

Regulation of Numbers of Macrophages in the Endometrium of the Sheep by Systemic Effects of Pregnancy, Local Presence of the Conceptus, and Progesterone

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ABSTRACT: Many species exhibiting hemochorial placentation experience an accumulation of macrophages in the endometrium during pregnancy. For the present study, it was tested whether macrophages also accumulate in the endometrium of the sheep, which is a species undergoing an epitheliochorial placentation. An additional objective was to determine whether regulation of endometrial macrophage number occurs via systemic or local signals and whether progesterone is one of these signals. The approach was to evaluate presence of macrophages immunohistochemically using antibodies to CD68 and CD14. Tissues examined were from cyclic ewes in the luteal phase of the estrous cycle, unilaterally-pregnant ewes at day 140 of pregnancy in which pregnancy was surgically confined to one uterine horn, ovariectomized ewes, and ovariectomized ewes treated with progesterone for 44 days. Macrophages were localized predominately to the stromal compartment of the stratum compactum region of the endometrium. In non-pregnant ewes, macrophages were not abundant regardless of physiological status. Increased numbers of endometrial macrophages were seen for both the pregnant and non-pregnant uterine horns of unilaterally pregnant ewes. Numbers of macrophages were higher in the endometrium from the pregnant uterine horn than from endometrium from the non-pregnant uterine horn. Results indicate that macrophages accumulate in the endometrium by day 140 of pregnancy in the sheep and that this induction is because of both systemic and local signals. Progesterone appears not to be an important regulator of numbers of endometrial macrophages.

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INTRODUCTION

In species with hemochorial placentation, there is an accumulation of macrophages in one or more compartments of the endometrium during mid- to late-pregnancy. Such a phenomenon has been reported in humans,^{1,2} mice,^{3–5} rats,^{6,7} and guinea-pigs.⁸ While, inappropriate activation or accumulation of endometrial macrophages is associated with pregnancy loss⁹ and reduced trophoblast invasiveness,¹⁰ macrophages may ordinarily function to blunt maternal immune responses

against the conceptus through production of prostaglandin E₂,¹¹ indoleamine 2,3-dioxygenase,^{12,13} and T helper (Th2) cytokines such as interleukin-4 (IL-4).¹⁴ Endometrial macrophages may also promote trophoblast growth through secretion of mitogenic cytokines,¹⁵ participate in induction of apoptosis and removal of apoptotic cells,^{4,10} and play a role in parturition.^{5,16}

It is not known whether accumulation of macrophages in the endometrium is a general phenomenon in placental mammals or, rather, is limited to species with hemochorial placentae. Macrophages are present in

the endometrium of the pig throughout pregnancy but it is not clear whether numbers are higher than in non-pregnant females.¹⁷ In sheep, which like the pig exhibit epitheliochorial placentation, cells positive for major histocompatibility complex (MHC) class II are present in the intercaruncular endometrium during pregnancy, especially in the stroma and in the connective tissue underlying epithelia.¹⁸ Numbers of positive cells in stromal areas appeared to decrease as pregnancy progressed. The objective of the present study was to determine whether accumulation of macrophages in uterine endometrium of the sheep is increased during pregnancy and to determine whether regulation of numbers of endometrial macrophages is a local or systemic phenomenon and under the influence of progesterone.

MATERIALS AND METHODS

Materials

Hybridoma cells producing monoclonal antibody to CD45RA (clone 73B) were purchased from European Collection of Cell Cultures (Salisbury, UK). Ascites fluid from these cells was produced by the Hybridoma Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Mouse anti-human CD68 (clone EBM11; clarified ascites fluid, 0.46 mg/mL) was obtained from Biomed (Foster City, CA, USA), mouse anti-sheep CD14 (clone MM61A; clarified ascites fluid, 1.0 mg/mL) was from VMRD (Pullman, WA, USA), and control mouse ascites fluid (clarified, clone NS1) was from Sigma Chemical (St Louis, MO, USA). Normal goat serum was purchased from Atlanta Biologicals (Norcross, GA, USA). The Histoscan Monoclonal Detector kit and mounting medium were obtained from Biomed. Tissue freezing medium was obtained from Triangle Medical Sciences (Durham, NC, USA). Prostaglandin F_{2α} was Lutalyse[®] from Pharmacia Animal Health (Kalamazoo, MI, USA). Isopentane, acetone, H₂O₂, and pre-cleaned glass microscope slides were from Fisher Scientific (Pittsburgh, PA, USA).

