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.
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 conceptus 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 signaling. 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 important role in spiral artery remodeling to facilitate placentaion [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 how these cells during the critical window of pregnancy recognition signaling in cattle.

Uterine natural killer cells constitute 70% of decidual lymphocytes during human early pregnancy and are characterized by a CD56bright CD16– phenotype [14]. In rodents and other mammals that do not express CD36, NKp46 (CD335), a highly conserved natural 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 pregnancy [19]. Limited data are available about the presence of NK cells in bovine endometrium [6]. Macrophages, CD4+ and CD8+ T lymphocytes were reported in the bovine uterus during the estrous cycle and pregnancy [5, 20, 21]. Cytotoxic CD8+ and gamma delta (γδ) T cells were also found in ovine endometrium during mid and late gestation, and γδ 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 placentaion [24]. Preeclampsia and other pregnancy com-lications 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 interleukin 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 in-

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 estrus synchronized using synthetic prostaglandin F2α analog (Cloprostenol 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 (D17; 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...
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 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 Vi-sociation of the endometrium into a single-cell suspension was carried out on ice cold RPMI (11 835–055; Gibco RPMI medium, Life Technologies, Grand Island, NY) for immune cell isolation.

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.

DNA 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 (Q7650–05, 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 P4 (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% PBS-F12 to stop the dye uptake. This was followed by centrifugation at 295 × g for 10 min at 4°C. Washing was done with 10% PBS-F12 medium two more times, and cells were resuspended to a final concentration of 2 × 10^5/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 3:1 for 4 h. After the
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. *: \( P < 0.05 \), **: \( P < 0.01 \), ***: \( P < 0.001 \).

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.

Results

Proportion of T and natural killer cells

Immunofluorescence analysis (Supplemental Figure S1–3) for CD3, γδ, 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
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.
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.

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.
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 compared to day 17 of the cycle and then decreased on day 20 of pregnancy ($P = 0.01$; 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 (Supplemental 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 peripheral T cells in the presence of CD45+ uterine immune cell secretions was evaluated. Uterine immune cells from cyclic heifers enhanced proliferation of autologous peripheral T cells in the presence of ConA ($P < 0.05$, Figure 7A), whereas 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 ($P = 0.07$) during pregnancy compared to day 17 of the estrous cycle and did not change between days 17 and 20 of pregnancy (Figure 8A). Abundance 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 treatment 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**
Dairy heifers were used for this study because they exhibit high fertility. Furthermore, reduced conception rates in mature, lactating dairy cows [49] are postulated to be due, in part, to failure to reprogram the uterine mucosal immune system. Clearly, an elevated proinflammatory environment is detrimental to establishment of pregnancy. For example, cattle with clinical and subclinical diseases, including mastitis, exhibited reduced fertility [37]. Determining the types and functional status of uterine immune cells is necessary 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 correspond to

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 < .005, ns: not significant.
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.
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.

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 interface 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 secondary 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 γδ and CD8 T cells cultured with IL15 or TGFβ [59–61]. More importantly, a recent study identified a unique subset of CD3+ NKp46+ cells in bovine PBMC that expressed CD8 surface protein [62]. Those authors demonstrated that these nonconventional NK T cells respond 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 properties as those nonconventional NKT cells in blood. Further studies
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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.

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 anergy 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 activation 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 stimulated proliferation of autologous T cells in the presence of ConA. Although the proliferation response in D17C and D17P were not different, 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 functions of immune cells in pregnant heifers. However, variability in
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 tolerance 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 during 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 LGAL3SBP at day 16 of pregnancy compared to the cycle. Galectin 15 is important in trophoblast proliferation, migration, and attachment in goats and sheep, but is not expressed in bovine endometrium [70, 71].

T helper 1 or Th2 bias in the uterine environment was investigated 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 differentiation 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 presence of P4 may bias the environment toward Th2 activation during early pregnancy. This is consistent with the concept that Th2 cytokines and cells are dominant during normal human pregnancy and an aberrant bias toward a Th1 environment leads to pregnancy complications including preclampsia 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 expression was not detected by PCR at day 16 of bovine early pregnancy [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.
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 placenta development 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 CD4+ 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 during bovine pregnancy is yet to be determined. Secretion of IFNG by uterine immune cells during early pregnancy could facilitate angiogenesis for placenta formation 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 conceptus survival in dairy heifers. This study established an increase in endometrial NKp46+ and CD8+ T cells during early pregnancy. Interestingly, uterine immune cells from pregnancy heifers had greater cytotoxic potential as measured by degranulation assay, which could be a direct effect of IFN. 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 inhibitory molecules, including CD274, LAIC3, 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 parthenogenetic 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 quantification (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 = 5), and day 20 pregnant (n = 5) uterus. The negative isotype controls for the respective areas is denoted as negative. Magnification ×400. P > 0.1.

**Supplemental Figure S2.** Immunofluorescence analysis of yδ ex-expression in uterus. Representative panel (A) and ImageJ quantification (B) for yδ 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. Magnification ×400. P > 0.1.

**Supplemental Figure S3.** Immunofluorescence analysis of CD4 expression in uterus. Representative panel (A) and ImageJ quantification (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. Magnification ×400. P > 0.1.

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