ABSTRACT

Cows in early lactation (EL) are purportedly immune suppressed, which renders them more susceptible to disease. Thus, the study objective was to compare key biomarkers of immune activation from i.v. lipopolysaccharide (LPS) between EL and mid-lactation (ML) cows. Multiparous EL (20 ± 2 DIM; n = 11) and ML (131 ± 31 DIM; n = 12) cows were enrolled in a 2 × 2 factorial design and assigned to 1 of 2 treatments by lactation stage (LS): (1) EL (EL-LPS; n = 6) or ML (ML-LPS; n = 6) cows administered a single LPS bolus from Escherichia coli O55:B5 (0.09 µg/kg of body weight), or (2) pair-fed (PF) EL (EL-PF; n = 5) or ML (ML-PF; n = 6) cows administered i.v. saline. After LPS administration, cows were intensely evaluated for 3 d to analyze their response and recovery to LPS. Rectal temperature increased in LPS relative to PF cows (1.1°C in the first 9 h), and the response was more severe in EL-LPS relative to ML-LPS cows (2.3 vs. 1.3°C increase at 4 h post-LPS; respectively). Respiration rate increased only in EL-LPS cows (47% relative to ML-LPS in the first h post-LPS). Circulating tumor necrosis factor-α (TNF-α), macrophage inflammatory protein (MIP)-1α, MIP-1β, and IFN-γ-inducible protein-10 increased within the first 6 h after LPS and these changes were exacerbated in EL-LPS relative to ML-LPS cows (6.3-, 4.8-fold, 57%, 93%, 10%, and 61% respectively). All cows administered LPS had decreased circulating iCa relative to PF cows (34% at the 6 h nadir), but the hypocalcemia was more severe in EL-LPS than ML-LPS cows (14% at 6 h nadir). In response to LPS, neutrophils decreased regardless of LS, then increased into neutrophilia by 24 h in all LPS relative to PF cows (2-fold); however, the neutrophilic phase was augmented in EL- compared with ML-LPS cows (63% from 24 to 72 h). Lymphocytes and monocytes rapidly decreased then gradually returned to baseline in LPS cows regardless of LS; however, monocytes were increased (57%) at 72 h in EL-LPS relative to ML-LPS cows. Platelets were reduced (46%) in LPS relative to PF cows throughout the 3-d following LPS, and from 24 to 48 h, platelets were further decreased (41%) in EL-LPS compared with ML-LPS. During the 3-d following LPS, serum amyloid A (SAA), LPS-binding protein (LBP), and haptoglobin (Hp) increased in LPS compared with PF groups (9-fold, 72%, and 153-fold, respectively), and the LBP and Hp responses were more exaggerated in EL-LPS than ML-LPS cows (85 and 79%, respectively) whereas the SAA response did not differ by LS. Thus, our data indicates that EL immune function does not appear “suppressed,” and in fact many aspects of the immune response are seemingly functionally robust.

Keywords: immune suppression, endotoxin, inflammation, periparturient

INTRODUCTION

Transition dairy cows (approximately 3 weeks before and post-calving) succumb to diseases and disorders at higher rates than other production stages, and their increased vulnerability is ostensibly attributed to immune suppression (Drackley, 1999; LeBlanc, 2014). Traditionally, homeorhetic alterations associated with the initiation of milk synthesis (e.g., increased nonesterified fatty acids [NEFA], hyperketonemia, hypocalcemia) have been inculpat for the periparturient immune suppression (Curtis, 1983; Goff and Horst, 1997; Ingvartsen and Moyes, 2013). Recent work has emphasized the role of immune activation in the periparturient period, where all cows, regardless of health status, encounter some degree of inflammation (especially in the first 2 weeks; Bertoni et al., 2008; Trevisi et al., 2015). The transition cow immune system has been alternatively described as dysregulated, where immune cell functions are seemingly suppressed, but inflammation is aggra-
vated (Sordillo et al., 2009; Trevisi and Minuti, 2018; LeBlanc, 2020).

