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Induced endometritis in early lactation compromises production and reproduction in dairy cows

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ABSTRACT

Objectives of this experiment were to study the effect of infusing utero-pathogenic bacteria to induce endometrial inflammation on productive performance in early lactation and subsequent reproduction. Although endometritis is associated with perturbed reproduction, numerous factors may contribute to the observed association. It was hypothesized that induced endometrial inflammation, resulting in localized and systemic inflammatory responses, compromises production and reproduction. Holstein cows without clinical disease and with less than 18% polymorphonuclear leukocytes (PMN) in endometrial cytology on d 31 ± 3 postpartum had their estrous cycle synchronized. Cows were blocked by parity and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as untreated controls (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48). Endometrial cytology was taken on d 2 and 7 after treatment to evaluate the proportion of PMN. Rectal temperature, dry matter intake, and yields of milk and components were measured in the first 7 d after treatment. Blood serum was analyzed for concentration of haptoglobin. Leukocytes were isolated from blood on d 2 and 7 after treatment and on d 19 after artificial insemination (AI) and mRNA was quantified for a select group of genes. Cows received AI and reproduction was followed for 300 d postpartum. Bacterial infusion induced endometrial inflammation with increased proportions of PMN in the endometrial cytology on d 2 (4.4 ± 0.7 vs. 26.3 ± 2.8%) and 7 (10.9 ± 1.7 vs. 17.4 ± 2.1%) after treatment, resulting in increased mean prevalence of subclinical endometritis (>10% PMN; 23.3 ± 6.3 vs. 80.9 ± 5.1%). Rectal temperature did not differ between CON and INF, but the concentration of haptoglobin in serum tended to increase in INF compared with CON (113 ± 14 vs. 150 ± 16 μg/mL). Induced endometrial inflammation reduced yields of milk (44.9 ± 0.8 vs. 41.6 ± 0.8 kg/d), protein (1.19 ± 0.03 vs. 1.12 ± 0.03 kg/d), and lactose (2.17 ± 0.04 vs. 2.03 ± 0.04 kg/d) and tended to reduce dry matter intake (20.7 ± 0.5 vs. 19.4 ± 0.6 kg/d) in the first 7 d after treatment. Indeed, the reduction in milk yield lasted 4 wk. However, treatment did not affect yields of energy-corrected milk or fat because treatment with INF increased the concentration of fat in milk (3.54 ± 0.10 vs. 3.84 ± 0.10%). Induced endometrial inflammation reduced pregnancy per AI at all inseminations (33.4 ± 5.1 vs. 21.6 ± 3.7%) and the hazard of pregnancy (0.61; 95% CI = 0.36–1.04), which extended the median days open by 24 d. Blood leukocytes from INF cows had increased mRNA expression of the pro-inflammatory gene *IL1B* on d 2 and 7 after treatment, but reduced expression of the IFN-stimulated genes *ISG15* and *MX2* on d 19 after AI. Induced endometrial inflammation depressed production and caused long-term negative effects on reproduction in lactating dairy cows.

Key words: dairy cow, induced endometritis, inflammation, reproduction

INTRODUCTION

Early lactation is characterized by an increased risk of diseases in dairy cows (Carvalho et al., 2019). Most diseases that affect cows in early lactation are of an inflammatory nature and have been linked with depressed reproductive performance because they are associated with delayed cyclicity (Santos et al., 2010), reduced embryo and conceptus development (Ribeiro et al., 2016), and increased risk of pregnancy loss (Ribeiro et al., 2016; Edelhoff et al., 2020). Uterine diseases such as metritis and endometritis are among the most prevalent periparturient diseases affecting dairy cows, and they are associated with an increased acute phase

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protein response and immune dysregulation (Galvão et al., 2010, 2011; Bromfield et al., 2015). One of the consequences of inflammation and immune system activation is hypophagia (Brown and Bradford, 2021), which is expected to reduce productive performance (Bell and Roberts, 2007). Thus, it is not surprising that cows that develop uterine disease have compromised productive and reproductive performance (Carvalho et al., 2019; Edelhoff et al., 2020). Nevertheless, postpartum cows often have multiple concurrent health problems, thus making it more difficult to discern the effects of endometrial inflammation from other associated disorders on production and reproduction.

Pregnancy is sensitive to maternal metabolic cues and health status of the dam. Although extensive literature exists linking uterine diseases and compromised reproduction, most of the data are epidemiological in nature. Recently, a model to induce endometrial inflammation has been described to study the effects on the reproductive tract (Piersanti et al., 2019). Uterine infusion of utero-pathogenic *Escherichia coli* and *Trueperella pyogenes* in nulliparous (Piersanti et al., 2019) and dry primiparous cows (Dickson et al., 2020) induced localized and systemic signs of inflammation with increased endometrial influx of PMN and concentration of haptoglobin in blood that lasted days to weeks after the treatment. A consequence of induced endometrial inflammation was a transient reduction in the proportion of oocytes or zygotes that developed to morula following in vitro embryo production (Dickson et al., 2020). Induced endometrial inflammation altered the transcriptome of oocytes (Piersanti et al., 2020) and several reproductive tissues (Horlock et al., 2020) days to weeks after the treatment, likely mediated by the pathogen-associated molecular patterns and damage-associated molecular patterns that affect numerous cellular pathways in the endometrium and ovaries (Bromfield et al., 2015; Sheldon et al., 2019). One of the consequences of uterine disease is the reduced survival of pregnancy that has been demonstrated after AI (Ribeiro et al., 2016) or embryo transfer (Ribeiro et al., 2016; Edelhoff et al., 2020). Nevertheless, a limitation in the literature is the lack of randomized experiments demonstrating that the depression in production and reproduction are indeed caused by inflammatory diseases that affect the uterus of dairy cows.

The hypothesis of the present experiment is that induced endometrial inflammation causes localized and systemic inflammatory responses that have long-term detrimental effects on reproduction in dairy cows. A second hypothesis was that induced endometrial inflammation depresses DMI and compromises productive performance in dairy cows. Thus, the objectives of the present experiment were to use a previously described model of induced endometritis to characterize endometrial inflammation, systemic inflammatory responses, blood metabolites, and investigate effects on DMI, production, and reproduction in dairy cows.

MATERIALS AND METHODS

All procedures used in the present experiment were approved by the University of Florida Institutional Animal Care and Use Committee (protocol number 201508884) and the experiment was conducted at the University of Florida Dairy Research Unit.

Bacterial Culture and Preparation of Inocula

Escherichia coli MS499 and *T. pyogenes* MS249 were collected and isolated from cows with metritis characterized previously (Goldstone et al., 2014a,b). *Escherichia coli* was cultured from frozen glycerol stocks on Luria-Bertani agar. The day before infusion, a single colony was picked from the plate and inoculated into Luria-Bertani broth containing 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. The culture was incubated overnight at 37°C with shaking at 200 rpm. The overnight culture was diluted 1:100 into 50 mL of fresh Luria-Bertani broth and incubated 7 h to reach stationary phase. After incubation, 1 mL of bacterial cells were collected by centrifugation at 10,000 × *g* for 5 min at 4°C and washed with sterile PBS. We prepared 9 batches of inoculum and diluted in PBS that resulted in a mean (± SD) of $5.19 \pm 1.09 \times 10^7$ cfu/mL *E. coli*.

Trueperella pyogenes MS249 was grown from frozen glycerol stocks on Trypticase Soy Blood agar at 37°C for 48 h. The day before infusion, a single colony was selected and inoculated into Bacto Brain Heart Infusion broth (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific) and cultured at 37°C with shaking at 200 rpm for 48 h to reach stationary phase. Nine batches of inoculum were prepared and diluted in PBS, resulting in a mean of $4.34 \pm 1.05 \times 10^7$ cfu/mL *T. pyogenes*.

Individual syringes containing each of the 2 inocula were kept and transported in ice to the farm on the day of treatment.

Sample Size Calculation, Experimental Design, and Treatments

A 2-tailed sample size was calculated using the POWER procedure in SAS version 9.4 (SAS/STAT, SAS Institute Inc.) for the hazard of pregnancy using data from Edelhoff et al. (2020) for an expected hazard ratio of 0.65 when cows are induced to develop endometrial inflammation relative to control cows ($\alpha =$

0.05; $\beta = 0.20$) assuming a coefficient of determination of 0.02, standard deviation of 0.85, and probability of event of 0.80 in the first 300 DIM. The sample size calculated was 75 experimental units. Additional cows were added to accommodate potential attrition during the experiment.

