Deviations in populations of peripheral blood mononuclear cells and endometrial macrophages in the cow during pregnancy

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Abstract

The presence of conceptus alloantigens necessitates changes in maternal immune function. Here, we used the cow to evaluate whether species with epitheliochorial placentation have changes in specific leukocyte populations during pregnancy similar to those reported in species with hemotrophic placentae. At days 33–34 of pregnancy, there was no effect of pregnancy status on the number of cells positive for CD8, CD4, $\gamma\delta$ T cell receptor, or the monocyte marker CD68 in the peripheral blood mononuclear cell (PBMC) population. There was, however, an increase in the proportion of CD4⁺ cells that were positive for CD25. There was no effect of status on the proportion of PBMCs that were CD8⁺ when comparing preparturient cows to nonpregnant cows. However, preparturient cows had an increased percentage of PBMCs that were $\gamma\delta$ T cells and CD4⁺CD25⁺ and a tendency for a lower percentage that were CD68⁺ cells. Using immunolocalization with anti-CD68, it was found that pregnant cows had increased numbers of CD68⁺ cells in the endometrial stroma as early as days 54–100 of gestation; this increase persisted through the last time examined (day 240 of gestation). Cells positive for CD68 were also positive for another macrophage/monocyte marker, CD14. In conclusion, pregnancy in the cow is associated with changes in peripheral and endometrial leukocyte numbers, which are similar to patterns observed in other species.

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Introduction

Pregnancy is an immunologically distinct period for the eutherian female because the presence of conceptus alloantigens necessitates changes in maternal immune function to prevent immunological destruction of the conceptus. These changes can be observed systemically and at the fetal–maternal interface. Maternal immune adjustments to pregnancy have been most clearly defined in the human and mouse. Both of these species have an invasive, hemotrophic placenta in which trophoblast invades the endometrium. Among the changes in immune function during pregnancy are an increase in circulating regulatory T cells (T_{reg}), defined as CD4⁺CD25⁺FOXP3⁺ cells, that function to down-regulate T lymphocyte function (Aluvihare *et al.* 2004, Somerset *et al.* 2004, Yang *et al.* 2008); a temporary anergy of maternal lymphocytes to conceptus MHC class I antigens (Tafari *et al.* 1995); and synthesis of immunosuppressive proteins at the maternal–fetal interface including interleukin 10 (IL10; Hanna *et al.* 2000, Murphy *et al.* 2005) and transforming growth factor β 1 (TGFB1; Suzuki *et al.* 1995, Gorivodsky *et al.* 1999, Simpson *et al.* 2002). In addition, pregnancy is characterized by an increase in the number of specific leukocyte populations within the uterus including macrophages (Hunt *et al.* 1985, Heikkinen *et al.* 2003, Cupurdija *et al.*

2004, Kim *et al.* 2007), $\gamma\delta$ T cells (Heyborne *et al.* 1992, Mincheva-Nilsson *et al.* 1992, 1997), and natural killer (NK) cells (Bilinski *et al.* 2008, Shigeru *et al.* 2008). These cells have been implicated in vascular remodeling (NK cells: Bilinski *et al.* 2008); immunosuppression through secretion of IL10 and TGFB1 ($\gamma\delta$ T cells: Suzuki *et al.* 1995, Nagaeva *et al.* 2002; NK cells: Murphy *et al.* 2005) and parturition (macrophages: Thomson *et al.* 1999, Mackler *et al.* 2000).

One of the characteristics of pregnancy in eutherian mammals is great diversity in placental anatomy and uterine function. At three separate times in mammalian evolution, epitheliochorial placentation arose as a specialization from an ancestral hemotrophic placenta: in lemurs, moles, and in the ancestor of cetartiodactyls, suidae, and perrisodactyls (Vogel 2005). Evolutionary pressure for development of this type of placentation could have involved increased efficiency of placental transport (Leiser *et al.* 1997) and increased maternal control over the vascular supply to the conceptus (Mess & Carter 2007). The epitheliochorial placenta might also have evolved as a strategy for immunological defense of the conceptus. The apposition of placental trophoblast and endometrial epithelium in species with epitheliochorial placentation has been compared with the immunological relationship between commensal bacteria and host organisms, with little immunological