We and others speculate that transition dairy cows are at an increased risk of LPS exposure via uterine, mammary, and intestinal epithelial hyperpermeability due to parturition, lactogenesis, and dietary changes that may exacerbate inflammation postpartum (Eckel and Ametaj, 2016; Horst et al., 2021). Prior reports allude to increased LPS tolerance during the transition period (Sheldon et al., 2020; Filipe et al., 2021), which is reduced immunogenicity following repeated or chronic exposure to LPS (Beeson, 1947). Some suggest LPS tolerance protects against infections or resulting immune activation (Wheeler et al., 2009; Petzl et al., 2011), while others speculate it dampens the immune system's ability to detect and mount an effective response (López-Collazo and del Fresno, 2013; Pena et al., 2014). Further, repeated LPS exposure is potentially causal to the development of several transition cow disorders (Zebeli et al., 2011). We speculated that transition cows develop LPS tolerance, which would curb the immune response relative to cows later in lactation, and this might explain, at least in part, their increased susceptibility to infections. A better understanding of the complex immune response and how it is temporarily regulated and coordinated during different physiological states could be relevant to animal health and periparturient management and nutrition. Therefore, objectives were to characterize different aspects of the immune response to an LPS challenge in early lactation (EL) and mid-lactation (ML) dairy cows and we hypothesized EL cows would be less immunogenic toward LPS than ML cows (demonstrated by an attenuated increase in rectal temperature, decreased circulating cytokines and acute phase proteins [APP], leukocytosis, and perturbations in ionized Ca ([iCa]) because of presumed prior LPS exposure during the periparturient period that may promote tolerance and hyporesponsiveness.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Iowa State University Animal Care and Use Committee. In brief, multiparous, non-pregnant EL (20 ± 2 DIM; 52 ± 6 kg daily milk yield; 2.5 ± 0.8 parity; n = 11) and ML (131 ± 31 DIM; 52 ± 5 kg daily MY; 2.8 ± 0.9 parity; n = 12) cows clinically healthy (<250,000 SCC on last test day with no reported clinical disease in the current lactation) and selected for similar milk yield were randomly assigned to 1 of 2 treatments by lactation stage (LS) equally distributed across 2 replicates to comprise a 2 × 2 factorial design: (1) EL (EL-LPS; n = 6) or ML (ML-LPS; n = 6) cows fed ad libitum and administered a single LPS bolus (0.09 µg/kg BW; approximately 15.3 µg/mL) from Escherichia coli O55:B5 in 4 mL sterile saline, i.v., or (2) control pair-fed (PF) EL (EL-PF; n = 5) or ML (ML-PF; n = 6) cows to LPS treatments of their respective LS and i.v. administered 4 mL of sterile saline. The LPS dose (0.09 µg/kg BW) and serotype were selected in an attempt to prevent maximum immune activation and allow potential LS differences to be exposed, based on prior research (Horst et al., 2019a; Abeyta et al., 2023). Cows were moved to individual box-stalls (4.57 × 4.57 m) at the Iowa State University Dairy Farm (Ames, IA) and acclimated for 4 d during which indwelling bilateral jugular catheters were inserted as described previously (Horst et al., 2020b). After acclimating, the study comprised 2 experimental periods (P). During P1 (3 d) baseline production data were collected and cows were fed ad libitum, and methods related to animal diet and milking regimen are reported in the companion paper (Opgenorth et al., companion paper). Treatments were administered at the beginning of P2 and cows were monitored for 3 d. Feed intake of LPS cows was recorded every 2 h such that PF cows would receive feed allotments every 2 h in an effort to maintain a similar pattern and amount of feed intake among treatments. To provide adequate time to calculate the PF regimen, the experimental timeline of PF cows began 2 d following LPS-infused cows.

During P2, blood samples were collected via jugular catheter at 0, 3, 6, 9, 12, 24, 36, 48, 60, and 72 h relative to i.v. treatment administration. Blood samples were collected and divided in 2 tubes containing K2EDTA (BD, Franklin Lakes, NJ), one for harvesting plasma, and another for complete blood count (CBC) analysis, and in a third tube containing a clotting agent (serum separator tubes; BD, Franklin Lakes, NJ) for harvesting serum. Serum samples were allowed to clot at room temperature before centrifugation. Plasma and serum were harvested after centrifugation at 1,500 × g for 15 min at 4°C before storing at −20°C until analysis. Plasma serum amyloid A (SAA), LPS-binding protein (LBP), and haptoglobin (Hp) concentrations were determined with commercially available kits (SAA, Tridelta Development Ltd., Kildare, Ireland; LBP, HyCult Biotech, Uden, the Netherlands; Hp, Life Diagnostics, West Chester, PA). The inter- and intra-assay coefficients of variation for SAA, LBP, and Hp were: 10.5 and 4.3%, 4.7 and 5.7%, and 8.2 and 2.8%, respectively. Whole blood (~3 mL) intended for CBC analysis was stored at 4°C until submission to the Iowa State University's Department of Veterinary Pathology. Serum exclusively from LPS cows were analyzed with
a MILLIPLEX Bovine Cytokine/Chemokine 15-plex kit (BCYT1–33K-PXBBK15; EMD Millipore Corporation, Billerica, MA, USA) utilizing antibodies to bovine IFN-γ, IFN-γ-inducible protein (IP)-10, IL-6, IL-8, IL-10, IL-36 receptor antagonist (RA), macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF)-A. The assay was performed according to the manufacturer’s instructions on a BioPlex 200 system (BioRad, Hercules, CA) with Bio-Plex Manager Software (v 6.1.1). Quality control values for each marker were consistently within the range indicated by the manufacturer. Additionally, blood was collected in heparinized tubes (BD, Franklin Lakes, NJ) and immediately analyzed for iCa with an iSTAT hand-held machine and cartridge (CG8+; Abbott Point of Care, Princeton, NJ). Rectal temperature and respiration rate were recorded hourly for the first 6 h, then at the 9th, and 12th h, and every 6 h thereafter. Rectal temperatures were measured using a digital thermometer (GLA M900 Digital Thermometer, San Luis Obispo, CA). Respiration rates were determined by counting flank movements for 15 s and multiplying the observed rate by 4 to obtain breaths per minute (bpm).