The experiment was a randomized complete block design. All cows included in the experiment had a live singleton calf, and did not have milk fever, retained placenta, or metritis. Furthermore, endometrial cytology was performed on d 31 ± 3 postpartum to exclude cows with >18% PMN, based on the threshold of subclinical endometritis suggested by Kasimanickam et al. (2004) for cows evaluated by 33 DIM. On d 31 ± 3 postpartum (Figure 1), cows received an i.m. injection of 100 μg of GnRH (OvaCyst, 50 μg/mL of gonadorelin diacetate tetrahydrate, Bayer HealthCare LLC) and those with a corpus luteum detected by ultrasonography on d 37 ± 3 postpartum and that met the inclusion criteria were enrolled in the experiment.

Eighty-five Holstein cows in early lactation met the inclusion criteria and were enrolled in the experiment at 37 ± 3 DIM. Cows in each weekly cohort were blocked by parity group, as primiparous or multiparous cows, and genomic breeding value for cow conception rate and, within block, randomly assigned to remain as untreated controls (**CON**; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *E. coli* and 4.34×10^8 cfu *T. pyogenes* at 37 ± 3 DIM to induce endometrial inflammation (**INF**; n = 48). Eleven weekly cohorts of cows were assigned to the experiment, and for every weekly cohort, the blocks contained 2 cows as follows: 1 cow was randomly assigned to CON and 1 to INF, except for 11 blocks that contained 3 cows, 1 randomly assigned to CON and 2 to INF. This randomization system was applied to result in more INF than CON cows because of the risk of INF result in very few pregnant cows. Cows received a second i.m. injection of 100 μg of GnRH at 37 ± 3 DIM (Figure 1) to ensure that they remained in the luteal phase during the days following enrollment in the experiment. The rationale to have CON in which cows remained untreated was to minimize induction of endometrial inflammation as infusion of sterile solutions into the uterus elicit an influx of PMN resulting in some cows presenting inflammatory response with mucopurulent or purulent vaginal discharge (Piersanti et al., 2019; Dickson et al., 2020).

At 31 DIM, CON and INF cows had, respectively, means (± SD; median) of 2.3 (± 1.0; 2) and 2.3 (± 1.3; 2) lactations ($P = 0.50$); 7.9 (± 5.2; 6.0) and 7.8% (± 5.5; 7.5) PMN in endometrial cytology ($P = 0.93$); 0.70 (± 0 .36; 0.70) and 0.65 (± 0.33; 0.70) PTA for metritis ($P = 0.48$); 2.34 (± 1.32; 2.40) and 2.27 (± 1.36; 2.00) PTA for cow conception rate ($P = 0.82$); and 1.30 (± 1.06; 1.30) and 1.17 (± 1.29; 1.10) PTA for daughter pregnancy rate ($P = 0.59$).

Intrauterine treatment in INF cows was performed as described by Piersanti et al. (2019) with modification. No endometrial scarification was performed before bacterial infusion to avoid excessive endometrial inflammation because of physical trauma. Cows were restrained and received a caudal epidural injection of 60 mg of lidocaine hydrochloride 2% (Aspen Veterinary Resources). The perineum and the exterior vulva were

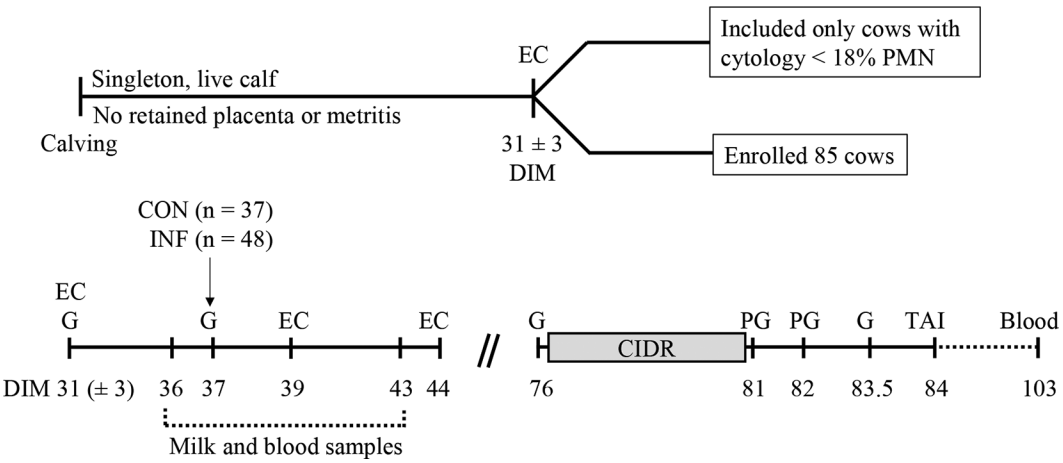

Figure 1. Diagram of the experiment. Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as controls (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48). CIDR = controlled-internal drug release containing progesterone; EC = endometrial cytology; G = GnRH (100 μg of gonadorelin; OvaCyst, 50 μg/mL of gonadorelin diacetate tetrahydrate, Bayer); PG = prostaglandin $F_{2\alpha}$ (25 mg of dinoprost; Lutalyse sterile solution, 5 mg/mL of dinoprost as tromethamine salt, Zoetis); PMN = polymorphonuclear leukocytes; TAI = timed artificial insemination.

cleaned with betadine soap (VEDCO, Povidone-iodine 0.75% solution) followed by a spray of 70% ethanol. A Neilson catheter (45 cm; Supplies for Farmers) covered in a sanitary sheath (IMV Technologies) was introduced into the reproductive tract and guided through the cervix to reach the uterine body via transrectal palpation. Once in the last ring of the cervix, the sheath was retracted to expose the catheter port and the solution was dispensed into the body of the uterus. The infusate was 30 mL total, including 10 mL of PBS containing *E. coli* MS499, 10 mL of PBS containing *T. pyogenes* MS249, and a final 10 mL of PBS to flush the catheter. During the experimental period, cows did not receive any additional treatments or medication. Investigators were not blind to treatments.

Endometrial Cytology and Counting of Cells

Endometrial samples were collected for cytology on d 2 and 7 after enrollment (Figure 1). The perineum and the exterior vulva were cleaned with betadine soap followed by a spray of 70% ethanol. An endometrial sample was collected using a cytobrush (Medscand Medical, Cooper Surgical) as previously described (Kasimanickam et al., 2004; Lima et al., 2015). The cytobrush was smeared onto a clean glass slide, air-dried, and stained with Rapid-Chrome Kwik-Diff (Thermo Fisher Scientific). A total of 200 cells, including endometrial epithelial cells, mononuclear leukocytes, and PMN, were counted at 40× magnification (Nikon Instruments) and the proportion of PMN was determined. Subclinical endometritis was defined as >10% PMN in endometrial cytology (Kasimanickam et al., 2004).

Rectal Temperature, Blood Sampling, and Serum Assays

Rectal temperature (AG-102 thermometer, AG-Medix) was measured twice daily, between 0600 to 0800 h and 1800 to 2000 h, from d 0 to 7 after enrollment.

Blood was sampled from the coccygeal vessels into evacuated tubes (Vacutainer, Becton Dickson) containing no additive once daily on d 0 to 6 after enrollment for serum separation. Tubes were kept at ambient temperature for 30 min to allow clotting and then placed on ice and transported to the laboratory within 3 h of collection. Tubes were centrifuged at 2,000 × *g* for 20 min at room temperature for serum separation, then aliquoted and frozen at −20°C until further analyses.

Concentrations of haptoglobin in serum were measured according to Makimura and Suzuki (1982) using a standard curve designed with serum with high and low concentrations of haptoglobin quantified using a commercial kit (Cow Haptoglobin ELISA, Hapt-11; Life Diagnostics Inc.). Intra- and interassay coefficients of variation were 8.4 and 10.2%, respectively. Haptoglobin was measured in serum collected before treatments, and on d 1 to 6 after treatments.

Concentrations of fatty acid in serum were evaluated using a commercial kit (NEFA-C kit, Wako Diagnostics Inc.) following the modifications by Johnson and Peters (1993). Intra- and interassay coefficients of variation were 2.2 and 3.6%, respectively. Concentrations of glucose in serum were measured using a commercial kit (Glucose Oxidase G520–480 kit, Teco Diagnostic Kit). Intra- and interassay coefficients of variation were 4.0 and 8.3%, respectively. Concentrations of glucose and fatty acids were measured in serum collected before treatments, and on d 1 to 3 after treatments.

