBW. On 0, 1, and 2 DRP weights were collected from the on-farm computer software from primiparous and multiparous cows. The 3 measurements were used to generate a mean calving body weight (cBW). To only account for the BWC related to tissue accretion or mobilization, the weight of the gravid uterus prepartum and the weight of the empty uterus right after calving were subtracted from the prepartum and cBW, respectively. The weight of the gravid uterus and empty uterus were calculated using NRC (2021) equations as follows:

Gravid uterine weight = \((Calf BW \times 1.825) \times e^{\left[\frac{0.0243 - (0.0000245 \times day of gestation)}{(280 - day of gestation)}\right]}\); 

Empty postpartum uterine weight = (Calf BW × 0.2288).

After excluding the weight of the gravid uterus from the ppBW and the weight of the empty uterus from the cBW, the BWC prepartum was calculated as the difference between cBW and ppBW divided by the number of days between measurements.

**Statistical Analysis**

All statistical analyses were conducted using JMP Pro 15 (JMP Statistical Discovery, SAS Inst. Inc., Cary, NC), SAS 9.4 (SAS/STAT, SAS Inst. Inc., Cary, NC), and RStudio 2022.07.0 (RStudio, PBC, Boston, MA).

The cow was considered the experimental unit. All continuous data were analyzed by ANOVA with linear mixed effects using the MIXED procedure of SAS (SAS/
To assess the effect of calcidiol treatment in primiparous cows, a model only including primiparous cows and containing the fixed effects of metritis (0 vs. 1), time of measurement (−14, 0, 3, and 7 DRP), calcidiol (0 vs. 1), and the interactions between metritis and time, and metritis and calcidiol was analyzed. There were no differences (\( P > 0.10 \)) between treatments in any of the variables measured. A chi-squared test was used to compare the proportion of cows that developed metritis in the control (n = 18/31) and calcidiol-treated groups (n = 19/33), and there were no differences between treatments (\( P = 0.98 \)).

After model fitting, all immune markers, cytokines, ppBW, BWC, and fatty acids were evaluated for the distribution of the residuals. The data with residuals with deviations from normality were subjected to power transformation according to the Box-Cox method (Piepho and Piepho, 2009) and reanalyzed. To report least squares means (LSM) and standard errors (SE), back transformation of the LSM and SE were performed according to Jorgensen et al. (1998).

For the immune markers, cytokines, and fatty acids, the full model contained the fixed effects of metritis (0 vs. 1), time of measurement (−14, 0, 3, and 7 DRP), parity (primiparous vs. multiparous), and the interactions between metritis and time, and metritis and parity. The models included the random effect of cow nested within metritis and the cytokines also included the random effect of plate (1 to 12). The repeated statement consisted of time (day) with the subject being cow nested within metritis. The covariance structure with the smallest Akaike information criterion was selected, and spatial power covariance structure resulted in the best fit for all variables. The approximate denominator degrees of freedom for the F tests were calculated using the Kenward-Roger method. All models contained the fixed effects of metritis, time, their interaction, and parity. A backward elimination procedure was applied when the interaction between metritis and parity had a \( P > 0.05 \). When either an effect of metritis, or an interaction between metritis and time was observed (\( P \leq 0.05 \)), nonparametric multifactor contrasts by time were performed using the ART-C procedure of the ARTool package as previously described (Elkin et al., 2021). To report means and SE, outliers were excluded using JMP Pro 15 and raw means and SE were reported.

Pearson correlations (\( r \)) were performed in JMP Pro 15 to evaluate the relationship between PMN counts and the MFI of CD62L on the PMN, between ppBW and BWC, between BWC and prepartum fatty acids, between the prepartum proportion of live cells and prepartum fatty acids, between prepartum fatty acids and DRP the prepartum sample was taken, and between the proportion of prepartum live cells and DRP the prepartum sample was taken. Pearson correlations were performed between the calving ranked values of IL-1β and calving fatty acids, between the prepartum live cells and prepartum ranked values of IL-8, and between the prepartum proportion of PMN and the prepartum ranked values of IL-8. All variables met the assumptions for Pearson correlation, except for IL-1β and IL-8, which were non-normally distributed. Spearman rank
correlations (rho) were performed for IL-1β and IL-8, and ranked values (Supplemental Table S1; https://doi.org/10.17632/jynj857t3z.1; Casaro et al., 2023) were presented in the figures. Statistical significance was considered at \( P \leq 0.05 \), and marginal significance was considered at \( 0.05 < P \leq 0.10 \).