Statistical Analysis

Data were analyzed with the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) as described in the companion paper (Opgenorth et al., companion paper). In brief, fixed effects of treatment group (LPS vs. PF), LS (EL vs. ML), time, their interactions (i.e., group × LS, group × time, LS × time, and group × LS × time) and replicate were analyzed with a spatial power covariance structure for rectal temperature, respiration rate, acute phase proteins, CBC parameters, and iCa. Pre-planned contrasts were assessed to isolate group and LS treatment comparisons (i.e., EL-LPS vs. ML-LPS, EL-LPS vs. EL-PF, and ML-LPS vs. ML-PF). Because of costs, cytokines were only measured in LPS administered cows; thus, effects of LS (EL vs. ML), time, their interaction, and replicate were analyzed with an autoregressive covariance structure for cytokines. A logarithmic transformation was performed for MIP-1β analysis. Time relative to LPS administration served as a repeated measure and included the subject of cow within group. Pre-planned contrasts were assessed to isolate group and LS treatment comparisons (i.e., EL-LPS vs. ML-LPS, EL-PF vs. EL-LPS, and ML-PF vs. ML-LPS). Baseline data (collected at 0 h) were analyzed separately without a repeated measure but included a random effect of cow within group. Data are reported as least squares means ± standard error of the mean and considered significant if P ≤ 0.05 and a tendency if 0.05 < P ≤ 0.10. Sample size was determined by considering logistical constraints and prior research (Al-Qaisi et al., 2020; Horst et al., 2020a). Post-hoc power analysis (PROC POWER; SAS Institute Inc., Cary, NC) considering a primary parameter of interest (rectal temperature) indicated statistical power of > 99% (α = 0.05) when comparing EL-LPS and ML-LPS results. The EL-PF group originally contained n = 6, but one EL-PF cow had increased SCS during P2 (presumably due to subclinical mastitis) and was excluded from all statistical analyses; thus, EL-PF n = 5.

RESULTS

Production, metabolite, and hormone responses are documented in our companion paper (Opgenorth et al., companion paper). Cows administered LPS had increased rectal temperature compared with PF groups in the first 9 h (1.1°C; P < 0.01; Figure 1A) and the fever was more severe in EL-LPS than ML-LPS cows (1.1°C at 4 h peak, respectively; P < 0.01). Additionally, respiration rate was acutely increased in EL-LPS cows (47% on h 1) compared with ML-LPS cows and overall respiration rate throughout the 3-d challenge increased in EL-LPS compared with ML-LPS cows (24%; P < 0.01; Figure 1B). The circulating cytokine response corroborated the febrile response in LPS cows as TNF-α, IL-6, IL-10, MIP-1α, MIP-1β, MCP-1, and IP-10 peaked at 3 h and decreased over time (Time P ≤ 0.05; Figures 2A-G). Cows in EL-LPS had increased TNF-α, IL-6, MIP-1α, MIP-1β, MCP-1, and IP-10 relative to ML-LPS (63-, 4.8-fold, 93%, 10%, 57%, and 61%, respectively; P ≤ 0.05). Concentrations of IL-10 did not differ by LS overall, but EL-LPS had numerically increased concentrations relative to ML-LPS cows (P = 0.11). Further, IFN-γ increased post-LPS compared with baseline but did not differ by LS (P > 0.68; Figure 2H). Concentrations of IL-8 and IL-36RA were decreased whereas VEGF-α tended to increase after LPS relative to baseline, but neither were affected by LS (P > 0.24; data not shown).