Blood Sampling and Isolation of Peripheral Blood Mononuclear Cells

Blood was sampled by puncture of the coccygeal vessels into 10-mL lithium-heparinized tubes (Vacutainer, Becton Dickinson) on d 2 and 7 after enrollment, and on d 19 after the first AI. The mononuclear leukocytes (**PBMC**) were isolated by Ficoll gradient centrifugation (Ficoll-Paque Premium, Cytiva). Briefly, 2 mL of whole blood diluted in equal volume of PBS was layered on 3 mL of Ficoll in 15-mL conical tubes and centrifuged at 500 × *g* for 40 min at 18°C. The layer containing mononuclear leukocytes was carefully aspirated and transferred into a 15-mL tube and washed twice with PBS at a 1 to 3 ratio of PBMC to PBS and centrifugation at 500 × *g* for 15 min at 18°C. The supernatant was discarded, the cell pellet was resuspended in 1 mL of PBS, and red blood cells were removed by hypotonic lysis. Thereafter, the contents were transferred into 2-mL Eppendorf tubes and centrifuged at 12,000 × *g* for 10 min at room temperature. The supernatant was discarded, and cell pellet was resuspended with 400 μL of Trizol (TRIzol LS Reagent, Invitrogen) and stored at −80°C until nucleic acid extraction.

RNA Extraction from Blood Mononuclear Cells and Gene Expression Analyses

Total RNA was extracted from PBMC by adding 80 μL of chloroform to the Trizol solution containing cells. Samples were centrifuged at 15,000 × *g* for 15 min at 4°C, and the colorless aqueous phase containing RNA was transferred to a new microtube. An equal volume of 100% pure ethanol was added to the microtube, samples were homogenized and then incubated for 3 min at room temperature. Purification of RNA was performed using the Quick-RNA 96 kit (Zymo Research) accord-

ing to the manufacturer's instructions. Purity and concentration were evaluated using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Samples had a mean (± SD) 260:280 nm ratio of 2.00 ± 0.06, and 260:230 nm ratio of 2.01 ± 0.20.

A total of 1 μg of purified RNA was used to synthesize cDNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time quantitative PCR was performed using iTag Universal SYBR Green Supermix (Bio-Rad Laboratories) to determine the RNA expression of genes listed in Supplemental Table S1 (https://figshare.com/s/3fa032e2b7d49eef4e49). The genes β-actin (*ACTB*) and *GAPDH* were used as references. A Bio-Rad CFX96 was employed with an initial denaturation step at 95°C for 20 s followed by 40 cycles of 95°C for 5 s, a specific annealing temperature (Supplemental Table S1) for 30 s and extension at 60°C for 30 s. The thermal profile for determining the melting curve started at 65°C with a gradual temperature increase (0.5°C/5 s) to 95°C. Primer efficiency ranged from 95.6 to 103.2%. Each sample was evaluated in duplicate, and the housekeeping genes were included in all plates. Statistical analyses were performed on delta cycle threshold (**dCt**) values as described by Steibel et al. (2009). Fold changes relative to the CON treatment were calculated using the method described by Yuan et al. (2006), whereby fold change was calculated from least squares means (**LSM**) difference according to the formula $2^{-\mathrm{ddCt}}$, where dCt = Ct target gene − Ct geometric mean of housekeeping genes, and ddCt = dCt INF − dCt CON.

Blood and Milk Sampling and Flow Cytometry

Blood was sampled by puncture of the coccygeal vessels into 10-mL lithium-heparinized tubes on d 0, 3, and 7 after enrollment. Red blood cells were removed from 200 μL of whole blood by hypotonic lysis. Cells were incubated for 30 min on ice with allophycocyanin conjugated anti-CD14 (Tuk4 clone, Thermo Fisher Scientific), fluorescein isothiocyanate conjugated anti-CD11b (CC126 clone, Bio-Rad), and phycoerythrin conjugated anti-CD62L (BAQ92A clone, Kingfisher Biotech Inc.) in 100 μL of PBS containing 0.5% BSA (Thermo Fisher Scientific) and 2 m*M* EDTA (Thermo Fisher Scientific). Cells were labeled with a fluorescent viability dye (7-AAD, Invitrogen) and analyzed according to size and granularity, viability, CD14 and CD11b expression, and CD62L expression (Merriman et al., 2017). The assays were performed using a flow cytometer equipped with 405-, 488-, and 633-nm lasers (BD Biosciences).

Milk was sampled on d −1, 3, and 7 relative to enrollment. Briefly, teats were dipped in antiseptic foam and cleaned by thorough scrubbing of the teat ends with alcohol swabs. After discarding the first strips of milk, a sample was collected into a 15-mL sterile conical tube. The tubes were kept at a 30° angle relative to the teat to avoid contamination. The tubes were immediately placed in ice and transported to the laboratory for flow cytometric analysis of CD14, CD11b, and CD62L cell markers. Briefly, somatic cells were isolated from 200 μL of whole milk by adding it in 2 mL of PBS with 2 m*M* EDTA (Thermo Fisher Scientific) and centrifugation at 650 × *g* for 5 min at 4°C. Milk cells were labeled for CD14, CD11b, and CD62L markers as described for blood leukocytes.

Blood granulocytes were segregated as $CD14^+$ or $CD14^-$ cells, and $CD14^-$ cells were considered neutrophils. Blood mononuclear cells labeled $CD14^+$ were considered monocytes, whereas those $CD14^-$ were considered lymphocytes. Milk cells were gated as described for peripheral blood leukocytes, except that mononuclear cells $CD14^+$ were considered macrophages (Supplemental Figure S1; https://figshare.com/s/3fa032e2b7d49eef4e49). The median fluorescence intensity of CD14, CD11b, and CD62L were quantified.

Intake of DM, and Yields of Milk and Milk Components

Cows were fed twice daily a diet containing (DM basis) 55% corn silage and 45% concentrate mixture with finely ground corn, citrus pulp, solvent-extract soybean meal, heat-treated soybean meal, Ca salts of palm fatty acids, and a mineral-vitamin-protein mixture. Cows were fed twice daily at 0700 and 1200 h and the amounts of feed offered and refused were measured daily from d −6 relative to enrollment to the first 7 d after enrollment. The DM content of corn silage and the concentrate mixture was measured weekly by drying samples in a forced-air oven at 100°C for 48 h. The DM content of the diet fed and refused was calculated based on the proportion of corn silage and concentrate and the respective DM of the 2 ingredients.

Cows were milked at 0630 and 1830 h daily and yields of milk were recorded automatically (AfiFlo milk meters, Afimilk) from d −6 relative to enrollment to the first 7 d after enrollment. Day of enrollment in the experiment was considered d 0. Samples of milk were collected from each milking on the day before enrollment, and again on d 1, 2, 3, 5, and 7 relative to enrollment in the experimental period for analyses of fat, protein, lactose, SNF, TS, urea N, and SCC by the DHIA Laboratory at the Southeast Milk (Belleview, FL). Milk yield and the corresponding concentrations of milk components from each of the 2 daily milkings were used to calculate the yields of milk components

and then back calculate their concentrations in milk on that day. Yields of milk corrected for 3.5% fat and energy, and the net energy content of milk were calculated as follows: 3.5% FCM (kg/d) = (0.4324 × milk yield) + (16.218 × fat yield); ECM (kg/d) = (0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield); milk NE_L (Mcal/d) = milk yield × [(0.0929 × fat %) + (0.0563 × protein %) + (0.0395 × lactose %)]. For the days in which milk was not sampled, d 4 and 6 of the experimental period, the concentration of components in milk used were the mean of the day preceding and following d 4 and 6 (e.g., on d 4, concentration of fat in milk was the mean of d 3 and 5). The SCS was calculated as follows: SCS = $[\log_{10}(SCC/12.5)]/\log_{10}(2)$. Additional daily milk yield was also recorded for the first 140 d after treatment.

