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Changes in Myeloid Lineage Cells in the Uterus and Peripheral Blood of Dairy Heifers During Early Pregnancy[1]

Manasi M. Kamat,[5] Sreelakshmi Vasudevan,[5] Samar A. Maalouf,[3,5] David H. Townson,[4,6] Joy L. Pate,[5] and Troy L. Ott[2,5]

[5]*Department of Animal Science, Center for Reproductive Biology and Health, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, Pennsylvania*
[6]*Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire*

ABSTRACT

Establishment of pregnancy requires interaction between the developing conceptus and the uterine mucosal immune system. Myeloid lineage cells (macrophages and dendritic cells) are key mediators of pregnancy in rodents and humans but relatively little is known regarding their role and distribution during early pregnancy in ruminants. We tested the hypothesis that myeloid lineage cell number, distribution, and function are altered during early pregnancy in dairy heifers. Dairy heifers were inseminated using sperm from a single bull (Day 0), and uteri and blood were collected at slaughter on Days 17 and 20 of pregnancy to investigate the response of myeloid lineage cells to the presence of a conceptus. Responses were compared to noninseminated heifers on Day 17 of the estrous cycle. Peripheral blood and uterine-derived immune cells were isolated magnetically and examined using flow cytometry. Immunohistochemical analysis was used to evaluate the spatial distribution of myeloid lineage cells in the endometrium and quantitative polymerase chain reaction was conducted to quantify abundance of mRNA transcripts associated with myeloid lineage cell function. Transcripts for major histocompatibility complex (*MHC*) II, cluster of differentiation (*CD*) 80, *CD86*, *CD163*, and indoleamine 2,3-dioxygenase (*IDO*) 1 were greater in endometrium of pregnant compared to cyclic heifers. Immunofluorescence analysis revealed increased labeling for MHCII and SIRPA in pregnant compared to cyclic heifers. There were approximately 50% more CD14$^+$CD11c$^+$ cells in the peripheral circulation of pregnant compared to cyclic heifers. A greater number of myeloid lineage cells were observed during early pregnancy, and this increase was most pronounced in and around the shallow glands. Furthermore, expression of molecules associated with a tolerogenic or alternatively activated phenotype of these cells also increased in pregnant heifers. The results support the hypothesis that myeloid lineage cells with a tolerogenic phenotype are involved in establishment of pregnancy in dairy heifers.

cattle, conceptus, dendritic cell, macrophage, pregnancy, uterus

INTRODUCTION

Low fertility reduces profitability and efficiency of animal production. Early embryonic loss is a major source of pregnancy failure in dairy cattle [1], with the majority of loss occurring during the first 4 wk of gestation [2]. Embryos can be successfully transferred into cyclic cattle as late as 15 days after the onset of estrus, but no later [3], indicating that the critical window for conceptus signaling to maintain pregnancy occurs between Days 15 and 18 of early pregnancy.

During this window, conceptus-secreted interferon tau (IFNT) prevents regression of the corpus luteum (CL), ensuring that progesterone production is maintained [4]. Furthermore, IFNT, a type I interferon, is postulated to be responsible for altering uterine immune cell function to sustain an environment supportive of embryo growth and placentation [5]. Not surprisingly, uterine immune cells are responsive to IFNT [6–9], as are immune cells in the systemic circulation [10, 11]. Nonetheless, relatively little is known about the effects of the conceptus on uterine immune cell populations and function during this critical window, and the role of the mucosal immune system during this period remains one of the least understood aspects of peri-implantation biology [12].

In mammals, the maternal immune system must be modulated to accommodate the semiallogeneic conceptus. Furthermore, immune modulation occurs not only in the uterus but also in the peripheral immune system [5, 13]. Both myeloid and lymphoid lineage cells participate in regulating trophoblast attachment/invasion in humans and rodents [14, 15]. Lymphoid lineage cells include T cells, natural killer (NK) cells, and B cells, and myeloid lineage cells include monocytes, macrophages, and dendritic cells (DCs). Both lineages are essential for successful pregnancy outcomes. For example, NK cells and macrophages are needed for spiral artery remodeling in rodents and humans, and T cells influence implantation and the establishment of immune tolerance [16, 17]. Nevertheless, relatively little is known about these cell types in ruminants, especially during early pregnancy. Several reports have quantified myeloid lineage cells during the estrous cycle or early pregnancy in beef heifers [6, 18, 19], with somewhat conflicting results. The reasons for the discrepancies between these three studies are not clear, but may be related to the specificity of antibodies used and/or that the temporal and spatial relationships were not examined. Clearly, determination of the temporal and spatial regulation of myeloid lineage cells will help identify potential immune cell targets for conceptus signals during early pregnancy.

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[2]Correspondence: Troy L. Ott, Department of Animal Science, 324 Henning Building, University Park, PA 16802. E-mail: tlo12@psu.edu
[3]Current address: INDIGO Biosciences Inc., State College, PA.
[4]Current address: Department of Animal and Veterinary Sciences, University of Vermont, Burlington, VT.

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Myeloid lineage cells arise from hematopoietic stem cells in the bone marrow and are composed of monocytes, macrophages, DCs, erythrocytes, and granulocytes [20]. Macrophages that express prostaglandin E2, interleukin (IL) 4, IL10, indoleamine 2,3-dioxygenase 1 (IDO1), arginase1, and mannose receptor C are considered to be tolerogenic or alternatively activated (M2) [21–23]. M2 macrophages promote an immune-privileged environment, resolving inflammation and facilitating tissue remodeling [24, 25]. Therefore, an abundance of macrophages at the maternal-fetal interface during early pregnancy conceivably contributes to a tolerogenic environment in the endometrium, promoting conceptus survival and development of the placenta. Conversely, macrophages that produce tumor necrosis factor alpha, IL6, and chemokine (C-C motif) ligand (CCL) 2 are characterized as proinflammatory or M1, and exhibit increased phagocytosis and activation of T-cell responses. Currently, the functional status of myeloid lineage cells in the uterus of the cow during early pregnancy is not clear, but likely extends beyond simply antigen presentation and phagocytosis [17].

DCs are present at the fetal-maternal interface during early pregnancy in cattle, rodents, and humans [19, 26, 27]. DCs also regulate T-cell responses and, depending on their activation status, they can induce either tolerogenic or inflammatory responses [28, 29]. Decidual DCs cultured with thymic stromal lymphopoietin (TSLP) produce CCL17 and IL10 [30], which are involved in DC migration [31] and induction of a tolerogenic environment, respectively [32]. Effects of DCs on T-cell response to placental antigens at the fetal-maternal interface may mediate tolerance towards the allogeneic conceptus [30]; however, this has not been studied during early pregnancy in the bovine endometrium.

Thus, both macrophages and DCs are likely to play important roles at the fetal-maternal interface to facilitate survival of the conceptus. This study tested the hypothesis that myeloid lineage cells increase in the uterus and peripheral blood during early pregnancy, and that their abundance is temporally and spatially regulated in the uterine wall (UW). Furthermore, we hypothesized that pregnancy results in increased expression of proteins associated with alternatively activated myeloid lineage cells to promote conceptus attachment and placentation.