recognition occurring unless the epithelial barrier is breached (Moffett & Loke 2006). There is some evidence, however, that the immunological adjustments to pregnancy in species with epitheliochorial placentation are similar to those seen in the mouse and human. In the sheep, for example, pregnancy is associated with an increase in the number of macrophages in stroma (Tekin & Hansen 2004) and in the number of $\gamma\delta$ T cells in the luminal epithelium and immediately adjacent stroma (Lee *et al.* 1992, Majewski *et al.* 2001). There is evidence for specific downregulation of maternal cytotoxic lymphocytes towards paternal antigens in pregnant mares (Baker *et al.* 1999), and there is accumulation of cells with NK activity during early pregnancy in the pig (Yu *et al.* 1993).

For the present series of experiments, the cow was used as a model to define the changes in immune function during pregnancy with a view to test whether the changes in immune function seen in human and mice are also seen in a species with epitheliochorial placentation. The specific hypotheses tested were that $CD4^+CD25^+$ cells increase in circulation during pregnancy, while circulating concentrations of $\gamma\delta$ T cells and macrophages decrease. Moreover, it was hypothesized that the reduction in the number of the latter two cell types reflects recruitment to the uterus. Identification of these or similar changes in immune function during pregnancy would strengthen the notion that existence of an epitheliochorial placenta does not minimize the need for immunological adjustments in pregnancy. Moreover, such changes could result in an altered immune function in the preparturient period when the female is susceptible to uterine infection and other immune challenges.

Results

Representative flow cytometry patterns

Typical dot plots are shown in Fig. 1. Analysis of peripheral blood mononuclear cells (PBMCs) by side and forward scatter resolved cells into two populations (Fig. 1A). The first, more abundant population was of small size and little granularity, and represents lymphocytes primarily. The second population, which represents monocytes primarily, was of large size and granularity. Expression of markers for T cells (CD4, CD8, and CD25) was based on the cells in the lymphocyte gate. Expression of $\gamma\delta$ T and CD68 was analyzed in both lymphocyte and monocyte regions. Cells positive for antibody could be readily distinguished from negative cells based on increased fluorescence when compared with cells stained with IgG (Fig. 1B–G). Cells positive for CD25 were resolved into two populations – a $CD25^{bright}$ and a $CD25^{dim}$ population (Fig. 1C). There were, however, no treatment differences in the percentage of $CD25^+$ cells that were bright and dim. Therefore, data were analyzed after pooling both populations.

Differences between pregnant and nonpregnant cows at days 33–34 of gestation in subpopulations of PBMCs

There were no differences between pregnant and nonpregnant cows in the proportions of PBMCs that were positive for CD4 and CD8 (Fig. 2A). Pregnant cows had, however, a higher ($P < 0.06$) percentage of PBMCs that were $CD25^+$ (Fig. 2A). Moreover, the percentage of $CD4^+$ cells that were also positive for CD25 was higher ($P < 0.05$) for pregnant cows (Fig. 2C).

There was no effect of pregnancy status on the proportion of PBMCs in the lymphocyte and monocyte gates that were $\gamma\delta T^+$ and $CD68^+$ cells (Fig. 2D and E).

Differences between preparturient and nonpregnant cows in subpopulations of PBMCs

There was no effect of pregnancy status on the percentage of cells in the lymphocyte gate that were positive for CD8 (Fig. 3A). However, the percentage of cells in the lymphocyte gate that were $CD4^+$, $CD25^+$, and $CD4^+CD25^+$ were higher ($P < 0.05$) for preparturient cows than for nonpregnant cows (Fig. 3A and B). The increase in the number of $CD4^+CD25^+$ cells represents an increase in $CD4^+$ cells and an increase in the proportion of $CD4^+$ cells that were $CD25^+$ because the number of $CD4^+$ that were $CD25^+$ cells was higher ($P < 0.05$) in preparturient cows (Fig. 3C). Preparturient cows also had a greater proportion of cells in the lymphocyte gate that were $\gamma\delta T^+$ ($P < 0.01$) and a tendency for a greater proportion of cells in the monocyte gate that were $\gamma\delta T$ cells ($P < 0.07$; Fig. 3D). There was also a tendency ($P < 0.10$) for the percentage of cells that were $CD68^+$ to be lower for preparturient cows for the monocyte gate (Fig. 3E).