Reproductive Management and Reproductive Responses

All cows were subjected to a timed AI protocol for first insemination starting at 76 ± 3 DIM and insemination performed at 84 DIM (Figure 1). Hormonal treatments and AI were performed in the morning except for the last GnRH preceding AI that was administered in the evening, approximately 12 h before insemination. Cows were fitted with pedometers (Afi Act II, Afimilk), and those that were detected in estrus by the farm were inseminated on the same day. Cows that were re-inseminated before pregnancy diagnosis were considered not pregnant to the previous AI. Pregnancy was diagnosed by transrectal ultrasonography on d 32 ± 3 after each AI based on the presence of an amniotic vesicle and an embryo with a heartbeat. Cows diagnosed as nonpregnant had the estrous cycle resynchronized for timed AI (d 0, 100 μg of GnRH; d 5 and 6, 25 mg of $PGF_{2\alpha}$; d 8, 100 μg of GnRH and timed AI). Pregnant cows on d 32 after AI were re-evaluated for pregnancy on d 74 d after AI. Reproduction was followed for the first 300 DIM or until a cow was sold, died or designated by the farm manager as "do not inseminate," whichever happened first. For calculations of 21-d cycle insemination rate or 21-d cycle pregnancy rate, a cow that passed the voluntary waiting period of 81 d postpartum was considered eligible and it was assumed that the length of the estrous cycle was 21 d. Any eligible cow was expected to be inseminated each 21-d interval until becoming pregnant or being sold, dead, or designated "do not inseminate" by 300 DIM, whichever happened first. Responses evaluated included the proportion of cows receiving AI, the DIM at first AI, the proportion of cows that became "do not inseminate," pregnancy per AI (**P/AI**) at first and at all AI, 21-d cycle insemination rate, 21-d cycle pregnancy rate, proportion of pregnant cows by 300 DIM, and the hazard of pregnancy. For survival analysis, a cow was considered pregnant based on the diagnosis on d 74 after AI, and those that remained nonpregnant were censored on the day when sold, dead, designated as "do not inseminate," or at 300 DIM, whichever happened first.

Statistical Analyses

The individual cow was the experimental unit in all statistical models. Continuous data were analyzed by ANOVA with linear mixed-effects models using the MIXED procedure of SAS (SAS/STAT, SAS Institute Inc.). Normality of residuals and homogeneity of variance were examined for each continuous dependent variable analyzed after fitting the statistical models. Responses that violated the assumptions of normality were subjected to power transformation according to the Box-Cox procedure (Box and Cox, 1964) using a macro for mixed models in SAS (Piepho, 2009). The LSM and respective standard errors of the means were back transformed for presentation of results according to Jørgensen and Pedersen (1998). The statistical models included the fixed effects of treatment (CON vs. INF), day of measurement, the interaction between treatment and day, the respective covariate, and the random effects of block and of cow nested within treatment. Measurements taken before treatment administration were used as covariate in the respective statistical analyses. Day was the term in the REPEATED statement, and the covariance structure was selected according to spacing between measurements and model fit assessed based on the smallest Akaike information criterion. When an interaction between treatment and day resulted in $P \leq 0.10$, then means at different time points were partitioned using the SLICE command of SAS. Responses with a single measurement per cow were analyzed with models that included the fixed effects of treatment and covariate, and the random effect of block.

Pregnancy at first AI, the proportion of cows that became do not inseminate, and the proportion of pregnant cows by 300 DIM were analyzed by generalized linear mixed-effects models using logistic regression with the GLIMMIX procedure of SAS (SAS/STAT, SAS Institute Inc.), fitting a binary distribution. The statistical models included the fixed effects of treatment and covariates collected pre-enrollment, and the random effect of block. The adjusted probabilities of P/AI or subclinical endometritis and associated standard error were computed using the inverse link function in SAS (ILINK) to return the estimates onto the scale of the data.

Table 1. Effect of induced endometrial inflammation in early lactation on endometrial polymorphonuclear cells, endometritis, rectal temperature, and blood metabolites in dairy cows (LSM ± SEM)

	Treatment[1]		*P*-value[2]		
Item	CON	INF	TRT	Day	TRT × day
Polymorphonuclear cells,[3] %	7.0 ± 1.1	21.5 ± 2.4	<0.001	<0.001	<0.001
d 2	4.4 ± 0.7	26.3 ± 2.8	<0.001	—	—
d 7	10.9 ± 1.7	17.4 ± 2.1	0.02	—	—
Subclinical endometritis,[4] %	23.3 ± 6.3	80.9 ± 5.1	<0.001	0.78	0.03
d 2	15.0 ± 6.8	86.7 ± 5.6	<0.001	—	—
d 7	34.4 ± 9.4	73.5 ± 7.6	0.003	—	—
Rectal temperature, °C	38.6 ± 0.08	38.6 ± 0.08	0.78	<0.01	0.51
Serum haptoglobin, μg/mL	113.0 ± 13.9	149.5 ± 16.8	0.06	0.08	0.72
Serum glucose, m*M*	4.35 ± 0.11	4.11 ± 0.11	0.03	0.57	0.30
Serum fatty acids, m*M*	0.33 ± 0.04	0.37 ± 0.04	0.35	<0.01	0.40

[1]Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as control (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48).

[2]TRT = effect of treatment; day = effect of day; TRT × day = interaction between TRT and day.

[3]Polymorphonuclear cells in endometrial cytology collected on d 2 and 7 after treatment.

[4]Subclinical endometritis based on >10% polymorphonuclear cells in endometrial cytology.

The prevalence of subclinical endometritis was analyzed by generalized linear mixed-effects models using logistic regression with the GLIMMIX procedure of SAS (SAS/STAT, SAS Institute Inc.), fitting a binary distribution. The statistical model included the fixed effects of treatment, day of measurement (2 vs. 7 after treatment), the interaction between treatment and day, and the proportion of PMN on the endometrial cytology before treatment, at 31 DIM, and the random effects of block and cow nested within treatment. The adjusted probability of subclinical endometritis and associated standard error were computed using the ILINK function as previously described.

For the 21-d cycle insemination rate, pregnancy per AI at all AI, and 21-d cycle pregnancy rate, the data were analyzed by generalized linear mixed-effects models using logistic regression with the GLIMMIX procedure of SAS (SAS/STAT, SAS Institute Inc.), fitting a binomial distribution. The statistical models included the fixed effects of treatment and the covariate, and the random effect of block. The adjusted probabilities and associated standard error were computed using the ILINK function as previously described.

Days open were analyzed with the Cox's proportional hazard regression using the PHREG procedure of SAS (SAS/STAT, SAS Institute Inc.). The model included the fixed effects of treatment and the genomic value for daughter pregnancy rate. The adjusted hazard ratio and respective 95% confidence intervals were calculated. Survival curves generated were adjusted for covariates in the statistical models using the BASELINE statement in PHREG of SAS. Model fit was assessed by plotting the martingale and deviance residuals against the estimates of the linear predictor. Proportionality of hazards among treatments was evaluated using the ASSESS statement for proportional hazard in PHREG. Proportionality of the hazard for the effect of treatment was not violated ($P = 0.52$). The median open days were calculated with the LIFETEST procedure of SAS (SAS/STAT, SAS Institute Inc.).

In all mixed-effects models, the Kenward-Roger method was used to approximate the denominator degrees of freedom for the F tests. Statistical significance was considered at $P \leq 0.05$, and tendency was considered at $0.05 < P \leq 0.10$.

RESULTS

Intrauterine Bacterial Infusion Induced Endometrial Inflammation

Treatment with INF increased ($P < 0.01$) the proportion of PMN in endometrial cytology on d 2 and 7 after enrollment, and increased ($P < 0.01$) the proportion of cows with subclinical endometritis compared with CON (Table 1; Figures 2A and 2B). Concentration of haptoglobin in serum tended to increase ($P = 0.06$) 32.3% in INF compared with CON cows, but treatment did not affect rectal temperature (Table 1; Figures 2C and 2D). Concentration of glucose in serum decreased ($P = 0.03$) in INF compared with CON; however, concentration of fatty acids in serum did not differ between CON and INF (Table 1; Figures 2E and 2F).

