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Research Article

Effects of early pregnancy on uterine lymphocytes and endometrial expression of immune-regulatory molecules in dairy heifers†

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Abstract

Natural killer (NK) cells are essential for establishment of human and rodent pregnancies. The function of these and other cytotoxic T cells (CTL) is controlled by stimulatory and inhibitory signaling. A role for cytotoxic cells during early pregnancy in cattle has not been described, but regulation of their function at the fetal–maternal interface is thought to be critical for conceptus survival. The hypothesis that the relative abundance of CTL and expression of inhibitory signaling molecules is increased by the conceptus during early pregnancy was tested. The proportions of lymphoid lineage cells and expression of inhibitory signaling molecules in the endometrium during early pregnancy in dairy heifers were determined using flow cytometry, immunofluorescence, and real-time PCR on days 17 and 20 of pregnancy and day 17 of the estrous cycle. Results revealed an increased percentage of NKp46$^+$ and CD8$^+$ cells in the uterus of pregnant heifers. Furthermore, a large percentage of uterine immune cells coexpressed these proteins. Compared to cyclic heifers, CD45$^+$ uterine cells from pregnant heifers exhibited greater degranulation. Endometrium from pregnant heifers had greater mRNA abundance for the inhibitory molecules, *CD274* and lymphocyte activating gene 3 *(LAG3)*, and greater cytotoxic T lymphocyte-associated protein 4 (CTLA4), molecules that can interact with receptors on antigen-presenting cells and induce lymphocyte tolerance. This study demonstrates a dynamic regulation of both cytotoxic immune cells and tolerogenic molecules during the peri-implantation period that may be required to support establishment of pregnancy and placentation.

Summary Sentence

Pregnancy increases abundance of natural killer and cytotoxic T cells and expression of immune inhibitory molecules in the endometrium of dairy heifers during early pregnancy

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Key words: pregnancy, endometrium, cattle, natural killer cells, cytotoxic T cells.

Introduction

Pregnancy is a unique immunological condition during which the semiallogeneic conceptus survives in the presence of the maternal immune system. Clearly, endometrial cells are modulated by concep-tus signals to facilitate maintenance of the corpus luteum (CL) and establishment of pregnancy [1]. Interferon tau (IFNT), produced by the conceptus, is hypothesized to be a key early mediator of this sig-naling. However, the effects of conceptus signals on the proportions and functions of endometrial immune cells are poorly understood [2]. In humans and rodents, natural killer (NK) cells play an im-portant role in spiral artery remodeling to facilitate placentation [3, 4]. The working hypothesis for this study is that conceptus signal-ing increases the proportion of uterine NK (uNK) cells and induces molecules that promote tolerance to facilitate conceptus growth and formation of the placenta. Reprogramming immune cell function is likely essential for pregnancy, and its failure or inadequate modulation likely contributes to embryo loss [5].

There are very few studies that describe changes in endometrial immune cells during early pregnancy in dairy cows [6–8], and most of the information available is extrapolated from human and rodent models [3, 9, 10]. Studies in these species support a role for NK cells and regulatory T cells during the peri-implantation period [11–13]. However, little is known about these cells during the critical window of pregnancy recognition signaling in cattle.

Uterine natural killer cells constitute 70% of decidual lympho-cytes during human early pregnancy and are characterized by a CD56^{bright} CD16^{-} phenotype [14]. In rodents and other mammals that do not express CD56, NKp46 (CD335), a highly conserved nat-ural cytotoxicity receptor, is regarded as the protein that defines NK cells [15]. In ruminants, NK cell activity was demonstrated in disso-ciated uterine endometrium during mid and late gestation [16–18]. Similar cells were identified in the porcine uterus during early preg-nancy [19]. Limited data are available about the presence of NK cells in bovine endometrium [6]. Macrophages, CD4^{+} and CD8^{+} T lymphocytes were reported to be present in the bovine uterus during the estrous cycle and pregnancy [5, 20, 21]. Cytotoxic CD8^{+} and gamma delta^{+} ($\gamma\delta$) T cells were also found in ovine endometrium during mid and late gestation, and $\gamma\delta^{+}$ T cells increased close to parturition [22]. In general, NK cells and T cells are thought to participate in growth of the conceptus, immunosuppression, and/or placental detachment at parturition [9].

Interferon tau, the conceptus-produced antiluteolytic signal in ruminants, increases cytotoxicity of porcine and ovine NK cells in vitro [23]. This suggests that IFNT could regulate the function of NK cells at the fetal–maternal interface, but also raises the question of how activation of NK cells promotes pregnancy.

In other species, NK cells mediate spiral artery remodeling to facilitate placentation [24]. Preeclampsia and other pregnancy com-plications have been attributed to disruption of uNK cell activity, including fetal growth retardation and second trimester miscarriage in women [12, 25, 26]. Cytotoxic granules in NK and other CTL con-tain granzymes and perforins that lyse target cells [27–29]. Clearly, increased cytotoxicity could put the conceptus at risk were there not compensatory signals. Tolerogenic cytokines, including inter-leukin 10 (IL10) and transforming growth factor beta (TGFB), can act as counterbalancing signals, reducing the cytotoxicity of CTL. Natural killer and T cells are also regulated by antigen-presenting cells (APC) via inhibitory receptor stimulation [30]. Interaction of CTLA4 with CD80 and CD86 costimulatory molecules on APC increases indoleamine 2,3-dioxygenase (IDO) expression and therefore promotes tolerance during pregnancy [31]. Similarly, interaction of programmed cell death 1 (PDCD1) with CD274 (aka programmed cell death ligand 1) can cause T-cell anergy in the endometrium [32]. Tolerogenic myeloid cells can also be induced via engagement of major histocompatibility complex antigen II (MHC II) with LAG3 on activated T and NK cells [33]. Such tilting of the balance toward tolerogenic factors and cytokines in the bovine endometrium would support the concept of T helper 2 (Th2) bias during pregnancy [34].

Establishing the identity, distribution, and function of uterine lymphocytes during early pregnancy is vital to the understanding of immune-mediated causes of infertility in cattle. Infections, including mastitis, are common in dairy cows and can reduce conception rates [35, 36]. Infections result in immune cell activation and produc-tion of inflammatory cytokines that could interfere with embryonic development, leading to peri-implantation embryo loss [37, 38]. Endometritis also causes delay in ovulation and reduces conception rates [39]. The hypothesis tested here is that conceptus signals in-crease the proportion of endometrial NK cells and induce expression of molecules that mediate immune tolerance to support establishment of pregnancy and uterine remodeling associated with placentation. To address this hypothesis, the proportions and distributions of uNK cells and T cells and expression of inhibitory molecules in the endometrium of dairy heifers during early pregnancy were characterized.

Materials and methods

Animals

All procedures involving animals were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (protocol #44524) and complied with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Postpubertal Holstein dairy heifers (350–450 kg) were estrous synchronized using synthetic prostaglandin $F_{2\alpha}$ analog (Clo-prostenol sodium, 500 mcg; Intervet, Summit, NJ). Estrous activity was monitored three times a day for 30 min each. On the day of estrus, the animals were either inseminated with frozen sperm from one bull of proven fertility or were not inseminated and served as nonpregnant controls. On day 17 of the estrous cycle (D17C; n = 12) and/or pregnancy (D17P; n = 9), animals were sacrificed for tissue collection in a USDA-inspected abattoir. A subset of inseminated animals was slaughtered at day 20 of pregnancy (D20P; n = 5). The inseminated animals were confirmed pregnant by the presence of a conceptus in the uterine flushing. A subset of animals was used for optimization studies and could not be included in the flow cytometry data due to differences in processing and labeling between runs as well as limitation in cell numbers for various assays. Plasma progesterone (P4) concentrations were measured to confirm a functional CL at the time of slaughter for both cyclic and pregnant heifers. In ad-dition, endometrial samples were assayed for interferon-stimulated genes (ISG; MX1, MX2, and ISG15) to further confirm pregnancy status (data not shown). Two of the D17P heifers were excluded from further analysis due to low ISG expression, which was similar to that seen in D17C heifers, suggestive that these animals were in the process of losing the pregnancy.

Tissue collection

On the day of tissue collection, 500 mL of blood was collected at exsanguination for isolation of peripheral blood mononuclear

Introducing process under study
What is known about how the process works
Knowledge gap
What is known within the knowledge gap
Hypothesis
Importance of work
Discussion of knowledge gap; narrowing of knowledge gap
What is known about one part of the hypothesized mechanism
What is known about another part of the hypothesized mechanism
How two parts of the hypothesized mechanism may interact; knowledge gap
What is known about how the hypothesized mechanism works
Continued: What is known about how the hypothesized mechanism works
Claim of the importance of this research
Support for the claim of importance
Hypothesis
How hypothesis was addressed

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cells (PBMC) using Ficoll paque (17–1440-02; GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient centrifugation [40]. The uterus was flushed with 30 mL cold phosphate buffered saline (PBS) for conceptus recovery. After flushing, each uterine horn was opened along the mesometrial side and full-thickness punch biopsies (8 mm; n = 5/uterus) from the middle of the uterine horn ipsilateral to the CL were obtained using a cork borer. The biopsies were immersed in OCT compound and frozen in isopentane-cooled over liquid nitrogen for immunofluorescence analysis. The remaining endometrium was dissected free of myometrium and was either snap-frozen in liquid nitrogen for mRNA quantification or transported to laboratory on ice cold RPMI (11 835–055; Gibco RPMI medium, Life technologies, Grand Island, NY) for immune cell isolation.