Immunolocalization of $CD68^+$ and $CD14^+$ cells in endometrium as affected by pregnancy status

Cells positive for CD68 were very abundant in the lamina propria of the endometrial stroma in pregnant cows (Fig. 4B and C). A fewer number of positive cells were present in the submucosa. There were no $CD68^+$ cells in the luminal or glandular epithelia. In contrast to the pattern in pregnancy, there were very few $CD68^+$ cells in the endometrium from nonpregnant cows (Fig. 4A). The pattern of $CD14^+$ cells was very similar to that for CD68 (Fig. 4D).

The high degree of staining for CD68 in pregnant endometrium made counting of individual cells impossible. Instead, a subjective score for staining intensity was used to determine the pregnancy status effects on the number of $CD68^+$ cells. Staining intensity was greater for pregnant cows than for nonpregnant cows at all stages of pregnancy examined ($P < 0.05$) for both the lamina propria (Fig. 5A) and submucosa (Fig. 5B).

Figure 1 Representative acquisition dot plots to analyze subpopulations of bovine peripheral blood mononuclear cells. (A) Forward (x-axis) and side (y-axis) scatter plots to analyze cells on the basis of size (x-axis) and granularity (y-axis). The lymphocyte (gate 1) and monocyte (gate 2) gates are circled. (B) Dual labeling for CD4 (y-axis) and CD8 (x-axis) among the cells in the lymphocyte gate. (C) Dual labeling of CD4 (y-axis) and CD25 (x-axis) for the cells in the lymphocyte gate. Note that CD25 labeling could be categorized as dim or bright. (D–G) Labeling of $\gamma\delta$ T cells (D and E) and CD68 (F and G) in the lymphocyte gate (D and F) and monocyte gate (E and G). For D–G, the x-axis represents fluorescence and the y-axis represents forward scatter (FSC).

The co-localization of CD68 and CD14 expression was analyzed by dual-color immunofluorescence. In one experiment, two-color immunofluorescent labeling of endometrial sections was performed using antibodies to CD68 and CD14. The majority of CD14⁺ cells were positive for CD68 (Fig. 6). In the second experiment, two-color immunofluorescent labeling of single-cell preparations of adherent endometrial cells revealed that cells that were labeled with CD14 were also labeled with CD68 (Fig. 7).

Discussion

Results presented here indicate that pregnancy in the cow, as in other species, is characterized by changes in immune cell populations in the periphery and uterus. During early pregnancy, at days 33–34 of gestation, there was an increase in the proportion of peripheral blood lymphocytes that were CD4⁺CD25⁺ cells. These cells, which could be analogous to the T_{reg} cells described as

increasing during pregnancy in mice and humans (Aluvihare *et al.* 2004, Somerset *et al.* 2004), were also present in higher amounts in preparturient cows when compared with nonpregnant cows. Late pregnancy was also associated with an increase in the proportion of $\gamma\delta$ T cells in peripheral blood and a tendency for a decrease in the proportion of cells (presumably monocytes) positive for CD68. The most striking change associated with pregnancy was large-scale recruitment of macrophages positive for CD68 and CD14 to the endometrial stroma.

Pregnancy-associated changes in immune cell populations are likely to be important for protection of the conceptus from maternal immune attack or for removal of cellular debris and microorganisms from the uterus following parturition. The observation that pregnancy causes changes in immune function in the cow that parallel what is seen in other species suggests that the trophoblast–maternal immunological relationship in species with epitheliochorial placentae is not an inert one, as has been implied (Moffett & Loke 2006), but one characterized by immunological adaptation.

Figure 2 Effect of pregnancy status at days 33–34 after insemination on populations of peripheral blood mononuclear cells as determined by flow cytometry. Data are least-squares means $\pm$ S.E.M. for results for nonpregnant (NP) and pregnant cows (P). (A) Percentage of cells in the lymphocyte gate positive for CD4, CD8, and CD25. (B) Percentage of cells in the lymphocyte gate that are positive for both CD4 and CD25. (C) Percentage of CD4⁺ cells that are also CD25⁺. (D) Percentage of cells in the lymphocyte gate (G1) and monocyte gate (G2) that are positive for $\gamma\delta$ T. (E) Percentage of cells in the lymphocyte gate (G1) and monocyte gate (G2) that are positive for CD68.