MATERIALS AND METHODS

Animals and Tissue Collection

All procedures involving animals were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (protocol #44524) and comply with the guide for the care and use of agricultural animals in agricultural research and teaching [33]. Postpubertal commercial Holstein dairy heifers (350–450 kg) were synchronized using a synthetic prostaglandin $F_{2\alpha}$ analog (cloprostenol sodium, 500 μg; Intervet). Heifers were monitored for estrus three times per day for 30 min each. On the day of estrus (Day 0), animals were either inseminated with frozen-thawed semen from one bull of proven fertility (n = 12 heifers for Day 17 of pregnancy and n = 7 heifers for Day 20 of pregnancy) or remained uninseminated and served as cyclic controls (n = 9 heifers for Day 17 of estrous cycle). Heifers were slaughtered at a commercial abattoir to generate the following study groups: Day 17 cyclic (n = 9), Day 17 pregnant (n = 9), and Day 20 pregnant (n = 5). As expected, some inseminated heifers were not pregnant (lacked conceptus or inappropriate conceptus size) and were excluded from further analyses. At slaughter, 500 ml jugular blood was collected with EDTA as an anticoagulant for isolation of peripheral blood mononuclear cells (PBMCs) [34]. The reproductive tracts were collected within 10 min of exsanguination and the uteri were flushed with 40 ml ice-cold PBS to recover conceptuses. Plasma samples were collected after centrifugation of blood at 1500 × *g* and presence of a functional CL was confirmed using a progesterone ELISA validated for use in dairy cattle [35]. Concentrations of progesterone >2 ng/ml were observed for all heifers on the day of slaughter. Pregnancy was confirmed by flushing a conceptus of appropriate size from the uterus. Endometrium (15 g) was trimmed from the uterine horn ipsilateral to the CL and stored in ice-cold RPMI medium 1640 lacking phenol red (11835-030; Life Technologies) containing 1% w/v bovine serum albumin (BSA; 2930; OmniPur; EMD Millipore) and 0.1% v/v gentamycin solution (10 mg/ml; Gibco, Life Technologies). Enzymatic dissociation of the endometrium was carried out in RPMI containing collagenase (1000 U/ml; Worthington Biochemicals) and dispase (4.8 mg/ml; Roche Diagnostics) for 1 h at 37°C in a flat-bottom AHB spinner flask (Bellco). Deoxyribonuclease (260 U/ml; Worthington Biochemicals) was added after 30 min of enzymatic dissociation. After the first digestion, the supernatant was collected and washed twice in PBS containing 4 ml of 2 mM EDTA (PBS-EDTA) with centrifugation at 350 × *g*, and the pellet was resuspended in PBS-EDTA and stored on ice. The tissue was digested for another hour under the same conditions and the pellet from the second digestion was combined with the first. The final pellet was washed in PBS-EDTA and passed through a 70-μm filter (22-363-548; Fisher Scientific) and its volume was adjusted to 2 ml with PBS-EDTA. Isolation of total immune cells was accomplished by adding 0.005 μg/μl anti-CD45 IgG2a (CACT-B51A; Washington State University Monoclonal Antibody Center) antibody for up to 1 billion total cells for 30 min at 4°C. This was followed by three washes with PBS-EDTA and the final pellet was resuspended in 2 ml PBS-EDTA. Magnetic beads conjugated with the secondary antibody (anti IgG2a+b; Catalog #130-047-202; Miltenyi Biotec) were added and incubated for 30 min according to the manufacturer's instructions. The mixture was passed through a 30-μm filter (preseparation filters; Catalog #130-041-407; Miltenyi Biotec). The filtrate was then sorted by magnet-activated cell sorting (MACS; Miltenyi Biotec) and the positive fraction was collected to yield $CD45^+$ cells. The viability of the cells was determined using the Guava ViaCount Flex reagent (4000-0040; EMD Millipore).

Flow Cytometry

The purity of $CD45^+$ cells was ascertained by labeling the $CD45^+$ fraction with the anti-IgG2a secondary antibody for 30 min at 4°C. The efficiency of the magnetic separation was determined by labeling the $CD45^-$ fraction with an antibody against CD45. Following labeling, both of these preparations were analyzed by flow cytometry. The proportion of $CD45^+$ immune cells in the endometrial homogenate was typically 10%–15% of the total endometrial cells isolated. Viability ranged from 60% to 65%, purity ranged from 90% to 95% of the $CD45^+$ cell population, and efficiency was greater than 95% as determined from the $CD45^-$ cell population (not shown). Proportions of the $CD45^+$ cells that expressed proteins characteristic of immune cell type were then determined using a Guava EasyCyte Plus (EMD Millipore).

The $CD45^+$ cells obtained from the magnetic separation were aliquoted into tubes at a final concentration of 1×10^6 cells/200 μl of PBS. These cells were then incubated for 30 min at 4°C with primary antibodies against CD14 (0.005 μg/μl; MM61A; Washington State University Monoclonal Antibody Center) and CD11c (0.005 μg/μl; BAQ153A; Washington State University Monoclonal Antibody Center) followed by three washes with PBS-EDTA at 250 × *g* for 10 min each at 4°C. The secondary antibodies, anti-IgG1 fluorescein isothiocyanate (FITC) (0.005 μg/μl; STAR132F; AbD Serotec) and anti-IgM R-phycoerythrin (RPE) (0.005 μg/μl; 102009; AbD Serotec) were added to each tube for 30 min at 4°C, followed by three washes with PBS-EDTA containing 5% goat serum, as described above. Percentage of $CD14^+CD11c^+$ cells was measured on a flow cytometer, after setting the gates on appropriate controls (cells with no antibody, with secondary antibody alone, or with a negative isotype antibody). Compensation for FITC and RPE was done using positive staining for CD3-IgG1 using anti-IgG1 RPE and anti-IgG1 FITC secondary antibodies.

Immunofluorescence Analysis

Full-thickness uterine biopsies (8 mm in diameter) were collected from the intercaruncular regions of the uterine horn ipsilateral to the CL using a cork borer. Biopsies were embedded in Optimal Cutting Temperature (Miles Laboratories, Inc.) and frozen over isopentane that was cooled in liquid nitrogen. Frozen sections (5-μm thickness; n = 5 heifers per treatment group and 2 serial sections per animal) were used for immunofluorescence labeling of proteins expressed by immune cell types. The sections were thawed at 37°C for 30 min followed by fixation in 100% ice-cold ethanol for 8 min. Details of primary antibodies used are included in Supplemental Table S1 (Supplemental Data are available online at wwwbiolreprod.org). The slides were briefly dipped in PBS and then washed for 5 min in PBS containing 0.1% BSA. The sections were incubated overnight with the primary antibody of interest in PBS containing 0.1% BSA and 20% goat serum (G9023; Sigma-Aldrich) in a humidified chamber at 4°C. For IDO1 labeling, the tissues were made

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permeable by adding 0.1% TritonX-100 (Sigma-Aldrich) to all the solutions. After 24 h, the slides were washed twice in PBS containing 0.1% BSA for 5 min each, followed by 1-h incubation with secondary antibody (anti-IgG Alexa Fluor 555), with the same diluent as the primary antibodies, in a humidified chamber at room temperature in the dark. The sections were then mounted using 4′,6-diamidino-2-phenylindole (P36931; gold prolong anti-fade; Invitrogen) and incubated in the dark for 24 h. The fluorescence signal was then captured using an Olympus BX51 microscope fitted with a DP71 camera (Olympus) and the appropriate microscope filters for green (U-MNB2; Olympus), red (U-N41004; Olympus), and blue (U-MNU2; Olympus) wavelengths. Exposure time was kept constant for each primary antibody and its appropriate negative-isotype antibody control. Percentage of area labeled was analyzed using ImageJ software available from the NIH [36]. For each animal, three fields per section in each of two sections were analyzed. For each area analyzed, labeling intensity and percentage of area labeled were measured in the following areas of the total UW: luminal epithelium (LE), shallow stroma (SS), shallow glands (SG), deep glands (DG), and myometrium (M).