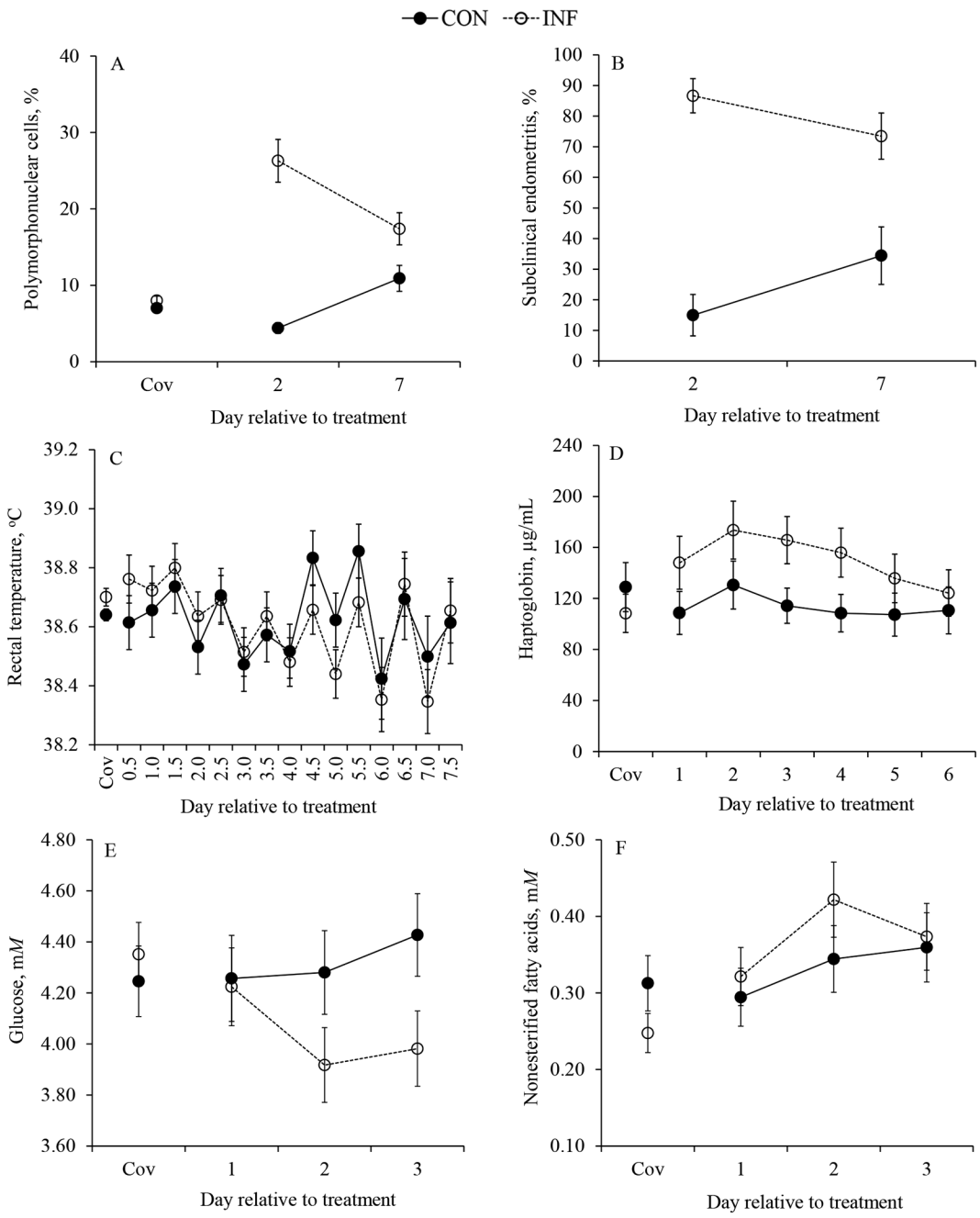

Figure 2. Proportion of polymorphonuclear leukocytes in endometrial cytology (A), prevalence of subclinical endometritis based on PMN >10% (B), rectal temperature (C), and serum concentrations of haptoglobin (D), glucose (E), and nonesterified fatty acids (F) in control (CON; n = 37) or cows that received an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase (INF; n = 48). Cov = covariate value measured before treatment administration. Values represent the LSM and error bars depict SEM.

Effect of Induced Endometrial Inflammation on Production Performance

Intake of DM tended ($P = 0.07$) to be less in INF compared with CON (Table 2; Figure 3A). Yields of milk, true protein, and lactose decreased ($P \leq 0.02$) in INF compared with CON, but treatment did not affect yields of fat or ECM because treatment with INF increased ($P = 0.02$) the content of fat in milk compared with CON (Table 2; Figures 3B and 3C). Indeed, reductions ($P < 0.05$) in milk yield caused by INF were observed in wk 1 (−3.3 kg/d), 2 (−2.5 kg/d), and 4

Table 2. Effect of induced endometrial inflammation in early lactation on production performance in the 7 d following treatment (LSM ± SEM)

	Treatment[1]		*P*-value[2]		
Item	CON	INF	TRT	Day	TRT × day
DMI, kg/d	20.7 ± 0.6	19.4 ± 0.5	0.07	0.33	0.63
Milk, kg/d	44.9 ± 0.8	41.6 ± 0.8	0.002	0.43	0.12
ECM, kg/d	43.1 ± 0.9	41.6 ± 0.8	0.16	0.91	0.15
ECM/DMI, kg/kg	2.11 ± 0.06	2.15 ± 0.05	0.67	0.72	0.10
3.5% FCM, kg/d	44.8 ± 1.0	43.5 ± 0.9	0.27	0.93	0.20
NE_L, Mcal/d	29.8 ± 0.6	28.9 ± 0.6	0.23	0.88	0.25
Fat					
%	3.54 ± 0.10	3.84 ± 0.10	0.02	0.73	0.96
kg/d	1.56 ± 0.05	1.58 ± 0.04	0.72	0.92	0.44
Protein					
%	2.66 ± 0.03	2.70 ± 0.03	0.14	0.89	0.98
kg/d	1.19 ± 0.02	1.12 ± 0.02	0.02	0.59	0.08
Lactose					
%	4.85 ± 0.03	4.87 ± 0.03	0.49	0.01	0.06
kg/d	2.17 ± 0.04	2.03 ± 0.04	0.009	0.30	0.09
SNF					
%	8.47 ± 0.04	8.53 ± 0.04	0.15	0.11	0.16
kg/d	3.79 ± 0.08	3.55 ± 0.07	0.01	0.42	0.12
SCS	2.69 ± 0.25	3.13 ± 0.22	0.17	0.08	0.03
Urea N, mg/dL	10.49 ± 0.40	9.86 ± 0.33	0.23	0.06	0.38

[1]Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as control (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48).

[2]TRT = effect of treatment; day = effect of day; TRT × day = interaction between TRT and day.

(−2.9 kg/d) relative to treatment, after which yield of milk no longer differed (Figure 4). A tendency ($P = 0.10$) for the interaction between treatment and day was observed for yield of ECM per kilogram of DMI, which was greater for INF than CON on experimental d 3 (Table 2; Supplemental Figure S2D; https://figshare.com/s/3fa032e2b7d49eef4e49). An interaction between treatment and day ($P = 0.03$) was observed for SCS because cows that received INF had greater SCS compared with CON on d 2 and 3 (Table 2; Figure 3D). Treatment did not affect the concentrations of urea N, SNF, or TS in milk, although yield of SNF decreased ($P = 0.01$) in INF compared with CON (Table 2).

Effect of Induced Endometrial Inflammation on PBMC mRNA Expression

Induced endometrial inflammation increased ($P < 0.01$) the mRNA expression of *IL1B* on d 2 and 7 after treatment compared with CON, but treating cows with INF did not affect the expression of *TNFA* or *IL6* (Table 3). The LSM for the dCT and respective standard error of the mean for genes investigated in PBMC on d 2 and 7 after enrollment are depicted in Supplemental Table S2 (https://figshare.com/s/3fa032e2b7d49eef4e49).

Expression of genes that encode pro-inflammatory cytokines did not differ between CON and INF on d

Table 3. Effect of induced endometrial inflammation on mRNA expression in peripheral blood mononuclear cells (PBMC) in the first week after treatment[1]

	d 2		d 7		*P*-value[2]		
Gene	CON	INF	CON	INF	TRT	Day	TRT × day
TNFA	1.00	1.00	1.02	1.09	0.75	0.53	0.72
IL1B	1.00	1.81	1.13	1.95	0.005	0.39	0.84
IL6	1.00	1.24	2.25	1.98	0.75	<0.001	0.20

[1]Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as control (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48). Blood samples were collected on d 2 and 7 after treatment for PBMC isolation and RNA extraction. Values are fold-change relative to d 2 in CON treatment.