Following LPS administration, cows had increased SAA concentrations relative to PF cows (9.0-fold; P < 0.01; Figure 3A), but this was not influenced by LS. Additionally, LBP increased following LPS in EL-LPS cows compared with EL-PF throughout the experiment (93%; P < 0.01; Figure 3B), but ML-LPS only had increased LBP at 12 h and a tendency for increased LBP at 24 h relative to ML-PF (99 and 67%; P = 0.03 and P = 0.09, respectively). Thus, overall circulating LBP between ML-LPS and ML-PF did not differ (P > 0.25), and throughout the 3 d post-LPS, EL-LPS had increased LBP compared with ML-LPS (85%; P
Haptoglobin increased in LPS relative to PF cows (153-fold; \( P < 0.01 \); Figure 3C), and EL-LPS had a more robust Hp response than ML-LPS (2.0-fold from 48 to 72 h; \( P < 0.01 \)).

Circulating neutrophil concentrations abruptly decreased at 3 h after LPS infusion regardless of LS and gradually increased thereafter and neutrophilia developed by 24 h in all LPS cows (2-fold increase in neutrophils in LPS relative to PF; Figure 4A). The neutrophilic period was more intensified in EL-LPS than ML-LPS cows (63% from 24 to 72 h; \( P < 0.01 \)). Circulating lymphocytes decreased at 3 h in LPS relative to PF cows (82%; \( P < 0.01 \); Figure 4B) and gradually returned to PF concentrations by 24 h regardless of LS. Monocytes followed a similar pattern as lymphocytes in the LPS groups, where concentrations were lowest at 3 h (~98% relative to PF cows; \( P < 0.01 \)) and returned to pre-LPS levels by 48 h regardless of LS (Figure 4C). However, post-hoc analysis at 72 h after LPS revealed EL-LPS had increased monocytes (2-fold relative to EL-PF; 57% relative to ML-LPS; \( P < 0.01 \)). Platelet concentrations in LPS cows decreased at 3 h (75% relative to PF; \( P < 0.01 \)), and from 24 to 48 h post hoc analysis revealed platelets were more decreased in EL-LPS than ML-LPS cows (41%; \( P = 0.02 \); Figure 4D). Mean platelet volume (MPV) increased dramatically 3 h after LPS administration in cows from both LS (46% relative to PF counterparts; \( P < 0.01 \); Figure 4E), but ML-LPS MPV decreased and did not differ from ML-PF by 6 h whereas in EL-LPS cows had maintained elevated MPV until 48 h such that throughout the experiment, MPV in EL-LPS was substantially increased relative to EL-PF and ML-LPS cows (28 and 15%, respectively; \( P < 0.01 \)).

Circulating iCa decreased in LPS relative to PF cows (34% at 6 h nadir; \( P < 0.01 \); Figure 5), but the hypocalcemia was more pronounced in EL-LPS than ML-LPS cows (14% at 6 h nadir and 8% overall; \( P < 0.01 \)).
Figure 2. Effects of lipopolysaccharide (LPS) and lactation stage (LS; early [EL] or mid-lactation [ML]) on (A) tumor necrosis factor (TNF)-α, (B) IL-6, (C) IL-10, (D) macrophage inflammatory protein-1α (MIP-1α), (E) ln MIP-1β, (F) monocyte chemoattractant protein (MCP)-1, (G) IFN-γ-inducible protein (IP)-10, and (H) IFN-γ. The 0 h baseline data represent an average of each LS before LPS administration but were not used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. 
DISCUSSION