Quantitative PCR

The uterine horn ipsilateral to the CL was cut open and 5 g endometrium was trimmed and flash frozen in liquid nitrogen for RNA extraction. Procedures for RNA extraction, cDNA synthesis, and quantitative PCR (qPCR) analyses were as previously described [37]. Primer details are included in Supplemental Table S2.

Statistics and Data Analysis

All data were analyzed using the MIXED procedures of SAS (v 9.3; Statistical Analysis Software). Following a significant *F* test, the following orthogonal comparisons were made: Day 17 cyclic versus Days 17 and 20 pregnant and Day 17 pregnant versus Day 20 pregnant. Pairwise comparisons were made using Student *t*-test with Bonferroni correction. For qPCR, all statistical analyses were performed on $2^{-\Delta Ct}$ values and results are expressed as $2^{-\Delta\Delta Ct}$ in figures [38]. Student *t*-test was used to compare percentage of $CD14^{+}CD11c^{+}$ cells in blood of Day 17 cyclic and Day 17 pregnant heifers and uteri of these same groups. Statistical significance was declared for *P* values less than 0.05 and statistical tendencies for *P* values between 0.05 and 0.1. In cases where heterogeneity of variance was present the data were log or square-root transformed and a normal distribution was confirmed. Least-square means with pooled standard errors were plotted on graphs. All graphs were created using GraphPad (Version Prism 5; GraphPad Software Inc.). Significance of $P \leq 0.01$ is indicated with **, $P \leq 0.05$ is indicated with *, and tendencies of $0.05 < P < 0.1$ are indicated with †.

RESULTS

Endometrial Abundance of $CD45^{+}$ Cells

There were no differences in the proportion of $CD45^{+}$ cells to total cells isolated nor the number of viable $CD45^{+}$ cells/g tissue among the three study groups ($P > 0.1$). Fewer ($P \leq 0.05$) $CD45^{+}$ cells (Fig. 1A) were detected in the UW of Day 17 pregnant and Day 20 pregnant compared to Day 17 cyclic heifers. There was a tendency for reduced percentage area labeled for $CD45^{+}$ in and around the DG in pregnant compared to cyclic heifers ($P = 0.06$) (Fig. 1A).

Expression of Dectin1, MRC1, NOS2, *and* NOS3

There were no differences in steady-state mRNA abundance of *Dectin1* and *MRC1* between statuses (Day 17 cyclic compared to both Day 17 and Day 20 pregnant) or days (Day 17 pregnant compared to Day 20 pregnant) ($P > 0.1$; Supplemental Fig. S1). Messenger RNA for *NOS2* and *NOS3* were detected, but expression was low and not different among statuses or days (data not shown).

Expression of MHC II, CD80, *and* CD86

Abundance of mRNA for *MHC II*, *CD80*, and *CD86* was greater ($P \leq 0.05$) in the endometrium from Day 17 and Day 20 pregnant heifers compared to Day 17 of the cycle (Fig. 2). *MHC II* mRNA abundance tended to increase ($P = 0.06$) on Day 20 compared to Day 17 of pregnancy (Fig. 2A). *CD80* and *CD86* mRNA abundance was greater ($P \leq 0.05$) on Day 20 compared to Day 17 of pregnancy (Fig. 2, B and C).

There was a tendency for an increase ($P = 0.06$) in total area labeled for MHC II (Fig. 3A) between Day 17 cyclic and Day 17 and Day 20 pregnant heifers. This difference was primarily due to the increase in labeling for MHC II in and around the SG during pregnancy compared to the estrous cycle ($P \leq 0.05$; Fig. 3A). Higher-magnification (×1000) image of the labeled cells clearly showed the large size and the irregular spindle-shaped morphology of the positively labeled cells, consistent with myeloid lineage cells (Fig. 3B).

Expression of Signal Regulatory Protein Alpha, CD163, and IDO1

There was a tendency for increased signal regulatory protein alpha (SIRPA) in endometrium from pregnant heifers compared with cyclic heifers ($P = 0.06$; Fig. 4A). Percentage of area labeled with SIRPA in the LE was less in pregnant compared to cyclic heifers ($P \leq 0.05$). Labeling for SIRPA in and around SG and DG in pregnant heifers had a tendency to be greater than in cyclic heifers ($P = 0.09$; Fig. 4A). Abundance of mRNA for *CD163* was greater ($P \leq 0.05$) in endometrium of pregnant compared to cyclic heifers (Fig. 5A). Cells expressing CD163, a protein involved in resolution of inflammation [39], were more abundant ($P \leq 0.05$) in endometrium from pregnant compared to cyclic heifers (Fig. 5B). Abundance of these cells did not differ between Days 17 and 20 of pregnancy (Fig. 5A). Abundance of *IDO1* mRNA was greater ($P \leq 0.01$) in pregnant heifers compared to cyclic heifers. Interestingly, *IDO1* expression tended to decrease ($P = 0.07$) between Days 17 and 20 of pregnancy (Fig. 6A). These results were consistent with percentage of area labeled for IDO1 using ImageJ quantification (Fig. 6B). Overall, there was greater ($P \leq 0.01$) IDO1 protein expression on Day 17 of pregnancy compared to Day 17 of the cycle across the entire UW. Between Day 17 and Day 20 of pregnancy, IDO1 decreased ($P \leq 0.01$) across the entire UW (Fig. 6B). The effects of pregnancy on IDO1 were most pronounced in and around the LE (Fig. 6C).

Abundance of $CD14^{+}CD11c^{+}$ Cells in Peripheral Blood and Endometrial Immune Cells

There was a greater ($P \leq 0.05$) proportion of monocytes in the PBMCs of Day 17 pregnant compared to Day 17 cyclic heifers (Fig. 7). We did not detect differences in these dual labeled cells in the endometrium, likely because of the large animal-to-animal variation (data not shown).

DISCUSSION

A number of studies have examined the transcriptome in the uterine endometrium during early pregnancy in cattle [40–42]. One consistent result from these studies is that genes and pathways involved in immune regulation are among the most strongly regulated in the earliest stages of pregnancy. This is perhaps not surprising given that one of the first signals emanating from the developing conceptus is a type I interferon, IFNT [43]. These studies have stimulated greater interest in the effects of the developing conceptus on the mucosal immune system. Our results support the hypothesis that the presence of a conceptus increases myeloid lineage cells in the uterus and peripheral circulation of dairy heifers around the time of