Animals

Multiparous ewes that were predominately of Rambouillet breeding ($n = 6$) were surgically prepared to limit pregnancy to one uterine horn. Briefly, one uterine horn was ligated surgically and the ovary ipsilateral to the ligated horn was also removed. After > 30 days of recovery, estrus was induced by injection of 10 mg prostaglandin F_{2α} and ewes were bred at the subsequent estrus to fertile rams to establish pregnancy. The pregnant uterus was recovered at day 140 of gestation. Endometrial tissue was collected at

slaughter from four cyclic ewes – all ewes were in the luteal phase of the estrous cycle at the time of recovery of the uterus as determined by the presence of corpus luteum. To determine regulation by progesterone, endometrial tissues were also examined from bilaterally ovariectomized ewes in which both uterine horns were ligated and where ewes were treated daily with i.m. injection of either progesterone (50 mg/mL) ($n = 4$) or an equivalent volume (2 mL) of sesame oil vehicle ($n = 4$) for 44 days. The ewes providing these tissues were from an experiment in which hybridoma cells were injected into one uterine horn¹⁹ – only endometrium from the uterine horn not receiving hybridoma cells was used for analysis.

Tissue Collection

The uterus was recovered after slaughter by captive-bolt stunning and exsanguination. For all ewes, tissue samples were excised from randomly determined regions of the intercaruncular endometrium. For unilaterally pregnant ewes, intercaruncular endometrium was collected separately from the pregnant horn and non-pregnant horn. Tissue samples (3–4 mm³) were snap-frozen in aluminum cassettes containing tissue-freezing medium by using liquid nitrogen cooled isopentane. Tissue blocks were stored at –70°C until sectioning.

Immunohistochemistry

Frozen 6 μm sections of uterine endometrium were prepared using a cryostat microtome (Micron Model HM 505 E, Walldorf, Germany). Sections were mounted onto pre-cleaned glass slides and allowed to air dry. For localization of CD68 and CD45RA, tissue was fixed at room temperature via sequential incubations with 95% (v/v) ethanol and 100% acetone followed by washing in 10 mM KPO₄, pH 7.4 containing 0.9% (w/v) NaCl (phosphate-buffered saline, PBS). For CD14, the ethanol step was omitted. The procedures for immunohistochemistry were carried out according to manufacturer's instructions. All steps were performed at room temperature, antibody incubations were performed in a humidified chamber, and cells were washed with staining buffer [PBS containing 2% (v/v) normal goat serum] between all steps. Briefly, sections were sequentially incubated with blocking buffer [PBS containing 2% (v/v) normal goat serum, 0.3% (v/v) H₂O₂] and tissue conditioner supplied in the kit before incubation with primary antibody overnight. Primary antibodies diluted in staining buffer were anti-CD68 (1:200 dilution), CD14 (1:500 dilution), and anti-CD45RA (naïve T cell marker, 1:500). Appropriately diluted control mouse ascites fluid was used as a negative control. Slides were incubated overnight with primary antibody, and then incubated sequentially

with biotinylated anti-mouse immunoglobulin for 30 min, streptavidin-alkaline phosphatase for 30 min, and 3-amino, 9-ethylcarbazole (all reagents from the kit) for 10 min. Slides were washed under tap water, counterstained with hematoxylin (1:20 dilution of the stain provided in the kit) and cover slips mounted. Slides were examined for staining using bright field microscopy with an epifluorescence microscope (Zeiss Axioplan 2, Göttingen, Germany) and photographed using a Sony CD Mavica 400 digital camera (San Diego, CA, USA) mounted on the microscope.

Statistical Analysis

The concentration of CD68⁺ cells in the stratum compactum of 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 by two independent persons. Data were analyzed by least squares ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, Release 8.02, Cary, NC, USA). The statistical model included effects of source of tissue (pregnant uterus–pregnant horn, pregnant uterus–non-pregnant horn, luteal phase of the estrous cycle, ovariectomized–progesterone, and ovariectomized–vehicle). Variation due to effects of tissue source were partitioned into individual degree of freedom (df) contrasts to further delineate differences between groups. The specific contrasts used were as follows: pregnant uterus–pregnant horn versus pregnant uterus–non-pregnant horn; pregnant groups versus non-pregnant groups; luteal phase versus ovariectomized groups; and ovariectomized–progesterone versus ovariectomized–vehicle).