**RESULTS**

From the 128 cows that were enrolled in the study, a total of 119 cows, 64 primiparous and 55 multiparous were included in the analysis. Of the 119 cows, 57 (48%) were diagnosed with metritis. Of the 64 primiparous, 37 (58%) were diagnosed with metritis, whereas of the 55 multiparous, 20 (36%) were diagnosed with metritis. Of the 57 (100%) cows with metritis, 10 (18%), 24 (42%), 19 (33%), and 4 (7%) cows were diagnosed at 3 ± 1, 7 ± 1, 10 ± 1, and 13 ± 1 DRP, respectively. The mean days from enrollment until parturition was 14 ± 6 d.

**PMN from Dairy Cows Increase the Expression of CD62L Before Extravasation**

There was an overall negative correlation between the PMN counts and the MFI of CD62L on the PMN (\( r = -0.55; 95\% \text{ CI} = -0.48 \) to \(-0.61; P < 0.01; \text{Supplemental Figure S2, https://doi.org/10.17632/jynj857t3z.1, Casaro et al., 2023} \)).

**Cows That Develop Metritis Have Less Peripheral Blood Total Leukocytes and Live Cells But More Single Cells**

There was a main effect of metritis on total cells, and cows that developed metritis had lesser total cells when compared with cows that did not develop metritis (\( P = 0.05; 8,047 ± 370 \text{ vs. } 9,038 ± 342 \text{ cells/μL; Supplemental Table S2; https://doi.org/10.17632/jynj857t3z.1; Casaro et al., 2023} \)). When analyzing metritis by day, total cells were lesser on d 3 in cows that developed metritis when compared with cows that did not develop metritis (\( P = 0.03; 7,395 ± 433 \text{ vs. } 8,665 ± 405 \text{ cells/μL; Supplemental Table S2} \)). There was a main effect of metritis on live cells as a proportion of total cells, and cows that developed metritis had lesser live cells as a proportion of total cells when compared with cows that did not develop metritis (\( P = 0.04; 7,746 ± 355 \text{ vs. } 8,747 ± 328 \text{ cells/μL; Supplemental Figure S3B} \)). When analyzing metritis by day, live cell counts were lesser on d −14 (\( P = 0.05; 7,493 ± 421 \text{ vs. } 8,625 ± 389 \text{ cells/μL; d 3 (} P = 0.03; 7,162 ± 417 \text{ vs. } 8,419 ± 389 \text{ cells/μL; and d 7 (} P = 0.05; 7,110 ± 417 \text{ vs. } 8,230 ± 389 \text{ cells/μL) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S3B). The metritis by day interaction (\( P = 0.04) \) showed that cows that developed metritis had greater single cells as a proportion of live cells on d 3 when compared with cows that did not develop metritis (\( P = 0.04; 93.6 ± 0.6 \text{ vs. } 92.0 ± 0.5\% ; \text{Supplemental Figure S3C} \)).

**Cows That Develop Metritis Have More Myeloid Cells Prepartum**

The metritis by day interaction (\( P = 0.01) \) showed that cows that developed metritis had greater myeloid cells as a proportion of single cells on d −14 when compared with cows that did not develop metritis (\( P < 0.01; 50.2 ± 1.5 \text{ vs. } 43.0 ± 1.4\% ; \text{Supplemental Table S2} \)).