Increased disease prevalence in early lactation is traditionally attributed to immune suppression (decreased neutrophil migration and effector functions; Newbould et al., 1976; Hill et al., 1979; Kehrli et al., 1989), which is purportedly caused by the periparturient metabolic and mineral milieu (i.e., increased NEFA, hyperketonemia, and hypocalcemia; Targowski and Klucinski, 1983; Goff and Horst, 1997; Ingvartsen and Moyes, 2013; Vanacker et al., 2022). However, numerous investigators have identified variable and elevated biomarkers of inflammation (i.e., cytokines and APP) in transition cows, even in the absence of clinical disease (Bertoni et al., 1997; Brodzki et al., 2015; Trevisi et al., 2015). The source of this inflammation can vary and is often not obvious. Parturition is an inflammatory event (e.g., via uterine contractions, cervical dilation, etc.; Norman et al., 2007) during which a large percentage of cows acquire some degree of uterine bacterial contamination (Paisley et al., 1986; Földi et al., 2006), and the dietary transitions and lactation onset increase epithelial barrier exposure to pathogen insult (Eckel and Ametaj, 2016). Although increased inflammation observed in transition cows demonstrates a normal response to pathogen exposure, repeated immune stimulation toward LPS develops a state of tolerance whereby leukocytes become less inflammatory (i.e., decreased cytokines, neutrophil adhesion, and fever; Beeson, 1947; Ziegler-Heitbrock, 1995). This hyporesponsiveness of the immune system is also observed in periparturient cows (i.e., delayed diapedesis of neutrophils; Hill et al., 1979; Frost and Booker, 1986; Mallard et al., 1998), which has been attributed to some transition cow morbidities in response to repeated LPS exposure (Zebeili et al., 2011). Thus, we hypothesized EL cows may develop a state of LPS tolerance during the transition period that would make them less immune-responsive toward LPS, which might partly explain their increased susceptibility to disease. In stark disagreement with our hypothesis, EL cows appeared to have a much more intense immune responses (based upon multiple metrics; see below) to LPS than ML cows.

Rectal temperature increased after LPS administration, demonstrating that immune activation was successfully induced. Further, the magnitude of fever was more severe in EL-LPS cows than in ML-LPS. The intense fever (40.7°C at 4 h peak) in EL-LPS cows agrees with previous LPS models where peak vaginal temperatures were similar to ours in EL cows (Brandão et al., 2016; Chandler et al., 2022), whereas we and others report much lower rectal temperatures (~39.5°C) in ML cows (Kvidera et al., 2017; Al-Qaisi et al., 2020; Horst et al., 2020a,b). Fever enhances leukocyte func-
tions and responsiveness toward LPS and is thus important for surviving an infection (Rosenspire et al., 2002; Lee et al., 2012). Interestingly, mounting a fever is energetically expensive (Kluger, 1979) and this may help explain the increased respiration rate in EL cows, ostensibly to provide increased oxygen to support the enhanced metabolic rate (Fischler and Reinhart, 1997). Endotoxin administration can also cause pulmonary edema and hemorrhage (Bahrami et al., 1997; Chen et al., 2014), but whether this contributed to the increased respiration rates in EL cows, or why bronchial vasculature would be more susceptible in EL cows is unknown. Nonetheless, overall, EL-LPS cows clearly had a more intense fever and respiratory response than cows in established lactation.

Leukocytes have toll-like receptor 4 (TLR-4), which, after recognizing LPS, initiates an immune response (i.e., increased cytokine production, leukocyte proliferation, and exertion of effector functions; Sabroe et al., 2005; Lu et al., 2008; Arango Duque and Desco-
teaux, 2014). Herein, increased circulating IL-6 and TNF-α after LPS agrees with others (Chandler et al., 2022; Choudhary et al., 2023) and demonstrates that leukocytes detected and responded to LPS. Further, EL-LPS cows had increased IL-6 and TNF-α relative to ML-LPS cows, indicating heightened responsiveness to LPS by immune cells in EL and this agrees with ex vivo cytokine responses (Brink et al., 2022). Moreover, because both cytokines contribute to the mounting of a fever (Conti et al., 2004), the elevated IL-6 and TNF-α ostensibly explains, at least in part, why EL cows had exacerbated febrile and hypophagic responses to LPS (described in the companion paper; Opgenorth et al., companion paper).