At both days 33–34 and late pregnancy, there were changes in the population of CD4⁺CD25⁺ cells in peripheral blood. At days 33–34, this change reflected an increased proportion of CD4⁺ cells that expressed CD25⁺. In preparturient cows, there was an increase in

CD4⁺CD25⁺ cells that resulted from an increase in the relative number of CD4⁺ in the periphery and an increase in the proportion of CD4⁺ cells that were positive for CD25. In both women and mice, a subpopulation of CD4⁺CD25⁺ that expresses the transcription factor FOXP3 is increased in peripheral blood during pregnancy (Aluvihare *et al.* 2004, Somerset *et al.* 2004). These cells have been identified as T_{reg} cells that can secrete cytokines such as IL4 that inhibit activation of cytotoxic T cells against alloantigens (Mjösberg *et al.* 2007). The importance of T_{reg} cells for pregnancy success is indicated by the observation that the percentage of CD4⁺CD25⁺ cells in PBMCs is reduced in women with unexplained recurrent spontaneous abortion (Yang *et al.* 2008). In mice, depletion of CD25⁺ cells decreased the ability of the female to sustain pregnancy (Aluvihare *et al.* 2004). It is not clear from the present results whether some or all of the CD4⁺CD25⁺ seen in peripheral blood of cows are T_{reg} cells. Antibodies to bovine FOXP3 are not available and several unsuccessful attempts were made to identify antibodies raised against FOXP3 in other species that crossreact with bovine.

The immune changes coincident with pregnancy seen here do not represent the earliest such changes in pregnancy. Ruminant species are unique among mammals in that they have evolved to use a type I interferon (interferon- τ) as the trophoblast signal that inhibits endometrial prostaglandin F-2 α synthesis and allows prolonged lifespan of the corpus luteum (Roberts *et al.* 2003). Interferon- τ has retained its immunomodulatory properties and changes in the expression of interferon-stimulated genes (*ISG*) have been described in PBMCs in cattle as early as day 16 of gestation (Han *et al.* 2006, Gifford *et al.* 2007). By day 32 of pregnancy, expression of *ISG15* in blood cells was similar to that for nonpregnant cows (Han *et al.* 2006), but it cannot be ruled out that changes in PBMCs seen at days 33–34 in the present study were caused by interferon- τ secretion by the conceptus earlier in pregnancy.

It was hypothesized that the relative number of $\gamma\delta$ T cells in peripheral blood would decline during pregnancy, especially in the preparturient period, because of recruitment to the uterus. Indeed, accumulation of $\gamma\delta$ T cells in the endometrium is a characteristic of pregnancy in humans (Mincheva-Nilsson *et al.* 1992, 1997), mice (Heyborne *et al.* 1992), and sheep (Lee *et al.* 1992, Meeusen *et al.* 1993, Majewski *et al.* 2001). In contrast to our hypothesis, the relative number of $\gamma\delta$ T cells in peripheral blood was not affected by pregnancy status at days 33–34 after insemination and was higher for preparturient cows than for nonpregnant cows. An increase in the relative number of $\gamma\delta$ T cells in peripheral blood 3 weeks before parturition followed by decrease to initial values at the time of parturition has been noted by others (Van Kampen & Mallard 1997).

Figure 3 Differences in the peripheral blood mononuclear cell populations between nonpregnant (NP) and preparturient cows (P) as determined by flow cytometry. Data are least-squares means $\pm$ S.E.M. (A) Percentage of cells in the lymphocyte gate positive for CD4, CD8, and CD25. (B) Percentage of cells in the lymphocyte gate that are positive for both CD4 and CD25. (C) Percentage of CD4⁺ cells that are also CD25⁺ (D) Percentage of cells in the lymphocyte gate (G1) and monocyte gate (G2) that are positive for $\gamma\delta$ T. (E) Percentage of cells in the lymphocyte gate (G1) and monocyte gate (G2) that are positive for CD68.