[2]TRT = effect of treatment; day = effect of day; TRT × day = interaction between TRT and day.

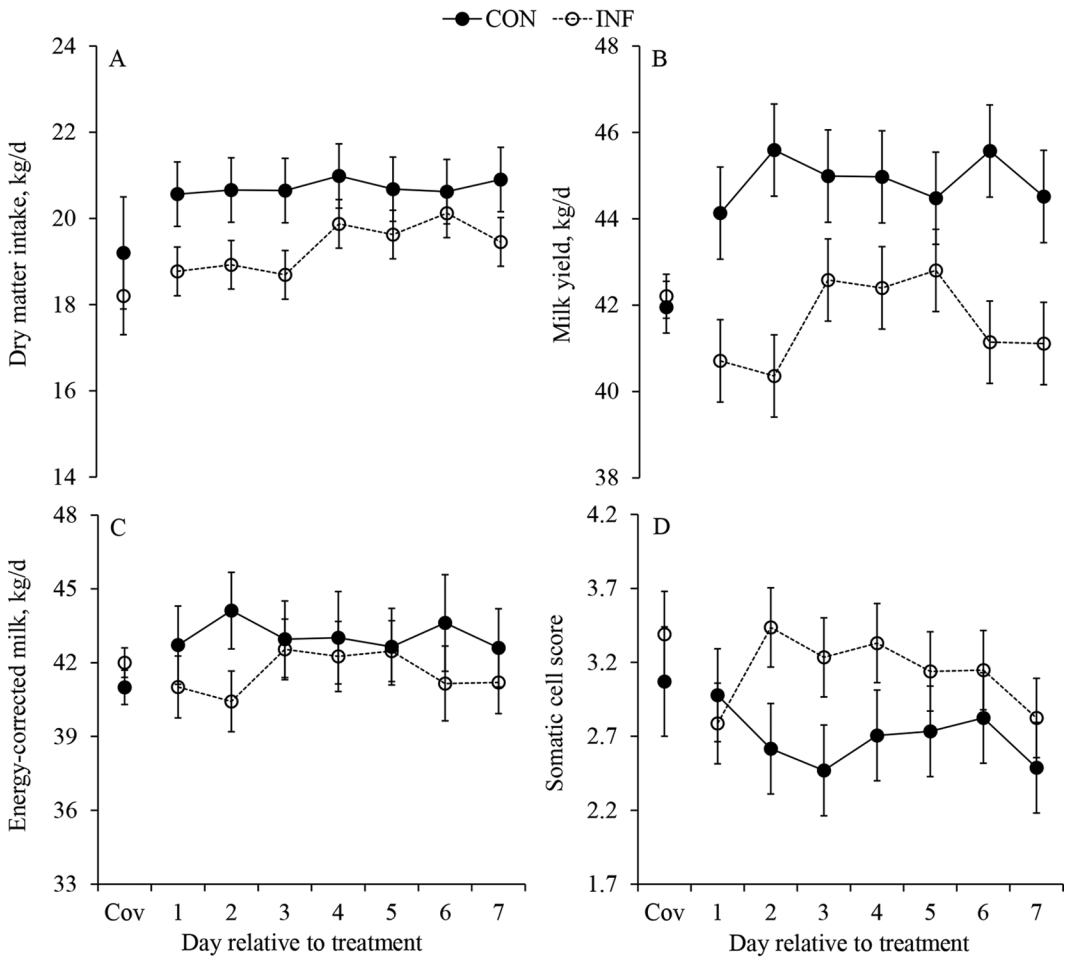

Figure 3. Dry matter intake (A), milk yield (B), ECM yield (C), and SCS (D) in control (CON; n = 37) or cows that received an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase (INF; n = 48). Cov = covariate value measured before treatment administration. Values represent the LSM and error bars depict SEM.

19 after insemination (Table 4). Pregnancy upregulated ($P < 0.01$) 2- to 5-fold the expression of the IFN-stimulated genes *ISG15*, *MX2*, and *RTP4* in PBMC, and the upregulation of *ISG15* and *MX2* was attenuated in INF compared with CON (Table 4). Treatment did not affect the expression of *RTP4*. The LSM for the dCT and respective standard error of the mean for the genes evaluated on d 19 after AI are depicted in Supplemental Table S3 (https://figshare.com/s/3fa032e2b7d49eef4e49).

Effect of Induced Endometrial Inflammation on Blood and Milk Immune Cell Populations

Treatment did not affect the majority of the leukocyte population evaluated in blood or milk on d 3 and 7 (Supplemental Table S4; https://figshare.com/s/3fa032e2b7d49eef4e49). Blood granulocytes were unaffected by treatment or the interaction between treatment and day. A tendency for interaction ($P = 0.06$) between treatment and day was observed for the percentage of blood mononuclear leukocytes because cows that received INF had a reduced proportion of those cells in blood on d 7 after treatment compared with CON. Induced endometrial inflammation tended to increase ($P = 0.08$) the percentage of $CD14^+$ PBMC; however, treatment did not affect expression of CD62L or CD11b in $CD14^+$ or $CD14^-$ PBMC.

Similar to observations in blood, treatment did not affect the proportion of granulocytes in milk, but the proportion of granulocytes expressing $CD14^+$ increased ($P = 0.04$) in INF compared with CON cows. Treatment did not affect the proportion of mononuclear leukocytes in milk or the proportion of those leukocytes expressing CD14. Within the $CD14^+$ cells, cows that received INF had reduced ($P = 0.05$) expression of CD11b and

Table 4. Effect of induced endometrial inflammation and pregnancy on mRNA expression in peripheral blood mononuclear cells (PBMC) on d 19 after insemination[1]

	Nonpregnant[2]		Pregnant[2]		*P*-value[3]		
Gene	CON	INF	CON	INF	TRT	PREG	TRT × PREG
TNFA	1.00	0.96	1.08	1.20	0.74	0.23	0.56
IL1B	1.00	1.63	1.33	1.20	0.37	0.98	0.24
IL6	1.00	0.97	0.60	0.75	0.62	0.09	0.56
ISG15	1.00	1.09	5.35	2.19	0.07	<0.001	0.03
MX2	1.00	0.99	3.79	1.94	0.03	<0.001	0.04
RTP4	1.00	0.99	2.39	1.40	0.17	0.003	0.19

[1]Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as control (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48). Blood was sampled on d 19 after AI for PBMC isolated for mRNA quantification. Values are fold-change relative to nonpregnant CON treatment.

[2]Pregnancy was diagnosed by ultrasonography on d 32 after insemination.

[3]TRT = effect of treatment; PREG = effect of pregnancy; TRT × PREG = interaction between TRT and PREG.

tended ($P = 0.06$) to reduce the intensity of CD11b expression. Treatment did not affect milk $CD14^-$ cells.

Effect of Induced Endometrial Inflammation on Reproduction

The DIM at first AI did not differ between CON and INF (Table 5). Treatment did not affect P/AI at first insemination, but induced endometrial inflammation with INF tended to reduce ($P = 0.06$) P/AI for all postpartum AI compared with CON. The number of AI and the 21-d cycle insemination rate did not differ between CON and INF, but treatment with INF reduced ($P = 0.05$) the 21-d cycle pregnancy rate compared with CON. Consequently, induced endometrial inflammation reduced ($P = 0.02$) the proportion of cows that became pregnant by 300 DIM. The risk of cows becoming "do not inseminate" tended to increase ($P = 0.06$) in INF compared with CON cows. Indeed, the hazard of pregnancy tended ($P = 0.07$) to be smaller for INF than CON resulting in 24 more median days open in INF compared with CON cows (Table 5; Figure 5). The reasons for cows to become do not inseminate included lymphosarcoma, hemorrhagic bowel syndrome, bad udder conformation, and cows with advanced lactation after multiple AI.