In addition to pyrogenic cytokines, IL-10 (an anti-inflammatory cytokine) increased after LPS, which corroborates others and suggests efforts to control the inflammatory response (Rennick et al., 1997; Choudhary et al., 2023). Interleukin-10 dampens macrophage pro-inflammatory cytokine production and macrophage maturation to limit tissue damage (Moore et al., 2001), which protects against the harmful effects of an over-activated inflammatory response. Although not significant \( (P = 0.11) \), IL-10 was numerically increased in EL-LPS compared with ML-LPS cows. In healthy EL cows, IL-10 is typically increased relative to cows in later lactation (Britti et al., 2015). Further, similar to TNF-α and IL-6, MCP-1, and IP-10 in part govern leukocyte function and migration into inflammatory sites (Fahey et al., 1992; Rollins, 1997; Dufour et al., 2002), and were unsurprisingly increased following LPS. The chemokines MIP-1α and MIP-1β are synthesized by a diversity of myeloid and lymphoid cells in response to LPS and they recruit macrophages and lymphocytes to enhance inflammation and wound healing (Chavas et al., 2015). Likewise, MCP-1 is produced by and attracts a variety of cells – most notably macrophages and monocytes (Yadav et al., 2010), but it also carries out protective roles through enhancing bacterial clearance and controlling pro-inflammatory cytokine synthesis (Zisman et al., 1997; Gomes et al., 2013). Lipopolysaccharide stimulates IP-10 production in macrophages, which recruit T cells (Loetscher et al., 1996; Kopydowski et al., 1999). Further, similar to TNF-α and IL-6, MIP-1α, MIP-1β, MCP-1, and IP-10 were elevated in EL-LPS relative to ML-LPS cows. Overall, cytokines were increased in LPS relative to PF cows, and many were further exacerbated in EL-LPS cows. This in vivo LS cytokine difference agrees with some (Sordillo et al., 1995; Røntved et al., 2015; Jahan et al., 2015), but not other (Revelo and Waldron, 2012) models of ex vivo LPS stimulation of either whole blood or isolated immune cells from periparturient cows. Reasons for the inconsistencies might be because Revelo and Waldron (2012) specifically analyzed isolated neutrophils whereas Sordillo et al. (1995) and Jahan et al. (2015) stimulated monocytes and whole blood, respectively. Although inconsistent (LeBlanc, 2020), some neutrophil functions that are associated with LPS-induced immune activation (i.e., microbicidal activities; Böhmer et al., 1992) are reduced in peripartum cows (Newbould, 1976; Kehrli et al., 1989; Dosogne et al., 2001), which might be attributed to their preference for increased tissue remodeling functions after parturition (i.e., extracellular matrix degradation; Burton et al., 2005). Although neutrophils secrete cytokines, most circulating cytokines are produced by macrophages and lymphocytes (Zhang and An, 2007). Thus, results herein suggest the enhanced cytokine response observed in EL-LPS cows is more likely due to hyperresponsiveness by immune cells other than neutrophils.
Increased SAA, LBP, and Hp concentrations following LPS agree with other LPS models (Kvidera et al., 2017; Horst et al., 2019b; Chandler et al., 2023) and further confirm that we successfully employed an immune activating event. In concert with other immune biomarkers, LBP and Hp were further increased in EL-LPS cows, indicating a more robust acute phase response. Interleukin-6 and TNF-α enhance hepatic positive APP production (Heinrich et al., 1990; Thomsen et al., 2013), so their amplified response in EL-LPS may explain an increased APP synthesis relative to ML-LPS cows. During LPS-induced immune activation, Hp exerts protective effects by controlling monocyte activation and cytokine production (Arredouani et al., 2005); likewise, LBP can neutralize LPS and reduce cytokine release from mononuclear cells during an acute phase response (Gutsmann et al., 2001). Thus, similar to IL-10, the exaggerated Hp and LBP in EL-LPS cows may indicate a negative feedback loop in an attempt to dampen the hyperinflammatory response. Interestingly, EL-LPS cows also had increased circulating BUN and MUN post-LPS compared with ML-LPS cows (Opgenorth et al., companion paper). Synthesizing APP requires a large amount of AA (Iseri and Klasing, 2014) and this demand occurs simultaneously with immune-induced inappetence (Horst et al., 2019b; Al-Qaisi et al., 2020). Skeletal muscle is catabolized to supply the necessary AA for APP synthesis; the excess AA not used for APP production are deaminated, and the amino group is converted into urea (Thomsen et al., 2013). Some skeletal muscle derived AA are also used for gluconeogenesis and the deaminated amino group also contributes to urea synthesis (see companion paper; Opgenorth et al., companion paper). Therefore, the temporal patterns of IL-6, LBP, Hp, BUN and MUN imply that EL cows coordinate physiology and metabolism to support a heightened immune response. Regardless, increased Hp and LBP in EL-LPS relative to ML-LPS cows provided more evidence that EL-LPS cows had a more robust immune response to LPS than ML-LPS cows.