Endometrium (15 g) was trimmed from the ipsilateral uterine horn and stored in ice-cold RPMI medium containing 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO) and 0.1% gentamycin (10 mg/mL; 15 710–072; Life technologies). Enzymatic dissociation of the endometrium into a single-cell suspension was carried out as previously described [5]. An aliquot of the resuspended cells was counted, and cell viability was estimated using Guava ViaCount Flex reagent (4000–0040; Millipore, Billerica, MA) using a Guava EasyCyte Plus (Millipore) [41].

Flow cytometry

Endometrial immune cells were immunomagnetically sorted from contaminating parenchymal cells using antibodies against CD45 (antibody CACTB51A, VMRD Inc., Pullman, WA) and an AutoMACS cell separator (MACS; Miltenyi Biotec Inc, San Diego, CA) as described previously [41]. The cells were prepared for dual-color flow cytometric analysis by indirect immunofluorescence. Primary antibodies used included anti-NKp46, anti-CD8β, anti-interferon gamma (IFNG) and anti-IL10 (Supplemental Table S1) and were purchased from WSU monoclonal antibody center (Pullman, WA), Biolegend (San Diego, CA), or AbD Serotec (Raleigh, NC). Cells were diluted to 5×10^5 cells/200 μL PBS/EDTA in 96-well plates (3997 Corning costar, Corning, NY), and antibody incubations and washes were done as described previously [5, 41]. Labeling for intracellular cytokines was done using BD cytofix/cytoperm reagents using the manufacturer's protocol (554 714, BD Biosciences, San Jose, CA). Cells were resuspended in ice-cold PBS/EDTA to a concentration of approximately 500 cells/μL for flow cytometric analysis. Unlabeled immune cells, cells incubated only with secondary antibodies, and cells incubated with isotype-matched antibody were used as controls to determine autofluorescence and nonspecific binding. A total of 10,000 CD45$^+$ cells were analyzed for each sample. Cell debris was excluded based on size.

Immunofluorescence

Frozen tissue was sectioned to 5 μm thickness. For cell surface protein staining, sections were thawed at 37°C in a humidified chamber for 30 min and fixed with 100% acetone for 8 min. Sections were then washed in PBS containing 0.1% BSA (PBS/BSA) and were incubated with primary antibodies at 4°C overnight. After washing with PBS/BSA, sections were incubated with secondary antibody (details listed in Supplemental Table S1) at room temperature for 1 h, were washed, and counterstained with Prolong gold antifade DAPI (P-36 931, Life Technologies). For intracellular cytokine staining, fixation was carried out by incubating slides in ice-cold acetone for 8 min. Sections were then incubated for 30 min in PBS/BSA containing 0.1% Triton X 100 (774 953 402; Sigma Aldrich) for permeabilization. The protocols for intracellular and cell surface staining were the same in all other aspects. The antibodies were diluted in PBS/BSA with 0.1% Triton X 100 along with 20% goat serum (Life technologies) for intracellular proteins or just with PBS/BSA containing 20% goat serum. Images were captured using Olympus BX-51 fluorescence photomicroscope (Tokyo, Japan) with the DP71 image capture software and the appropriate microscope filters for green (U-MNB2, Olympus), red (U-N41004, Olympus), and blue (U-MNU2, Olympus) wavelengths. Tissue sections were divided into five areas and images were captured from (1) luminal epithelium (LE), (2) shallow stroma (SS), (3) shallow glands (SG), (4) deep glands (DG), and (5) myometrium (M), and percent area labeled was quantified using Image J software (NIH, USA; [5]). The average percent area labeled for all areas analyzed is represented as uterine wall (UW). Percent area labeled and mean intensity were recorded for each image and were used to quantify the protein localization. Quantification included three images/area, two sections/animal, and four to five animals/treatment.

RNA isolation and real-time PCR

Total RNA was isolated from 1 g of frozen endometrium and amount and quality assessed. DNase treatment was carried out using the RQ1 RNase free DNase kit (M6101; Promega Corporation, Madison, WI). Complementary DNA synthesis was done using Dynamo cDNA synthesis kit utilizing random hexamer primers (F470L; Thermoscientific, Waltham, MA). Primers and annealing temperatures used for real-time PCR are listed in Supplemental Table S2. Primers were validated by sequencing amplicons (Genomics core facility, Huck Institute of Life Sciences, Penn State University). Real-time PCR was done using SYBR Green (QT650–05, Bioline USA, Taunton, MA) using Ribosomal protein L19 (RPL19) or beta-actin as the reference gene as previously described [42].

Proliferation assay

Uterine CD45$^+$ cells were isolated by magnetic sorting as described previously [5]. One million cells were resuspended in 2 mL of X-VIVO 10 medium (04–380Q; Lonza, Allendale, NJ, USA) with added insulin, transferrin, and selenium (ITS-G; 354 351; Corning) and P_4 (10 ng/mL; Q2600; Steraloids Inc, Wilton, NH, USA). The cells were labeled with CFSE (21 888; Sigma) as described previously [43]. Cells were cultured with or without concanavalin A (40 μg/mL; ConA; 234 567, Calbiochem, La Jolla, CA) for 72 h [44] and analyzed for loss of CFSE as an indicator of proliferation using a flow cytometer.

Degranulation assay

Uterine and blood CD45$^+$ cells were labeled with PKH26 dye (MINI26; Sigma Aldrich) according to manufacturer's protocol. Briefly, 1×10^6 cells were pelleted by centrifugation at 295 × g for 10 min at 4°C and resuspended in 1.5 mL of diluent C and 3 μL of PKH26 dye. The cells along with dye were incubated for 7 min at room temperature. Incubation was followed by addition of 10% FBS-F12 to stop the dye uptake. This was followed by centrifugation at 295 × g for 10 min at 4°C. Washes were done with 10% FBS- F12 medium two more times, and cells were resuspended to a final concentration of 2×10^4/mL in medium containing X-VIVO 10 with ITS-G and P4. Unlabeled K562 erythroleukemia cells (ATCC CCL-243, Manassas, VA, USA) were used as target cells for the assay. Degranulation assay was carried out by incubating CD45$^+$ immune cells that had been labeled with PKH26 with and without unlabeled K562 target cells at an effector: target ratio of 5:1 for 4 h. After the

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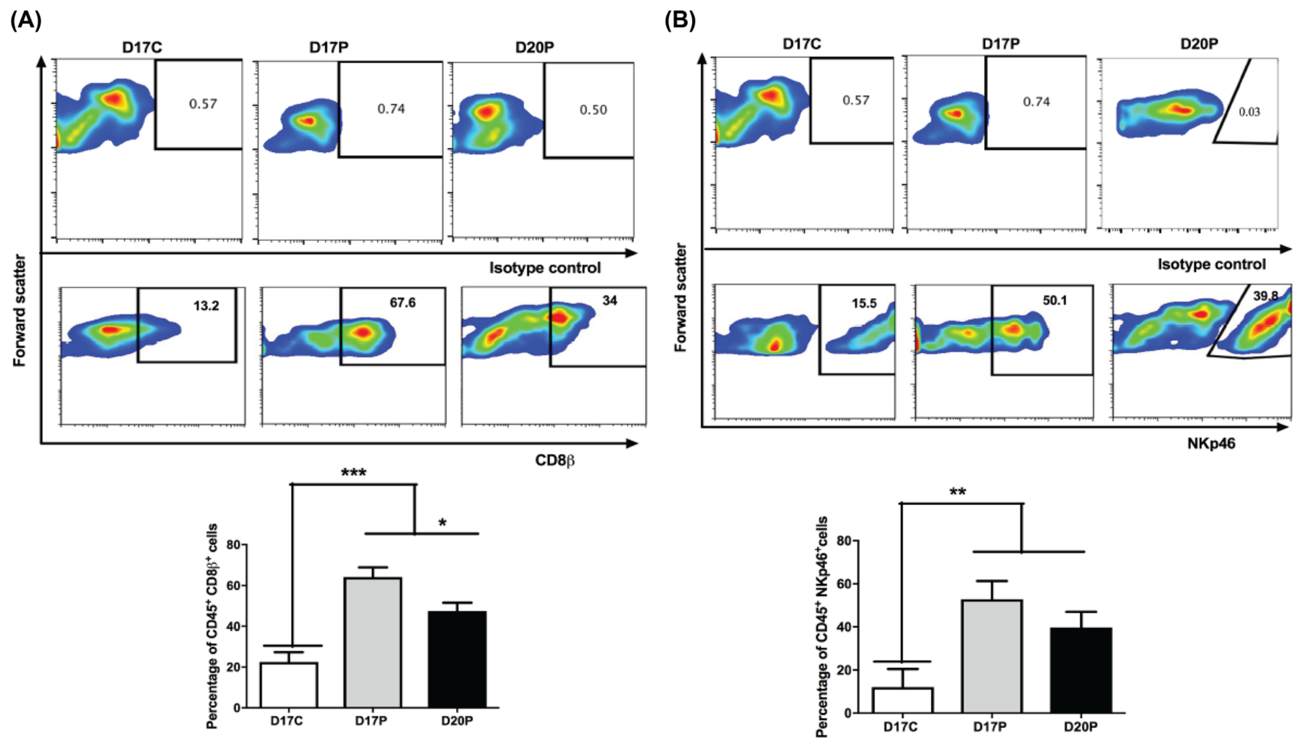

Figure 1. Percentage of CD8β $^{+}$ and NKp46^{+} cells in endometrial CD45^{+} cells. Representative flow cytometric analysis showing percentage of cells that express CD8β (A) and NKp46 (B) of endometrial CD45^{+} cells from day 17 cyclic (n = 3), day 17 pregnant (n = 3), and day 20 pregnant (n = 4) heifers. Because the samples were collected and analyzed on different days, isotype controls for specific antibodies were used as negative controls for confirming antibody staining. This approach corrects for the differences in flow cytometric (voltage) settings between runs and confirms specific labeling. Orthogonal comparison for status (D17C vs D17P and D20P) and days of pregnancy (D17P vs D20P) are indicated by lines. *: $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

incubation, cells were labeled with mouse antihuman CD107a FITC (11–1079-42; Affymetrix eBioscience, San Diego, CA, USA) for 45 min and washed two times with PBS-EDTA. The cells were read on a flow cytometer and were analyzed for PKH26^{+} CD107a^{+} cells. The surface expression of CD107a on effector cells was used as an indicator of degranulation [45]. Mouse antihuman isotype control FITC (Biolegend) was used as a negative control for labeling.