RESULTS

In cyclic ewes, CD68⁺ cells were scattered throughout the endometrium (Fig. 1, top left panel). Cells were present within the stroma, along the basement membrane of the luminal and glandular epithelium and, less abundantly, in the luminal and glandular epithelium. Abundance varied from the occasional cell to fairly numerous. Cells were large in appearance and varied in shape from round to irregular. CD68 staining often appeared to be localized in cytoplasmic granules. In unilaterally pregnant animals, the appearance of CD68⁺ cells was not altered but there was a change in the number and localization of cells (Fig. 1, middle left and right panels). In particular, there was a large increase in the number of CD68⁺ cells and these were located almost exclusively in the stroma, particularly in the stratum compactum region underneath the luminal epithelium. The pregnancy-associated increase in cell

number and change in distribution occurred in both the pregnant and non-pregnant uterine horns, although increases in number were more pronounced for the pregnant uterine horn.

Pregnancy-associated changes in numbers and localization of macrophages could not be mimicked by progesterone treatment. Indeed, macrophages were not abundant in ovariectomized animals regardless of whether they were treated with progesterone (see Fig. 1, top right).

The numbers of CD68⁺ cells in endometrium was often too high to allow counting individual cells. Thus, relative abundance of CD68⁺ cells in the endometrium was estimated on a subjective scale ranging 0–5. These scores were subjected to ANOVA and results are shown in Fig. 2. There were no significant differences in staining intensity between cyclic ewes, ovariectomized ewes, and ovariectomized ewes treated with progesterone. However, staining intensity was greater for both horns of the unilaterally pregnant ewes than for cyclic ewes ($P < 0.001$) and was greater for the pregnant uterine horn of unilaterally pregnant ewes than for the non-pregnant uterine horn ($P < 0.001$).

The distribution of CD68⁺ cells was distinct from that for CD45RA⁺ lymphocytes, which were located in the luminal and glandular epithelium and in the subepithelial stroma at the base of the epithelium (Fig. 1, bottom right panel). In contrast, CD14⁺ cells (examined in a subset of three unilaterally pregnant, two cyclic, one ovariectomized and one progesterone-treated ewe) exhibited a localization pattern and pregnancy-associated changes that mimicked those of CD68 (Fig. 1, lower left panel). Staining for CD14 was often more diffuse than for CD68, suggesting release of CD14 and absorption to extracellular matrix.

DISCUSSION

Present results demonstrate that the ewe undergoes an increase in numbers of macrophages in the endometrium during pregnancy. Thus, endometrial accumulation of macrophages during pregnancy, which has been described heretofore only in species with hemochorial placentae,^{1–8} is a phenomenon that is not limited to species with one type of placentation. Function of these macrophages during pregnancy is not known but evidence exists to indicate a role in immunoprotection of the conceptus,^{11–14} apoptosis,^{4,10} trophoblast growth,¹⁵ and parturition.^{5,16} In sheep, it is possible that endometrial macrophages participate in the increase in number and granularity of $\gamma\delta$ T cells seen in endometrial luminal epithelium^{20,21} because autologous monocytes can stimulate proliferation of bovine $\gamma\delta$ T cells.²²