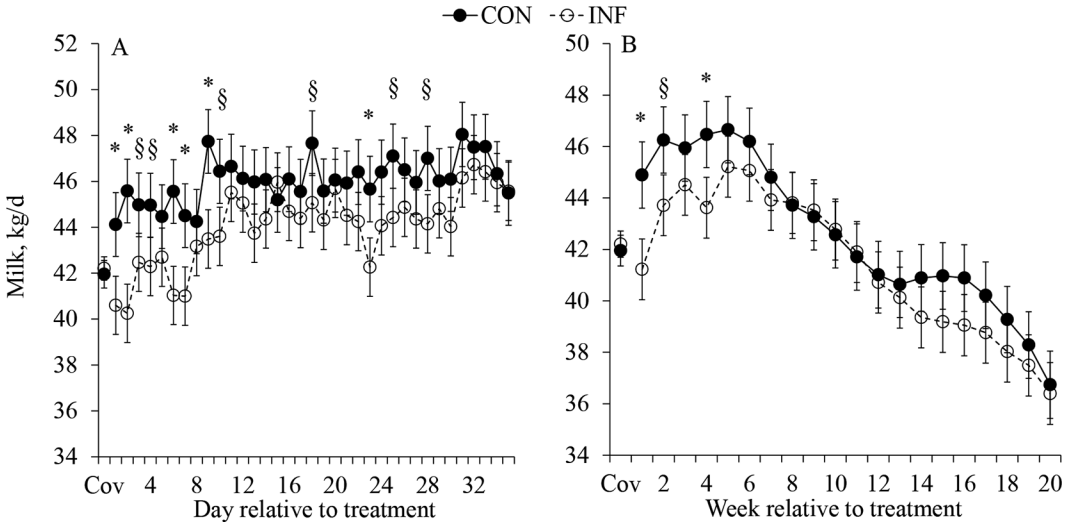

Figure 4. Yields of milk in the first 35 (A) and 140 d (B) after treatment in control (CON; n = 37) or cows that received an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase (INF; n = 48). Cov = covariate value measured before treatment administration. Within day or week relative to treatment, * denotes statistical difference ($P < 0.05$) and § denotes a tendency for statistical difference ($P < 0.10$). Values represent the LSM and error bars depict SEM.

Table 5. Effect of induced endometrial inflammation in early lactation on reproductive performance in dairy cows (LSM ± SEM, unless otherwise specified)

	Treatment[2]		
Item[1]	CON	INF	*P*-value
DIM at first AI	84.4 ± 1.1	84.8 ± 1.1	0.77
Pregnant at first AI, %			
d 32	29.4 ± 8.0	28.3 ± 7.0	0.92
d 74	23.7 ± 7.4	23.7 ± 6.6	0.99
Pregnancy per AI at all AI, %	33.4 ± 5.1	21.6 ± 3.7	0.06
Number of AI	2.64 ± 0.28	2.84 ± 0.25	0.59
21-d cycle insemination rate, %	60.5 ± 3.6	58.0 ± 2.8	0.62
21-d cycle pregnancy rate, %	21.0 ± 3.4	12.9 ± 2.3	0.05
Pregnant by 300 DIM, %	88.2 ± 5.7	62.0 ± 8.0	0.02
Do not inseminate, %	11.3 ± 5.7	30.1 ± 7.9	0.06
Days open			
Median (95% CI)	150 (115–163)	174 (146–223)	—
Adjusted HR (95% CI)	Referent	0.61 (0.36–1.04)	0.07

[1]Pregnancy status based on the diagnosis on d 74 after each AI for inseminations within the first 300 d postpartum. Nonpregnant cows were censored by 300 d postpartum or when they became do not inseminate or were sold or died, whichever happened first. HR = hazard ratio.

[2]Holstein cows at 37 ± 3 DIM were blocked by parity group and genomic breeding value for cow conception rate and, within block, assigned randomly to remain as control (CON; n = 37) or to receive an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48).

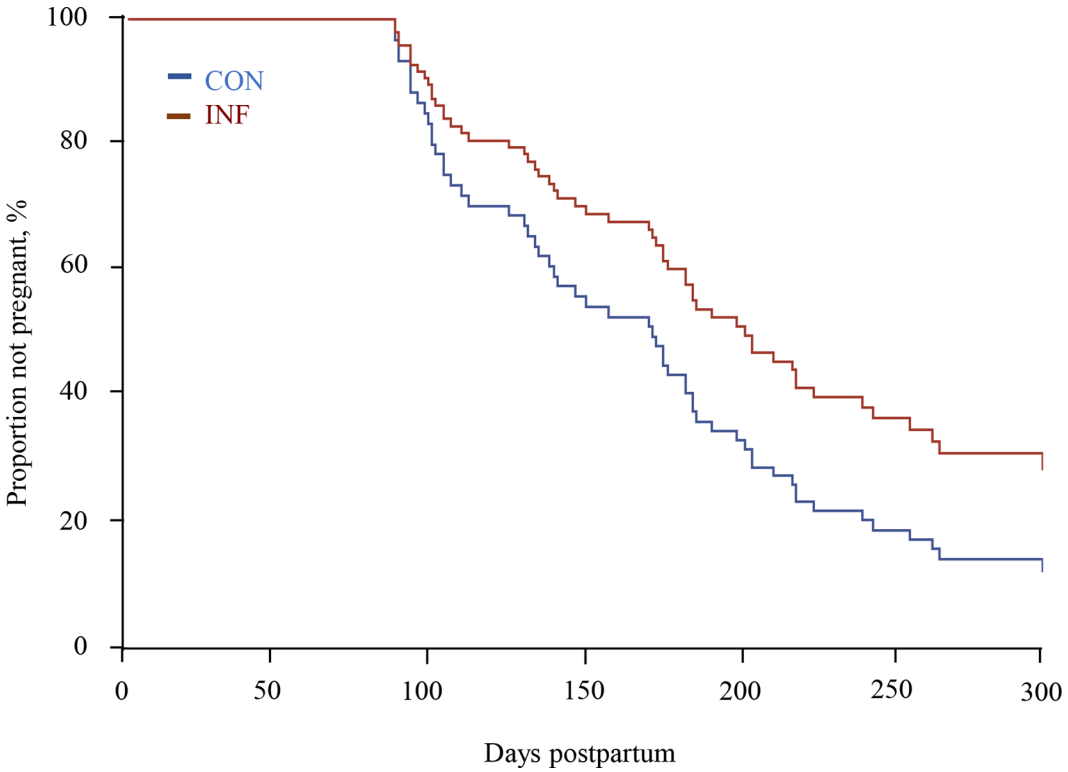

Figure 5. Survival curves for days open in cows that remained as controls (CON; n = 37) or those that received an intrauterine infusion of 5.19×10^8 cfu *Escherichia coli* and 4.34×10^8 cfu *Trueperella pyogenes* during the luteal phase to induce endometrial inflammation (INF; n = 48). Effect of treatment ($P = 0.07$). Median days open (95% CI) were 150 (115 to 163) in CON and 174 (146 to 223) in INF cows.

DISCUSSION

The current experiment documented the negative effects of induced endometritis in early lactation dairy cows on production and reproduction by applying a previously described model used in nulliparous (Piersanti et al., 2019) or primiparous dry cows (Dickson et al., 2020). Induced endometrial inflammation with *E. coli* and *T. pyogenes* resulted in an influx of PMN into the endometrium and caused a systemic response with a tendency for increased haptoglobin concentrations in serum. The induced endometrial inflammation upregulated expression of *IL1B* in PBMC in the days following treatment, but reduced expression of the pregnancy-induced IFN-stimulated genes *ISG15* and *MX2* on d 19 of gestation, 2 mo after treatment. The latter suggests reduced secretion of IFN-τ likely related to compromised conceptus development or reduced leukocyte sensitivity to IFN-τ (Ribeiro et al., 2016). Indeed, induced endometrial inflammation compromised reproduction in cows by decreasing P/AI and the rate of pregnancy, which extended days open, but also reduced DMI and milk yield. Findings of the present experiment corroborate those of epidemiological studies that document the associations between uterine disease and depressed production and reproduction in dairy cows (LeBlanc et al., 2002; Bell and Roberts, 2007; Sheldon et al., 2019). The current findings provide specific evidence that endometrial inflammation is an important underlying factor in the association between uterine disease and depressed production and reproduction in dairy cows.