Circulating leukocytes and platelets were markedly altered following LPS administration and this additionally confirms that systemic immune activation was achieved. The LPS-administered cows developed an early neutropenic response followed by neutrophilia before returning to baseline concentrations, and this pattern agrees with previous models (Lehtolainen et al., 2003; Horst et al., 2019b, 2020a). The neutropenia phase did not differ by LS, but EL-LPS had a more pronounced neutrophilic period than ML-LPS cows and this agrees with results from an intramammary LPS model (Lehtolainen et al., 2003). Once endothelial cells and circulating leukocytes are activated by LPS or inflammatory mediators, expression of adhesion molecules (i.e., L-selectin) increases to allow neutrophils to migrate out of circulation into inflammatory sites (Maeger, 1999; Zhou et al., 2005). Increased circulating neutrophils over time presumably consists of immature neutrophils that migrate from bone marrow in response to acute immune activation (Diez-Fraile et al., 2003). Further, bovines have a smaller segmented (i.e., mature) neutrophil storage capacity relative to other species (Stockham and Scott, 2008), and therefore, the neutrophilic phase described herein ostensibly consists of even a larger percentage of underdeveloped neutrophils. Immature circulating neutrophils are less effective, especially when evaluated using ex vivo functionality assays (Pillay et al., 2010; Leliefeld et al., 2016), and this has profound implications for our understanding of parturient “immune suppression” (Horst et al., 2021). Reasons why EL-LPS cows had a more pronounced and prolonged neutrophilia phase are not clear, but a key mediator of bone marrow neutrophil production and secretion is granulocyte colony-stimulating factor (Furze and Rankin, 2008), which is released following both LPS and TNF-α exposure (Koeffler et al., 1987; Dale et al., 1995). The acute and more robust increase in TNF-α from EL-LPS cows likely contributed to, at least in part, the exaggerated neutrophilia in EL cows.

Lymphocytes and monocytes likewise decreased after LPS administration in both LS groups and this pattern following immune activation was expected (Richardson et al., 1989); however, similar to the neutrophil pattern, EL-LPS cows had increased monocytes compared with ML-LPS cows at 72 h, which alludes to heightened responsiveness to LPS (Yates et al., 2011). Monocytosis that developed in EL-LPS cows likely indicates increased tissue demand for macrophage replacement (Chen et al., 2018). Platelets were decreased whereas MPV was increased in LPS-administered cows, and this pattern is consistent with other reports following LPS and *Escherichia coli* mastitis in dairy cows and septic human patients (Aydemir et al., 2012; Hagiwara et al., 2014; Horst et al., 2019b). Platelets and MPV are negatively correlated after LPS: decreased platelets explain increased aggregation, while increased MPV indicates upregulated platelet release from bone marrow (Yilmaz et al., 2008). The pattern of reduced platelets but increased MPV was more pronounced in EL-LPS than in ML-LPS cows, indicating EL-LPS had augmented platelet proliferation and aggregation. Platelets are more sensitive to LPS activation than leukocytes (Brooks et al., 2007), but reasons why EL-LPS appear to have increased platelet activation are not clear. Altogether, perturbations in leukocyte and platelet parameters support the interpretation that EL-LPS cows had a heightened immune activation.
Lastly, LPS administration acutely decreased circulating iCa and this is a highly conserved response across species (Carlstedt et al., 2000; Toribio et al., 2005; Al-Quisi et al., 2020; Horst et al., 2020a). Cows in both LS developed transient hypocalcemia, but EL-LPS cows had a more intense decrease in iCa than ML-LPS cows. The magnitude and pattern of hypocalcemia was similar to that described in early lactation (Chandler et al., 2023), but the severity of this response depends upon the LPS dose and LS (Kvidera et al., 2017; Horst et al., 2020). Decreased iCa after LPS appears to be influenced by increased disease severity (Zivin et al., 2001) rather than metabolic differences by LS since pre-LPS iCa concentrations in EL-LPS and ML-LPS cows were similar. Immune-induced hypocalcemia is seemingly a strategy the animal purposefully initiates to facilitate a non-immune system approach of endotoxin clearance. Hypocalcemia is thought to promote endotoxin sequestration into lipoproteins for eventual hepatic clearance and this route presumably reduces the endotoxin load that TLR-4 presenting cells need to detoxify, a scenario that apparently minimizes collateral damage from excessive inflammation (Weinstock et al., 1992; Birjimohun et al., 2007; Horst et al., 2021). A concept that is reinforced by intervening experimentation demonstrating blunted inflammation following i.v. lipid infusion (Gordon et al., 2005; Horst et al., 2020b). Interestingly, some circulating lipoprotein components are decreased in EL relative to ML cows (Raphael et al., 1973; Takahashi et al., 2003; Kessler et al., 2014). Regardless, the exaggerated hypocalcemia in EL-LPS cows agrees with increased rectal temperature, cytokines, and APP and provides additional evidence that the EL cow immune system was more intensely engaged in response to LPS than ML-LPS cows. Interestingly, iCa did not decrease in the PF group, which suggests that suboptimal DMI is not responsible for periparturient hypocalcemia as has been suggested (Seely et al., 2021). Regardless, our observations have interpretive implications for the causes, consequences, and potential intervention strategies of post-calving circulating Ca patterns.