**Cows That Develop Metritis Have More PMN Extravasation Postpartum**

The metritis by day interaction (\( P = 0.01) \) showed that cows that developed metritis had greater PMN as a proportion of single cells on d −14 when compared with cows that did not develop metritis (\( P = 0.01; 28.5 ± 1.3 \text{ vs. } 23.9 ± 1.2\% ; \text{Supplemental Figure S4A, https://doi.org/10.17632/jynj857t3z.1; Casaro et al., 2023} \)). There was a main effect of metritis on PMN counts, and cows that developed metritis had lesser PMN counts when compared with cows that did not develop metritis (\( P = 0.02; 1,131 ± 51 \text{ vs. } 1,293 ± 48 \text{ cells/μL; Supplemental Figure S4B} \)). When analyzing metritis by day, PMN counts were lesser on d 3 in cows that developed metritis when compared with cows that did not develop metritis (\( P = 0.01; 628 ± 87 \text{ vs. } 938 ± 83 \text{ cells/μL; Supplemental Figure S4B} \)). The metritis by day interaction (\( P = 0.01) \) showed that cows that developed metritis had greater proportion of PMN...
expressing CD62L on d 3 when compared with cows that did not develop metritis ($P = 0.02; 98.7 ± 0.6\text{ vs. } 96.7 ± 0.6\%$; Supplemental Table S2). There was a main effect of metritis on PMN CD62L MFI, and cows that developed metritis had greater PMN CD62L MFI when compared with cows that did not develop metritis ($P < 0.01; 4.218 ± 119 \text{ vs. } 3.747 ± 110 \text{ MFI}; \text{Supplemental Figure S4C}$). When analyzing metritis by day, PMN CD62L MFI was greater on d 3 ($P < 0.01; 5.279 ± 191 \text{ vs. } 4.427 ± 181 \text{ MFI}$) and d 7 ($P < 0.01; 6.004 ± 191 \text{ vs. } 5.121 ± 181 \text{ MFI}$) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S4D).

When analyzing metritis by day, monocytes as a proportion of single cells were greater on d −14 ($P = 0.05; 2.242 ± 100 \text{ vs. } 1.956 ± 100 \text{ MFI}$) and d 7 ($P < 0.01; 6.6 ± 1.3 \text{ vs. } 2.369 ± 100 \text{ MFI}$; Supplemental Figure S4D). When analyzing metritis by day, PMN CD11b MFI was greater on d 3 ($P < 0.01; 5.279 ± 191 \text{ vs. } 4.427 ± 181 \text{ MFI}$) and d 7 ($P < 0.01; 6.004 ± 191 \text{ vs. } 5.121 ± 181 \text{ MFI}$) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S4C). There was a main effect of metritis on PMN CD11b MFI, and cows that developed metritis had greater PMN CD11b MFI when compared with cows that did not develop metritis ($P < 0.01; 91.6 ± 0.6 \text{ vs. } 94.4 ± 0.4\%$; Supplemental Figure S5D). When analyzing metritis by day, the proportion of monocytes expressing CD11b and cows that developed metritis had lesser proportion of monocytes expressing CD11b when compared with cows that did not develop metritis ($P < 0.01; 92.6 ± 0.4 \text{ vs. } 94.4 ± 0.4\%$; Supplemental Figure S5D). When analyzing metritis by day, the proportion of monocytes expressing CD11b was lesser on d 3 ($P < 0.01; 91.6 ± 0.6 \text{ vs. } 94.4 ± 0.4\%$) and d 7 ($P < 0.01; 87.7 ± 0.6 \text{ vs. } 91.8 ± 0.5\%$) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S5D). There was a main effect of metritis on the proportion of monocytes expressing MHCIi, and cows that developed metritis had lesser proportion of monocytes expressing MHCIi when compared with cows that did not develop metritis ($P = 0.05; 75.8 ± 1.6 \text{ vs. } 80.0 ± 1.5\%$; Supplemental Table S2). When analyzing metritis by day, the proportion of monocytes expressing MHCIi was lesser on d 7 in cows that developed metritis when compared with cows that did not develop metritis ($P = 0.02; 72.3 ± 1.9 \text{ vs. } 78.5 ± 1.8\%$; Supplemental Table S2).

### Cows That Develop Metritis Have Less Monocyte Extravasation and Antigen Presentation Capacity Postpartum

There was a main effect of metritis on monocytes as a proportion of single cells, and cows that developed metritis had greater monocytes as a proportion of single cells when compared with cows that did not develop metritis ($P < 0.01; 6.9 ± 0.3 \text{ vs. } 5.7 ± 0.2\%$; Supplemental Figure S5A). When analyzing metritis by day, monocytes as a proportion of single cells were greater on d −14 ($P = 0.02; 5.4 ± 0.3 \text{ vs. } 4.4 ± 0.3\%$), d 3 ($P < 0.01; 11.6 ± 0.7 \text{ vs. } 8.8 ± 0.5\%$), and d 7 ($P < 0.01; 8.0 ± 0.5 \text{ vs. } 6.0 ± 0.3\%$) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S5A). There was a main effect of metritis on monocytes expressing CD11b, and cows that developed metritis had greater monocytes expressing CD11b, and cows that developed metritis had lesser monocyte CD62L MFI when compared with cows that did not develop metritis ($P = 0.02; 72.3 ± 1.9 \text{ vs. } 78.5 ± 1.8\%$; Supplemental Table S2).