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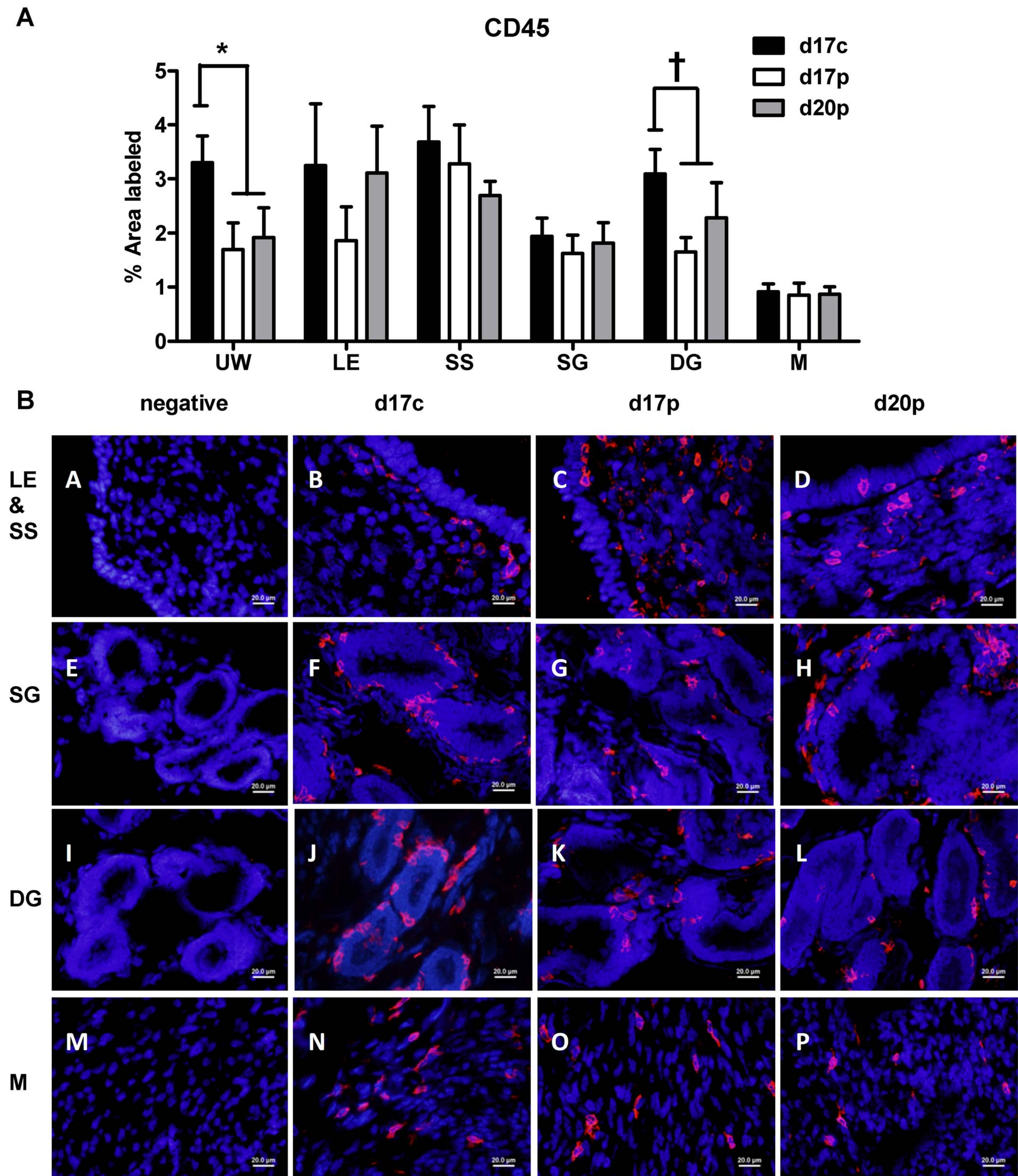

FIG. 1. **A**) Percentage of area labeled for $CD45^+$ cells in the following areas: LE, SS, SG, DG, and M. UW, average percentage of area labeled for all areas. †$0.05 < P < 0.1$; *$P \leq 0.05$. Bars show least-square means ± pooled SEM. **B**) Representative images of immunofluorescence detection of CD45 in the endometrium during the estrous cycle and early pregnancy. Images **A**, **E**, **I**, and **M**: negative isotype controls; images **B**, **F**, **J**, and **N**, Day 17 cyclic; images **C**, **G**, **K**, and **O**: Day 17 pregnant; images **D**, **H**, **L**, and **P**: Day 20 pregnant. Images **A**–**D**: LE and SS; **E**–**H**: SG; **I**–**L**: DG; **M**–**P**: M. Original magnification ×400.

maternal recognition of pregnancy. In addition, a number of molecules associated with tolerance were increased in the endometrium of pregnant heifers that are likely to alter the functional state of uterine myeloid lineage cells.

Myeloid lineage cells are known to be modified during pregnancy in humans and rodents to support establishment and maintenance of pregnancy [14, 27, 29]. The presence of myeloid lineage cells and their changes during pregnancy in

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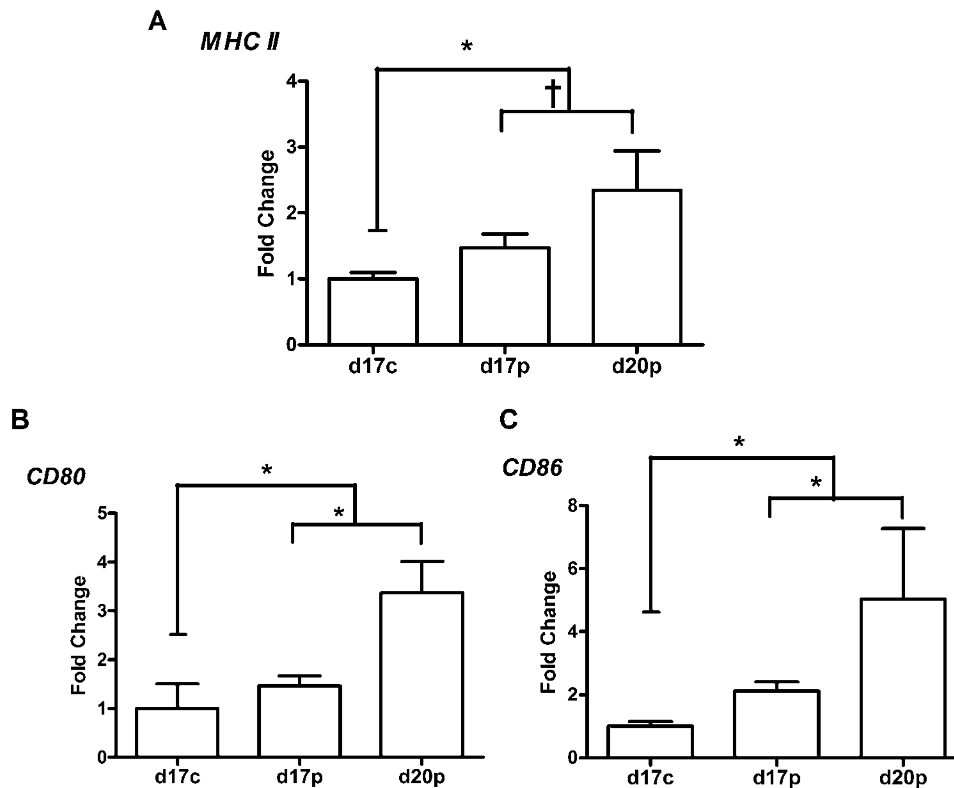

FIG. 2. *MHC II* (**A**), *CD80* (**B**), and *CD86* (**C**) mRNA abundance in endometrium. †$0.05 < P < 0.1$; *$P \leq 0.05$. Bars show least-square means ± SEM.

dairy cattle have been reported mainly after Day 30 of pregnancy, with most studies focusing on mid to late gestation (>50 days) [44–46]. Changes in myeloid lineage cells during early pregnancy are less clear. CD14 is a protein expressed on monocytes, on macrophages, and to a lesser extent on DCs [47]. In one report, no changes were detected in $CD14^+$ cells (macrophages) up to Day 16 of pregnancy in beef heifers [6]. This is supported by another study in Simmental beef heifers on Day 18 of pregnancy, where no increase in CD14 was detected [18]. However, another study detected more $CD14^+$ cells in endometrium on Days 13 and 16 of pregnant compared to cyclic beef heifers [19]. The reasons for these discrepancies are not clear, but they may be due to different technical approaches. Furthermore, they were conducted using beef heifers, and none examined changes after Day 18 of pregnancy.