The recruitment of macrophages to the pregnant uterus has been described in many species such as humans (Heikkinen *et al.* 2003, Cupurdija *et al.* 2004, Kim *et al.* 2007), mice (Hunt *et al.* 1985), and sheep (Tekin & Hansen 2004). Present results indicate that this

process occurs in the cow also with numbers increasing by days 54–100 of pregnancy. For preparturient cows, there was a decline in the relative number of CD68⁺ cells in peripheral blood and this change could reflect increased recruitment to the uterus. That the CD68⁺ cells in endometrial stroma are macrophages is indicated by the co-expression of CD14, which is also expressed in tissue macrophages such as in pulmonary alveoli (Yang *et al.* 1995). The signals for movement of monocytes from the blood to the uterus and their differentiation once resident in the endometrium are not known. Mouse trophoblast can cause migration of blood monocytes *in vitro* (Fest *et al.* 2007) and change monocyte cytokine prolif and activation in response to lipopolysaccharide.

The accumulation of large number of macrophages in the endometrium during pregnancy strongly suggests an important role for these cells in the uterus. One possibility is that uterine macrophages participate in parturition by promoting placental detachment. Evidence for immunological participation in the parturition process is indicated by the increased incidence of retained placentae in cows, which share MHC class I antigens with their conceptus (Joosten *et al.* 1991). Retained placenta in cattle has also been related to decreased activity of macrophages in the placentomal area (Miyoshi *et al.* 2002). The postpartum uterus is characterized by abundant lochia and microorganisms (Lewis 1997, Thatcher *et al.* 2006), and macrophages may participate in the involution process whereby these materials are removed in the postpartum period. Finally, it may be that macrophages function during pregnancy to promote survival of the allogeneic conceptus. In human, placental macrophages express markers of alternative activation (Cupurdija *et al.* 2004) and markers such as stabilin-1 that have been suggested to be involved in an anti-inflammatory function (Politz *et al.* 2002). It has been proposed that clearance of apoptotic trophoblast cells by uterine macrophages alters macrophage cytokine secretion to reduce inflammation and promote conceptus survival (Mor *et al.* 2006). Also, macrophage-associated stabilin-1 can bind to and process placental lactogen *in vitro*, suggesting regulation of extracellular levels of placental lactogen by alternatively activated macrophages (Kzhyshkowska *et al.* 2008).

There is evidence that the preparturient dairy cow is immunosuppressed with a decline in the number of CD4⁺, CD8⁺, and $\gamma\delta$ cells in peripheral blood (Van Kampen & Mallard 1997, Kimura *et al.* 1999, 2002) and reduced proliferation and interferon- γ secretion by mitogen-stimulated lymphocytes (Detilleux *et al.* 1995, Nonnecke *et al.* 2003). There was little evidence of immunosuppression in the present study. Preparturient cows did not have reduced proportions of CD8⁺ cells in peripheral blood when compared with nonpregnant cows and the number of CD4⁺ and $\gamma\delta$ T cells was increased. Karcher *et al.* (2008) also did not observe declines in CD8⁺ cells as parturition approached and

Figure 4 Immunolocalization of cells positive for (A–C) CD68 and (D) CD14 in bovine endometrium. Represented are sections of endometrium from a nonpregnant cow (A) and pregnant cows at days 75 (C) and 209 (B and D) of pregnancy. Note that fetal chorion has been retained for panel B only. Brown, immune reaction product; blue, hematoxylin counterstain. LE, luminal epithelium; LP, lamina propria; Myo, myometrium; UG, uterine gland; SM, submucosa; CE, chorionic epithelium.

there was a tendency for an increase in the proportion of $\gamma\delta$ T cells as parturition approached. In the present study, the only direct evidence for reduced immune competency in preparturient cows was the tendency for a reduction in the number of CD68⁺ cells. It is possible, however, that the increase in the number of CD4⁺CD25⁺ and $\gamma\delta$ T cells seen in preparturient cows is an indication for immunosuppression in the preparturient period if some of these cells are T_{reg} cells.