Transwell assay

Suppression of proliferation of autologous peripheral T cells by uterine CD45^{+} cells was analyzed. PBMCs were isolated and magnetically sorted using antibodies against CD2 and gamma/delta TCR (TCR1-N12gamma). The positive (T cell) fraction was used as target cell population for the immunosuppression assay. These cells were labeled with CFSE. The CFSE-labeled peripheral T cells (target) were cultured in 24-well inserts (353 095; Corning) with unlabeled CD45^{+} uterine cells (effector) in the lower chamber of 24-well plates (353 226; Corning) with or without ConA stimulation for 72 h. Loss of CFSE from the target cells was analyzed using flow cytometry.

Data analysis

Data were analyzed using MIXED procedures of SAS (SAS v 9.3). Following a significant F test, status differences were examined using preplanned orthogonal contrasts (D17C vs D17P & D20P and D17P vs D20P) and are indicated with horizontal line over both D17P and D20P bars when compared to D17C (for status) and separate horizontal line over D17P and D20P (for day of pregnancy). For qPCR, all statistical analyses were performed on $\Delta\Delta$Ct values [46], and data are depicted as fold change ($2^{-\Delta\Delta Ct}$). For immunofluorescence (IF) images, percent area labeled in each region among treatment groups was analyzed. Pairwise comparisons were conducted using Bonferroni correction to maintain an experiment-wise type I error rate of $P < 0.05$. Statistical significance was declared for P-values <0.05 and tendencies for P-values between 0.05 and 0.1. In cases where data were not normally distributed, the data were log or square root transformed and normal distribution was confirmed. Least square means with pooled standard error means were plotted on graphs. For in vitro studies, including degranulation, proliferation, and immunosuppression, a Student t-test was used to compare D17C and D17P. All graphs were created using GraphPad (Version Prism 5; GraphPad Software Inc., La Jolla, CA).

Results

Proportion of T and natural killer cells

Immunofluorescence analysis (Supplemental Figure S1–3) for CD3, $\gamma\delta$, and CD4 protein showed that expression did not change with status or day of pregnancy. The percentage of CD45^{+} cells expressing CD8 in endometrium was greater ($P < 0.001$) in pregnant compared to cyclic heifers (Figure 1A), and this percentage decreased ($P < 0.05$) between day 17 and day 20 of pregnancy (Figure 1A). Similarly, the percentage of CD45^{+} cells that expressed NKp46^{+} cells was greater ($P < 0.05$) in pregnant compared to cyclic heifers, but did not change between day 17 and day 20 of pregnancy (Figure 1B). The flow cytometry scatter plots for CD8 and NKp46 labeling are shown in supplemental figures S4 and S5, respectively. The temporal and spatial patterns of NKp46^{+} uterine cells were further assessed using IF labeling (Figure 2A). There was an increase ($P < 0.01$) in the percent

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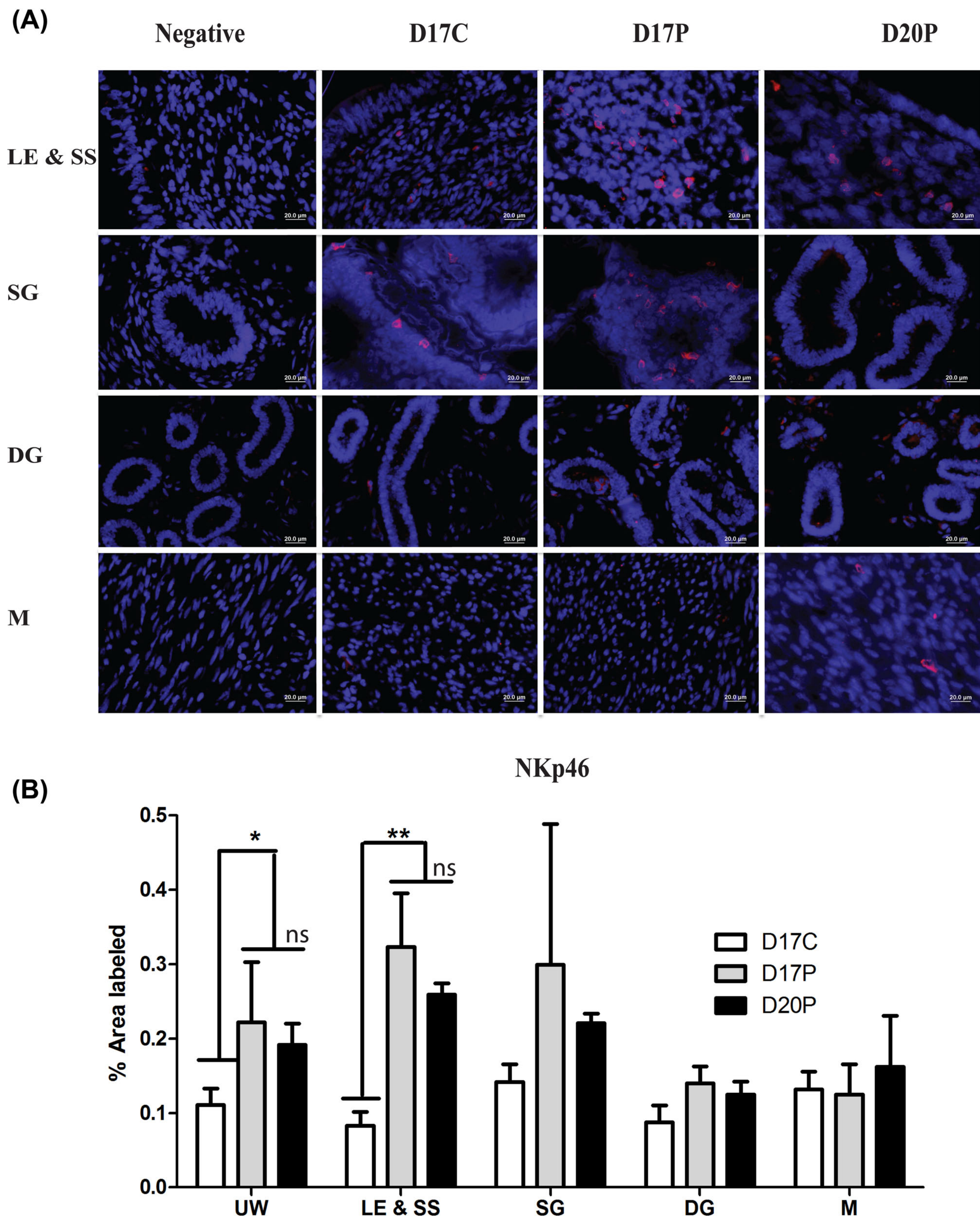

Figure 2. Immunofluorescence analysis of NKp46 expression in uterus. Representative panel (A) and ImageJ quantification (B) for NKp46 IF (% area labeled) in uterine wall (UW), luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M) in day 17 cyclic (n = 5), day 17 pregnant (n = 4), and day 20 pregnant (n = 3) uterus. Negative isotype controls for the respective areas are denoted as negative. Magnification ×400. Orthogonal comparison for status (D17C vs D17P and D20P) and days of pregnancy (D17P vs D20P) are indicated by lines. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