Macrophages and DCs can be identified based on expression of surface proteins including CD14, CD11b, CD11c, MHC II, CD80, and CD86. Here we show that abundance of *MHCII*, *CD80*, and *CD86* mRNA was greater in pregnant compared to Day 17 cyclic heifers, with abundance in pregnant animals on Day 20 greater than on Day 17. Furthermore, pregnancy increased the number of MHC II^+ and $SIRPA^+$ cells and affected their spatial distribution in the uterus. The increase in MHC II^+ and $SIRPA^+$ cells was most pronounced in and around the SG. Interestingly, $SIRPA^+$ cells decreased in and around the LE of pregnant heifers. Spatial regulation of SIRPA, a marker for tolerogenic cells, is consistent with prior work [19] and supports the hypothesis that conceptus signals regulate immune cell activation status during early pregnancy [18, 19]. Mansouri-Attia et al. [19] also detected spatial regulation of SIRPA (CD172a), but in that report the increase was detected only in the deep stroma. Furthermore, in that study $SIRPA^+$ cells were more abundant than $CD14^+$ cells (presumably macrophages) in the endometrium during the estrous cycle and early pregnancy. In contrast, our results identified $MHCII^+$ cells as the most abundant immune cell type in the endometrium, being at least three times more abundant on Day 20 of pregnancy than $SIRPA^+$ cells. This was substantiated by an increased abundance of mRNA for *MHCII* in the endometrium in pregnant compared to cyclic heifers. The reasons for the differences in cell abundance between previously published work and the current study could be due to use of beef versus dairy heifers, the precise days of pregnancy examined (Day 16 vs. Days 17 and 20 in this report) or differences in technical approaches. For instance, it is possible that the $CD14^+$ cells identified in those studies represent the same population identified using MHCII antibodies in the current study. This is an area that will require further study. Overall, results presented here support previous work [19] and the hypothesis that myeloid lineage cells increase in the endometrium during early pregnancy, in contrast to other reports [6, 18].

Upregulation of SIRPA in the uteri of pregnant heifers indicates the presence of macrophages and DCs in the uterus [19, 48]. The receptor SIRPA binds to its ligand, CD47, and activates immunoreceptor tyrosine-based inhibitory motifs that control differentiation of DCs into mature DCs. Interaction of these two molecules generates the ‘‘do not eat me’’ signal in tumors [49]. The interaction also blocks phagocytosis by preventing myosin-IIA accumulation at the phagocytic synapse [50]. Increased expression of SIRPA was reported in pregnant beef heifers on Day 16, but only in the $CD11c^+$ fraction, indicating that this increase was predominantly in DCs [19]. Whether SIRPA functions to regulate immune responses in the uterus is not clear because the presence of CD47 in the uterus has not been determined in ruminants.

Although the greater abundance of *CD80* and *CD86* mRNA in the endometrium shown here supports the conclusion that

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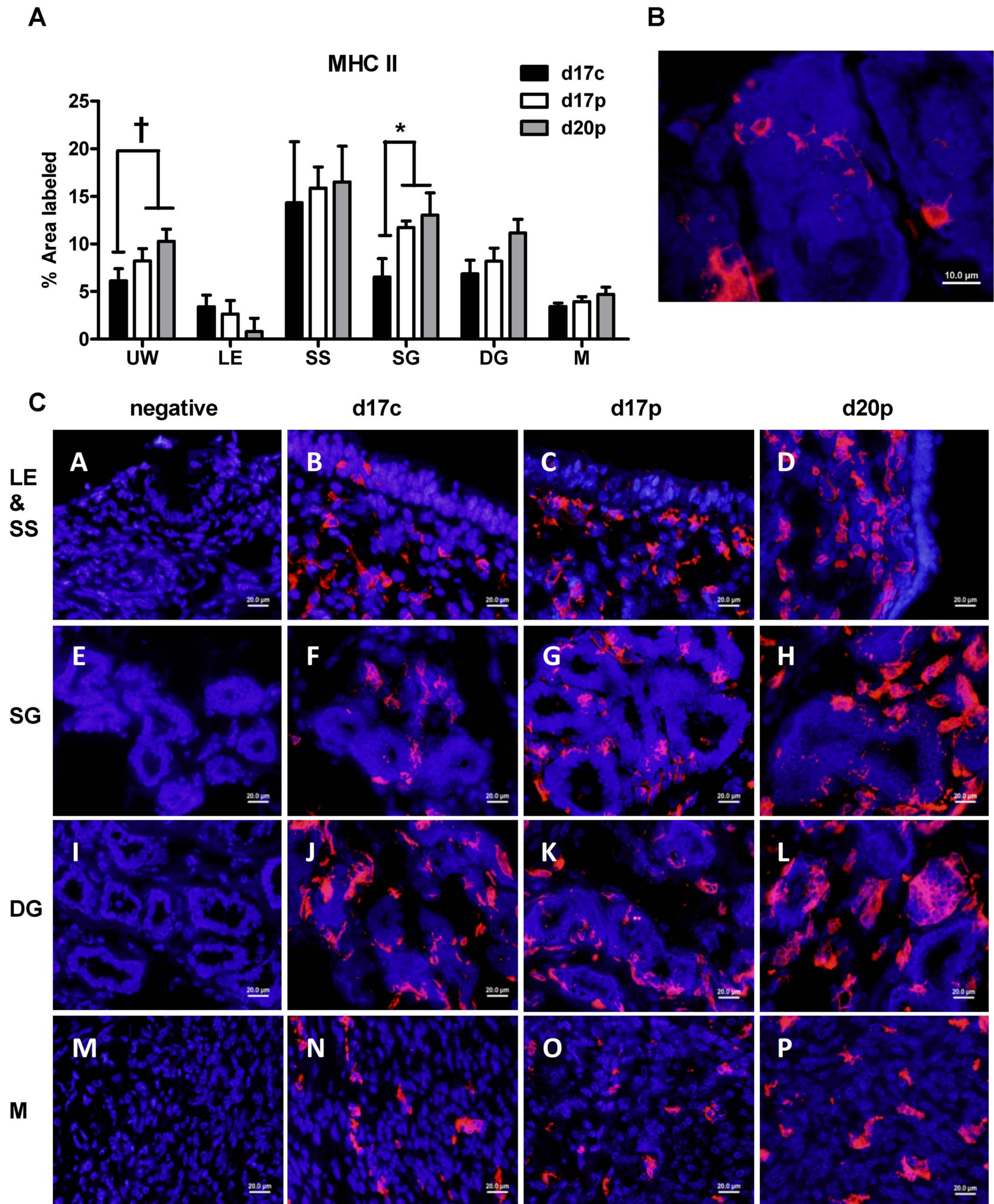

FIG. 3. **A**) Percentage of area labeled for MHC II$^+$ cells in the following areas: LE, SS, SG, DG. and M. UW, average percentage of area labeled for all areas. †$0.05 < P < 0.1$; *$P \leq 0.05$. Bars show least-square means ± SEM. **B**) Picture for MHC II staining. Magnification ×1000. **C**) Representative images of immunofluorescence detection of MHC II in the endometrium during the estrous cycle and early pregnancy. Images **A**, **E**, **I**, and **M**: negative isotype controls; images **B**, **F**, **J**, and **N**, Day 17 cyclic; images **C**, **G**, **K**, and **O**: Day 17 pregnant; images **D**, **H**, **L**, and **P**: Day 20 pregnant. Images **A**–**D**: LE and SS; **E**–**H**) SG; **I**–**L**) DG; **M**–**P**) M. Original magnification ×400.