Our hypothesis that EL cows would be less responsive to LPS was based on results indicating decreased TLR-4 expression and antigen-presenting machinery (2 indicators of LPS tolerance; Nomura et al., 2000; Wolk et al., 2000) in rumen epithelial cells, milk somatic cells, and circulating and lymph-resident leukocytes in early postpartum cows (Sordillo et al., 1995; Catalini et al., 2010; Cui et al., 2013; Mukherjee et al., 2013; Minuti et al., 2015). Clearly, our tenet could not have been more wrong, and reasons why EL cows had such a heightened immune activation toward LPS are not obvious. We speculate that the incongruity between the purported decreased TLR-4 expression in multiple cell types and the exaggerated immune response described herein could be the result of a phenomenon referred to as “stacked stressors” (Paterson et al., 2003; Murphy et al., 2004) where an initial immune event primes the immune system to undergo a hyperinflammatory response during a second insult (called the “two-hit” hypothesis; Moore et al., 1993). A transition cow encounters multiple stressors within a short window of time, and these include nutritional (dietary shifts), physiological (parturition, uterine involution; gastrointestinal tract hypertrophy), psychological (pen moves, routine disruptions, overcrowding) and potential pathogenic exposure at multiple endothelia (clinical and subclinical metritis and mastitis; gastrointestinal tract). An infectious stress would also include a plethora of different types of pathogen associated molecular patterns (stemming from the diverse microbiome of the GIT, mammary gland and uterus) and this may help explain the lack of tolerance observed in the current study. Irrespective of the mechanism(s), many aspects of the immune system are clearly highly sensitive to endotoxin in early lactation and having a better appreciation for these LS differences will likely have important implications for transition cow management, nutrition, and veterinary care.

There are some aspects of our experimental design that warrant consideration. The first is that the EL cows were approximately 20 DIM and while still in the arbitrary “transition” period, we may have missed the “suppressed” window. The second is that we utilized a single i.v. pulse of LPS and this approach and route clearly do not accurately reflect normal immune activation that typically accompanies the periparturient period (Horst et al., 2019a). The third is that the basal “inflammatory tone” was not measured before experimental enrollment. However, we utilized milk SCC, calving-ease score, and clinical veterinarian assessments in an attempt to ensure that clinically healthy cows from both LS were utilized in the trial. Besides, “basal” inflammation (at least acutely) is represented by the time zero data points. Regardless of the aforementioned limitations, the experimental design was robust enough to identify multiple differences in how EL and ML cows respond to immune activation.

Multiple prior reports describe EL cows as immune-suppressed (Lloyd, 1983; Kehrli et al., 1989; Sordillo, 2005), but it is becoming increasingly axiomatic of how dynamic the periparturient immune system is (Trevisi and Minuti, 2018). While some immune functions are considered suppressed (Wells, 1977; Kehrli et al., 1989), others are upregulated (Mann et al., 2019; Minuti et al., 2020). The hyperactive inflammatory response observed herein undoubtedly agrees with this latter supposition.
Like the transition cow, newborns are presumably immune suppressed, but are similarly hyperresponsive to LPS (Zhao et al., 2008; Brook et al., 2017). Harbeson et al. (2018) challenged the infant immune suppression paradigm by explaining how neonatal immune suppression is an easily described yet imprecise term to explain the complex problem of increased neonatal disease susceptibility. Likewise, we find it difficult to understand how evolution would favor an immune suppressive or dysregulated state in EL: a physiological scenario that would clearly not benefit survival or fitness of both the mother and offspring (Horst et al., 2021). Regardless of the natural selection speculation, our data further proffs the notion that the term “immune-suppressed” does not comprehensively or accurately describe many aspects of the transition cow’s immune system.

CONCLUSION

Early lactating cows have a much more pronounced immune response to endotoxin than ML cows and this is characterized by exacerbated changes in multiple variables. Overall, EL cows appear to have a very dynamic immune response, which does not harmonize with the dogma of periparturient immunosuppression. Elucidating why certain aspects of the immune system are highly sensitive to an pathogen in early lactation is necessary to fully understand the coordinated immune defense strategy of periparturient cows. A better appreciation for the aforesaid is a prerequisite for developing interventions that simultaneously benefit the cow and the farmer.

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