### Cows That Develop Metritis Have Greater B-Cell Activation

There was a main effect of metritis on the proportion of B-cells expressing CD62L, and cows that developed metritis had lesser proportion of B-cells expressing CD62L when compared with cows that did not develop metritis ($P < 0.01; 9.1 ± 1.2 \text{ vs. } 14.2 ± 1.1\%$; Supplemental Figure S6A). When analyzing metritis by day, the proportion of B-cells expressing CD62L was lesser on d −14 ($P = 0.01; 10.0 ± 1.3 \text{ vs. } 14.5 ± 1.2\%$), d 0 ($P < 0.01; 6.6 ± 1.3 \text{ vs. } 12.5 ± 1.2\%$), d 3 ($P < 0.01; 8.4 ± 1.3 \text{ vs. } 13.7 ± 1.2\%$), and d 7 ($P = 0.01; 11.4 ± 1.3 \text{ vs. } 16.0 ± 1.2\%$) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S6A). There was a main effect of metritis on the proportion of B-cells expressing CD11b, and cows that developed metritis had greater proportion of B-cells expressing CD11b when compared with cows that did not develop metritis ($P = 0.03; 72.2 ± 2.2 \text{ vs. } 65.5 ± 2.0\%$; Supplemental Figure S6B). When analyzing metritis by day, the proportion of B-cells expressing CD11b was greater on d −14 ($P = 0.01; 75.1 ± 2.4 \text{ vs. } 66.8 ± 2.1\%$), d 0 ($P = 0.04; 75.2 ± 2.3 \text{ vs. } 68.6 ± 2.1\%$), and d 3 ($P = 0.03; 71.1 ± 2.3 \text{ vs. } 64.0 ± 2.2\%$).
2.1%) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S6B). There was a main effect of metritis on B-cell CD62L MFI, and cows that developed metritis had lesser B-cell CD62L MFI when compared with cows that did not develop metritis ($P < 0.01; 162 \pm 15$ vs. $232 \pm 14$ MFI; Supplemental Table S2). When analyzing metritis by day, B-cell CD62L MFI was lesser on d $-14$ ($P < 0.01; 164 \pm 17$ vs. $236 \pm 16$ MFI), d 0 ($P < 0.01; 127 \pm 17$ vs. $203 \pm 16$ MFI), d 3 ($P < 0.01; 147 \pm 17$ vs. $225 \pm 16$ MFI), and d 7 ($P < 0.01; 207 \pm 17$ vs. $260 \pm 16$ MFI) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Table S2).

**Cows That Develop Metritis Have Less CD4+ T-cell Activation Postpartum**

The metritis by day interaction ($P = 0.01$) showed that cows that developed metritis had greater proportion of CD4+ T-cell expressing CD62L on d 3 when compared with cows that did not develop metritis ($P < 0.01; 47.0 \pm 1.0$ vs. $42.7 \pm 0.9$%; Supplemental Table S2). The metritis by day interaction ($P < 0.01$) showed that cows that developed metritis had greater CD4+ T-cell CD62L MFI on d 3 when compared with cows that did not develop metritis ($P < 0.01; 278 \pm 25$ vs. $193 \pm 13$ MFI; Supplemental Figure S6C).

**Cows That Develop Metritis Have Greater Plasma Pro-Inflammatory Cytokine Concentrations**

All cytokine results are summarized in Figure 2. There was a main effect of metritis on IL-1β concentration, and cows that developed metritis had greater IL-1β concentration when compared with cows that did not develop metritis ($P = 0.02; 11.7 \pm 1.4$ vs. $6.7 \pm 0.9$ pg/mL; Supplemental Figure S7A, https://doi.org/10.17632/jynj857t3z.1, Casaro et al., 2023). When analyzing metritis by day, IL-1β concentration was greater on d 0 ($P = 0.01; 16.9 \pm 4.6$ vs. $6.5 \pm 1.6$ pg/mL) and d 7 ($P = 0.03; 19.5 \pm 6.0$ vs. $8.1 \pm 1.9$ pg/mL) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S7A). There was a main effect of metritis on IL-8 concentration when compared with cows that did not develop metritis ($P < 0.01; 2.574.9 \pm 1.224.6$ vs. $738.4 \pm 163.4$ pg/mL) in cows that developed metritis when compared with cows that did not develop metritis (Supplemental Figure S7B). There was a main effect of metritis on IFN-γ, and cows that developed metritis had greater IFN-γ concentration when compared with cows that did not develop metritis ($P = 0.02; 2.0 \pm 0.1$ vs. $1.5 \pm 0.1$ pg/mL; Supplemental Figure S7C). When analyzing metritis by day, IFN-γ concentration was greater on d 0 (in cows that developed metritis when compared with cows that did not develop metritis ($P = 0.02; 2.1 \pm 0.2$ vs. $1.5 \pm 0.1$ pg/mL; Supplemental Figure S7C).