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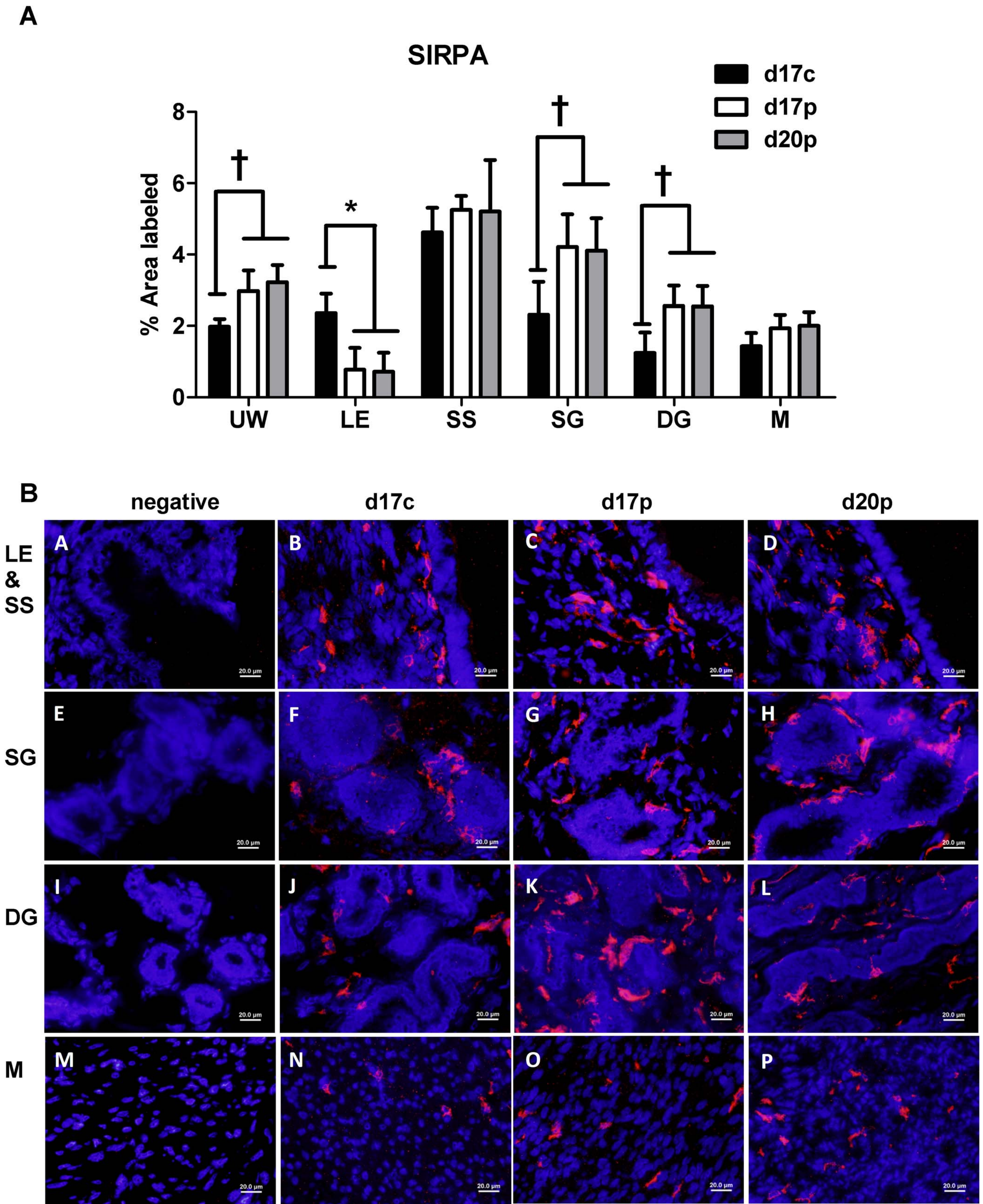

FIG. 4. **A**) Percentage of area labeled for SIRPA$^+$ cells in the following areas: LE, SS, SG, DG, and M. UW, average percentage of area labeled for all areas. †$0.05 < P < 0.1$; *$P \leq 0.05$. Bars show least-square means ± SEM. **B**) Representative images of immunofluorescence detection of SIRPA in the endometrium during the estrous cycle and early pregnancy. Images **A**, **E**, **I**, and **M**: negative isotype controls; images **B**, **F**, **J**, and **N**: Day 17 cyclic; images **C**, **G**, **K**, and **O**: Day 17 pregnant; images **D**, **H**, **L**, and **P**: Day 20 pregnant. Images **A**–**D**: LE and SS; **E**–**H**: SG; **I**–**L**: DG, **M**–**P**; M. Original magnification ×400.

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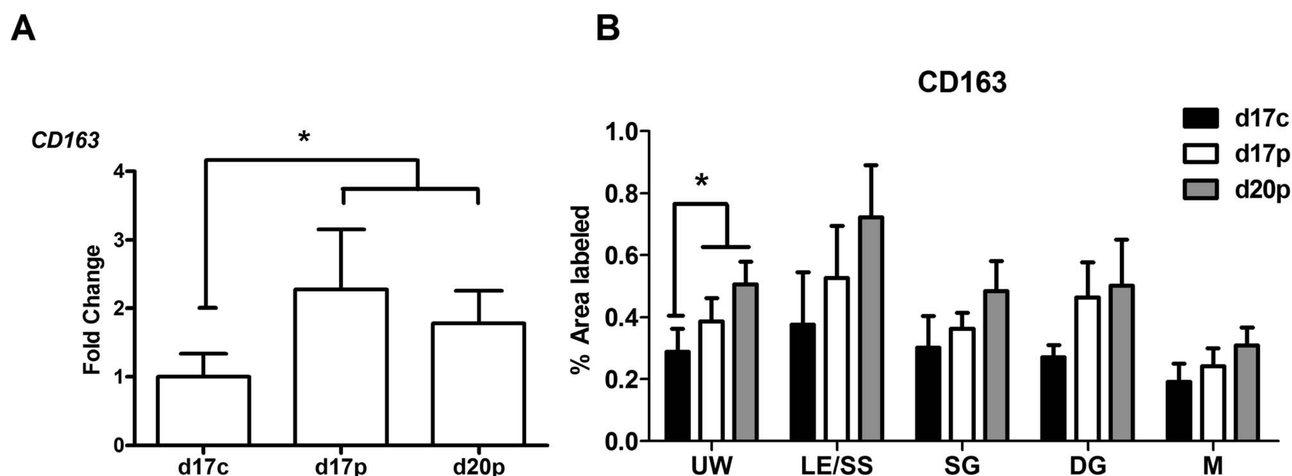