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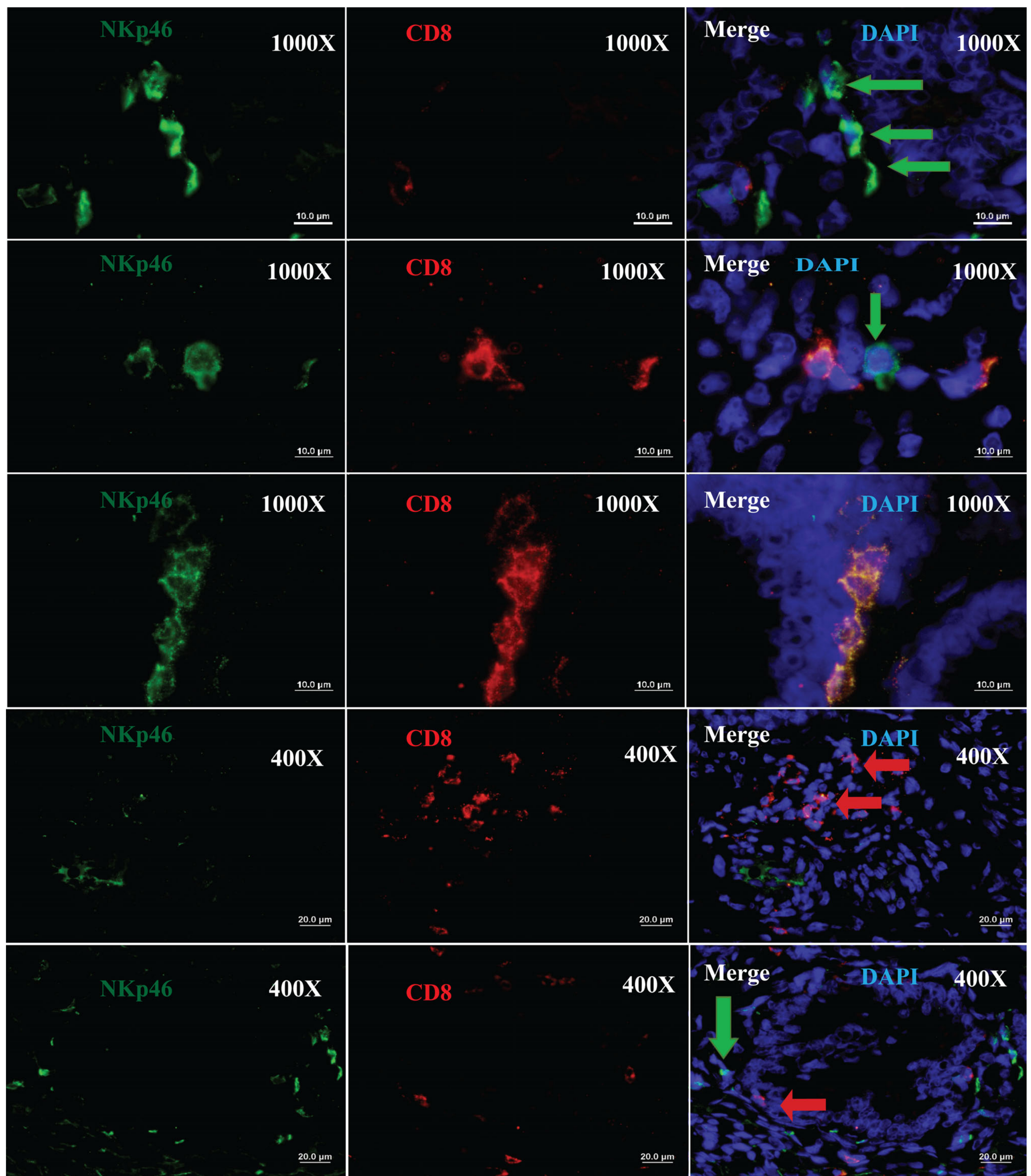

Figure 3. Colocalization of NKp46 and CD8 protein on uterine cells. Representative immunofluorescence images showing colocalization of NKp46 (green) and CD8α (red). Single labeled cells for NKp46 and CD8 are denoted with green and red arrows, respectively. Nuclei are counterstained with DAPI (blue). Overlay of all three channels indicated as merge. Magnification is ×1000 or ×400 indicated on the images.

area labeled (Figure 2B) with NKp46 in the LE and SS during pregnancy. Because of the high percentages of NKp46$^+$ and CD8$^+$ cells, it was hypothesized that these proteins were coexpressed on a subset of uterine immune cells. Coexpression of CD8 and NKp46 proteins was confirmed in the endometrium using dual label IF staining (Figure 3). Although there were single labeled NKp46$^+$ and CD8$^+$ cells, single labeled cells accounted for <20% of total cells in the sections examined.

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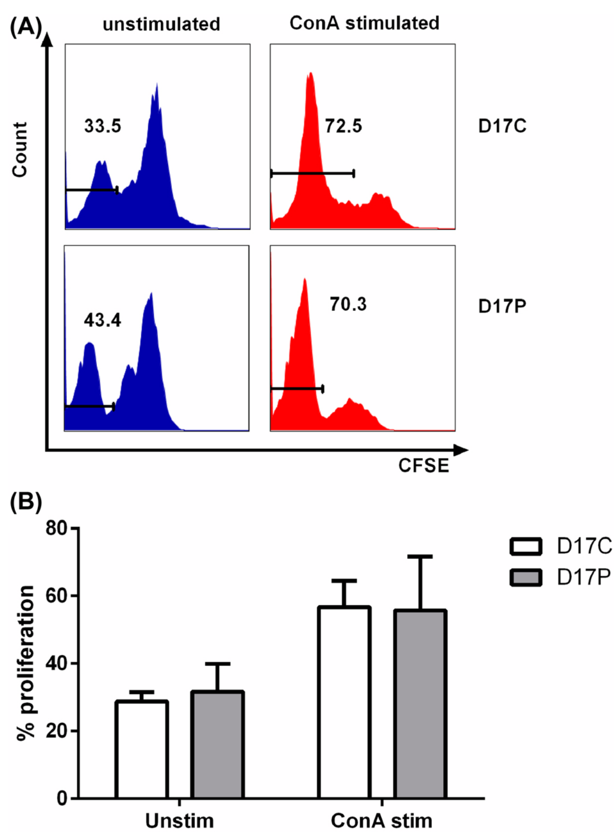

Figure 4. Proliferation of uterine immune cells ex vivo. Representative flow cytometric histograms (A) for CFSE intensity of $CD45^+$ uterine cells after 72 h of culture with or without ConA stimulation. (B) Percent proliferation of $CD45^+$ uterine cells from day 17 cyclic (D17C; n = 4) and day 17 pregnant (D17P; n = 4) animals. Student *t*-test compared D17C vs D17P. $P > 0.1$.

Effect of pregnancy on proliferation of uterine immune cells

To evaluate the proliferative potential of uterine immune cells during pregnancy, $CD45^+$ immune cells were labeled with CFSE and cultured with or without stimulation for 72 h. Although total $CD45^+$ immune cells proliferated in response to ConA, there was no effect of pregnancy status on proliferation of cells ($P > 0.1$; Figure 4).

Degranulation potential of uterine immune cells

Uterine immune cells were cultured with K562 (target) cells for 4 h, and degranulation was assessed by surface expression of CD107a. Incubation of target cells with uterine $CD45^+$ cells from pregnant heifers elicited a greater degranulation response compared to cyclic heifers ($P < 0.05$; Figure 5A and B). In contrast, degranulation of peripheral blood immune cells was unaffected by pregnancy status ($P > 0.1$; Figure 5B). Furthermore, mRNA abundance of the key cytotoxic granule protein, granzyme A *(GZMA)*, was greater in total endometrial RNA on day 17 of pregnancy compared to day 17 of the estrous cycle and decreased 3-fold ($P < 0.05$) by day 20 of pregnancy (Figure 5C).

Expression of immune regulatory proteins

Cytotoxic T cells and NK cells can be regulated through interaction with antigen-presenting cells including macrophages. Therefore, the presence of mRNA for the inhibitory surface receptors, *LAG3* and *CD274*, in the endometrium was assessed. On day 17 of pregnancy, endometrial *CD274* mRNA abundance was 18-fold greater com-pared to day 17 of the cycle and then decreased on day 20 of preg-nancy ($P = 0.01$; Figure 6A). Lymphocyte activation gene-3 mRNA abundance tended to be greater on day 20 compared to day 17 of pregnancy ($P = 0.06$; Figure 6B). Cytotoxic T lymphocyte-associated protein-4 expression was greater across the entire UW in pregnant compared to cyclic animals ($P < 0.01$; Figure 6D). Labeling for CTLA4 was evident on immune cells as well as uterine parenchymal cells, especially the luminal and glandular epithelial cells (Supple-mental Figure S6).

Effect of endometrial immune cell secretions on peripheral T cells

To evaluate if inhibitory factors secreted by uterine immune cells from pregnant heifers conferred immunosuppressive properties, a transwell proliferation assay was conducted. Proliferation of periph-eral T cells in the presence of $CD45^+$ uterine cell secretions was evaluated. Uterine immune cells from cyclic heifers enhanced pro-liferation of autologous peripheral T cells in the presence of ConA ($P < 0.05$, Figure 7A), while there was no effect of the secretions from uterine immune cells from pregnant animals on the proliferation of T cells ($P > 0.1$, Figure 7B).

Endometrial expression of immune function genes

To further understand the functional status of uterine immune cells during pregnancy, abundance of mRNA for several immune-mediating proteins in the endometrium was investigated. Abundance of galectin-1 *(LGALS1)* mRNA tended to be lower ($P = 0.07$) dur-ing pregnancy compared to day 17 of the estrous cycle and did not change between days 17 and 20 of pregnancy (Figure 8A). Abun-dance of interleukin 15 *(IL15)* mRNA tended to be greater ($P = 0.09$) in pregnant compared to cyclic heifers (Figure 8B). The transcription factors, GATA binding protein 3 (*GATA3)* and T-box 21 (*TBX21)*, were investigated to determine if there was a bias toward Th1 or Th2 signaling in early pregnancy. No difference was detected with status or day of pregnancy for *TBX21*, which is a Th1 transcription factor (Figure 8C). However, abundance of the Th2 transcription factor, *GATA3*, tended to be greater ($P = 0.06$) in pregnant compared to cyclic heifers and then tended to be greater ($P = 0.06$) at day 20 compared to day 17 of pregnancy (Figure 8D).