Moffett & Loke (2006) compared the maternal–fetal immunological relationship in species with epitheliochorial placenta to the immunological relationship between commensal bacteria and host organisms. In this view, little immunological recognition of the conceptus occurs unless the epithelial barrier is breached. The current results indicate that development of the epitheliochorial placenta during evolution has not changed the occurrence of several immunological adjustments to pregnancy. Like the human and mouse, there is an increase in CD4⁺CD25⁺ lymphocytes in peripheral blood and accumulation of macrophages in the endometrium. These pregnancy-associated changes in immune function indicate that, rather than being an immunologically inert tissue, the bovine conceptus is a tissue whose presence requires maternal immunological adjustments. These adjustments can be detected as early as day 16 (Han *et al.* 2006, Gifford *et al.* 2007), are present at days 33–34 of pregnancy, and are still occurring close to parturition. Thus, the maternal immune system in the cow is constantly adjusting to the conceptus. Given this pattern of immunological change, which is also seen in other species with epitheliochorial placenta (Lee *et al.* 1992, Yu *et al.* 1993, Baker *et al.* 1999, Majewski *et al.* 2001, Tekin & Hansen 2004), it seems more likely that evolution of an epitheliochorial placenta occurred because of increases in

efficiency of placental transport (Leiser *et al.* 1997) or increased maternal control over the vascular supply to the conceptus (Mess & Carter 2007) rather than to change the fundamental characteristics of the maternal–fetal immunological relationship.

Materials and Methods

Materials

Tissue culture medium-199 (TCM-199), normal goat serum, BSA Fraction-V, Dulbecco's PBS (DPBS), and Hoescht 33342 were purchased from Sigma–Aldrich. The Fico-Lite 1077 was from Atlanta Biologicals (Norcross, GA, USA). The Zenon Alexa Fluor 488 mouse IgG1 labeling kit, Zenon Alexa Fluor R-phycoerythrin mouse IgG1 labeling kit, Zenon Alexa Fluor 647 mouse IgG2a labeling kit, and the mounting medium (Prolong Antifade Kit) were obtained from Invitrogen Molecular Probes. Paraformaldehyde (8%, w/v) was purchased from Electron Microscopy Sciences (Fort Washington, PA, USA).

Hybridoma cells producing monoclonal antibodies against bovine CD8 (clone 7C2) and ovine $\gamma\delta$ T (clone 86D) were purchased from European Type Cell Culture Collection (Salisbury, UK). These monoclonal antibodies were obtained as culture supernatants of hybridoma cell cultures prepared by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research.

Mouse anti-human CD68 (clone EBM11; clarified ascites fluid, 2.3 μ g/ml) was obtained from Biomed (Foster City, CA, USA); mouse anti-bovine CD14 (clone MM61A; clarified ascites fluid, 10 μ g/ml), mouse anti-bovine CD4 (clone CATC 138A; clarified ascites fluid, 10 μ g/ml), and mouse anti-bovine CD25 (clone CATC 108A; clarified ascites fluid, 10 μ g/ml) were from VMRD (Pullman, WA, USA). Control mouse ascites fluid (clarified, clone NS1) was from Sigma–Aldrich. Normal goat serum was purchased from Pel-Freez Biologicals (Rogers, AR,

Figure 5 Effect of pregnancy status on the intensity of staining for CD68 in the lamina propria (A) and submucosa (B) of the endometrium. Shown are data for nonpregnant cows ($n=7$) and pregnant cows at days 54–100 ($n=7$), 101–200 ($n=8$), and 201–240 of pregnancy ($n=5$). Intensity was estimated subjectively using a scoring system from 0 to 5. Data are least-squares means $\pm$ S.E.M. Bars with different letters are different from each other ($P<0.05$).

USA). The Histoscan Monoclonal Detector kit and mounting medium were obtained from Biomed. Tissue freezing medium was obtained from Biotech Medical Corporation (Kuala Lumpur, Malaysia). Lab-Tek Glass Chamber Slides were obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

Flow cytometric analysis of PBMCs

Animals

Cows were maintained at the University of Florida Dairy Research Unit at Hague, Florida. The first experiment was designed to determine the differences in the peripheral blood

lymphocyte populations between the nonpregnant and pregnant cows at days 33–34 of gestation. A total of 33 lactating Holstein cows were subjected to timed artificial insemination using a modified Presynch–OvSynch procedure (Brusveen *et al.* 2008) for insemination at days 233 ± 21 after calving (range 127–389). Cows were diagnosed for pregnancy using transrectal ultrasound examination at days 33–34 after insemination and a blood sample was collected by coccygeal venipuncture into heparinized tubes and used for flow cytometry. Blood samples were collected from a total of 18 nonpregnant and 15 pregnant cows.