FIG. 5. **A**) *CD163* mRNA abundance in endometrium. $^{*}P \leq 0.05$. Bars show least-square means ± SEM. **B**) Percentage of area labeled for $CD163^{+}$ cells in the following areas: LE, SS, SG, DG, and M. UW, average percentage of area labeled for all areas. $^{*}P \leq 0.05$. Bars show least-square means ± SEM.

myeloid lineage cells increase during early pregnancy, the precise identity of these cells (macrophage or DC) remains to be determined. Clearly, both of these cell types are targets for IFN signaling. Type I interferons increase CD86 expression on DCs [51] and pregnancy-induced MHC II expression was detected in the endometrium on Day 17 [41], linking increases in these molecules to conceptus signaling. Myeloid cells typically present antigen to T cells to induce an immune response. However, myeloid cells that express CD80 and CD86 can also interact with cytotoxic T lymphocyte-associated

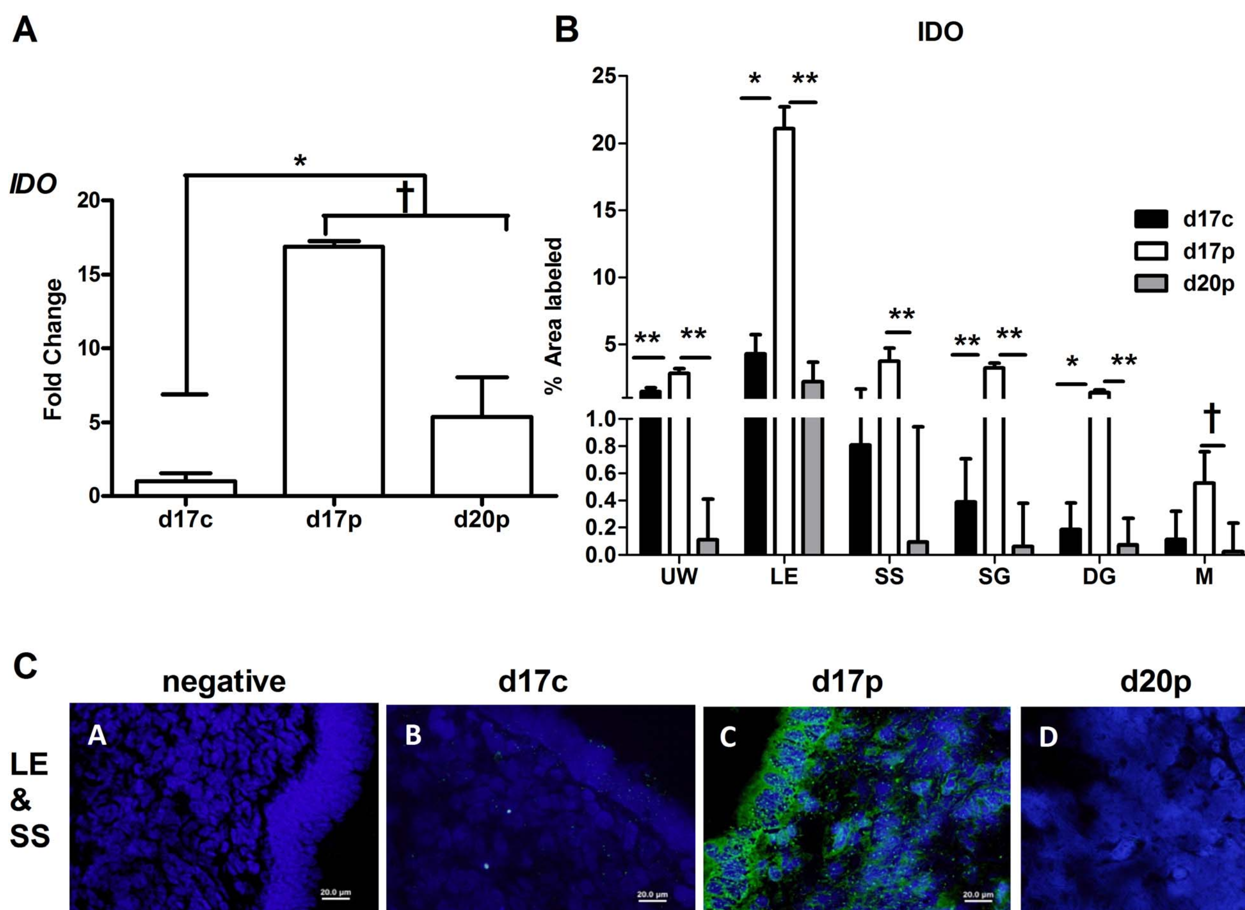

FIG. 6. **A**) *IDO1* mRNA abundance in endometrium. †$0.05 < P < 0.1$; $^{*}P \leq 0.05$. Bars show least-square means ± SEM. **B**) Percentage of area labeled for $IDO1^{+}$ cells in the following areas: LE, SS, SG, DG, and M. UW, average percentage of area labeled for all areas. †$0.05 < P < 0.1$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. Bars show least-square means ± SEM. **C**) Representative images of immunofluorescence detection of IDO1 in the LE and SS during the estrous cycle and early pregnancy. Image **A**: negative isotype control; Image **B**: Day 17 cyclic; image **C**: Day 17 pregnant; image **D**: Day 20 pregnant. Original magnification ×400.

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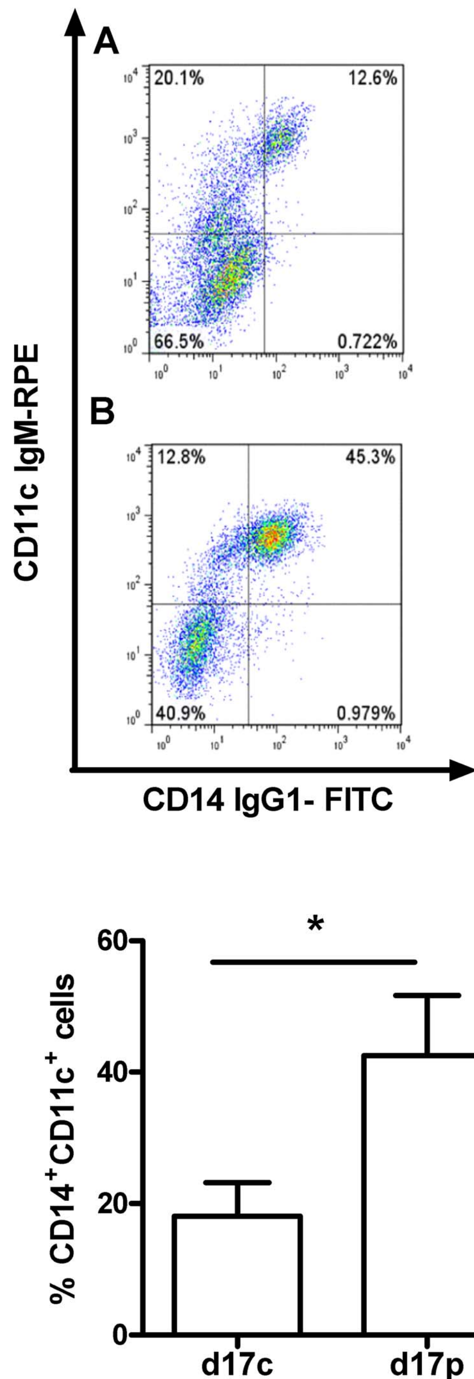

FIG. 7. Representative flow cytometry dot plots of $CD14^{+}D11c^{+}$ cells for Day 17 cyclic (**A**) and Day 17 pregnant (**B**) animals. Percentage of $CD14^{+}CD11c^{+}$ cells from total CD45 sorted PBMCs of Day 17 cyclic (n = 4) and Day 17 pregnant animals (n = 3). $^{*}P \leq 0.05$. Bars show least-square means ± SEM.

protein (CTLA) 4 on effector T cells, and MHC II can interact with lymphocyte activation gene (LAG) 3 on T regulatory cells to induce inhibitory responses [52–54]. Increased CTLA4 in pig endometrium on Day 14 of pregnancy was detected by transcriptomic analysis [55] and we hypothesize that the conceptus is similarly inducing inhibitory signaling in the bovine uterus during early pregnancy.