**Greater IL-8 Is Associated with Greater Cell Death and Prepartum PMN**

There was a negative correlation between the prepartum live cells and the prepartum IL-8 (rho = $-0.23; P < 0.01$; Supplemental Figure S8A, https://doi.org/10.17632/jynj857t3z.1, Casaro et al., 2023). There was a positive correlation between the prepartum IL-8 and the prepartum PMN proportion (rho = 0.25; $P < 0.01$; Supplemental Figure S8B).

**Cows That Develop Metritis Have Greater Prepartum BW, ppBW Loss, and Circulating Fatty Acids at Calving**

There was a main effect of metritis on ppBW, and cows that developed metritis had greater ppBW when compared with cows that did not develop metritis ($P = 0.02; 716.89 \pm 9.57$ vs. $684.58 \pm 10.00$ kg; Figure 3A). There was a main effect of metritis on BWC, and cows that developed metritis were losing more weight when compared with cows that did not develop metritis ($P < 0.01; -0.45 \pm 0.10$ vs. $0.23 \pm 0.11$ kg/d; Figure 3B). There was a main effect of metritis on fatty acids, and cows that developed metritis had greater fatty acids when compared with cows that did not develop metritis ($P = 0.01; 1.61 \pm 0.04$ vs. $1.42 \pm 0.04$ peak heights; Figure 3C). When analyzing metritis by day, fatty acids were greater on d 0 in cows that developed metritis when compared with cows that did not develop metritis ($P < 0.01; 1.96 \pm 0.05$ vs. $1.65 \pm 0.05$ peak heights; Figure 3C).

**BW Loss Is Associated with ppBW and Plasma IL-1β**

There was a negative correlation between BWC and ppBW ($r = -0.44$; 95% CI = $-0.58$ to $-0.27$; $P < 0.01$; Figure 4A). There was a negative correlation between BWC and prepartum fatty acids ($r = -0.30$; 95% CI = $-0.47$ to $-0.12$; $P < 0.01$; Figure 4B). The
A negative correlation between the prepartum live cells as a proportion of total cells and prepartum fatty acids was marginally significant ($r = -0.19$; 95% CI = $-0.36$ to 0.00; $P = 0.06$; Figure 4C). There was a positive correlation between prepartum fatty acids and DRP ($r = 0.45$; 95% CI = 0.28 to 0.59; $P < 0.01$; Figure 4D), and a negative correlation between the prepartum proportion of live cells and DRP ($r = -0.21$; 95%...
CI = −0.38 to −0.04; \( P < 0.01 \); Figure 4E). There was a positive correlation between calving fatty acids and calving IL-1β (rho = 0.21; \( P = 0.03 \); Figure 4F).

**DISCUSSION**

Herein, we explored the metabolic and immune profiles peripartum to try to unravel the immune and metabolic changes associated with acute postpartum uterine infection.

We observed that cows that developed metritis had greater ppBW, more activated B-cells pre- and postpartum, and more cell death pre- and postpartum than cows that did not develop metritis. In dairy cows, BW is strongly correlated with BCS, a measure of adipose tissue deposition (Holling et al., 2004); hence, we speculate that heavier cows were fatter. In nonruminants, necrotic-like adipocyte death is a pathological hallmark of obesity (Cinti et al., 2005). Upon cellular stress or injury, damaged or dying cells release DA and activate immune cells by different pathways, including toll-like receptors (TLR) stimulation (Roh and Sohn, 2018; Gong et al., 2020). During obesity, dying adipocytes release cell-free DNA, which reaches the peripheral blood and serves as an endogenous ligand for TLR-9 (Guillerey et al., 2012). In mice models, TLR-9 stimulation of B-cells causes the rapid shedding of CD62L, leading to B-cell activation (Morrison et al., 2010). Furthermore, activated B-cells express high levels of CD11b, which enhance their migratory ability (Kawai et al., 2005). Activated B-cells infiltrate the adipose tissue and together with T-cells and M1 macrophages surround the necrotic adipocytes in so called “crown like structures,” which leads to local and systemic persistent low-grade inflammation (Winer et al., 2014).