IL10 and interferon gamma expression by uterine immune cells

Endometrial *IL10* mRNA and protein expression by uterine immune cells was greater ($P < 0.05$) in pregnant compared to cyclic heifers and did not change between day 17 and day 20 of pregnancy (Figure 9A and B). Interferon gamma mRNA was detected in all three treat-ment groups, but no differences in abundance in endometrial mRNA were evident among the groups (Figure 9C). However, there was an increase ($P < 0.05$) in the percentage of uterine $CD45^+$ $IFNG^+$ cells between days 17 and 20 of pregnancy, as assessed by flow cytometry (Figure 9D).

Discussion

Little is known regarding changes in immune cell types and functions during the critical period of maternal recognition of pregnancy in dairy cattle. For successful pregnancy, conceptus signals must block the luteolytic mechanism, reprogram uterine immune cells to induce

Knowledge gap

Summary of process under investigation

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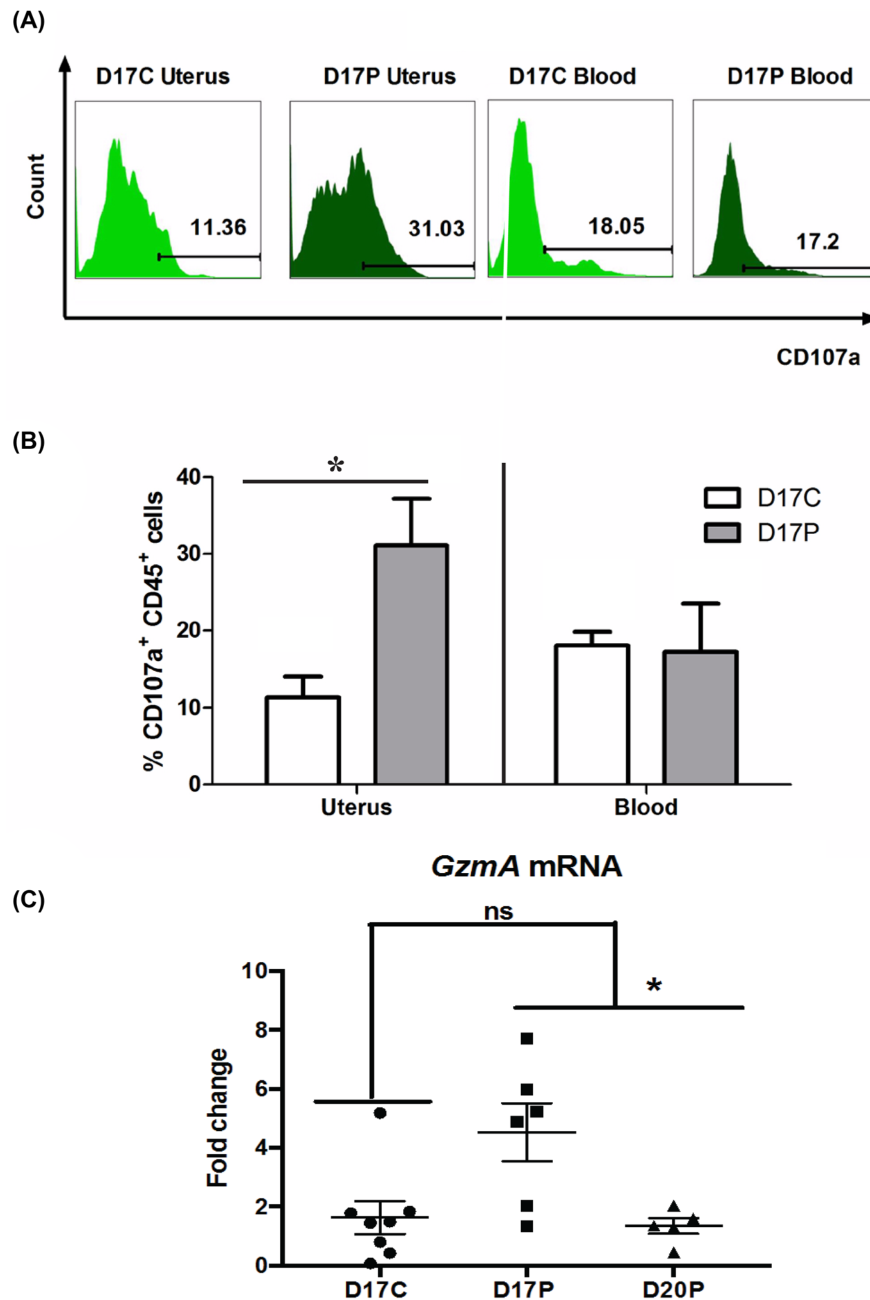

Figure 5. Uterine and peripheral immune cell degranulation and endometrial mRNA abundance of GZMA. Representative flow cytometric histograms (A) and mean ± SEM (B) CD107a surface expression on PKH26$^+$ uterine and peripheral CD45$^+$ cells from day 17 cyclic (n = 4) and day 17 pregnant (n = 4) animals. Student *t*-test compared status effect (D17C vs D17P) *: $P < 0.05$. (C) Fold change of endometrial mRNA for GZMA in day 17 cyclic (n = 8), day 17 pregnant (n = 6), and day 20 pregnant (n = 4) animals. Orthogonal comparison for status (D17C vs D17P and D20P) and days of pregnancy (D17P vs D20P) are indicated by lines. *: $P < .0.05$, ns: not significant.

What was hypothesized

Basis for hypothesis

Rationale for study subject

tolerance to conceptus alloantigens, and participate in the extensive uterine remodeling required for placentation. It was hypothesized that pregnancy would result in increased NK cells and induction of mediators of immune tolerance in the endometrium. This was based on prior work in rodents and humans [4, 10, 47] and limited work in ruminants [17, 18, 48].

Dairy heifers were used for this study because they exhibit high fertility. Furthermore, reduced conception rates in mature, lactat-ing dairy cows [49] are postulated to be due, in part, to failure

to reprogram the uterine mucosal immune system. Clearly, an ele-vated proinflammatory environment is detrimental to establishment of pregnancy. For example, cattle with clinical and subclinical dis-eases, including mastitis, exhibited reduced fertility [37]. Determining the types and functional status of uterine immune cells is neces-sary to understand their roles in early pregnancy. Embryos can be transferred into cyclic cattle no later than days 15–16 after the onset of estrus [50], indicating that conceptus signaling must be initiated before day 17 of pregnancy in cattle. Days 15–16 also corresponds

Continued: Rationale for study subject

Need for this research

What was already known about process under investigation

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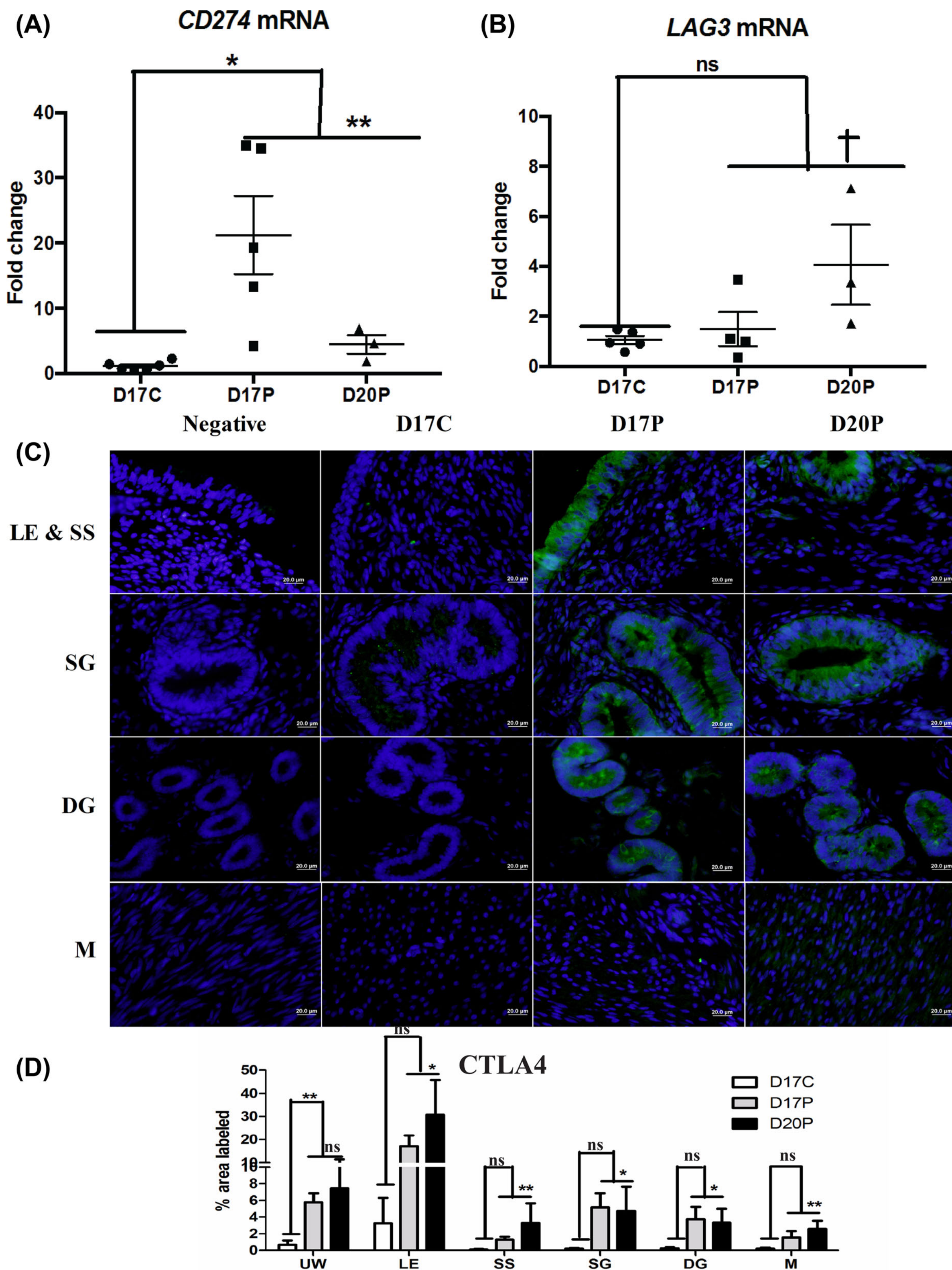