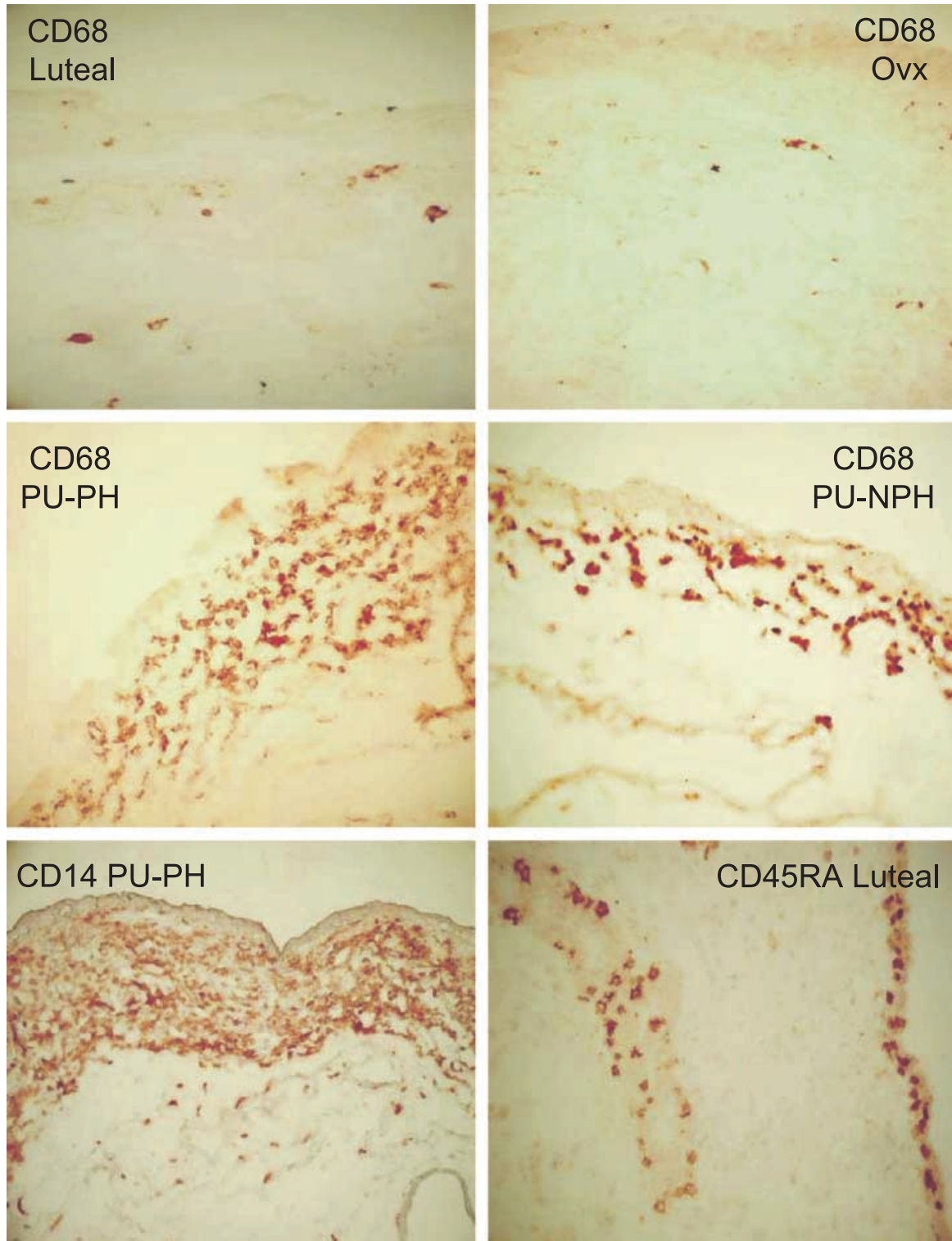


Fig. 1. Representative patterns of localization of cells positive for CD68, CD14 and CD45RA in the endometrium of the sheep. All images are at the same magnification (original magnification 400 \times). Abbreviations used are Ovx, ovariectomized-vehicle treated; PU-PH, pregnant uterus-pregnant horn and PU-NPH, pregnant uterus-non-pregnant horn. Luteal refers to a cyclic ewe in the luteal phase of the estrous cycle.

The accumulation of macrophages during pregnancy was limited spatially to the stromal compartment of the endometrium. This is a different region of the

endometrium than the epithelial compartments where lymphocytes are located (as demonstrated by the immunohistochemical localization of CD45RA⁺ cells

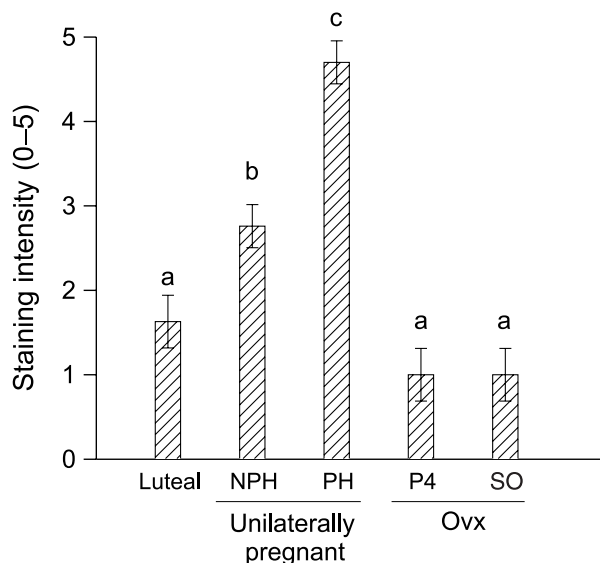


Fig. 2. Effect of pregnancy status and progesterone treatment on numbers of CD68⁺ macrophages in the endometrium. Numbers were estimated visually using a subjective scoring system from 0 to 5. Differences between treatments were determined by ANOVA. Data are least squares mean \pm S.E.M. Bars with different letters (a–c) are different from each other ($P < 0.001$) as determined by use of orthogonal contrasts to separate treatment effects into single df comparisons. Abbreviations are as follows: NPH, non-pregnant horn; PH, pregnant horn; P4, progesterone-treated; SO, sesame-oil, and Ovx, ovariectomized.