The greater level of fatty acid accumulation in adipose cells during obesity activates NADPH oxidase, enhancing the production of reactive oxygen species (ROS), and their release to circulation (Furukawa et al., 2004). Elevated ROS upregulate the mRNA expression of NADPH oxidase, augmenting ROS production, leading to systemic oxidative stress, which is characterized by cell death (Furukawa et al., 2004).

During prepartum, we observed that cows that developed metritis had greater proportions of PMN and monocytes, and greater IL-8 concentration when compared with cows that did not develop metritis. Furthermore, we observed that as cell death increased, IL-8 levels also increased, and that IL-8 was positively correlated with the proportion of PMN. Cell damage-associated OS triggers IL-8 production by peripheral blood leukocytes, which stimulate neutrophil migration from the bone marrow to the peripheral blood (Mulder et al., 2021) and triggers the production of C-C motif ligand-2 (CCL2) by vascular cells, which stimulate monocyte migration from the bone marrow to the peripheral blood (Camps et al., 2014). The greater ppBW together with the greater B-cell activation, lesser proportion of live cells, greater IL-8, and greater proportion of PMN and monocytes suggest that cows that will develop metritis are experiencing systemic low-grade inflammation in the prepartum period.

Cows that developed metritis were losing more weight prepartum, had more fatty acids at calving, and had greater IL-1β and IFN-γ concentrations when compared with cows that did not develop metritis. Moreover, we observed that heavier cows were losing more weight prepartum, had more blood fatty acids, and that blood fatty acids were positively correlated with cell death, and IL-1β concentration. Adipocytes from obese cows produce more leptin than those from nonobese cows (Kuhla et al., 2016). Leptin is secreted into circulation and is sensed by neurons located in the arcuate nucleus of the hypothalamus causing feed intake reduction (Ahiina and Antwi, 2008). Although most cows have feed intake reduction around parturition, cows that develop metritis have a sharper feed intake reduction in...
the last weeks prepartum, which leads to greater fatty acid mobilization (Hammon et al., 2006; Pérez-Báez et al., 2019). Consequently, this excessive fatty acid mobilization enhances the production of ROS (Schönfeld and Wojtczak, 2008), exacerbating the ongoing systemic inflammatory response. Systemic inflammation is characterized by greater peripheral blood pro-inflammatory cytokines such as IL-1β, which is also sensed by neurons located in the arcuate nucleus of the hypothalamus exacerbating the depression in feed intake (Kuhla, 2020). On top of that, adipose tissue T-cells surrounding necrotic adipocytes produce high levels of IFN-γ (Rocha et al., 2008), which enhances lipolysis (Memon et al., 1992), exacerbating fatty acid mobilization, leading to a vicious cycle of cell damage, immune cell activation and cytokine secretion, feed intake depression, and lipolysis. Indeed, both the decrease in the proportion of live cells and the greater B-cell activation are maintained at parturition, that together with the greater levels of IL-1β and IFN-γ suggests that cows that will develop metritis are undergoing a persistent inflammatory state by the day of calving.

In the current study, postpartum cows that developed metritis had monocytes with lesser activation, lesser MHCII, and greater counts, which suggests less monocyte activation and extravasation. Contrarily, cows that developed metritis had greater PMN activation and a sharp decrease in PMN counts, which suggests more PMN extravasation. Because the literature regarding the role of CD62L on PMN extravasation is contradictory (Swain et al., 1998; Momose et al., 1999; Diez-Fraile et al., 2002, 2003; Ivetic, 2018; Björkman et al., 2019), we investigated the correlation between the PMN counts and the MFI of CD62L on the PMN. The observed negative correlation between the PMN counts and the MFI of CD62L on the PMN, indicates that PMN increase the expression of CD62L before leaving the blood vessel.