Figure 6. Endometrial mRNA abundance of CD274, LAG3, and IF analysis of CTLA4 expression in the uterus. Fold change of endometrial mRNA for CD274 (A; D17C n = 6, D17P n = 5, and D20P n = 3) and LAG3 (B; D17C n = 5, D17P n = 4, and D20P n = 4). Representative panel (C) and ImageJ quantification (D) for CTLA4 IF (% area labeled) of uterine wall (UW), luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M) in day 17 cyclic (n = 4), day 17 pregnant (n = 4), and day 20 pregnant (n = 5) uterus. The negative isotype controls for the respective areas are denoted as negative. Magnification ×400. Orthogonal comparison for status (D17C vs D17P and D20P) and days of pregnancy (D17P vs D20P) are indicated by lines. †0.05 > $P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, ns: not significant.

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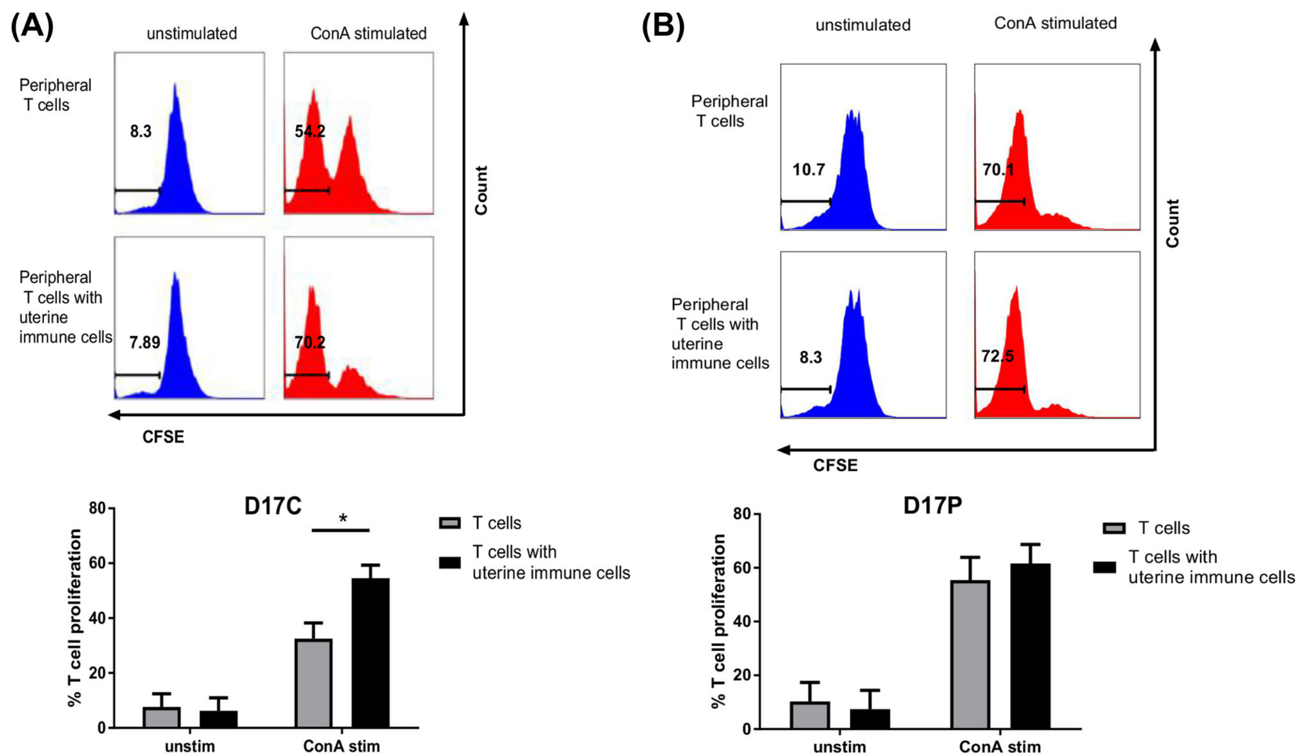

Figure 7. Effect of endometrial immune cells secretions on peripheral T-cell proliferation. Representative flow cytometric histograms (A, C) and mean percent proliferation (B, D) of autologous peripheral T cells in the presence and absence of uterine immune cells secretions (n = 4) with or without ConA stimulation in day 17 cyclic (A, B) and day 17 pregnant (C, D) animals. Student *t*-test compared D17C vs D17P.*: $P < 0.05$.

Rationale for methods, connecting methods to the focus of this study

Summary of focus of this study; area of future research

Key finding: what results showed about hypothesis

Similarity to other research; 1st category of evidence that supports hypothesis

Difference between this result and other research

Suggested reason for difference

Details about methods and results of this study that relate to difference

How methods may have affected results that relate to difference

Fact from similar research on other species

to the peak IFNT secretion per cell by the bovine conceptus [51–53]. Therefore, day 17 of pregnancy and the estrous cycle was chosen to identify the proximal changes in immune function during a time when plasma P4 concentrations are similar between the two statuses. Day 20 of pregnancy was included to examine immune responses 4–5 days after signaling is established. Between day 16 and day 19, the conceptus is rapidly elongating, forming the first binucleate gi-ant cells, establishing an allantois and functional yolk sac, as well as vitelline circulation [51, 54–56]. While this study focused on the crit-ical peri-implantation period prior to conceptus attachment, future studies will address these same questions after day 20 of pregnancy during a period of rapid placental growth and development.

Results of this study provide evidence that uterine lymphoid cells respond to maternal and/or embryonic signals during early preg-nancy in dairy heifers. Similar to results in human, rodent, and porcine endometrium, NKp46$^+$ cells constituted about half of the CD45$^+$ cells in the endometrium of pregnant heifers. This differs from the findings of Oliveira et al. [6], who detected a decrease in NKp46$^+$ cells when comparing day 16 of pregnancy to day 16 of cycle. This difference could be attributed to differences in NKp46 antibodies (ovine vs. bovine) used as well as days of pregnancy ex-amined (day 16 vs days 17 and 20). In this study, both IF analysis and flow cytometric data demonstrated a greater percentage of NKp46$^+$ cells in the pregnant uterus than the cyclic uterus. The NKp46 anti-body was used for both flow and IF experiments and appeared to be more robust in staining for flow cytometry detection compared to IF. This could be the reason for apparent low number of cells identified by IF in uterus while flow data suggests around 50% of CD45$^+$ cells to be NKp46$^+$ in the pregnant uterus. During early human and rodent pregnancy, uNK cells are abundant around trophoblast cells and are thought to be required for trophoblast attachment and invasion [4]. Endometrial NKp46$^+$ cells were observed in luminal epithelium and shallow stroma where they may facilitate placental vascularization and the attachment process by producing angiogenic proteins. However, this remains to be determined.

Similar to results with NKp46, a population of CD8$^+$ cells were more abundant in pregnant compared to cyclic heifers and their numbers declined modestly from day 17 to day 20 of pregnancy. The presence of CD8$^+$ T cells in the endometrium is documented in human pregnancy [57]. These cells persist in the fetal–maternal in-terface throughout pregnancy and exhibit an inhibitory phenotype, lacking expression of CD28. Whether the bovine CD8$^+$ T cells in the pregnant endometrium are inhibitory remains to be determined. Because a high percentage of NKp46$^+$ cells and CD8$^+$ cells were present in the uterus, coexpression of these two proteins on the same cells was investigated. Although not quantified, the majority of uterine CD45$^+$ cells coexpressed NKp46 and CD8 proteins with fewer than 20% of cells expressing only one of these two proteins. Boysen et al. [58] reported that bovine NK cells coexpress CD8 in the sec-ondary lymphoid organs, but this is the first report of such cells in the endometrium. There is, however, evidence for expression of NK cell receptors, including NKp46, on $\gamma\delta$ and CD8 T cells cultured with IL15 or TGFB [59–61]. More importantly, a recent study identified a unique subset of CD3$^+$ NKp46$^+$ cells in bovine PBMC that ex-pressed CD8 surface protein [62]. Those authors demonstrated that these nonconventional NK T cells responded to both T-cell receptor (TCR) and NK signaling and had a diverse TCR repertoire, unlike conventional NKT cells. It is therefore possible that the NKp46$^+$ cells identified in this study have similar phenotypic and functional prop-erties as those nonconventional NKT cells in blood. Further studies