In the present experiment, a cocktail of utero-pathogenic *E. coli* and *T. pyogenes* previously characterized (Goldstone et al., 2014a,b) was used to induce endometrial inflammation. Although the cocktail includes *T. pyogenes*, which is considered one of the main bacteria identified as risk factor for clinical endometritis (Prunner et al., 2014), it lacked other often identified groups of microorganisms that colonize the uterus during uterine diseases such as *Bacteroides*, *Porphyromonas*, and *Fusobacterium* (Jeon et al., 2015, 2018). Nevertheless, infusion of only *T. pyogenes* during the luteal phase has been shown to cause endometrial inflammation with loss of the tissue architecture (Lima et al., 2015), and infusion of a similar cocktail containing the same pathogens into the uterine lumen of dairy heifers and cows has been shown to induce inflammation and perturb numerous aspects of reproduction (Horlock et al., 2020; Piersanti et al., 2020; Dickson et al., 2022). Also, CON cows received no sham treatment, with only sterile PBS, to minimize any induction of endometrial inflammatory. Infusion of sterile solutions into the uterus have been shown to elicit an influx of PMN resulting in some cows presenting inflammatory response with mucopurulent or purulent vaginal discharge (Piersanti et al., 2019; Dickson et al., 2020). Because of that, the present experimental approach allows inferences to be made relative to endometrial inflammation caused by the pathogens used and their associated molecular patterns, which is typically observed during spontaneous endometritis in dairy cows (Prunner et al., 2014; Bromfield et al., 2015).

One of the consequences of uterine diseases, particularly metritis, is a depression in appetite that is thought to mediate the reduced productive performance in dairy cows (Bell and Roberts, 2007; Pérez-Báez et al., 2019). In fact, cows that develop metritis, endometritis, or both have reduced DMI starting prepartum and continuing postpartum, before the diseases are diagnosed (Hammon et al., 2006). The reduction in DMI prepartum observed by Hammon et al. (2006) in cows that eventually developed metritis, endometritis, or both suggests that inadequate intake of nutrients prepartum might be a risk factor for uterine diseases, although the same association was not observed in a larger subsequent study (Pérez-Báez et al., 2019). In the present experiment, induced endometrial inflammation tended to reduce DMI, which helps explain the reduced yields of milk, lactose, and protein. Induced endometrial inflammation increased signs of systemic immune responses in the week following treatment with increased PBMC expression the pro-inflammatory cytokine *IL1B* and concentrations of haptoglobin in serum. These systemic responses suggest that peripheral immune cells sensed the pro-inflammatory stimuli in the uterus, and cytokines released during an inflammatory response induce symptoms of sickness including a reduction in appetite (Dantzer and Kelley, 2007). Hypophagia that follows disease is perhaps a protective mechanism to contain the inflammatory response (Brown and Bradford, 2021). Greer et al. (2005) conducted an elegant experiment using a nematode model in sheep to study the effects of activation of the immune system on appetite. Authors used immunologically naïve lambs to remain as control or to receive *Trichostrongylus colubriformis* larvae with or without concurrent administration of glucocorticoids weekly. Gastrointestinal infection by *T. colubriformis* in naïve lambs reduced DMI compared with controls; however, administration of glucocorticoid, to suppress the immune response, prevented the depression in intake in immunologically naïve lambs infected with *T. colubriformis* (Greer et al., 2005). Inflammation not only reduces nutrient intake, but also increases energy expenditure to mount a response against infection (Colditz, 2002). Taken together, activation of the immune response caused by induced endometrial inflammation reduced DMI and milk yield. The lack of effect of treatment on ECM yield is explained by the increased concentration

of fat in milk in INF compared with CON cows. It was initially thought that the increase in milk fat content would be mediated by increased lipomobilization and release of fatty acids from the adipose tissue to be taken up by the mammary gland, although we were unable to detect an effect of treatment on concentrations of fatty acids in serum of cows.

Another possibility for the reduced milk yield was the increase in SCS in INF cows. Increased SCS suggests an inflammatory response in the mammary gland, which is often associated with reduced milk yield (Fernandes et al., 2021). Intramammary infusion of inflammatory cytokines increased the SCC in milk and, consequently, reduced milk yield (Watanabe et al., 2008). Abundance of *IL1B* mRNA in PMBC in the first week after treatment increased in INF compared with CON cows. Bacterial endometritis results in release of pathogen-associated molecular patterns and damage-associated molecular patterns that likely mediate the increase in pro-inflammatory cytokines (Sheldon et al., 2019). In 2 experiments with lactating goats, intrauterine infusion of LPS increased the concentrations in milk of IL-1β and IL-6 and the SCC (Purba et al., 2020a,b). Perhaps pro-inflammatory cytokines or bacterial products released in response to induced endometrial inflammation might have had access to the systemic circulation and affected the mammary gland with implications to SCS.

Induced endometrial inflammation had long-term negative effects on reproduction in dairy cows, in particular a reduction in P/AI that resulted in reduced rate of pregnancy, extended days open, and a reduced proportion of pregnant cows by 300 DIM. Not surprisingly, a larger proportion of INF cows became "do not inseminate" which, combined with the reduced rate of pregnancy, would eventually increase culling from the herd, all factors that contribute to the economic burden attributed to uterine diseases (Pérez-Báez et al., 2021). Infusion of pathogenic bacteria into the uterus of cows causes massive tissue damage not compatible with pregnancy (Lima et al., 2015; Sheldon et al., 2019). *Escherichia coli* contains LPS that are powerful immunogens that dysregulate endometrial cell function (Sheldon et al., 2019). *Trueperella pyogenes* produces pyolysin, which is a cholesterol-dependent cytolysin that damages cell membranes (Sheldon et al., 2019), induces cytolysis of bovine endometrial stromal cells (Amos et al., 2014), and pyroptosis in leukocytes (Liang et al., 2022). Infusion of LPS into the uterus, mammary gland, or directly into the bloodstream resulted in reduced LH secretion (Peter et al., 1989; Lavon et al., 2008), which could compromise dominant follicle development and ovulation. Cows diagnosed with endometritis had follicular fluid with presence of large concentrations of LPS (Herath et al., 2007), which has been suggested to compromise steroidogenesis, induce premature oocyte activation, impair oocyte quality, and reduce the ovarian reserve (Herath et al., 2007; Bromfield et al., 2015). Induction of endometrial inflammation has been shown to impair the developmental capacity of bovine oocytes based on reduction in development to the morula stage following in vitro embryo production (Dickson et al., 2020). Such changes in the follicle and oocyte provide mechanistic data that help explain the reduced P/AI observed herein.

It is well established that inflammatory diseases are linked with impaired early embryo and peri-implantation conceptus development (Ribeiro et al., 2016). Day 16 concepti from cows previously diagnosed with inflammatory diseases were less developed resulting in reduced IFN-τ concentration in the uterine flush (Ribeiro et al., 2016). Indeed, expression of IFN-stimulated genes in PBMC was attenuated in early pregnancy in cows previously diagnosed with disease (Ribeiro et al., 2016). Expression of *ISG15* and *MX2* were less in pregnant INF cows compared with pregnant CON cows, suggesting less IFN-τ secretion by the conceptus. It is possible that induced endometritis compromised conceptus development that might result in reduced maintenance of pregnancy in dairy cows (Ribeiro et al., 2016; Edelhoff et al., 2020). Furthermore, endometrial inflammation, based on the diagnosis of subclinical endometritis, has been linked with reduced fertilization in dairy cows (70.1 vs. 83.7%; Cerri et al., 2009), which would contribute to the reduced P/AI. One of the consequences of the model used herein is the long-term changes in gamete and reproductive tissue mRNA expression that lasted weeks to months (Horlock et al., 2020; Piersanti et al., 2020; Dickson et al., 2022). It is possible that such changes in tissue gene expression might compromise the machinery needed for oocyte competence or endometrial cell function, such as secretion of histotroph, and subsequent placentation that help explain the reduced rate of pregnancy documented in the present experiment.

CONCLUSIONS

Endometrial inflammation was successfully induced in early lactation dairy cows characterized by increased proportion of PMN in endometrial cytology and prevalence of subclinical endometritis. Induced endometrial inflammation resulted in systemic effects with increased expression of the pro-inflammatory gene *IL1B* in blood leukocytes, tendentially increased serum concentrations of haptoglobin, tended to reduce DMI, and decreased serum glucose concentrations with negative effect on milk yield, although ECM remained unaffected. Pregnancy upregulated IFN-stimulated genes in blood

leukocytes, but the stimulation on *ISG15* and *MX2* was markedly reduced in cows induced to develop endometrial inflammation, thus suggesting impaired conceptus development, which helps explain the reduced pregnancy per AI and rate of pregnancy. The findings of this randomized experiment corroborate those from observational studies that have documented negative effects of uterine inflammatory diseases on production and reproduction in dairy cows.

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