in luminal and glandular epithelium). The implications for macrophage–lymphocyte interactions are not clear although the two cell types can presumably communicate through cytokine secretion. No efforts were made to evaluate localization of macrophages in the caruncular portion of the endometrium that, with the fetal cotyledons, form the placentomes where the majority of gas and nutrient exchange takes place. Previous experiments indicate that leukocytes are very sparse in this tissue.¹⁸

Of interest in this study was whether the signal leading to accumulation of macrophages in the endometrium was the result of a locally acting trophoblast-derived molecule or through a signal of conceptus or maternal origin that can act systemically. This question was examined through the use of the unilaterally pregnant ewe model whereby the conceptus is confined locally to one uterine horn. Pregnancy proceeds normally in the pregnant uterine horn²³ and the array of proteins produced by the endometrium is similar in the pregnant and non-pregnant uterine horns.²⁴ Using this model, it was demonstrated previously that the accumulation of $\gamma\delta$ T cells in the endometrial epithelium does not depend upon locally acting conceptus signals.²⁵ In the present study, accumulation of macrophages did not require the

local presence of the conceptus because the increased accumulation of CD68⁺ and CD14⁺ cells in endometrial stroma during pregnancy occurred in both the pregnant and non-pregnant uterine horn. Thus, at least some of the signals that lead to accumulation of macrophages in the endometrium act systemically. Nonetheless, there was greater accumulation of macrophages in the pregnant uterine horn than in the non-pregnant horn. Accordingly, locally derived signals from the conceptus also contribute to macrophage accumulation in the endometrium.

At least in the mouse, the endometrium expresses genes for many molecules that could cause increased macrophage accumulation including colony-stimulating factor 1, monocyte chemoattractant protein-1, macrophage inflammatory protein 1 (MIP1) and regulated on inactivation, normal T expressed and secreted protein (RANTES).^{4,26,27} Expression of these genes is under hormonal control involving actions of progesterone and estrogen.²⁷ It is likely that the increase in endometrial macrophages in the non-pregnant uterine horn of unilaterally pregnant ewes is the result of induction of expression of one or more chemokine genes in the endometrium by endocrine signals from the mother or placenta.

There was no evidence in the present study that progesterone was one of the hormonal signals driving accumulation of endometrial macrophages as numbers of macrophages in endometrium of ovariectomized ewes treated with progesterone was low. It is possible that more prolonged exposure to progesterone could lead to different results but the 44 day treatment regimen is sufficient to induce secretion of ovine uterine serpin, the major progesterone-induced endometrial protein in sheep²⁸ and to inhibit xenograft rejection in utero.¹⁹ In mice, progesterone injections restored numbers of endometrial macrophages in ovariectomized animals²⁹ but high concentrations of progesterone reduced macrophage migration into the uterus and inhibited macrophage function.^{30–32}

The local stimulatory effect of the presence of the conceptus on endometrial macrophage numbers seen in the present study may reflect the actions of chemokines or cytokines produced by the placenta. For example, mouse placenta produces MIP1 α and MIP1 β ³³ and human trophoblast produces macrophage migration inhibitory factor.³⁴ Regulation of macrophages by the placenta may be complex as there is evidence that signals from the conceptus may inhibit macrophage function to provide immunoprotection to the conceptus. In humans, secretory human leukocyte antigen-G (HLA-G) from trophoblast has been hypothesized to inhibit endometrial macrophage function through activation of the inhibitory receptors immunoglobulin-like transcripts 2 and 4.³⁵

In conclusion, the phenomenon of accumulation of macrophages in the endometrium during pregnancy is not limited to species with hemochorial placentae but also occurs in the sheep, a species that undergoes epitheliochorial placentation. Regulation of macrophage accumulation is dependent upon both locally acting and systemic molecules. There was no evidence that progesterone was one of these signals.

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