Specific result from this study and how it may relate to the hypothesis

Remaining knowledge gap

2nd category of evidence

Similar research on other species

Remaining knowledge gap

Rationale for method

Result of investigation

Comparison of result to other research

Related findings from other research

Possible explanation for how hypothesized process works in this study based on findings from other research

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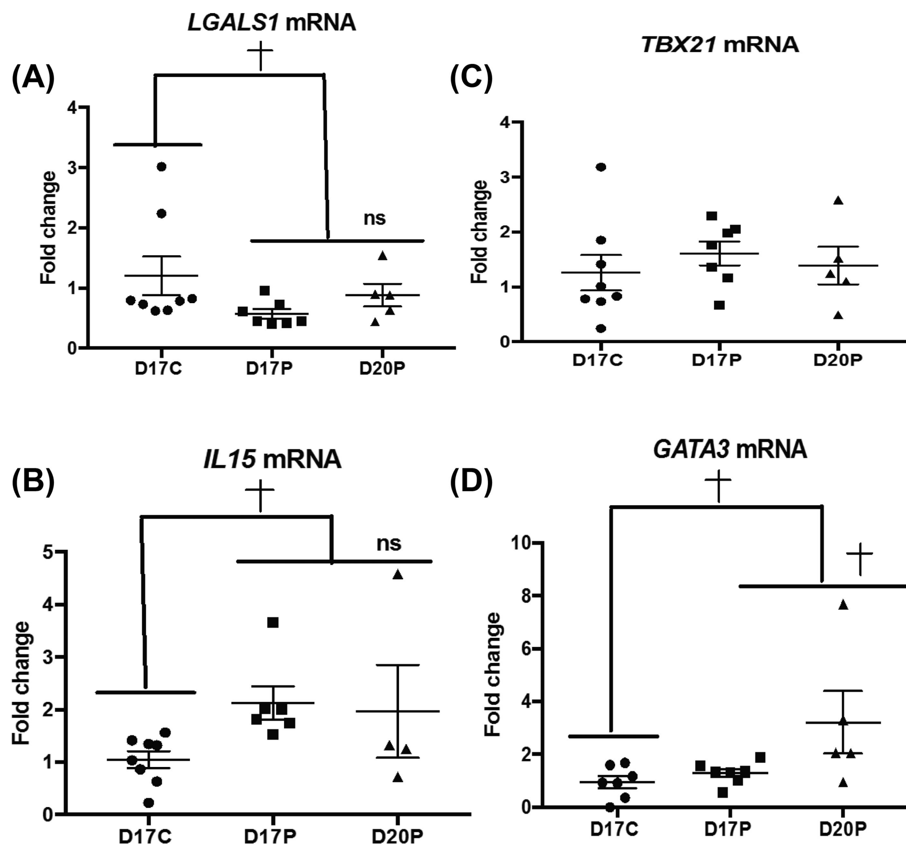

Figure 8. Endometrial mRNA abundance of LGALS1, IL15, TBX21, and GATA3. Fold change of endometrial mRNA for LGALS1 (A; D17C n = 8, D17P n = 6, and D20P n = 4),), IL15 (B; D17C n = 8, D17P n = 6, and D20P n = 4), TBX21 (C; D17C n = 8, D17P n = 7, and D20P n = 5), and GATA3 (D; D17C n = 7, D17P n = 7, and D20P n = 5). Orthogonal comparisons for status (D17C vs D17P and D20P) and day of pregnancy (D17P vs D20P) are indicated by lines. †: $0.05 > P < 0.1$, ns: not significant.

Remaining knowledge gap

3rd category of evidence and how it supports the hypothesis

Related fact from other research

Hypothesized detail about evidence

4th category of evidence from this study and how it relates to other evidence from this study

Possible explanation for how hypothesized process works based on evidence from this study, and follow-up idea

Rationale for investigating follow-up idea, a 5th category of evidence

are needed to determine the lineage and phenotypic characteristic of this population in the bovine uterus.

The increased degranulation in uterine immune cells from preg-nant heifers in the current study indicates greater cytotoxic potential of these cells during pregnancy. Prior work showed that exposure to IFNT can increase cytotoxicity of NK cells [23], and the cells isolated from pregnant heifers in this study were exposed to IFNT in vivo prior to collection. We did not determine which cell population(s) among the $CD45^+$ cells degranulated; however, we hypothesize that it was the $NKp46^+$ and/or $CD8^+$ cells because they constituted the greatest percentage of $CD45^+$ during pregnancy. Coincident with the increase in degranulation, there was greater abundance of *GZMA* mRNA in the endometrium on day 17 of pregnancy. Messenger RNA for *GZMA* was 3-fold greater on day 17 in pregnant com-pared to cyclic heifers and then declined on day 20 compared to day 17 of pregnancy. The pattern of change was similar to the expres-sion pattern of $CD8^+$ T cells. Therefore, degranulation of cytotoxic granules containing GZMA by $CD8^+$ T cells and/or $NKp46^+$ cells could occur during early pregnancy. We postulate that degranulation is associated with uterine remodeling or a process of reducing the toxicity of these cells as pregnancy progresses. However, excessive release of cytotoxic granules could be harmful for pregnancy, and thus there are most likely mechanisms in place to regulate immune cell cytotoxicity.

The presence of cytotoxic cells in the pregnant uterus led to the investigation of potential inhibitory protein expression that could regulate their functions. The abundance of *CD274* and *LAG3* mRNA as well as CTLA4 protein in the endometrium was greater in pregnant compared to cyclic heifers. These immune-mediating factors were expressed on both uterine parenchymal and immune cells. Expression of MHC II protein as well as *CD80* and *CD86* costimulatory genes increased in the bovine endometrium during early pregnancy [5]. Interaction of lymphocytes with MHC II^+ APC can modulate the functions of both APC and lymphocytes. For example, CD274 expressed on APC, and parenchymal cells, could induce T-cell an-ergy via engagement of PDCD1 [32]. Similarly, LAG3, expressed on activated T and NK cells, could interact with MHC II to induce tolerogenic myeloid cells and thus regulate proliferation and activa-tion of effector T and NK cells [33, 63]. Cytotoxic T lymphocyte-associated protein 4 interacts with CD80/86, blocking its interaction with CD28 and causing T-cell anergy [64]. These interactions confer immunosuppressive properties to immune cells [65]. Interestingly, uterine immune cells from cyclic, but not pregnant, heifers stim-ulated proliferation of autologous T cells in the presence of ConA. Although the proliferation response in D17C and D17P were not dif-ferent, it is possible that IFNT exposure in the pregnant animals led to a more robust response in the peripheral T cells from the pregnant animals. The supplementation of uterine secretions from pregnant heifers, therefore, had minimal influence on proliferation unlike that seen in cells from cyclic heifers. Furthermore, direct contact between target and effector cells may be needed for immunosuppressive func-tions of immune cells in pregnant heifers. However, variability in

Result of investigation

Possible explanation for how hypothesized process works based on other research

Related, surprising result from this study

Possible explanation for surprising result

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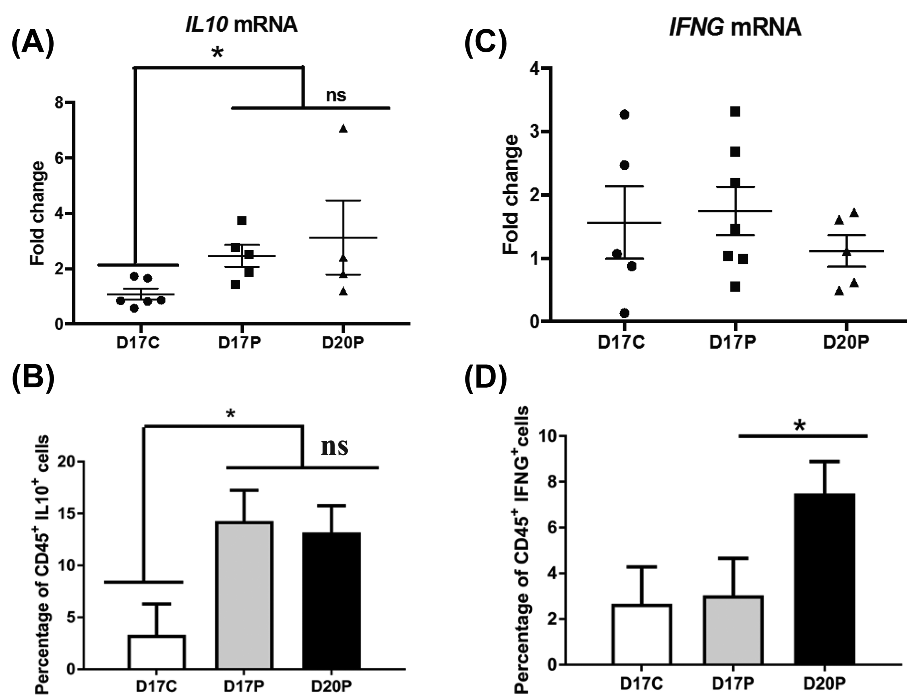

Figure 9. Endometrial mRNA abundance and uterine immune cell expression of IL10 and IFNG. Fold change of endometrial mRNA transcripts for IL10 (A; D17C n = 6, D17P n = 5, and D20P n = 4) and IFNG (C; D17C n = 5, D17P n = 7, and D20P n = 5). Percentage of IL10$^+$ (B) and IFNG$^+$ (D) cells of the total CD45 sorted uterine immune cell population in day 17 cyclic (n = 3), day 17 pregnant (n = 3), and day 20 pregnant (n = 4) endometria. Orthogonal comparison for status (D17C vs D17P and D20P) and day of pregnancy (D17P vs D20P) are indicated by lines. * = $P < 0.05$.