The second experiment was designed to determine the differences between the nonpregnant and preparturient cows. A coccygeal blood sample was collected from a total of eight nonpregnant cows that were non-lactating and at random stages of the estrous cycle and from eight pregnant cows that were non-lactating and were at an average of 281.3 ± 2.9 days of gestation (range 273–289). The preparturient cows were at an average of 4.9 ± 1.7 days before parturition (range 1–14 days).

Isolation of PBMCs

Blood (10 ml) was centrifuged at 600 *g* for 30 min to obtain the buffy coat. This layer was mixed with 2 ml TCM-199 and the cell suspension was transferred to the top of 2 ml Fico/Lite LymphoH placed in a 15 ml conical tube. Cells were centrifuged at 600 *g* for 30 min. Mononuclear cells were collected at the top of the Fico/Lite, centrifuged at 600 *g* for 10 min, resuspended in DPBS, and used to determine cell concentration and viability by trypan blue exclusion using a hemacytometer. The cells were then resuspended to a final concentration of 5×10^7 /ml.

Flow cytometry

The cells (5×10^6) were placed into 13×100 mm polyethylene tubes in staining buffer (DPBS supplemented with 0.1% (w/v) BSA and 0.1% (w/v) sodium azide), washed twice with 2 ml staining buffer, and resuspended in the smallest volume possible with staining buffer. The cells were then stained for single-color analysis using anti- $\gamma\delta$ and anti-CD68 antibodies and for dual-color analysis with anti-CD4 and anti-CD8 and anti-CD4 and anti-CD25. A mouse IgG was used in the same dilution of the primary antibody as a control to nonspecific antibody staining. The antibodies, including the IgG control, were tagged with Fab fragments against mouse IgG conjugated to Alexa Flour 488, Alexa Flour R-phycoerythrin and Alexa Flour 647 using the Zenon Mouse Labeling IgG kits as per manufacturer's instructions. The labeled antibody complex was then diluted in the staining buffer to a final concentration of 10 μ g/ml at room temperature for 30 min. After incubation, the samples were washed with 2 ml staining buffer, and resuspended with DPBS containing 4% (w/v) paraformaldehyde for fixation. Before analysis, the cells were washed once with 1 ml staining buffer and resuspended in 300 μ l staining buffer. The flow cytometry profiles were obtained on Fluorescent Analysis Cell Sorter 'FACSCalibur' using CELLQuest flow cytometry software (Becton-Dickinson, Franklin Lakes, NJ, USA). The cell populations analyzed were gated on the basis of forward and side scatter at the lymphocyte and monocyte regions.

Figure 6 Co-localization of CD68 and CD14 in endometrium at day 209 of pregnancy. The top panels represent sections that were dual labeled with anti-CD14 (red) and anti-CD68 (green). Blue indicates nuclear stain. The bottom panels represent sections incubated with red- and green-labeled isotype controls.

Immunohistochemistry for CD68 and CD14

Uteri were obtained from pregnant and nonpregnant cows of various breeds at a local abattoir. Fetal crown–rump length was measured to estimate the fetal age (Noden & de Lahunta 1990). Reproductive tracts from nonpregnant cows were used only if a corpus luteum was present. Tissues from a total of 20 pregnant cows (estimated fetal ages ranging from 54 to 240 days of pregnancy) and 7 nonpregnant cows were collected. Samples of intercotyledonary uterine endometrium ipsilateral to the corpus luteum were snap frozen in Tissue-Tek OCT embedding compound. Tissues from pregnant and nonpregnant cows were processed in parallel to avoid confounding physiological stage with procedural replicate.

For immunohistochemistry, 5 µm tissue sections were prepared with a cryostat microtome. Sections were placed onto precleaned glass slides, fixed in ice-cold acetone for 10 min and air dried. The sections were rehydrated in histochemistry buffer at 4 °C (10 mM NaPO₄ (pH 7.4) containing 0.9% (w/v) NaCl supplemented with 1% (v/v) normal goat serum) for 30 min.