The role of DCs during pregnancy is widely studied [27]. Conditional ablation of uterine DCs impairs proliferation and differentiation of endometrial stromal cells and hinders angiogenesis [56]. Uterine DCs treated with conceptus-derived TSLP increased CD80 and CD86 expression and the production of anti-inflammatory cytokines IL10 and transforming growth factor beta [57, 58]. Increased $CD14^{+}$ cells in the bovine endometrium on Days 13 and 16 of pregnancy and $CD172a^{+}CD11c^{+}$ cells in the stroma on Day 16 of pregnancy support the presence of DCs in cattle [19]. Clearly the activation status of uterine DCs and their cytokine secretion during early pregnancy needs to be determined.

Expression of CD45, or leukocyte common antigen, is considered a universal marker for immune cells [59]. The CD45 protein belongs to a family of transmembrane glycoproteins that exhibit phosphatase activity [60]. In the current study, total $CD45^{+}$ cells were fewer in the endometrium of pregnant heifers compared to cyclic heifers, with the decrease most prominent in and around the DG. This spatial regulation of CD45 during pregnancy could be due to greater expression of steroid receptors in the DG of ruminants during early pregnancy [61]. Similar reductions in $CD45^{+}$ cells were observed in the stroma of the zona basalis of Day 18 pregnant heifers [18]. Both myeloid and lymphoid lineage cells contribute to the $CD45^{+}$ cell populations in the uterus [62, 63]. What is not presently clear is whether the pregnancy-induced decrease in $CD45^{+}$ cells constitutes an actual loss of immune cells from the endometrium or, alternatively, a downregulation of CD45 expression on immune cells as has been described in various models of infection [59].

We demonstrated a greater proportion of $CD14^{+}CD11c^{+}$ cells in the circulation of Day 17 pregnant heifers compared to Day 17 cyclic heifers. We postulate that the $CD14^{+}CD11c^{+}$ cells in the periphery are monocytes that will migrate to the endometrium and differentiate into DCs. The greater proportion of dual-positive monocytes in peripheral blood could be targets for the increased CCL2 and CCL8 expression in the endometrium during early pregnancy [19, 41]. These chemotactic proteins induce migration of monocytes into tissues [64], and might be necessary to enhance the proportion of myeloid lineage cells in the endometrium and prepare the uterus for later stages of pregnancy [65, 66].

In addition to quantifying and describing the uterine spatial distribution of myeloid lineage cells during early pregnancy, we examined their functional status by determining expression of key molecules associated with their activation state. One of the best-described molecules involved in an immune response is the tryptophan-catabolizing enzyme, IDO1 [67]. This immunomodulatory enzyme catabolizes the essential amino acid L-tryptophan to N-formylkynurenine. Reduced tissue concentrations of tryptophan are postulated to inhibit proliferation of T cells by halting division of host cells at the G1 phase of the cell cycle [67, 68]. The enzyme is produced by both classically and alternatively activated macrophages and regulatory immune cells, and is induced in response to IFNG [69]. Expression and activity of IDO1 are greater in the uterus during early pregnancy in cattle [18]. Furthermore, N-formylkynurenine binds the arylhydrocarbon receptor and induces differentiation of T regulatory cells [70]. Our results showed a striking increase in *IDO1* mRNA and protein in Day 17 pregnant heifers compared to Day 17 cyclic heifers, similar to that reported by Groebner et al. [18]. Interestingly, both *IDO1* mRNA and protein abundance decreased by Day 20 of pregnancy, which has not been described previously. We hypothesize this reduction is mediated by IFNT-stimulated interferon regulatory factor (IRF) 2 expression, which inhibits the expression of a number of interferon-stimulated genes in the endometrium [71, 72]. Both IRF1 and IRF2 bind to the interferon-stimulated response elements and gamma-activated

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sequence elements in the IDO1 gene promoter [73, 74]. IFNT induces IDO1 expression by endometrial cells in vitro [18], presumably by activation of IRF1. We postulate that negative feedback by IRF2 decreases IDO1 gene transcription and protein expression. However, a direct effect of IFNT-induced IRF2 to reduce endometrial IDO1 expression remains to be experimentally tested. The functional significance of the acute reduction in IDO1 by Day 20 of pregnancy needs to be determined. However, the reduction could assist in the process of attachment of the conceptus, perhaps involving components of inflammatory signaling including IL1B and IFNG, as seen in the sow [75–77]. Clearly this abrupt increase and then decrease in IDO1 expression, which coincides with the onset of conceptus attachment and placentation, is an intriguing phenomenon that merits further study.

We also examined the effects of pregnancy on expression of other genes associated with alternatively activated (M2) macrophages, including *CD163* (scavenger receptor), *MRC1*, and *dectin1* [46, 78]. The CD163 protein tended to increase in uterine tissues of pregnancy compared to those from Day 17 cyclic heifers. This molecule participates in the production of heme metabolites by delivering hemoglobin to macrophages [79, 80], and is induced by anti-inflammatory cytokines including IL10 [39]. Consistent with these observations, another study identified increased expression of galectin-3 on Day 16 of pregnancy in concepti and uterine flushes [81]. Galectin-3 promotes alternative activation of macrophages, further supporting the concept of M2 macrophage accumulation in the bovine uterus during early pregnancy. Both MRC1 and dectin1 are upregulated in $CD14^{+}$ cells isolated from endometrium of pregnant (>160 days of pregnancy) cows when compared to $CD14^{+}$ cells from the peripheral circulation of the same animals [46]. Although our study did not detect a difference in *MRC1* or *dectin1* mRNA abundance between pregnant and cyclic heifers, this is likely due to the early stages of pregnancy examined. Furthermore, differences in mRNA abundance of less abundant transcripts are likely more difficult to detect when using total endometrial RNA compared to a pure $CD14^{+}$ population [46].

In summary, these results support the hypothesis that pregnancy induces increased myeloid lineage cells in dairy heifers. This increase was evident by upregulation of genes and proteins expressed on myeloid cells including *CD80, CD86*, and MHC II. Simultaneously, we detected increased expression of immunomodulatory molecules including CD163, IDO1, and SIRPA, supporting a tolerogenic/alternatively activated phenotype for these cells. An increase in the proportion of putative DC precursors ($CD14^{+}CD11c^{+}$ cells) was observed in the peripheral circulation of Day 17 pregnant heifers, although the significance of this observation remains unknown. Consistent with previous reports [18, 82] there was an overall decrease in $CD45^{+}$ immune cells in the endometrium of pregnancy, although it is not clear if this reflects a reduction in the number of mucosal immune cells or simply a downregulation of CD45 expression. Overall, the results support a role for conceptus-regulated myeloid cells during early pregnancy in dairy heifers.

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