Prolonged inflammatory responses are known for leading to immune tolerance (Rogovskii, 2020). This
refractory state is particularly associated with impaired monocyte and macrophage responses (Fernández-Ruiz et al., 2014), and enhanced neutrophils migration to infectious sites (Ariga et al., 2014). In fact, human peripheral blood monocytes pre-exposed to DAMPs had impaired cytokine production after LPS challenge, suggesting that monocytes’ exposure to DAMPs can cause immune tolerance, leading to monocytes refractory to subsequent stimulation (Fernández-Ruiz et al., 2014). Indeed, we observed that monocytes from cows that developed metritis had a reduced pro-inflammatory response after stimulation with *Escherichia coli* (Galvão et al., 2012). Therefore, it is plausible that cows that developed metritis were undergoing some degree of immune tolerance, hence, their monocytes were accumulating in the peripheral blood, and their PMN were being recruited to the uterus.

Upon arrival to the site of inflammation PMN release granule proteins, such as azurocidin and cathepsin 3, which exert chemotactic activity on monocytes launching their extravasation and recruitment to the inflammatory site (Soehnlein et al., 2009). Nonetheless, it is well established that neutrophils from cows with metritis have lesser phagocytosis and bactericidal activity (Hammon et al., 2006; Alhussien et al., 2021). Therefore, it is likely that other neutrophil functions such as granule proteins secretion could be impaired, thus, decreasing monocytes recruitment to the uterus and causing their accumulation in the blood. Whether monocytes are not being activated and recruited to fight the uterine pathogens because of immune tolerance or neutrophil impairment is to our knowledge currently unknown and deserves further investigation. Nonetheless, upon arrival to tissues, monocytes’ main role is to replenish tissue macrophages (Auffray et al., 2009). As professional phagocytes, monocytes, and macrophages orchestrate the clearance of invading pathogens, apoptotic cells, and dead neutrophils, playing a key role in

**Figure 5.** Illustration of our working hypothesis. We propose that greater prepartum adiposity in cows that developed metritis leads to persistent systemic inflammation, which was demonstrated by greater B-cell activation, greater pro-inflammatory cytokine concentration, and greater cell damage. Postpartum, we observed a dysregulated immune response, with greater PMN cell activation and extravasation but lesser monocytes and CD4⁺ T-cell activation and extravasation, which suggests postpartum immune tolerance. This dysfunctional immune response, with overall systemic inflammation followed by immune tolerance, may lead to failure to prevent bacterial infection, and development of metritis. ROS = reactive oxygen species.
the resolution of inflammation (Parihar et al., 2010). Therefore, we hypothesize that this process was impaired in cows that developed metritis, affecting the resolution of the inflammatory process.

Furthermore, we observed lesser postpartum CD4+ T-cells activation in cows that developed metritis when compared with cows that did not develop metritis. The lesser monocytes with less MHCII expression arriving at the uterus may lead to less antigen presentation to CD4+ T-cells at the lymph nodes, causing an increase in the proportion of peripheral blood naive CD4+ T-cells, thus, driving the traffic of lymphocytes away from the uterus and toward lymphoid organs (Ivetic et al., 2019). Once activated CD4+ T-cells arrive to the site of infection, they provide beneficial immune responses against offending pathogens (Goswami and Awasthi, 2020). Therefore, a decrease in CD4+ T-cells recruitment also impairs pathogen clearance.

Cows with metritis had less total cell counts after parturition, most likely driven by the sharp decrease in PMN counts. Given that cows with metritis had less total cell counts after parturition, they also had lesser single cells. When looking at the proportion of single cells from the live cells; however, cows with metritis had greater proportion of single cells. Upon activation, leukocytes increase their expression of adhesion molecules, which confers them the ability not only to interact with the endothelium, but also to interact and adhere to other immune cells (Golias et al., 2011). Therefore, it is plausible that because cows with metritis had lesser monocyte and CD4+ T-cell expression of adhesion molecules, these cells were not interacting with each other and with other immune cells, thus, leading to a greater proportion of single cells.

CONCLUSIONS

Herein, we observed that cows that developed metritis had greater ppBW, BW loss, and greater fatty acids concentrations at calving, which in turn were positively correlated with IL-1β concentrations. Cows that developed metritis had persistent systemic inflammation, which was demonstrated by greater B-cell activation, greater pro-inflammatory cytokine concentrations, and greater cell damage pre- and postpartum. Postpartum, we observed greater PMN cell activation and extravasation but lesser monocytes and CD4+ T-cells activation and extravasation. We propose that greater prepartum adiposity in cows that developed metritis may lead to systemic inflammation pre- and postpartum and immune tolerance postpartum, which may lead to failure to prevent bacterial infection, and development of puerperal metritis. Our working hypothesis is illustrated in Figure 5. This hypothesis needs to be tested in future experiments.

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