Procedures for immunohistochemistry were carried out according to the manufacturer's instructions. Briefly, sections were sequentially incubated with peroxidase-blocking buffer (histochemistry buffer with 0.3% (v/v) H₂O₂) for 5 min and washed three times in histochemistry buffer. The tissue conditioner supplied in the kit was applied to the samples for 4 min before incubation with primary antibody overnight at 4 °C; primary antibodies used were anti-CD68 (2.3 µg/ml) and anti-CD14 (10 µg/ml) in histochemistry buffer. As controls, other sections were incubated with IgG (10 µg/ml) in histochemistry buffer. Slides were then sequentially incubated with secondary antibody (biotinylated anti-mouse immunoglobulin as supplied in the kit) for 30 min, streptavidin–alkaline phosphatase reagent (from kit) for 30 min, and hematoxylin (as supplied in the kit) for 2 min. Slides were washed under tap water, cover slips were mounted, and the slides were examined for staining using light microscopy.

The concentration of CD68⁺ cells in the endometrial stroma was estimated subjectively according to a scale ranging from 0 (no positive cells) to 5 (very intense accumulation of positive cells). Evaluation was performed blindly with respect to treatment.

Figure 7 Co-localization of CD68 and CD14 in adherent cells isolated from dispersed endometrial cells from a pregnant cow at day 166 of pregnancy. Cells were labeled with anti-CD14 (red), anti-CD68 (green), and Hoescht 33342 (blue) for nuclei. Photomicrographs represent images obtained using differential interference contrast as well as fluorescence with the green and blue filters (CD68), red and blue filters (CD14), or with all three colors merged (Merge).

Two-color immunofluorescence for CD68 and CD14

Uterine sections were prepared as described previously. Sections were incubated with blocking buffer (DPBS supplemented with 20% (v/v) goat serum) for 20 min followed by incubation overnight at 4 °C with anti-CD68 (2.3 µg/ml), anti-CD14 (10 µg/ml), and isotype controls (10 µg/ml) labeled using the Zenon labeling system as described previously. The sections were then washed three times for 5 min using PBS and then labeled with Hoescht 33342 reagent (2.3 µg/ml) for 15 min. Sections were washed an additional three times for 5 min each, cover slips mounted using Prolong Antifade reagent, and slides examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) with a 40× objective and using Zeiss filter set 02 (DAPI filter), Zeiss filter set 03 (FITC filter), and Zeiss filter set 15 (rhodamine filter). Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Two-color immunofluorescence for CD68 and CD14 in endometrial adherent cells

Two-color immunofluorescence was performed using preparations of dispersed adherent cells from endometrium of a pregnant cow at day 166 of pregnancy. The uterus was obtained from a local abattoir and transported to the laboratory on ice (~2 h). A sample of the intercaruncular region was taken for isolation of stromal endometrial cells. Endometrial cells were removed from intercaruncular areas of the endometrium by mechanically scraping the inner surface of the endometrium with a sterile surgical blade. Cell scrapings were collected into a 50 ml sterile culture tube containing 50 ml TCM-199 supplemented with type I collagenase at 150 U/ml. Cells were incubated at 37 °C for 1 h under gentle rotation. Cells in suspension were then filtered through a sterile 100 µm cell strainer into 50 ml sterile culture tubes and centrifuged at 110 g for 5 min. The cell pellet was resuspended with 5 ml TCM-199 supplemented with 10% (v/v) fetal bovine serum and the cell number was determined using a hemacytometer. The cell suspension was placed into eight-well sterile chamber slides (Lab-Tek Glass Chamber Slides; Electron Microscopy Sciences) with a cover and incubated at 37 °C for 2 h to allow cells to adhere. The wells were washed three times with DPBS to eliminate non-adherent cells. The remaining adherent cells were fixed in DPBS containing 4% (w/v) paraformaldehyde for 15 min. The wells were washed three times in DPBS and two-color immunofluorescence staining was performed as described above.

Statistical analysis

Data were analyzed by least-squares ANOVA using the General Linear Model procedure of SAS (SAS for Windows, version 9.3; SAS Institute Inc., Cary, NC, USA). For the flow cytometry studies, the model included the effect of physiological status (pregnant versus nonpregnant), date samples were collected (i.e., replicate), and the interaction. For intensity of CD68 staining in endometrium, cows were grouped into four groups based on the stage of pregnancy (nonpregnant, $n=7$; days 54–100 of pregnancy, $n=7$; days 101–200 of pregnancy,

$n=8$; and days 201–240 of pregnancy, $n=5$). The mathematical model included the effect of stage of pregnancy.

Declaration of interest

There is no conflict of interest that would prejudice its impartiality.

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