T-cell response to ConA among cows cautions overinterpretation of these results and requires further studies to clarify mechanisms.

Abundance of *LGALS1*, which is secreted by uNK cells in other species [66], was also evaluated. Galectin-1 is an immunoregulatory glycan-binding protein that induces tolerogenic dendritic cells and T cells in the human uterus. Galectin-1 can also induce toler-ance by causing apoptosis of activated T cells [67]. Ovine midgestation placentas express LGALS1 protein and addition of LGALS1 decreased ovine T-cell proliferation in vitro [68]. Results from this study showed that LGALS1 mRNA abundance was lower during early pregnancy compared to the cycle. Therefore, LGALS1 may not be important for maintaining fetal–maternal tolerance during early pregnancy in dairy heifers. Of course, it is possible that other galectin family members are involved in immune regulation dur-ing early pregnancy. For example, the presence of galectin-9, 15 (LGALS9, LGALS15), and galectin-3 binding protein (LGAL3BP) were reported in the ruminant uterus during pregnancy [69]. The authors of that study examined days 5–16 of the estrous cycle and gestation and did not detect changes in *LGALS1* mRNA abundance with pregnancy. However, they observed an increase in *LGALS9* and *LGALS3BP* at day 16 of pregnancy compared to the cycle. Galectin 15 is important in trophoblast proliferation, migration, and attach-ment in goats and sheep, but is not expressed in bovine endometrium [70, 71].

T helper 1 or Th2 bias in the uterine environment was investi-gated by analyzing abundance of Th1 and Th2 transcription factors. T-box 21 is a Th1 transcription factor that regulates expression of the key Th1 cytokine IFNG. Interferon gamma directs differentia-tion of Th1 lineage cells by activating Th1 genes and repressing Th2 programming [72]. During pregnancy, TBX21 null mice exhibited defective NK cell maturation, but normal uNK cells [73] indicating that Th1 cells may not be critical for uNK development. Endometrial TBX21 and *IFNG* mRNA abundance did not change with day or pregnancy status in this study. The T helper 2 transcription factor, GATA-3, is required for differentiation of Th2 cells and for Th2 cytokine production by differentiated Th2 cells [74]. GATA binding protein 3 tended to be greater ($P = 0.06$) in the endometrium of pregnant heifers and also tended to be greater ($P = 0.06$) in day 20 compared to day 17 of pregnancy. Effects of pregnancy and P4 on TBX21 and GATA3 in PBMC of cattle were reported in one study [75]. More *GATA3* mRNA was present in PBMC from mid gestation cows, compared to nonpregnant cows (luteal phase of cycle), when treated with P4 (10 μg/mL). Progesterone decreased the abundance of *TBX21* mRNA in PBMC from both pregnant and nonpregnant animals suggesting a role for P4 in suppressing Th1 signaling. These results, along with *GATA3* mRNA abundance in the endometrium from pregnant heifers, suggest that conceptus secretions and pres-ence of P4 may bias the environment toward Th2 activation during early pregnancy. This is consistent with the concept that Th2 cy-tokines and cells are dominant during normal human pregnancy and an aberrant bias toward a Th1 environment leads to pregnancy complications including preeclampsia and preterm labor [76].

The cytokine response to pregnancy was analyzed to understand the tolerogenic and inflammatory milieu in the endometrium. There are conflicting results regarding expression of the tolerogenic cytokine, IL10, in the bovine endometrium. Interleukin 10 expres-sion was not detected by PCR at day 16 of bovine early preg-nancy [8]. However, Oliveira et al. [6] did detect *IL10* expression between day 13 and day 16 of pregnancy in beef cattle, but the mRNA transcript abundance did not change [6]. This study showed that *IL10* mRNA and CD45$^+$ IL10$^+$ cells were more abundant in the pregnant endometrium compared to the cyclic endometrium.

Remaining knowledge gap
Definition of a 6th category of evidence that was investigated
Rationale for investigating it
Result of investigation
Interpretation of result (did not appear to be a part of the hypothesized process)
Possible explanation for how hypothesized process works based on other research
A 7th category of evidence that was investigated
Rationale for investigating it
Result of investigation
Definition of term
Result of investigation, using term
Fact from other research
Result of investigation
Possible explanation for how hypothesized process works based on evidence from this study and other research
An 8th category of evidence that was investigated
Conflicting results from other research on this topic
Result of investigation

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How result relates to hypothesis

Rationale for a 9th category of evidence that was investigated

Result of investigation

How result relates to hypothesis

Remaining knowledge gap

Possible explanation for how hypothesized process works based on evidence

Restatement of objective

Restatement of evidence (Categories 1 and 2) that supported hypothesis

Restatement of evidence (Category 3) that supported hypothesis

Restatement of evidence (Categories 5 and 8) that supported hypothesis

Affirmation that results support hypothesis

Importance of this research to industry

Direction of future research

Interleukin 10 maintains an immunotolerant environment by suppressing fetal-specific T-cell proliferation [77]. Interferon gamma mRNA was analyzed because IFNG plays an important role in placentation in human [78], rodent [79], and porcine [80] endometrium. Total *IFNG* mRNA abundance did not change in the uterus with pregnancy. However, an increase in the percentage of CD45$^+$ IFNG$^+$ uterine immune cells was detected between day 17 and day 20 of pregnancy. Interferon gamma is known to suppress development of Th17 inflammatory cells [81] as well as to facilitate angiogenesis during pregnancy [79]. Whether IFNG suppresses Th17 cells dur-ing bovine pregnancy is yet to be determined. Secretion of IFNG by uterine immune cells during early pregnancy could facilitate an-giogenesis for placentation and IL10 could regulate inflammatory factors to ensure conceptus survival.

In conclusion, this study tested the hypothesis that tolerogenic lymphocytes are induced in response to pregnancy to ensure con-ceptus survival in dairy heifers. This study established an increase in endometrial NKp46$^+$ and CD8$^+$ T cells during early pregnancy. Interestingly, uterine immune cells from pregnant heifers had greater cytotoxic potential as measured by degranulation assay, which could be a direct effect of IFNT. The abundance of functional cytotoxic cells could indicate a role for these cells in pregnancy establishment in cattle, possibly for angiogenesis. However, results also provide evidence for a complex and dynamic regulation of immune function at the conceptus maternal interface during early pregnancy. The in-hibitory molecules, including *CD274, LAG3,* and CTLA4, increased and may control inflammatory responses to ensure fetal survival by inducing tolerogenic macrophages and T-cell anergy. Increase in IL10 expression by uterine immune cells during pregnancy further support the presence of such regulatory mechanisms. Thus, results are consistent with the hypothesis that pregnancy requires a balance of inflammatory and tolerogenic responses. Any perturbation of this balance, for example by infections, could result in reduced fertility as seen in mature, lactating dairy cows. Future studies will investigate whether the functions of the lymphoid cells, including NKp46$^+$and CD8 T cells, and their interaction with endometrial myeloid cells are altered in subfertile dairy cows compared to fertile dairy heifers.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Immunofluorescence analysis of CD3 expression in uterus. Representative panel (A) and ImageJ quan-tification (B) for CD3 IF (% area labeled) of uterine wall (UW), luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M) in day 17 cyclic (n = 5), day 17 pregnant (n = 4), and day 20 pregnant (n = 4) uterus. The neg-ative isotype controls for the respective areas is denoted as negative. Magnification ×400. $P > 0.1$.

Supplemental Figure S2. Immunofluorescence analysis of $\gamma\delta$ ex-pression in uterus. Representative panel (A) and ImageJ quantifica-tion (B) for $\gamma\delta$ IF (% area labeled) of uterine wall (UW), luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M) in day 17 cyclic (n = 5), day 17 pregnant (n = 5), and day 20 pregnant (n = 5) uterus. The nega-tive isotype controls for the respective areas is denoted as negative. Magnification ×400. $P > 0.1$.

Supplemental Figure S3. Immunofluorescence analysis of CD4 expression in uterus. Representative panel (A) and ImageJ quan-tification (B) for CD4 IF (% area labeled) of uterine wall (UW), luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M) in day 17 cyclic (n = 5), day 17 pregnant (n = 5), and day 20 pregnant (n = 5) uterus. The negative isotype controls for the respective areas is denoted as negative. ImageJ quantification for CD4 immunofluorescence (% area labeled) is shown in B. Magnification ×400. $P > 0.1$.

Supplemental Figure S4. Flow cytometric analysis of CD8. Flow cytometry scatter plots showing percentage of cells that express CD8β of endometrial CD45$^+$ cells from day 17 Cyclic (n = 3), day 17 pregnant (n = 3), and day 20 pregnant (n = 4) heifers.

Supplemental Figure S5. Flow cytometric analysis of NKp46. Flow cytometry scatter plots showing percentage of cells that express NKp46 of endometrial CD45$^+$ cells from day 17 cyclic (n = 3), day 17 pregnant (n = 3), and day 20 pregnant (n = 4) heifers.

Supplemental Figure S6. CTLA4 expression on parenchymal and immune cells in uterus. Representative images of CTLA4 (green) labeling in uterine section is shown in ×1000 magnification. Nuclei are counterstained with DAPI (blue).

Supplemental Table S1. List of antibodies.

Supplemental Table S2. List of primers.

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